Genetic Engineering of Horticultural Crops

Edited by Gyana Ranjan Rout | K.V. Peter

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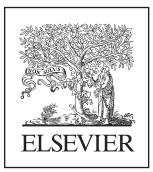

ACADEMIC PRESS

An imprint of Elsevier

Academic Press is an imprint of Elsevier
125 London Wall, London EC2Y 5AS, United Kingdom
525 B Street, Suite 1800, San Diego, CA 92101-4495, United States
50 Hampshire Street, 5th Floor, Cambridge, MA 02139, United States
The Boulevard, Langford Lane, Kidlington, Oxford OX5 1GB, United Kingdom

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Library of Congress Cataloging-in-Publication Data
A catalog record for this book is available from the Library of Congress

British Library Cataloguing-in-Publication Data
A catalogue record for this book is available from the British Library

ISBN: 978-0-12-810439-2

Publisher: Andre G. Wolff
Acquisition Editor: Nancy Maragioglio
Editorial Project Manager: Billie Jean Fernandez
Production Project Manager: Punithavathy Govindaradjane
Cover Designer: Christian J. Bilbow

Typeset by TNQ Books and Journals

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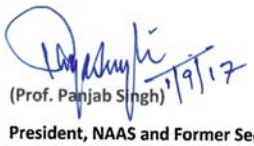
Foreword

Malnutrition, hidden hunger, and resultant stunting are rampant in India, despite self-sufficiency in food grain production. Past achievements in agricultural production transformed India from a "ship to mouth" existence to the present "farm to ship for export" position, which has given it national honor and pride. Appropriate public policy, use of science and technology, enlightened farmers, a well-organized public distribution system, and ever-growing demand for food grains has resulted in the much applauded Green Revolution. India is presently promoting a Nutrition Revolution through home gardens and multiple cropping systems. Despite all efforts, productivity of horticultural crops—fruits, vegetables, tubers, plantation crops, medicinal and aromatic plants, mushrooms, and bamboos—is one of the lowest, except for a few crops. Both biotic and abiotic factors limit production and the problem of deficit is further accelerated by huge harvest and postharvest losses. Genetic engineering is a scientific technology used in developed and many developing countries to increase production, reduce losses, and improve quality. The present edited book *Genetic Engineering of Horticultural Crops*, with 17 chapters authored by 58 scientists from 30 research institutes from India, China, Japan, and Turkey, reveals scientific advances made in this modern science of biotechnology and molecular biology. Contributions from different experts on various issues relating to genetic engineering of horticultural crops are of high value and worth reading. I congratulate all the contributing authors for their time, patience, and commitment to science. I appreciate the efforts made by Professors Gyana Ranjan Rout and K.V. Peter for coordinating and compiling this important publication. I am sure the researchers, academia, students, and others will take advantage of this important and informative publication.



(Prof. Panjab Singh)

President, NAAS and Former Secretary (DARE) and DG, ICAR

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Preface

It gives me great pleasure in writing this Preface for *Genetic Engineering of Horticultural Crop*, edited by Prof. G.R. Rout (Professor and Head, Department of Agricultural Biotechnology, College of Agriculture, Orissa University of Agriculture and Technology, Bhubaneswar, India) and Dr. K.V. Peter (Director, World Noni Research Foundation, Perungudi, Chennai, India). I found the chapters in this book carefully designed to focus on the various transgenic research activities and tools, which address crop productivity through the introduction of novel gene(s) with high nutrient qualities, resistance to pests and diseases, and improvement of stress tolerance. The book also discusses insights into key technologies, such as markers for trait(s) identification and transfer of these (various) genetic traits toward improving crop productivity. It also examines the latest trends in transgenic advances in a variety of crops, thus providing foundational information for future crop improvement research programs.

Genetic Engineering of Horticultural Crops covers a complete range of topics on the status of horticultural crop research, including identifying the need for transgenic traits, gene silencing in horticultural crops, transgenic research in fruit crops, spices, medicinal and aromatic plants, vegetables (with special reference to brinjal), root and tuber crops, nuts, and floriculture crops, transgenic development for biotic and abiotic stress management in horticultural crops, and, lastly, biosafety and bioregulatory mechanisms in transgenic crops. I am pleased to note that the latest developments in molecular biology research, including the role of marker-free technologies such as genome editing, have also been discussed in this book, which makes it not only an up-to-date book, but also a much-needed book in this area, covering topics that are essential for the development of transgenic (horticultural) crops. I am sure this book will become a great companion to researchers, postgraduate students, and plant biotechnologists, providing insights into the various gene transfer technologies relevant for sustainable agriculture and food production systems.

I congratulate Academic Press, an imprint of Elsevier, United States, for the excellence and great outlook of the book.

Best regards,

Dr. Kodi Isparan Kandasamy

BSc (UKM, Malaysia), MSc and PhD (London, UK)
Senior Vice President (Agriculture Biotechnology)

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CHAPTER

1 STATUS OF HORTICULTURAL CROPS: IDENTIFYING THE NEED FOR TRANSGENIC TRAITS

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1. INTRODUCTION

Although conventional plant breeding techniques have made considerable progress in the development of improved varieties, it has not been able to keep pace with the increasing demand for vegetables and fruits in developing countries. Therefore an instant need is felt to incorporate transgenic technology to speed up crop improvement programs. Three major constituents are involved in crop improvement programs, i.e., in vitro culture protocol development, marker-assisted breeding, and genetic engineering. Agricultural biotechnology has offered wonderful scope and potential to conventional methods for crop improvement, crop protection, crop quality management, and other horticultural traits. Biotechnology explores various opportunities in fruit production by providing new genotypes for breeding purposes, a supply of healthy and disease-free planting material, improvement in fruit quality, enhanced shelf-life, availability of biopesticides, biofertilizers, etc. The expression of undesirable genes can be blocked by the application of antisense gene technology and RNA-interference (RNAi) technology. Eventually, biotechnological interventions that could increase the efficiency of horticultural crop improvement are essential to generate plants with several desirable traits.

1.1 BASIC CONCEPT OF GENETICALLY MODIFIED CROPS

Genetically modified (GM) crops are products of the introduction of one or more characterized genes in a crop plant using recombinant DNA technology (RDT). The inserted gene is known as a transgene and the plants containing transgenes are often called GM crops or transgenic crops. GM crops are a possible solution for the widely discussed current problems of food and nutritional security. In contrast, conventionally bred cultivars result from artificial selection, random mutagenesis, or intra- or interspecific (rarely intergeneric) hybridization leading to the transfer of a number of uncharacterized genes from the same or a related alien species. Therefore the major difference between GM crops and conventionally bred cultivars is in the technology for transfer, and in the nature of genes transferred. Despite this debate among the proponents and opponents of GM crops, the global land area occupied by GM crops has been continuously increasing during the last 20 years, reaching a record 181.5 million ha globally in 2014, at an annual growth rate of between 3% and 4%, up 6.3 million ha from 175.2 million ha in 2013. The year 2014 was the 19th year of commercialization, when growth continued after a remarkable 18 consecutive years of increases; notably 12 of the 18 years were double-digit growth rates. In other words, biotech crops are the fastest adopted crop technology in the world.

Genetic Engineering of Horticultural Crops. http://dx.doi.org/10.1016/B978-0-12-810439-2.00001-5

The first commercially grown GM vegetable crop was tomato (called Flavr Savr), modified to ripen without softening by the Californian company Calgene, which took the initiative to obtain approval for its release in 1994. Currently, a number of food crops such as soybean, corn, cotton, Hawaiian papaya, potatoes, rapeseed (canola), sugarcane, sugar beet, field corn, as well as sweet corn and rice have been genetically modified to enhance their yield and durability, etc. Scientists are also working on oil-yielding crops and medicinal plants for the cosmetics industry, crops with altered nutritional value, and even crops that produce pharmaceutical drugs.

The basis of RDT is a key set of enzymes and techniques that allow DNA to be manipulated and modified precisely (Fig. 1.1). The fundamentals of RDT include:

1. Cutting of DNA with sequence-specific bacterial endonuclease (restriction endonuclease) to generate defined DNA fragments and using the enzyme DNA ligase to join them;
2. Separating nucleic acid on the basis of size by gel electrophoresis;
3. Detecting specific sequences in complex mixtures by nucleic acid hybridization;
4. Introducing DNA into cells;
5. Amplification of specific DNA molecules either by molecular cloning or using polymerase chain reaction.

Molecular cloning is an in vivo technique for producing large quantities of a particular DNA molecule (recombinant DNA molecule). The cloning process involves:

1. Introduction of the recombinant vector into a suitable host cell;
2. Selective propagation of cells containing the vector;
3. Extraction and purification of the cloned DNA molecule (recombinant DNA molecule).

1.2 PRODUCTION OF GENETICALLY MODIFIED CROPS WITH SPECIAL REFERENCE TO HORTICULTURAL PLANTS

With the rapid advances in biotechnology, a number of GM crops or transgenic crops carrying novel traits have been developed and released for commercial agriculture production. Commercial cultivation of transgenic crops started in the early 1990s. Herbicide tolerance and insect resistance are the main traits that are currently under commercial cultivation, and the main crops are: soybean, maize, canola, and cotton. A new and rigorous comprehensive global metaanalysis of 147 published biotech crop studies over the last 20 years confirmed the significant and multiple benefits that biotech crops have generated from 1995 to 2014. Millions of farmers, both large and small, worldwide have concluded that the returns from planting biotech crops are high, hence repeat planting is virtually 100%; good returns on their investment is the critical test applied by demanding farmers when judging the performance of any technology. Out of 28 countries involved in commercial cultivation of biotech crops, 20 were developing and only eight were industrial countries (Table 1.1, Fig. 1.2). More than half the world's population, ~60% or ~4 billion people, live in the 28 countries planting biotech crops. Bangladesh, one of the smaller and poorest countries in the world, approved and commercialized Bt brinjal in record time in 2014. Vietnam and Indonesia moved toward planting their first biotech crops in 2015 for a total of nine biotech countries in Asia. The choice of GM crops varies among the developing countries, with insect-resistant cotton being the most important commercially produced transgenic crop in Asian and African countries, while herbicide-resistant soybean followed by insect-resistant corn are predominant in the Latin American continent. In most cases, these GM technologies are proprietary, developed by the

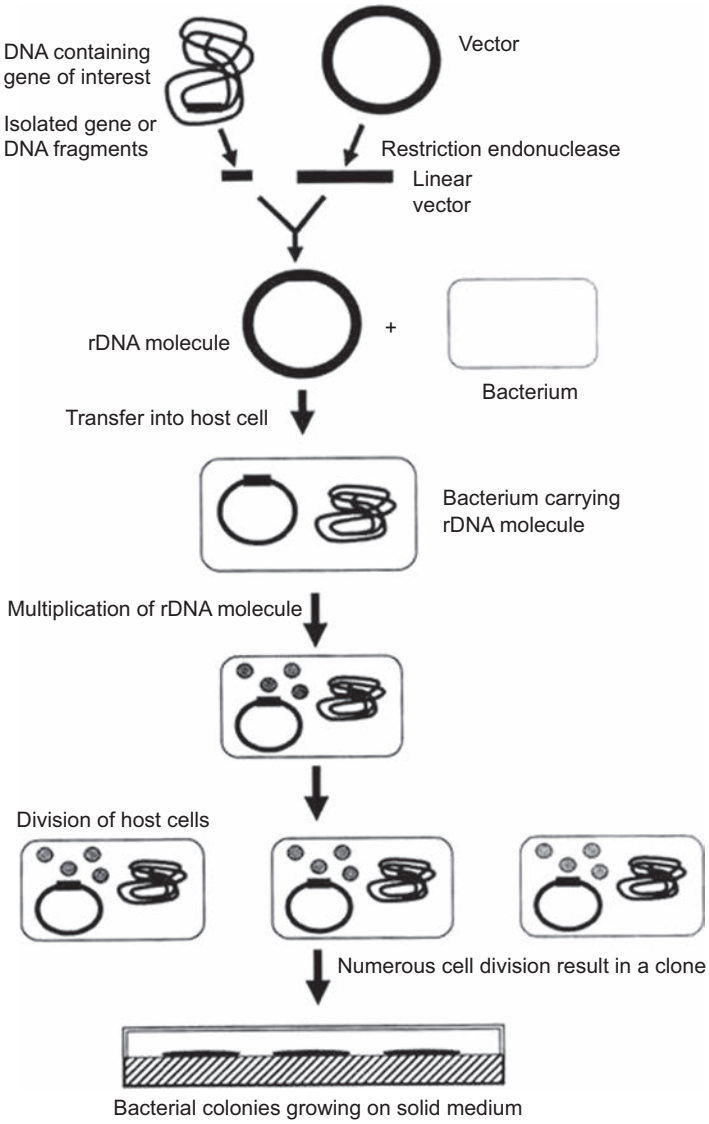

FIGURE 1.1 Basic steps of recombinant DNA technology.

private sector, and released for commercial production through licensing agreements. The cultivation and commercial production of GM crops are capital intensive because of the high costs of seed and technology. Nevertheless, their cultivation has increased, mainly because of the benefits accrued from lower labor and production costs, reduction in the use of chemical inputs, and improved economic gain.

Research on GM potato, cucumber, carrot, eggplant, sweet corn, and other vegetables in many countries of the world is aimed at resistance to viruses, bacteria, fungi, and insects, at tolerance to herbicides, at improvement of economic properties, prolongation of the consumption time, improvement

Table 1.1 Global Area of Biotech Crops During the Year 2014: by Country (Million Hectares)[b]

Rank	Country	Area (Million Hectares)	Biotech Crops
1	USA[a]	73.1	Maize, soybean, cotton, canola, sugarbeet, alfalfa, papaya, squash
2	Brazil[a]	42.2	Soybean, maize, cotton
3	Argentina[a]	24.3	Soybean, maize, cotton
4	India[a]	11.6	Cotton
5	Canada[a]	11.6	Canola, maize, soybean, sugar beet
6	China[a]	3.9	Cotton, papaya, poplar, tomato, sweet pepper
7	Paraguay[a]	3.9	Soybean, maize, cotton
8	Pakistan[a]	2.9	Cotton
9	South Africa[a]	2.7	Maize, soybean, cotton
10	Uruguay[a]	1.6	Soybean, maize
11	Bolivia[a]	1.0	Soybean
12	Philippines[a]	0.8	Maize
13	Australia[a]	0.5	Cotton, canola
14	Burkina Faso[a]	0.5	Cotton
15	Myanmar[a]	0.3	Cotton
16	Mexico[a]	0.2	Cotton, soybean
17	Spain[a]	0.1	Maize
18	Colombia[a]	0.1	Cotton, maize
19	Sudan[a]	0.1	Cotton
20	Honduras	<0.05	Maize
21	Chile	<0.05	Maize, soybean, canola
22	Portugal	<0.05	Maize
23	Cuba	<0.05	Maize
24	Czech Republic	<0.05	Maize
25	Romania	<0.05	Maize
26	Slovakia	<0.05	Maize
27	Costa Rica	<0.05	Cotton, soybean
28	Bangladesh	<0.05	Brinjal/eggplant
	Total	181.5	

[a]19 biotech mega-countries growing 50,000 ha, or more, of biotech crops.
[b]Rounded off to the nearest hundred thousand.
Courtesy: James, C., 2014. Global Status of Commercialized Biotech/GM Crops. The International Service for the Acquisition of Agri-biotech Applications (ISAAA), summary available at: http://www.isaaa.org/.

of nutrition values, and seedlessness of fruits. The development of transgenic fruit cultivars is in progress. Papaya resistant to papaya mosaic virus is grown in the United States and China (James, 2011). Biotech grapevine resistant to viral, bacterial, and fungal diseases with abiotic stress tolerance and health benefits was developed in South Africa. Biotech banana, apple, pear, and strawberry cultivars are under development. The result of the international research conducted over the past 20 years is the development of HoneySweet plum highly resistant to plum pox virus (PPV). GM HoneySweet plum

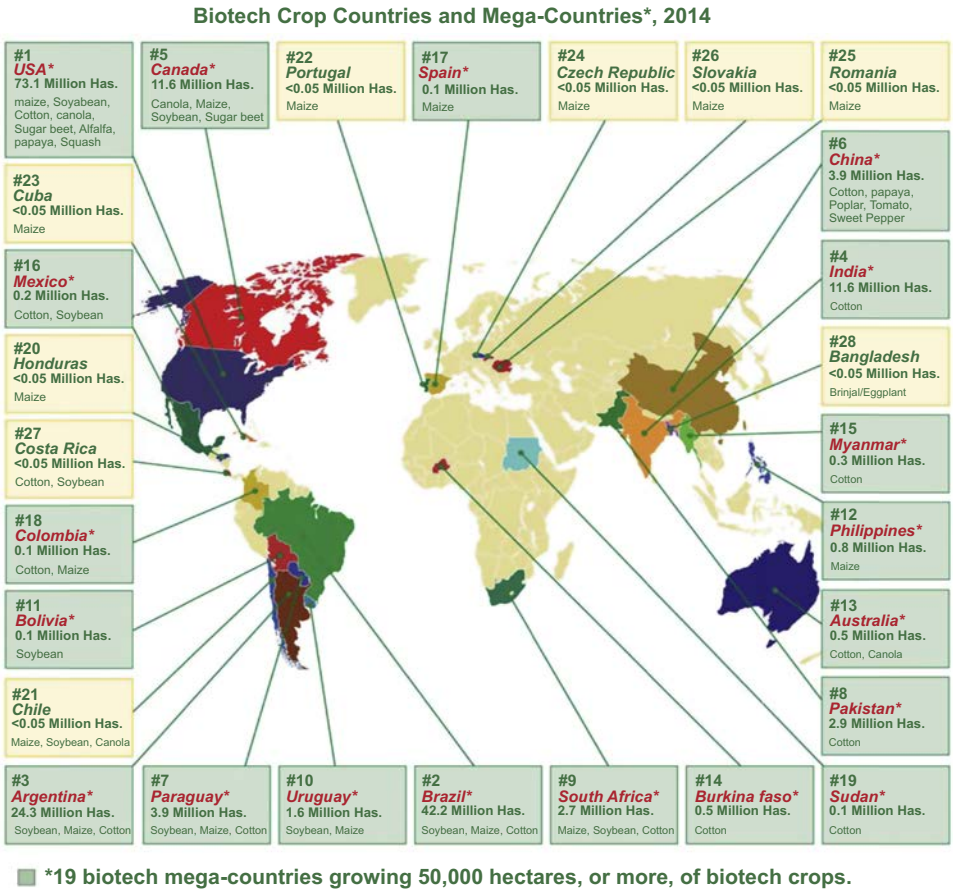

FIGURE 1.2 Global map of biotech crops countries and mega-countries in 2014.

Courtesy: James, C., 2014. Global Status of Commercialized Biotech/GM Crops. The International Service for the Acquisition of Agri-biotech Applications (ISAAA), summary available at: http://www.isaaa.org/.

resistant to PPV was deregulated in the United States in 2010. Plums (*Prunus domestica*) are an important source of vitamins, minerals, and phytonutrients and contain specific compounds that support good digestive function and bone health. Sharka disease is the most devastating disease of plum and is responsible for the reduction or loss of plum production in many areas of Europe (Cambra et al., 2006).

Papaya was genetically modified to resist the ringspot virus. "Sun Up" is a transgenic red-fleshed Sunset papaya cultivar that is homozygous for the coat protein (CP) gene papaya ringspot virus (PRSV); "Rainbow" is a yellow-fleshed F_1 hybrid developed by crossing "Sun Up" and transgenic yellow-fleshed "Kapoho" (Gonsalves, 2004). In the early 1990s *The New York Times* stated that Hawaii's papaya industry was facing disaster because of the deadly PRSV. Its single-handed savior was a breed engineered to be resistant to the virus. Without it, the state's papaya industry would have collapsed. Today, 80% of Hawaiian papaya is genetically engineered, and there is still no conventional or organic method to control ringspot

virus (Ronald and McWilliams, 2010). The GM cultivar was approved in 1998. In China, a transgenic PRSV-resistant papaya was developed by South China Agricultural University and was first approved for commercial planting in 2006; as of 2012, 95% of the papaya grown in Guangdong province and 40% of the papaya grown in Hainan province were genetically modified. The NewLeaf potato, brought to market by Monsanto in the late 1990s, was developed for the fast food market. It was withdrawn in 2001 after retailers rejected it and food processors faced export problems. As of 2005, about 13% of the zucchini (a form of squash) grown in the United States was genetically modified to resist three viruses; that strain is also grown in Canada. In 2011, Badische Aniline and Soda Company (BASF) requested the European Food Safety Authority's approval for cultivation and marketing of its Fortuna potato as feed and food. The potato was made resistant to late blight by adding resistant genes blb1 and blb2 that originate from the Mexican wild potato (*Solanum bulbocastanum*). In February 2013, BASF withdrew its application. In 2013, the United States Department of Agriculture (USDA) approved the import of a GM pineapple that is pink in color and that "overexpresses" a gene derived from tangerines and suppresses other genes, increasing production of lycopene. The plant's flowering cycle was changed to provide more uniform growth and quality. The fruit does not have the ability to propagate and persist in the environment once it has been harvested. According to Del Monte's submission, the pineapples are commercially grown in a "monoculture" that prevents seed production because the plant's flowers are not exposed to compatible pollen sources. Importation into Hawaii is banned for "plant sanitation" reasons (Perkowski, 2013). In 2014, the USDA approved a GM potato developed by J.R. Simplot Company that contained 10 genetic modifications that prevented bruising and produced less acrylamide when fried. The modifications eliminate specific proteins from the potatoes, via RNA interference, rather than introducing novel proteins. In February 2015, Arctic Apples were approved by the USDA, becoming the first GM apple approved for sale in the United States. Gene silencing is used to reduce the expression of polyphenol oxidase, thus preventing the fruit from browning.

1.3 INTERNATIONAL AND NATIONAL STATUS

There has been a consistent increase in global areas planted to transgenic crops from 1996 to 2014, which was assisted by 18 million farmers in 28 countries. About 181.5 million ha was planted (James, 2014) to transgenic crops in 2014 with high market value, such as herbicide-tolerant soybean, maize, cotton, and canola; insect-resistant maize, cotton, potato, and rice; and virus-resistant squash and papaya. With genetic engineering, more than one trait can be incorporated or stacked into a plant. Transgenic crops with combined traits are also available commercially. These include herbicide-tolerant and insect-resistant maize, soybean, and cotton. Notably, Bangladesh approved Bt brinjal for the first time on October 30, 2013, and in record time less than 100 days after approval small farmers commercialized Bt brinjal on January 22, 2014. Innate potato, another food crop, was approved in the United States in November 2014. It has lower levels of acrylamide, a potential carcinogen in humans, and suffers less wastage from bruising; potato is the fourth most important food staple in the world. A safer product and decreased wastage in a vegetatively propagated and perishable crop can contribute to higher productivity and food security. Also in November 2014, a new biotech alfalfa (event KK179) with up to 22% less lignin, which leads to higher digestibility and productivity, was approved for planting in the United States. The first biotech drought-tolerant maize, planted in the United States in 2013 on 50,000 ha, increased over fivefold to 275,000 ha in 2014 reflecting high acceptance by US farmers. Importantly, a new 2014 comprehensive global metaanalysis on 147 published biotech crop studies over the last 20 years worldwide confirmed the significant and multiple benefits that biotech crops have generated

from 1995 to 2014; on average GM technology adoption has reduced chemical pesticide use by 37%, increased crop yields by 22%, and increased farmer profits by 68%. These findings corroborate earlier and consistent results from other annual global studies, which estimated increases in crop productivity valued at US$133.3 billion for the period 1996–2013.

Biotech crops were grown commercially in all six continents of the world. Of the 28 countries planting biotech crops in 2015, 19 countries planted 50,000 ha or more to biotech crops (Table 1.2).

Table 1.2 Global Area of Biotech Crops During the Years of 2014 and 2015: By Country (Million Hectares)

Rank	Country	2014	2015
1	USA[a]	73.1	70.9
2	Brazil[a]	42.2	44.2
3	Argentina[a]	24.3	24.5
4	India[a]	11.6	11.6
5	Canada[a]	11.6	11.0
6	China[a]	3.9	3.7
7	Paraguay[a]	3.9	3.6
8	Pakistan[a]	2.9	2.9
9	South Africa[a]	2.7	2.3
10	Uruguay[a]	1.6	1.4
11	Bolivia[a]	1.0	1.1
12	Philippines[a]	0.8	0.7
13	Australia[a]	0.5	0.7
14	Burkina Faso[a]	0.5	0.4
15	Myanmar[a]	0.3	0.3
16	Mexico[a]	0.2	0.1
17	Spain[a]	0.1	0.1
18	Colombia[a]	0.1	0.1
19	Sudan[a]	0.1	0.1
20	Honduras	<0.1	<0.1
21	Chile	<0.1	<0.1
22	Portugal	<0.1	<0.1
23	Vietnam	<0.1	<0.1
24	Czech Republic	<0.1	<0.1
25	Slovakia	<0.1	<0.1
26	Costa Rica	<0.1	<0.1
27	Bangladesh	<0.1	<0.1
28	Romania	<0.1	<0.1
	Total	181.5	179.7

[a]19 biotech mega-countries growing 50,000 ha, or more, of biotech crops.
Courtesy: James, C., 2014. Global Status of Commercialized Biotech/GM Crops. The International Service for the Acquisition of Agri-biotech Applications (ISAAA), summary available at: http://www.isaaa.org/.

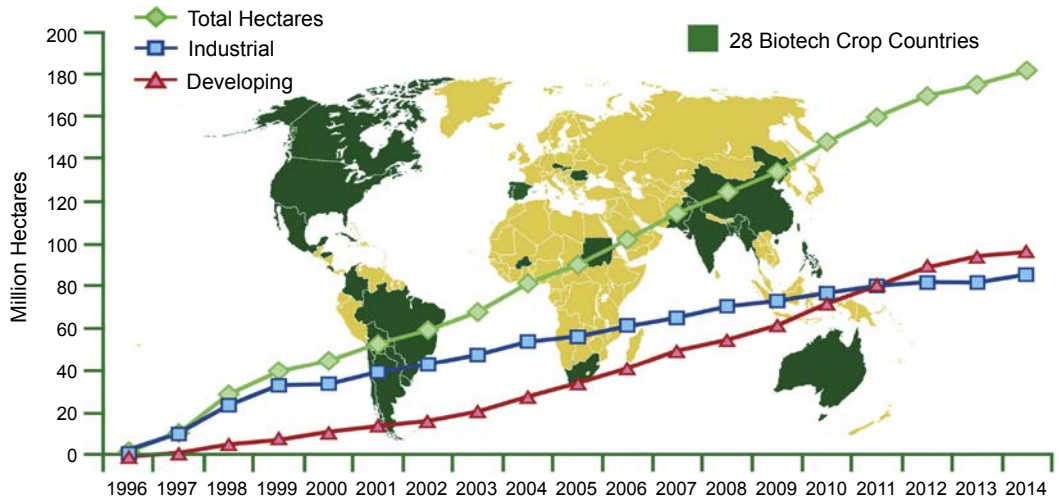

FIGURE 1.3 Global area of biotech crops million hectares (1996–2014).

Courtesy: James, C., 2014. Global Status of Commercialized Biotech/GM Crops. The International Service for the Acquisition of Agri-biotech Applications (ISAAA), summary available at: http://www.isaaa.org/.

These megacountries include the United States, Brazil, Argentina, India, Canada, China, Paraguay, Pakistan, South Africa, Uruguay, Bolivia, Philippines, Australia, Burkina Faso, Myanmar, Mexico, Spain, Colombia, and Sudan. Bangladesh approved a biotech crop (Bt brinjal) for planting the first time on October 30, 2013, and it was recorded that less than 100 days after the approval, commercialization was initiated on January 22, 2014 when 20 very small farmers planted their first crop of Bt brinjal; a total of 120 farmers planted 12 ha of Bt brinjal in 2014. This approval by Bangladesh is important in that it serves as an exemplary model for other small, poor countries. Also, very importantly, Bangladesh has broken the impasse experienced in trying to gain approval for commercialization of Bt brinjal in both India and the Philippines.

Remarkably, in 2014 global biotech crop hectarage continued to grow for the 19th consecutive year of commercialization; 18 million farmers in 28 countries planted more than 181 million ha in 2014, up from 175 million in 27 countries in 2013 (Figs. 1.3 and 1.4). Innate potato, another food crop, which was approved for the commercial cultivation in the United States in November 2014. It has lower levels of acrylamide, a potential carcinogen in humans, and suffers less wastage from bruising; potato is the fourth most important food staple in the world. A safer product and decreased wastage in a vegetatively propagated and perishable crop, can contribute to higher productivity and food security. Also in November 2014, a new biotech alfalfa (event KK179) with up to 22% less lignin, which leads to higher digestibility and productivity, was approved for planting in the United States. Importantly, a new 2014 comprehensive global metaanalysis, on 147 published biotech crop studies over the last 20 years worldwide confirmed the significant and multiple benefits that biotech crops have generated from 1995 to 2014; on average GM technology adoption has reduced chemical pesticide use by 37%, increased crop

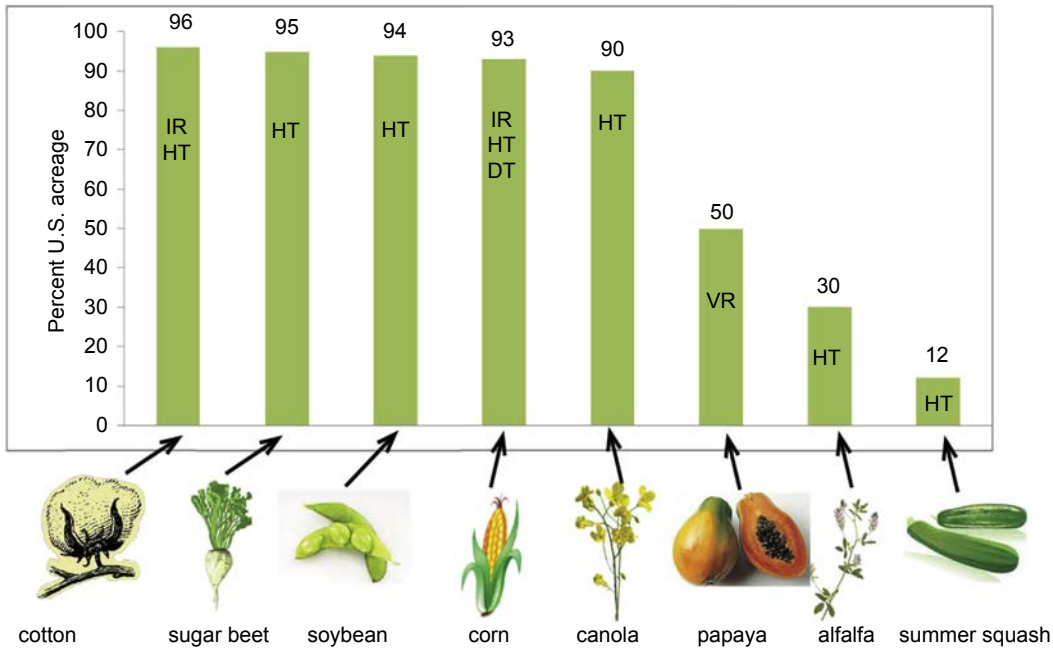

FIGURE 1.4 Currently grown genetically modified crops in the United States.

Traits for which they are modified, and percent of total acreage of the crop that is planted to GM varieties. *DT*, drought tolerant; *HT*, herbicide tolerant; *IR*, insect resistant; *VR*, virus resistant.

Courtesy: Byrne, P., 2014. Genetically Modified (GM) Crops: Techniques and Applications. Colorado State University.

yields by 22%, and increased farmer profits by 68%. These findings corroborate earlier and consistent results from other annual global studies which estimated increases in crop productivity valued at US$133.3 billion for the period 1996–2013.

2. NEED FOR GENETICALLY ENGINEERED CROPS IN THE PRESENT SCENARIO

2.1 CROP PRODUCTION AND PRODUCTIVITY

The efficacy of transgenic plant varieties in increasing production and lowering production costs is already demonstrable. In 1996 and 1997, the cultivation of virus-, insect-, and herbicide-resistant plants accounted for a 5%–10% increase in yield as well as for savings on herbicides of up to 40% and on insecticides of between $60 and $120 (US dollars) per acre (James, 1998). However, these increases in productivity, impressive as they are, probably have a limited impact on the global food supply because the products currently available on the market are suitable only for large mechanized farms practicing intensive agriculture. In fact, most of the transgenic crops that have been produced to date, especially by the private sector, are aimed either at reducing production costs in agricultural areas that already have high productivity levels or at increasing the value of the final product (e.g.,

improving the oil quality of seed crops). In a global sense, a more effective strategy to ensure sufficient levels of food production would be to increase productivity in developing countries, especially in areas of subsistence farming, where an increase in food production is urgently needed and where crop yields are significantly lower than those obtained in other areas of the world. In developing countries in the tropics and subtropics, crop losses caused by pests, diseases, and poor soils are made worse by climatic conditions that favor insect pests and disease vectors and by the lack of economic resources to purchase high-quality seeds, insecticides, and fertilizers. In addition to low productivity levels, postharvest losses in tropical areas are very high because of the favorable climate for fungal and insect infestation and the lack of appropriate storage facilities. Despite efforts to prevent pre- and postharvest crop losses, pests destroy over half of all world crop production. Postharvest loss caused by insects, the majority of which occurs in the developing world, is estimated to be 15% of the world's production. It is possible that many of these problems could be alleviated by plant biotechnology. A major advantage of plant biotechnology is that it often generates strategies for crop improvement that can be applied to many different crops. Genetically engineered virus resistance, insect resistance, and delayed ripening are good examples of strategies that could potentially benefit a diversity of crops. Insect-resistant plant varieties, using the dendotoxin of *Bacillus thuringiensis*, have been produced for several important plant species, including tomato, potato, walnut, and maize; these are already under commercial production. It is envisaged that these strategies can be used for many other crops important for tropical regions and other regions in the developing world. Genetically engineered delayed ripening, although only tested on a commercial scale for tomato, has an enormous potential application for tropical fruit crops, which suffer severe losses in developing countries because they ripen rapidly and because there is a lack of appropriate storage conditions and efficient transport systems for them to reach the final consumer.

2.2 ABIOTIC STRESS MANAGEMENT

High temperature, high light intensity, humidity, drought, frost, and salinity are the major abiotic stresses that reduce the yield and quality of fruit by affecting the vegetative and reproductive stages of growth and development. Nevertheless, abiotic stresses remain the greatest constraint to crop production. Research on genetic modification of various horticultural crops for improved abiotic stress tolerance has been explored with transformed tomato plants with a DNA cassette containing an *Arabidopsis* C repeat/dehydration-responsive element binding factor 1 (CBF1) complementary DNA (cDNA) and a nos terminator, driven by a cauliflower mosaic virus 35S promoter (Tsai-Hung et al., 2002). These transgenic tomato plants were more resistant to deficit water stress than the wild-type plants. Plants when exposed to abiotic stress conditions produce several pathogenesis-related proteins to compensate the effect of stress conditions. Among these proteins, osmotin is one of the important ones released during abiotic stress conditions. Husaini and Abdin (2008) reported the overexpressed tobacco osmotin gene in strawberry (*Fragaria* x *ananassa* Duch.) and found that the transgenic strawberry plants exhibited tolerance to salt stress. Also Subramanyam et al. (2011) expressed the tobacco osmotin gene in *Capsicum annuum* and the transgenic chilli plants exhibited improved salt tolerance. Cheng et al. (2009) developed transgenic tomato plants expressing the yeast SAMDC gene, which improved the efficiency of CO_2 assimilation and protected the plants from high-temperature stress (38°C) as compared to the wild-type plants. A bacterial mannitol-1-phosphate dehydrogenase (mtlD) gene driven by the constitutive cauliflower mosaic virus (CaMV) 35S promoter was transferred into tomato plants in

an attempt to improve abiotic stress tolerance (Khare et al., 2010). Drought (polyethylene glycol in medium) and salinity (sodium chloride in medium) tolerance tests revealed that transgenic lines exhibited a higher tolerance for abiotic stresses than nontransformed plants.

2.3 BIOTIC STRESS MANAGEMENT

Pathogens cause losses in 10%–16% of the global harvest (Chakraborty and Newton, 2011). This loss can be minimized by using transgenic technology. In traditional agriculture, only individuals of the same species or eventually closely related species can be crossbred. If in this naturally available gene pool, resistance to biotic stress does not exist, traditional breeders cannot create resistance or introgress this trait into new varieties. Therefore it is necessary to search for alternative sources of genes in other completely unrelated plant species or in microbial organisms. Besides, traditional methods are resource- and time-consuming and germplasm dependent (Roy et al., 2011). Also using chemical spray may have adverse effects on human health and the environment, including beneficial organisms, and may lead to the development of chemical-resistant insects and weeds (Wahab, 2009). Plant genetic engineering has been made possible during the last three decades. Currently, transgenic plants with herbicide, insect pest, and virus disease resistance are cultivated in more than 175.2 million ha globally (James, 2013), while in 1996 only 1.7 million ha of land were under transgenic crops. Out of the 29 countries currently contributing to the cultivation of transgenic plants, 21 are developing countries and eight are industrial. During the 1996–2014 period, cumulative economic benefits from transgenic plants were high in developing countries at US$47.9 billion compared to US$59 billion generated by industrial countries.

The major achievements in biotic stress management are as follows.

2.3.1 Pest Resistance

The bioinsecticidal δ-endotoxin gene (Bt gene) isolated from *B. thuringiensis* is currently in use to make plants resistant to insect pests. Progress in engineering insect resistance in horticultural plants has been attained by the use of insect control protein genes of *B. thuringiensis*. Insect resistance was first reported in tomato using Bt genes in 1987. Transgenic Bt tomato plants exhibited resistance against *Spodoptera litura* and *Heliothis virescens* (Fischhoff et al., 1987). Fruit trees such as persimmon transgenic for the *cry I* gene were found resistant to *Plodia interpunctata* and *Monema flavescens* (Tao et al., 1997). Potato varieties engineered for resistance to Colorado potato beetle were in commercial production for several years and were technically and agronomically successful, allowing significant reductions in insecticide use (Shelton et al., 2002). Chakrabarty et al. (2002) transformed cauliflower var. Pusa Snowball K-1 with a synthetic *cry IAb* gene and the transgenic plants indicated the effectiveness of the transgene against infestation by diamondback moth (*Plutella xylostella*) larvae during insect bioassays. Paul et al. (2005) developed transgenic cabbage (*Brassica oleracea* var. *capitata*) with a synthetic fusion gene of *B. thuringiensis* encoding a translational fusion product of *cry1B* and *cry1Ab* δ-endotoxins and found the transgenic plants resistant to *P. xylostella*. Transgenic technology has also been found to deliver resistance against various nematodes. Roderick et al. (2012) developed transgenic plantain (*Musa* sp.) cv. "Gonja manjaya" plants expressing a maize cystatin gene that inhibits the digestive cysteine proteinases and a synthetic peptide that disrupts nematode chemoreception. The best level of resistance exhibited by the transgenic plants against the major pest species *Radopholus similis* was 84% for the cystatin, 66% for the peptide, and 70% for the dual defense.

2.3.2 Disease Resistance

One of the major constraints limiting the production of fruit crops is diseases caused by several fungi, bacteria, and viruses. Conventional breeding seems to have limited application because of nonavailability of resistant gene(s) in gene pools of a particular crop. Genetic engineering of disease resistance in crops has become popular and valuable in terms of cost and efficacy. In fruit crops, the CP-mediated approach to engineer virus resistance has been in application to introduce resistance against diseases such as PPV, citrus tristeza virus, and grape fan leaf virus, etc. Papaya is grown in many tropical countries, but its cultivation is being threatened by PRSV, a disease that is considerably lowering its yield. Using biotechnological interventions, the CP gene of the virus has been transferred to papaya to confer PRSV resistance. Since 1998, GM papayas have been cultivated in Hawaii, USA, which had shown considerable resistance to PRSV. PRSV-resistant transgenic papaya varieties "Sun Up" and "Rainbow" have now occupied >80% shelf space in the US market. Also transgenic papaya plants with the mutated replicase (RP) gene from PRSV showed high resistance or immunity against PRSV in the field (Xiangdong et al., 2007). Praveen et al. (2010) developed transgenic plants of tomato with an AC4 gene–RNAi construct and the transgenic plants were found to show the suppression of tomato leaf curl virus activity. Yu et al. (2010) transformed commercial watermelon cultivars with an untranslatable chimeric construct containing truncated zucchini yellow mosaic virus CP and PRSV WCP genes.

HoneySweet plum is highly resistant to PPV, the most devastating disease of plums and other stone fruits. HoneySweet was deregulated in the United States in 2010. HoneySweet (aka C5) has been evaluated for 11 years (2002–12) in a regulated field trial in the Czech Republic for resistance to PPV, prune dwarf virus (PDV), and apple chlorotic leaf spot virus (ACLSV), all of them being serious diseases of plum. Even under the high and permanent infection pressure produced through grafting, PPV has been detected only in HoneySweet trees in several leaves and fruits situated close to the point of inoculum grafting. The lack of infection spread in HoneySweet demonstrates its high level of PPV resistance. Coinfections of PPV with PDV and/or ACLSV had practically no influence on the quantity and quality of HoneySweet fruits, which are large, sweet, and of a high eating quality. In many respects, they are superior to the fruits of the well-known cultivar Stanley. Many fruit growers and fruit tree nurseries in the Czech Republic are supportive of the deregulation of HoneySweet plum to help improve plum production and control the spread of PPV.

RNAi technology is being used quite successfully in controlling various bacterial and viral diseases in plants by switching off the expression of certain endogenous genes. Transgenic tomato plants expressing hairpin RNA (hpRNA) constructs against *Agrobacterium iaaM* and *ipt* oncogenes were found to be resistant to crown gall disease (Escobar et al., 2001). Using an hpRNA gene silencing strategy, transgenic poinsettia plants resistant to poinsettia mosaic virus have been developed by Clarke et al. (2008). RNAi technology has been found to impart resistance to various bacterial plant diseases. For imparting bacterial and fungal resistance, various genes such as chitinase, glucanase, attacin, osmotin, cercopin, defensing, etc. are being transferred into various horticultural crops globally. The HcrVf2 gene from a wild apple conferred scab resistance to a transgenic cultivated variety of apple by Belfanti (2004). Faize et al. (2004) developed transgenic apple plants with a wheat puroindoline-b (*pin B*) gene under a CaMV35S promoter and observed that the expression of *pin-b* gene reduced scab susceptibility in transgenic apple plants. Girhepuje and Shinde (2011) developed transgenic tomato plants expressing a wheat endochitinase gene and during disease screening the transgenic plants exhibited enhanced resistance to *Fusarium oxysporum.* Rivera-Domínguez et al. (2011) carried out genetic transformation of mango (*Mangifera indica*) cv. Ataulfo embryos with the defensin J1 gene.

2.3.3 Herbicide Resistance

The herbicide glyphosate is a potent inhibitor of the enzyme 5-enolpyruvylshikimate-3-phosphate synthase (EPSP) in higher plants. Shah et al. (1986) developed a cDNA clone encoding EPSP synthase from a cDNA library of a glyphosate-tolerant *Petunia hybrida* cell line (MP4-G) that overproduces the enzyme. This cell line was shown to overproduce EPSP synthase messenger RNA as a result of a 20-fold amplification of the gene. A chimeric EPSP synthase gene was constructed with the use of the cauliflower mosaic virus 35S promoter to attain high-level expression of EPSP synthase and introduced into petunia cells. Transformed petunia cells as well as regenerated transgenic plants were tolerant to glyphosate. Transgenic pineapple plants transformed with the bar gene for bialaphos resistance were developed by Sripaoraya et al. (2006) and evaluated for tolerance to the herbicide Basta. Seven months after transfer to the field, plants were found tolerant to 1600 mL/rai of Basta X (stock concentration 15% w/v glufosinate ammonium), this being twice the dose recommended for field application of the herbicide. Transgenic plants tolerant to glufosinate ammonium should facilitate more effective weed control in pineapple plantations without damage to the crop.

2.4 NUTRITIONAL VALUE

One of the main claims of the biotechnology industry since GM crops were first commercialized in the United States in 1996 has been that a "second generation" of genetic modification will bring real consumer benefits, for example, by improving the nutritional value of foods. First-generation GM crops are aimed at farmers by inserting genes for herbicide tolerance and insect resistance, which were sold by the biotechnology companies as a way to cut inputs and labor (which is disputed). Consumers in the United Kingdom, the rest of the European Union, and around the world rejected them. Their main use is therefore in animal feed and, more recently, in biofuels, neither of which are obvious or labeled at the point of sale. The biotech industry now hopes to boost their market with second-generation nutritionally enhanced GM crops, which it is claimed will alleviate malnutrition and improve health. Many biotech proponents go even further and claim that consumers will be able to obtain foods specifically developed to prevent common diet-related diseases.

2.5 SHELF-LIFE AND QUALITATIVE TRAITS

A distinction is made in the genetic manipulation of plants between input traits and output traits. Input traits involve changing the agricultural characteristics of plants, offering the farmer technical advantages in cultivation. These include traits that affect the growth of the plant, such as herbicide or insect resistance or tolerance to drought, cold, or lack of nutrients. Output traits are the qualitative or quantitative improvement of characteristics relating to the condition of plants or the substances they contain. For example, attempts are being made to use gene technology to give plants and parts of plants a longer shelf-life once they have been harvested (Flavr Savr tomato). It was observed that excessive softening is the main factor limiting fruit shelf-life and storage. Other goals are to achieve a higher vitamin or protein content. Output traits aim to provide advantages that are of personal benefit to the end consumer, and offer improved processing quality to companies that carry out the further processing of the products.

Transgenic plants modified in the expression of cell wall-modifying enzymes have been used to investigate the role of particular activities in fruit softening during ripening. Fruit ripening has been modified by altering the activity of cell wall enzymes such as polygalacturonases that are involved in tissue softening and deterioration. The biosynthesis of ethylene (the fruit-ripening hormone) has also been blocked in several ways to delay fruit ripening. Calgene Inc., USA (1994), developed the first commercialized

transgenic plant, a long shelf-life tomato (Flavr Savr) by the suppression of the polygalacturonase (PG) gene by antisense strategy (Fig. 1.5). The Flavr Savr tomatoes have improved flavor and total soluble solids, in addition to enhanced shelf-life. However, this Flavr Savr variety was withdrawn from the market 3 years later because of its disease susceptibility and lack of productivity. Later on, other tomato varieties with increased shelf-life were developed through antisense RNA inhibition of 1-aminocyclopropane-1-carboxylate (ACC) synthase or ACC oxidase and two ethylene precursors. Delayed leaf senescence has been achieved in tobacco and petunia by manipulation of cytokinin synthesis (Clark et al., 2003). Researchers at Horticultural Research International, UK, have identified the genes that control the taste, smell, and color of strawberries. As a result, it would now be possible to create super strawberries that will taste sweeter using transgenic approaches. Nambeesan et al. (2010) expressed a yeast spermidine synthase (ySpdSyn) gene under constitutive (CaMV35S) and fruit-ripening-specific (E8) promoters in *Solanum lycopersicum* (tomato). The ySpdSyn transgenic fruits had a longer shelf-life, reduced shriveling, and delayed decay symptom development in comparison with the wild-type fruits. Crop maturity indicated by the percentage of ripening fruits on the vine was delayed in a CaMV35S-ySpdSyn genotype, with fruits accumulating higher levels of the antioxidant lycopene. Notably, whole-plant senescence in the transgenic plants was also delayed compared with wild-type plants. Zhang et al. (2011) developed transgenic tomato plants by silencing the expression of the mitochondrial APX gene by an RNAi mechanism and observed increased vitamin C content in the transgenic tomato fruits.

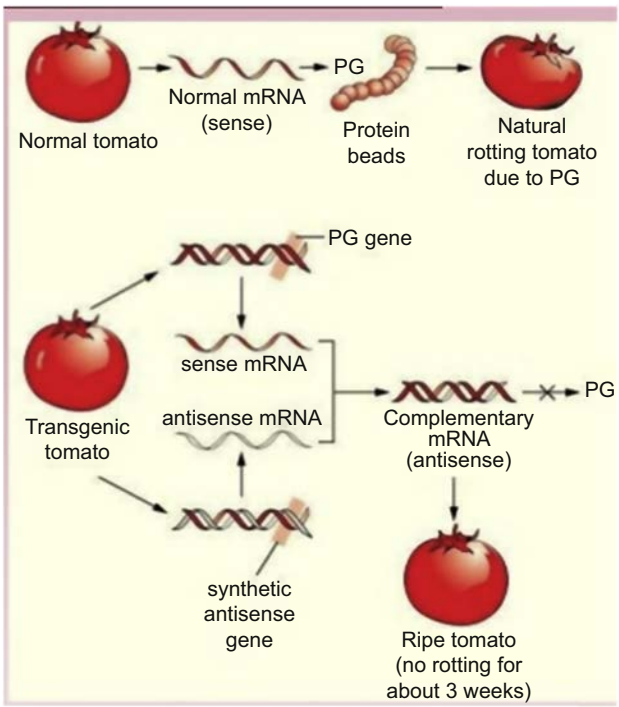

FIGURE 1.5 Making of Flavr Savr tomato.

Courtesy: http://www.google.com/Images.

3. TRANSGENIC RESEARCH IN MAJOR HORTICULTURE CROPS

3.1 FRUIT CROPS

Fruits are one of the major sources of vitamins, essential nutrients, antioxidants, and fibers in the human diet. During the last two to three decades, genetic engineering methods based on the use of transgenes have been successfully adopted to improve fruit plants and focused mainly on enhanced tolerance to biotic and abiotic stresses, increased fruit yield, improved postharvest shelf-life of fruit, reduced generation time, and production of fruit with higher nutritional value. However, the development of transgenic fruit plants and their commercialization have been delayed by many regulatory and social hurdles. Nowadays, new genetic engineering approaches, i.e., cisgenesis or intragenesis, receive increasing interest for the genetic modification of plants. The absence of selectable marker genes in the end product and the introduced gene(s) derived from the same plant or plants sexually compatible with the target crop should increase consumers' acceptance.

3.2 VEGETABLE CROPS

Several companies are working on tomatoes that can be vine ripened and shipped without bruising. Others are trying to improve tomatoes that are processed for catsup, soups, pastes, or sauces by genetically engineering them to contain more solids, be thicker, and to contain more lycopene, which provides the red color. These transgenic tomatoes will have higher viscosity (thickness and texture), higher soluble solids, better taste, improved color, and higher vitamin content. The objectives for fresh market tomatoes include enhancing overall flavor, sweetness, color, and health attributes. Genetic engineering is also being used to develop potatoes with more starch and less water to prevent damage when they are mechanically harvested. A potato with less water content may absorb less oil when it is fried, producing healthier french fries or potato chips.

3.3 FLORICULTURAL CROPS

The global flower industry thrives on novelty. Genetic engineering is providing a valuable means of expanding the floriculture gene pool, therefore promoting the generation of new commercial varieties. The commercialization of genetically engineered flowers is currently confined to novel coloring. In general, engineered traits are valuable to either the consumer or the producer. At present only consumer traits appear able to provide a return capable of supporting what is still a relatively expensive molecular breeding tool. The biosynthesis of floral pigments, particularly anthocyanins, has been elucidated in great detail in model flowers such as petunia. This knowledge is now being applied to an understanding of a wide range of other flowers and provides a means of targeting color modification in these species. The expression of genes transferred across genera is not always predictable and so requires considerable trial and error to arrive at stable phenotypes of commercial interest. The manipulation of metabolic pathways, often requiring introduction of multiple genes, can also be problematic. This is a reflection of the complexity of interactions within and between cells at a gene and gene product level. An understanding of gene function is an essential step in engineering novel traits. The production of novel flower color has been the first success story in floriculture genetic engineering. Other traits that have received attention include floral scent, floral and plant morphology, senescence of flowers both on the plant and postharvest, and disease resistance.

3.4 MEDICINAL AND AROMATIC PLANTS

Biotechnology offers promising tools for the creation of novel crop varieties with improved nutritional value, resistance to herbicides, pests, diseases, pollutants, and adverse climatic conditions. Using RDT, it is now possible to manipulate the levels and composition of active pharmaceuticals and essential oils in medicinal and aromatic plants. However, to accomplish this task, it is first crucial to understand the biochemical pathways and the pattern of expression of the genes responsible for the synthesis of specific natural products. Novel pathways can be introduced into target plants to confer them with novel traits, and, in parallel, endogenous genes can be "turned off" using the proper DNA constructs. Thus it is possible to manipulate biosynthetic pathways to cause either the accumulation of valuable metabolites or to prevent their degradation. In addition, the organ specificity or temporal restrictions for the production of natural products can be overcome using genetic engineering. The way transgenic plants are obtained and how the implementation of molecular biology methodologies has improved crops and plant products is described. Exciting possibilities to use transgenic plants for the production of industrial enzymes and other materials, and different examples illustrating methods to either boost the levels of pharmacologically active compounds or change essential oil composition in medicinal and aromatic plants, using RDT are discussed. The genetic engineering is a new emerging opportunity for the full exploitation of the biosynthetic potential of aromatic and medicinal plants.

4. BENEFITS OF COMMERCIALIZATION OF TRANSGENIC HORTICULTURAL CROPS IN DEVELOPING COUNTRIES

4.1 INCREASED PRODUCTION AND PRODUCTIVITY

In 2014, the largest review yet concluded that GM crops' effects on farming were positive. The study found that herbicide-tolerant crops have lower production costs, while for insect-resistant crops the reduced pesticide use was offset by higher seed prices, leaving overall production costs about the same. The yields increased 9% for herbicide tolerance and 25% for insect-resistant varieties. Farmers who adopted GM crops made 69% higher profits than those who did not. GM crops help farmers in developing countries, increasing yields by 14%. The researchers considered a number of studies that were not peer reviewed, and a few that did not report sample sizes. They attempted to correct for publication bias by considering sources beyond academic journals. The large data set allowed the study to control for potentially confounding variables such as fertilizer use.

4.2 ENHANCED CROP PROTECTION

The herbicide tolerance transgene confers tolerance to a specific herbicide. This trait allows farmers to apply an herbicide that acts on a wide range of weeds while not affecting the modified crop. Herbicide tolerance is currently the most commonly used GM trait worldwide. Herbicide-tolerant crops are mainly grown in developed countries with the primary aim of reducing applications of herbicides. The trait has also been achieved using other methods, particularly mutation breeding and gene transfer from wild relatives. In insect/pest resistance a transgene produces toxins to specific insects that feed on the crop. Such genes have been widely used and are already leading to substantial reductions in the use of pesticides and insecticides. Insect-resistant potato and maize varieties are being grown in both developed and developing countries. The biotech grapevine which is resistant to viral, bacterial, fungal diseases as well as abiotic

stress tolerances were developed in South Africa. The other biotech crops like banana, apple, pear, and strawberry cultivars are under the development. The results of the international research done over the past 20 years is the development of HoneySweet plum highly resistant to PPV. Examples of crops in which these traits are being introduced include coffee, bananas, cassava, potato, sweet potato, beans, papaya, squash, and melon. In some cases, the transgenes used are genes that occur naturally in the same species. Abiotic stress resistance is the ability of some plants to survive in harsh climatic or soil conditions and is sometimes associated with specific groups of genes. These genes can be isolated and introduced into crops. Such applications promise to be particularly valuable for developing countries, where abiotic stresses such as drought, heat, frost, and acidic or salty soils are common. Transgenic research on crops such as coffee, potato, brassica, and tomato varieties are currently in different stages of development.

4.3 IMPROVEMENTS IN SHELF-LIFE

Transgenic horticultural crops providing direct benefits to the consumer have also been reported. Calgene's Flavr Savr tomato silenced the gene encoding polygalacturonase, an enzyme implicated in fruit softening. The expectation was that the tomato would soften and delay ripening until a later stage of maturity. This later harvest, in principle, would permit greater development of flavor compounds and better taste. This product, first marketed in 1994, was a success with consumers but failed economically for a number of reasons. Regulatory agencies also approved several other delayed-ripening tomato varieties based on strategies targeted to block the ethylene biosynthetic pathway (ACC deaminase and antisense/cosuppressed ACC synthase) that is essential for ripening. None of these products are currently marketed, despite their technical feasibility and potential consumer benefits. Rather, they were preempted without a biotech approach utilizing the naturally occurring *rin* mutant of tomato that delays fruit ripening. Heterozygous plants produce fruits that ripen at a significantly slower rate than normal fruits.

4.4 IMPROVED NUTRITIONAL VALUE

The importance of fruits and vegetables in the human diet cannot be overemphasized. Many reviews have reported the wide range of determinants of desirable quality attributes in fruits and vegetables such as nutritional value, flavor, color, texture, processing qualities, and shelf-life. An understanding of the fundamental processes that influence fruit set, maturation, and ripening are required to manipulate fruit and vegetable yield and quality. Bapat (2010) revealed the constraints surrounding the extensive reproductive cycle in some fruits and vegetables that have long juvenile periods, a complex reproductive biology, high degree of heterozygosity, inter- and intraincompatibility, and sterility of breeding of fruit and vegetable plants such as tomatoes, oranges, etc. for improvement. Studies found that tomato plants transformed with the yeast SAMDC gene under the control of E8 promoter showed improvement in tomato lycopene content, better fruit juice quality, and improved vine life (Bapat, 2010). Ethylene response factors (ERFs) play an important role in modulating ethylene-induced ripening in fruits. These ERFs belong to a multigene family and are transcriptional regulators. These mediate ethylene-dependent gene expression by binding to the GCC motif found in the promoter region of ethylene-regulated genes. Modulation of expression of these individual ERFs in tomato has demonstrated their role in plant development and ripening. The sense and antisense LeERF1 transgenic tomatoes under the control of CaMV35 promoter were developed. Overexpression of LeERF1 in the tomatoes caused the

typical ethylene triple response on etiolated seedlings. Antisense LeERF1 fruits showed longer shelf-life compared with wild-type tomato. A new and important set of genes regulating different developmental processes involve microRNAs (miRNAs). Yin (2008) and Zhang et al. (2008) identified a set of miRNAs and their targets from tomatoes that were associated with the phase change from vegetative to generative growth. In addition, high-throughput Pyrosequencing has revealed miRNAs targeting genes that are involved in fruit ripening (Moxon et al., 2008).

4.5 QUALITY IMPROVEMENT

Dandekar et al. (2002) reported differential regulation of ethylene with respect to fruit quality components in apples. A direct correlation between ethylene and aroma production during apple ripening has been reported by Wang et al. (2007). However, not all components of fruit quality are under the direct control of ethylene. Two MdERFs (ethylene response factors) were isolated from ripening apple fruit and expressed exclusively in ripening fruit, whereas MdERF1 was expressed predominantly in ripening fruit with a small degree of expression in nonfruit tissues. The transcription of MdERFs was regulated positively by the ethylene signaling system. In a related study with two cultivars of apple, Zhu (2006) characterized the expression patterns of AAT and ACS gene family members to examine the relationship with volatile ester production during pre- and postharvest ripening. They found that differential expression of AAT genes contributed to phenotypic variation of volatile ester biosynthesis in the apple cultivars.

5. FUTURE PROSPECTIVES AND CONCLUSION

GM foods have the potential to solve many of the world's hunger and malnutrition problems and to help protect and preserve the environment by increasing yield and reducing reliance upon chemical pesticides and herbicides. Yet there are many challenges ahead for governments especially in the areas of safety testing, regulation, international policy, and food labeling. Many people feel that genetic engineering is the inevitable wave of the future and that we cannot afford to ignore a technology that has such enormous potential benefits. It has been estimated that demand placed on world agricultural production by 2050 will double assuming moderately high income growth taken together with expected population growth. However, we must proceed with caution to avoid causing accidental harm to human health and the environment as a result of our passion for this powerful technology. Genetic modification has increased production in some horticultural crops but the evidence we have suggests that the technology has so far addressed too few challenges in a few crops of relevance to production systems in many countries; even in developed countries a lack of perceived benefits for consumers and uncertainty about their safety have limited their adoption.

It was evident that developed biotechnological approaches have the potential to enhance the yield, quality, and shelf-life of fruits and vegetables to meet the demands of the 21st century. However, the developed biotech approaches for fruits and vegetables were more academic jargon than commercial reality. To make sure that the current debates and complexities surrounding the registration and the commercialization of GM fruits and vegetables are adequately addressed, various stakeholders in the industry (policymakers, private sectors, agriculturalists, biotechnologists, scientists, extension agents, farmers, and the general public) must be engaged in policy formulations, seed embodiments, and

product development. The full benefit of the knowledge can be reaped if there is total commitment by all stakeholders regarding increased and sustained funding, increased agricultural R&D, and less cost and time for registration and commercialization of new traits.

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CHAPTER

GENETIC ENGINEERING OF HORTICULTURAL CROPS: PRESENT AND FUTURE

2

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1. INTRODUCTION

Horticulture is one of the important sectors of agriculture, which consists of fruits, flowers, vegetables, spices, tuber crops, mushrooms, bamboo, plantation crops, and medicinal and aromatic plants (Sonah et al., 2011). Biotechnological tools have revolutionized conventional plant breeding methods by providing new genotypes for breeding purposes, supplying healthy and disease-free planting material, improving fruit quality, enhancing shelf-life, increasing availability of biopesticides, biofertilizers, etc. Recent advancements in molecular biology and genetic transformation encompass broad areas of biology from the utilization of living organisms or substances from those organisms to make or modify a product, to improving plants or developing microorganisms for specific uses. Integration of specially desired traits through genetic engineering has been possible in some horticultural crops (Thakur et al., 2012). The major areas of biotechnology include tissue culture, genetic engineering, molecular diagnostics, and molecular markers/breeding, as well as development of beneficial microbes that can be adopted for improvement of horticultural crops. Mutagenesis research for horticultural crops has provided considerable knowledge regarding mutagen effects, mutation induction methods, and improved traits, and their heritability as well as current scientific and technical advances can presently deliver mutation induction with new possibilities to contribute to horticultural crop improvement (Mba, 2013; Oladosu et al., 2016). Genetic engineering is a set of techniques that enable target DNA identification from different sources, its isolation and recombination, to introduce new characteristics that are not available in nature in an organism (Agarwal et al., 2016). Genetic engineering offers numerous potentially useful genetic manipulations for the improvement of horticultural crops (Fig. 2.1). Genome editing using artificial nucleases is more precise than conventional crop breeding methods or standard genetic engineering (transgenic or genetically modified) methods (Khatodia et al., 2016). By editing only a few of the billions of nucleotides at a predefined location, researchers can generate targeted mutations in DNA at the interface or insert new sections in the same way as they are made in traditional breeding and crossing experiments (Kim and Kim, 2014). This chapter highlights the present scenario of genetic engineering of horticultural crops.

Genetic Engineering of Horticultural Crops. http://dx.doi.org/10.1016/B978-0-12-810439-2.00002-7

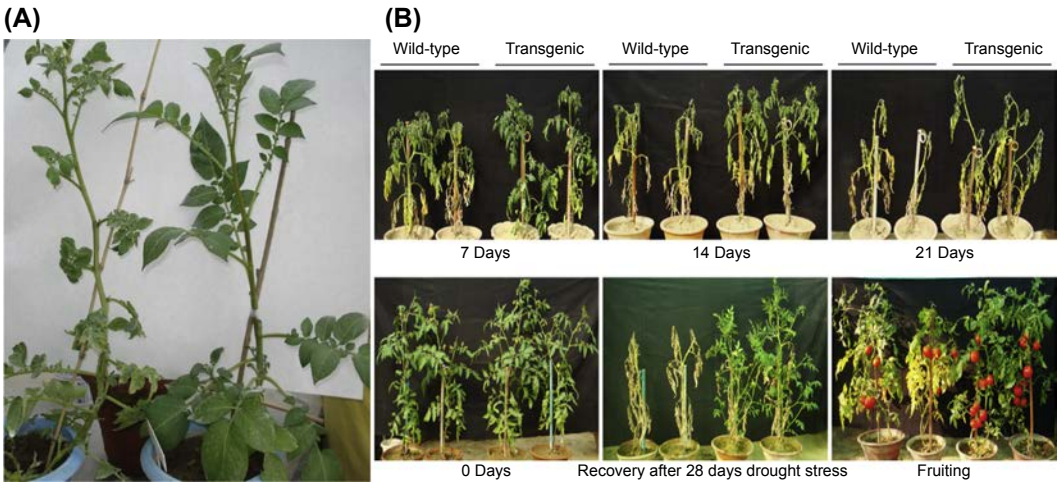

FIGURE 2.1

Plant showing different transgenic activity. (A) Potato plants showing RNAi transgenic Kufri Pukhraj with resistance to potato apical leaf curl disease; (B) Tomato plants showing higher drought tolerance.

Courtesy: Dr. S.K. Chakraborthy

2. TRADITIONAL BREEDING TECHNOLOGY

Much before the application of genetic engineering in horticulture crops, plant breeders used classical breeding technology for crop improvement. Initially, through the selection and reproduction of beneficial and observable phenotypes, farmers and plant breeders modified the underlying genotype of plants to adapt them for satisfying the needs of the human diet, medicinal requirements, and aesthetic purposes. To obtain higher yields and better quality varieties, many approaches have been used in crop breeding, such as various forms of hybridization and mutation breeding, and developing transgenics by genetic engineering (Xiong et al., 2015).

Conventional plant breeding develops new plant varieties by the process of selection, and targets improvement of the already present genetic potential of a plant species. It employs processes that occur in nature, such as sexual and asexual reproduction. The product of conventional breeding emphasizes certain characteristics that preexist in the gene pool of the species without introducing new genes. Plant breeders target accumulation of favorable alleles for a significant role in stress tolerance/resistance, nutritional quality, or other agronomic and horticultural traits in a plant genome. Genes that contribute to stress tolerance/resistance or other traits can be obtained from local germplasm resources, or through introduced landraces, or breeding lines from other breeding programs, wild species, or genera (Varshney et al., 2011). Plant tissue culture techniques proffer a substitute method of vegetative propagation of horticultural crops (Alizadeh et al., 2010). Micropropagation or clonal propagation through tissue culture was one of the first and widest applications of biotechnology because it was economical in time and space and provided disease-free and elite propagules. Production of virus-free planting material using meristem culture has been made possible in many horticultural crops (Venkat Raman et al., 2015; Krishna et al., 2016). The uniformity of individual plants within a clone population is a major advantage of clonal cultivars in commercial production (Krishna and Singh, 2013). The applications of plant

tissue culture go well beyond the bounds of agriculture and horticulture. It has found applications in environmental remediation and industrial processing. With rapid population growth, the total acreage of fruits, vegetables, and various ornamental plants could not have met the demands of the people in developing countries (Akin-Idowu et al., 2009). However, plant tissue culture may lead to genetic variations in undifferentiated cells, isolated protoplast, calli, tissues, and morphological traits of in vitro-raised plants as well as generate variability or somoclonal variations because of gene mutations or changes in epigenetic marks (Bairu et al., 2011; Currais et al., 2013).

3. MOLECULAR BREEDING TECHNOLOGY

The development of molecular markers, derived from research in molecular genetics and genomics, offers great promise for plant breeding. The use of DNA markers in plant breeding is called marker-assisted selection (MAS) and is a component of the new discipline of "molecular breeding" (Collard and Mackill, 2008). It can be used in plant breeding for the precision transfer of genomic regions of interest (foreground selection) and to accelerate the recovery of the recurrent parent genome (background selection) (Ibitoye and Akin-Idowu, 2011). A molecular marker provides a systematic basis to traditional breeding by enhancing its precision and genotype-based selection. The discovery and application of molecular markers for valuable traits reduces the requirement for complex phenotypic analysis and the time required for the development of new commercial varieties. DNA markers that are tightly linked to agronomically important genes (called gene tagging) may be used as molecular tools for MAS in plant breeding (Collard and Mackill, 2008). DNA markers are especially useful to determine the genetic makeup of plants, screening of parental germplasm for genetic variation, development of genetic linkage maps, and tagging of genes controlling the important traits as well as for selection of quantitative traits that prove difficult to select because of phenotypic assessment alone (Ibitoye and Akin-Idowu, 2011). In addition, a better understanding of the genetic and genomic control of horticultural traits achieved through molecular markers can help to design more efficient breeding strategies and map-based isolation of genes. Concomitantly, markers can provide clones of specific genes for genetic engineering of horticultural crop species (Collard et al., 2005; Ibitoye and Akin-Idowu, 2011). Markers closely linked to major genes responsible for the expression of important traits (disease/pest resistance, fruit/nut quality, self-incompatibility, etc.) have been developed in many horticultural crops and are being used for MAS (Dirlewanger et al., 2004; Zhu and Barritt, 2008; Bliss, 2010). The most widely used markers in major cereals are called simple sequence repeats (SSRs) or microsatellites (Gupta and Varshney, 2000). Expressed sequence tags have been widely used for the generation of SSRs and single nucleotide polymorphism (SNP) markers in several horticultural crops (Frary et al., 2005; Ekué et al., 2009). SNPs have been identified in many species (Gilchrist et al., 2006; Hyten et al., 2008) including grapes (Velasco et al., 2007) and apples (Chagné et al., 2008) and used successfully for the construction of genetic linkage maps (Salmaso et al., 2008; Vezzulli et al., 2008). During the last decade, linkage maps have been prepared for several horticultural crops by using various types of mapping populations and molecular markers (Sonah et al., 2011). Many quantitative trait loci (QTL) have been identified in fruit and vegetable crops for several important traits, for instance, fruit shape, fruit weight, and fruit sugar content in tomato (Frary et al., 2000; Liu et al., 2002); resistance to Ro1 *Globodera rostochiensis*, cold-sweetening, and flavonoid 3,5-hydroxylase gene in potato (Jung et al., 2005; Li, 2005; Paal et al., 2004); the scab resistance gene

in apple (Xu and Korban, 2003); nematode resistance in sugar beet (Cai, 1997); the virus resistance e1F4E gene in pepper (Ruffel et al., 2002); the lycopene β cyclase gene in papaya (Blas et al., 2010); the orange (Or) gene in cauliflower (Lu et al., 2006). Thus this approach can also be used for other important horticultural traits in the future. The cloned QTL can be transferred to cultivated varieties using MAS (Sonah et al., 2011).

4. TRANSGENIC TECHNOLOGY

Transgenic crops, commonly referred to as genetically modified, contain a foreign gene or genes for desired traits, which have been artificially inserted into its genome. This enables breeders to bring favorable genes, creating novel phenotypes that are often previously not available, into already elite cultivars, improving their value considerably and offering unique opportunities for controlling insects and pathogens (Silva Dias and Ortiz, 2014; Xiong et al., 2015). The inserted gene sequences are known as the transgene, and the method involved in gene transfer is called genetic transformation. At present, there are two general approaches for plant transformation: (1) vectorless or direct genetic transformation such as the particle bombardment method that delivers the DNA into cells using microscopic gold or tungsten particles coated with DNA; and the protoplast transformation method that involves DNA introduction into protoplasts using polyethylene glycol-mediated DNA uptake and electroporation, and liposome (containing plasmid DNA) fusion (Agarwal et al., 2016; Venkat Raman et al., 2015); (2) genetic transformation through *Agrobacterium tumefaciens*, which naturally transfers part of the tumor-inducing plasmid that is transfer DNA into plants (Finer, 2010). The major achievements of transgenic plant technology up to now concern biotic and abiotic stress resistance, herbicide tolerance, and improved product quality (Tarafdar et al., 2014).

4.1 TRANSGENIC RESEARCH IN HORTICULTURAL CROPS

The first commercially grown genetically modified crop was Flavr Savr tomato, which was released by Calgene in 1994 for its delayed ripening trait so that the fruits stayed firm for longer after harvest. This tomato contains an antisense version of a glycoside hydrolase gene that encodes the polygalacturonase, which can dissolve pectin present in the plant cell wall, resulting in slower softening and decay. Furthermore, the transgenic approach has been applied to tomato crops to manipulate fruit ripening, texture, and nutritional quality (Silva Dias and Ortiz, 2014) targeting various genes such as S-adenosylmethionine synthase, 1-aminocyclopropane-1-carboxylate (ACC) synthase, ACC oxidase (Oeller et al., 1991; Bolitho et al., 1997), pectin methylesterase (Thakur et al., 1996), deoxyhypusine synthase (Wang, 2005), lycopene β-cyclase (Rosati et al., 2000), NADP-dependent glutamate dehydrogenase (Kisaka and Kida, 2003), and amino acid decarboxylases (Tieman, 2006). Transgenic broccoli plants with reduced ethylene synthesis were also produced by silencing ACC oxidase and ACC synthase genes of the ethylene biosynthesis pathway (Higgins et al., 2006). By using transgenic methods, it is possible to alter plant genomes to improve the nutritional value of crops such as starch composition in potato (Takaha et al., 1998; Lorberth et al., 1998). Transgenic tomato plants produced to synthesize novel flavonoids and for folate biofortification were also reported (Schijlen et al., 2006; Diaz de la Garza et al., 2007).

Similarly, the improved nutritive value in potato might be achieved by reducing the polyphenol oxidase activity (associated with reduced wound-inducible browning), expressing a nonallergenic seed albumin gene (obtained from *Amaranthus hypochondriacus*), enhancing the level of carotenoid and lutein (through the phytoene synthase gene of *Erwinia uredovora*), decreasing the amount of reducing sugars (through expression of a bacterial-derived transgene coding for phosphofructokinase), and expressing the seed protein AmA1 (amaranth albumin 1) (Arican and Gozukirmizi, 2003; Chakraborty et al., 2000; Chakraborty et al., 2010; Ducreux, 2004; Navrátil et al., 2007).

Transgenic technology appears to be favorable for improving the sensory traits and shelf-life of melon fruit (Li et al., 2006), and Lu et al. (2006) developed transgenic cauliflower with β-carotene accumulation and Wahlroos et al. (2005) produced oilseed *Brassica rapa* with increased histidine content. Introducing plant genes to enhance innate plant defense mechanisms, e.g., activating phytoalexins, proteinase inhibitors, or toxic proteins, and invoking the hypersensitive reaction effectively reduces the fungal infection in several horticultural crops. Attempts have been made to engineer tomato, eggplant, potato, broccoli, and cabbage for fungal resistance, for instance, overexpression of pathogenesis-related genes, such as glucanase, chitinase, and thaumatin (singly and in combination), and a yeast desaturase gene in eggplant exhibited increased resistance to Verticillium wilt (Xing and Chin, 2000; Rajam and Kumar, 2007). Likewise, resistance in the transgenic tomato to blight (*Phyophthora infestans*) (Thomzik et al., 1997) and resistance against phytopathogenic fungus *Sclerotinia sclerotiorum* (*Sclerotinia* stem rot or white mold) in transgenic tobacco and tomato plants (Kesarwani et al., 2000) as well as transgenic potato plants expressing soybean β-1,3-endoglucanase gene exhibiting enhanced plant resistance to late blight (Borkowska et al., 1998) were also developed using transgenic approaches.

Virus-resistant plants are made by two approaches: (1) transferring the viral coat protein gene, or (2) genes of other proteins, which prevent the replication and movement of virus, for example, designing of transgenic papaya resistant against *Papaya ringspot virus* by using replicase gene (Chen et al., 2001; Verma et al., 2016) or by the RNA silencing method. Transgenic tobacco plants resistant against *Tobacco etch virus* (Lindbo et al., 1993) and *Potato virus Y* (Ghosh et al., 2002) have been developed. To date, several horticultural crops such as sweet potato resistant to *Sweet potato feathery mottle virus* and *Sweet potato chlorotic stunt virus*, cassava resistant to geminiviruses, potato resistant to *Potato virus Y*, tomato resistance to potyviruses and geminiviruses, lettuce resistant to *Lettuce tospovirus*, Chinese cabbage resistant to *Turnip mosaic virus*, and water melon resistant to *Zucchini yellow mosaic virus* have been generated using transgenic technology (Chellappan et al., 2004; Lin et al., 2004; Okada et al., 2001; Ghosh et al., 2006; Pang et al., 1996; Wu et al., 2009; Zhandong et al., 2007).

Transgenic approaches using antibacterial proteins such as lytic peptides, lysozymes, and iron sequestering glycoproteins have been applied to generate transgenic horticultural crops resistant to bacterial infections (Tarafdar et al., 2014). Use of antimicrobial peptides constitutively expressed in plant tissues has been recommended for the genetic engineering of plants for resistance against fungal and bacterial pathogens. Genetically transformed tomato with the *Arabidopsis* NPR1 gene has significant levels of enhanced resistance to bacterial wilt and moderate resistance to bacterial spot (Lin et al., 2014). Similarly, citrus plants transformed with *hrpN* gene, provoking the hypersensitive response and systemic acquired resistance (SAR) in plants (Barbosa-Mendes et al., 2009); *attacin A* gene, encoding attacin A which is an antimicrobial peptide (Cardoso et al., 2010); *AtNPR1* gene, which is key positive

regulator of SAR (Zhang and Voytas, 2011); *pthA-nls* gene, encoding three nuclear localizing signals (Yang et al., 2011); *Shiva A* and *Cecropin B* genes, encoding a bivalent antibacterial peptide (He et al., 2011) have showed significant resistance against *Xanthomonas axonopodis* pv. *citri/citrus* causing canker disease.

4.2 TRANSGENIC HORTICULTURAL CROPS FOR INSECT RESISTANCE

Insects can cause damage both in field conditions and during storage of plant products. New varieties of several horticultural crops have been developed utilizing a gene from the bacterium *Bacillus thuringiensis* (Bt) to produce a crystalline protein called *Bt* toxin, which is potentially toxic to a variety of herbivorous insects (Tsaftaris et al., 2000). Various cry genes [*cry1A*, *cry1Ab*, *cry1Ac*, *cry1A(b)*, *cry1Ab3*, *cry1Ba1*, *cry1C*, *cry1Ba1*, *cry1Ia3*, and *cry9Aa*] from Bt have been introduced into eggplant (Goggin et al., 2006), cabbage (Jin et al., 2000; Bhattacharya et al., 2002; Christey et al., 2006), cauliflower (Kuvshinov et al., 2001; Chakrabarty et al., 2002; Christey et al., 2006), garlic (Zheng et al., 2004), broccoli (Cao et al., 2001; Christey et al., 2006), tomato (Mandaokar et al., 2000), Chinese cabbage (Cho et al., 2001), choy-sum (Xiang et al., 2000), and potato (Cooper et al., 2009) for the development of transgenic plant resistance against numerous insects such as *Leptinotarsa decemlineata*, *Helicoverpa armigera*, *Macrosiphum euphorbiae*, *Bemisia tabaci*, etc. In addition to cry genes, cowpea trypsin inhibitor confers insect resistance in cauliflower (Iingling et al., 2005) and Chinese cabbage (Zhao et al., 2006).

4.3 TRANSGENIC HORTICULTURAL CROPS FOR ABIOTIC STRESS

Abiotic stress is the stress caused by environmental conditions such as heat, drought, salinity, cold, nutrient deficiency, and metal toxicity, which reduces growth and yield below the optimum levels. Most of the abiotic stresses are interconnected and lead to increased antioxidant capacity of the tissues or accumulation of compatible solutes through control of the genes involved in these mechanisms (Rai and Shekhawat, 2014). The most consistently successful approaches are the transfer of genes involved in signaling and regulatory pathways and introduction of genes encoding enzymes that catalyze the conversion of a naturally occurring substrate into a product with osmoprotective properties (Wang et al., 2003; Bhatnagar-Mathur et al., 2008). Genetic engineering for improved abiotic stress tolerance by expressing transcription factors has been achieved in a number of horticultural crops. For instance, overexpression of the MusaWRKY71 gene from *Musa* spp. cv. Karibale Monthan (ABB group), encoding a WRKY transcription factor protein, provides multiple abiotic stress tolerance in banana (Shekhawat et al., 2011). Similarly, overexpression of (DREB)1b, a cold-inducible transcription factor from *Arabidopsis thaliana*, was able to improve tolerance to cold stress in grapevine (Jin et al., 2009). Pasquali et al. (2008) demonstrated an improved tolerance to cold and drought stress in transgenic apple through overexpression of the cold-inducible Osmyb4 gene from rice, encoding a transcription factor belonging to the Myb family. Transformed tomato plants with the AVP1 gene, a vacuolar H+ pyrophosphatase, from *A. thaliana* showed enhanced performance under soil water deficit because of a strong and large root system allowing better use of limited available water (Park et al., 2005). Tolerance against osmotic stress induced by salt, drought, and chilling (stress) was achieved in lettuce by transforming the lettuce cv. "Grand Rapids" with a mutated P5CS gene for delta-1-pyrroline-5-carboxylate synthase, which catalyzes two steps of proline biosynthesis in plants (Pileggi et al., 2001). The

transformation of the bacterial mannitol-1-phosphodehydrogenase (mtlD) gene, which is involved in mannitol synthesis, in eggplants expressed stress against drought, salinity, and cold (Prabhavathi et al., 2002). Bhattacharya et al. (2004) induced salt tolerance in cabbage through the introduction of the bacterial glycine betaine biosynthesis (BetA) gene, which is involved in biosynthesis of glycine betaine.

5. BIOFARMING

Biofarming refers to the production of proteins and biomolecules, such as antigens, antibodies, and enzymes, that are of immense importance in therapeutics and pharmaceutical and industrial applications in transgenic plants at the agricultural scale (Dalal et al., 2006). Among horticultural crops, potato and tomato are the most commonly used host systems for biofarming. Plant delivery of vaccines has attracted much attention because this strategy offers several advantages over vaccine delivery by injection (Pascual, 2007; Yadav et al., 2013). Vaccines produced in edible parts of plants, e.g., grain, tuber, or fruit, are known as edible vaccines. Several attempts were made to express antigens related to various diseases in edible parts of plants, such as overexpression of the hepatitis E virus (HEV) open reading frame 2 partial gene in tomato plants, to investigate its expression in transformants and the immunoactivity of expressed products, and to explore the feasibility of developing a new type of plant-derived HEV oral vaccine (Ma et al., 2003). Similarly, hepatitis B surface antigen (HBsAg) (Richter et al., 2000) in potato, cholera toxin B subunit with an endoplasmic reticulum retention signal in tomato (Jani et al., 2002), loop-forming B-cell epitope (H386-400) of the measles virus hemagglutinin protein in carrot (Bouche et al., 2003), rabies virus epitopes fused with tobacco mosaic virus in spinach (Modelska et al., 1998), a gene for VP1 protein, and a coat protein of enterovirus 71 (EV71) in tomato were successfully expressed (Chen et al., 2006). Potato and tomato have also been transformed for bioproduction of therapeutically valuable proteins and antibodies, for instance, expression of recombinant isoform of human acetylcholinesterase in transgenic tomato (Mor et al., 2001), synthesis of human alpha-interferon protein in transgenic potato plants (Sawahel, 2002), and production of antibodies in transgenic tobacco, soybean, and potato (Artsaenko et al., 1998; Ko et al., 2003; Yadav et al., 2013). Production of industrial enzymes, chemicals, and raw materials can be carried out in plants either by introducing a foreign protein gene or by modifying the plants' metabolic pathways to generate sufficient end product, by-product, or a novel biomolecule. A cellulose gene endoglucanase (E1) from *Acidothermus cellulolyticus* was successfully transformed in transgenic potatoes, resulting in E1 protein accumulation that was up to 2.6% of total leaf soluble protein (Dai et al., 2000). Human milk proteins such as lactoferrin and casein have also been expressed in transgenic potatoes (Chong et al., 1997; Chong and Langridge, 2000). Similarly, overexpressing of the *Nephila clavipes* dragline protein gene in tobacco and potato has been generated to obtain large quantities of spider dragline silk (Scheller et al., 2001). In another study, overexpression of adenylate kinase in potato increases the adenylate content (Regierer et al., 2002) and Monellin has been expressed in lettuce and tomato transgenic plants (Peñarrubia et al., 1992). In addition, potatoes have been successfully engineered by introducing either the microbial fructosyltransferase genes from *Bacillus subtilis* (sacB) or *Streptococcus mutans* (ftf) or plant gene 1-SST (sucrose:sucrose 1-fructosyltransferase) and 1-FFT (fructan:fructan 1-fructosyltransferase) from globe artichoke (*Cynara scolymus*) for the production of the novel carbohydrate fructans (Van der Meer et al., 1994; Hellwege et al., 2000; Sharma and Khurana, 2016).

6. GENOME EDITING TECHNOLOGY

Targeted genome editing using engineered nucleases has the potential to accelerate basic research as well as plant breeding by providing molecular scissors or artificially engineered nucleases to target and digest DNA in a precise and predictable manner (Weeks et al., 2016). Genome editing with site-specific nucleases allows reverse genetics genome engineering and targeted transgene integration experiments to be carried out at specific locations in the genome of both model and crop plants, as well as in a variety of other organisms. The four steps necessary for modifying a plant gene through genome engineering include: (1) designing and developing an engineered nuclease construct, (2) delivering the construct and perhaps donor molecule into the plant (typically by genetic transformation), (3) inducing nuclease expression, and (4) screening the plants for the desired DNA sequence change (Curtin et al., 2012). A key step in genome editing is the introduction of targeted DNA double-strand breaks (DSBs) using an engineered nuclease, stimulating cellular DNA repair mechanisms (Kim and Kim, 2014). Different genome modifications can be achieved by custom-designed endonucleases, which enable site-directed mutagenesis via a nonhomologous end-joining (NHEJ) repair pathway and/or gene targeting via homologous recombination (HR) to occur efficiently at precise sites in the genome. Sequence modifications then occur at the cleaved sites if the donor DNA is not present, which can include deletions or insertions (indels), that result in frameshift mutations if they occur in the coding region of a gene, effectively creating a gene knockout in most cases of NHEJ. Alternatively, when the DSB generates overhangs, NHEJ can mediate the targeted introduction of a double-stranded DNA template with compatible overhangs (Cristea et al., 2013; Maresca et al., 2013). If the donor sequences are available, resolution of DSB occurs by HR, resulting in knock-in and could lead to point mutations and gene replacement (Fig. 2.2) (Bibikova et al., 2002; Bortesi and Fischer, 2015; Khatodia et al., 2016). Thus the repair of these DSBs by endogenous systems results in targeted genome modifications. Here, we review recent advances in site-specific nuclease technologies and discuss applications of these reagents for targeted genome engineering and analysis in horticultural crops. Precise genetic engineering can be achieved in higher organisms through genome editing with nucleases such as zinc-finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), and the clustered regularly interspaced short palindromic repeat (CRISPR)/Cas system. A brief comparison of genome editing technologies is described in Table 2.1.

6.1 ZINC-FINGER NUCLEASES

ZFNs are fusions of the nonspecific nuclease domain from the FokI restriction endonuclease with zinc-finger proteins. ZFNs bind to a target sequence, thereby dimerizing FokI nuclease, which induces targeted DNA DSBs that stimulate DNA damage-response pathways. The binding specificity of the designed zinc-finger domain directs the ZFNs to a specific genomic site (Kim and Kim, 2014; Gaj et al., 2013; Miller et al., 2007). Successful uses of ZFNs for gene editing in plants were initially reported in *A. thaliana* and tobacco (*Nicotiana tobacum*) in 2005 (Lloyd et al., 2005; Wright et al., 2005). ZFN-mediated targeted mutagenesis in plants involves sequence modification of preintegrated reporter constructs such as enzyme β-glucuronidase (GUS) gene mutagenesis in *Arabidopsis* (Tovkach et al., 2009). Cai et al. (2009) used an engineered tobacco cell culture system with a preintegrated reporter construct to show that the ZFNs can facilitate site-specific cleavage and transgene integration following cotransformation with an appropriately designed donor DNA (Cai et al., 2009). Evidence was reported for the role of ZFNs in heritable mutations at targeted endogenous loci of plants, e.g., a

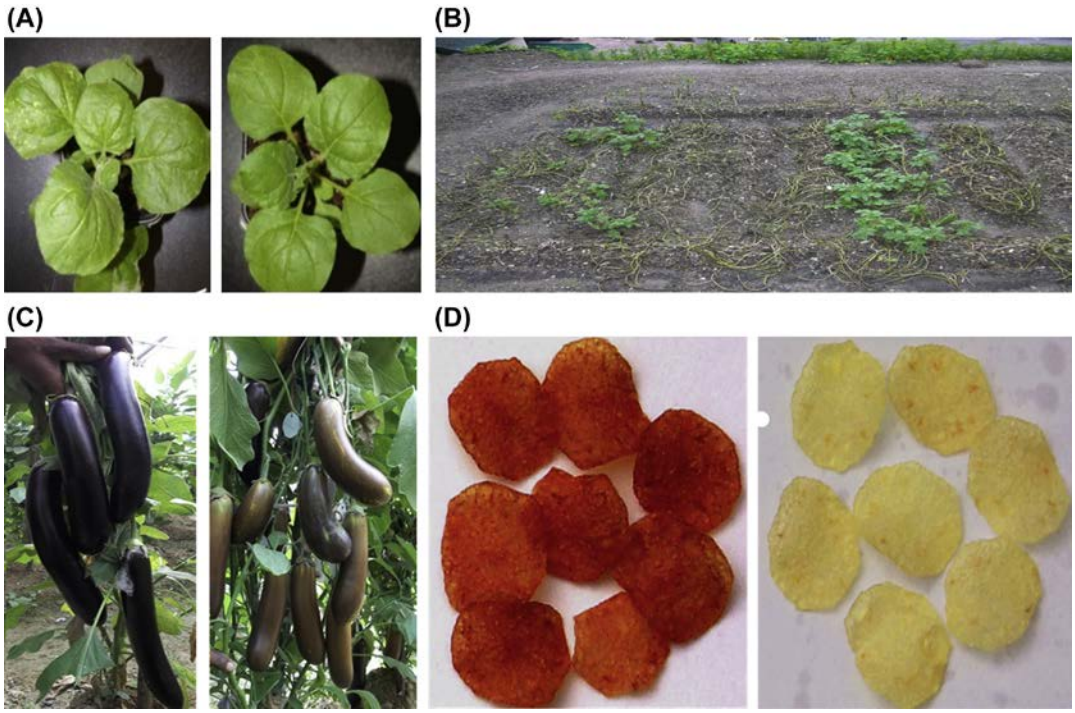

FIGURE 2.2

Plants showing different transgenic activity. (A) Replicase-mediated transgenic *Nicotiana benthamiana* showing resistance against *Papaya ringspot virus*; (B) Potato field response of RB-transgenic Katahdin (green) and nontransgenic control; (C) fruits of different Cry1Ac transgenic brinjal; and (D) RNAi-mediated silencing of invertase in potato tubers under cold stress.

Courtesy: Dr. Major Singh

gene encoding ZFNs targeting two paralogous DICER-LIKE genes (DCL4a and DCL4b) was transformed in soybean using *Agrobacterium* (Curtin et al., 2011) and in a similar study the genes encoding ZFNs designed to cleave within the ABA-INSENSITIVE-4 (ABI4) gene were stably integrated into *Arabidopsis* (Osakabe et al., 2010). In addition to small, NHEJ-induced mutations, ZFNs can drive the excision of larger targeted genome sequences from an integrated target construct concomitant with ZFN-mediated insertion of a heterologous DNA stretch specified by the donor DNA such as the deletion of the intervening 2.8-kb sequence of the green fluorescent protein (GFP) gene in tobacco (Cai et al., 2009; Petolino et al., 2010). Targeted cleavage at the IPK1 locus and precise "trapping" of the IPK1 promoter using ZFNs resulted in site-specific integration and herbicide resistance in *Zea mays* (Shukla et al., 2009). Until now, there have been no reports of ZFN applications in horticultural crops.

6.2 TRANSCRIPTION ACTIVATOR-LIKE EFFECTOR NUCLEASES

TALENs are fusions of the FokI catalytic domain and a central DNA-binding domain derived from transcription activator-like effector (TALE) proteins. TALEs contain multiple 33–35 amino acid repeat domains each of which recognizes a single nucleotide at the target site. Recognition sites are located on

Table 2.1 Key Differences Between Transcription Activator-Like Effector Nucleases (TALENs), Zinc-Finger Nucleases (ZFNs), and Clustered Regulatory Interspaced Short Palindromic Repeats/Cas (CRISPR/Cas) Systems

	TALENs	ZFN	CRISPR/Cas
Target DNA recognition	Protein: DNA	Protein: DNA	(gRNA-Cas9): DNA
Nuclease	FokI	FokI	Cas9
Construct	Proteins containing DNA-binding domains that recognize specific DNA sequences down to the base pair	Zinc-finger DNA binding motifs in a ββα configuration, the α-helix recognizes 3 bp segments in DNA	20 nt crRNA fused to atracrRNA and Cas9 endonuclease that recognize specific sequences to the base pair
Key components	TALE–Fok I fusion protein	ZF–Fok I fusion protein	gRNA and Cas9 protein
Function mode	TALE proteins recognize target DNA sequences R dimerization of FokI nucleases induce DSBs of DNA R B	ZF proteins recognize target DNA sequences R dimerization of FokI nucleases induce DSBs of DNA R DSBs are repaired by NHEJ or HDR	gRNA recognizes target DNA sequence next to an NGG motif R Cas9 induces DSBs of DNA R DSBs are repaired by NHEJ or HDR
Success rate	High (>99%)	Low (~24%)	High (~90%)
Average mutation rate	High (~20%)	Low or variable (~10%)	High (~20%)
Specificity-determining length of target site	30–40 bp	18–36 bp	22 bp (total length 23 bp)
Off-target effects	Low	High	Variable
Design feasibility	Difficult: • Need a customized protein for each gene sequence • Low delivery efficiency • Large-scale screening, time-consuming, and expensive to be constructed		Easy: • All-in-one gRNA-Cas9 vector system • Multigene editing is feasible • Protospacer adjacent motif next to target sequence required
References	Moscou and Bogdanove (2009), Boch et al. (2009), Gaj et al. (2013), Kim and Kim, 2014, and Xiong et al. (2015)	Perez-Pinera et al. (2012), Gaj et al. (2013), Kim and Kim (2014), and Xiong et al. (2015)	Mali et al. (2013), Cong et al. (2013), Jiang et al. (2013), Kim and Kim (2014), and Xiong et al. (2015)

crRNA, *CRISPR RNA;* DSBs, *double-strand breaks;* gRNA, *guide RNA;* HDR, *homology directed repair;* NHEJ, *nonhomologous end-joining;* tacrRNA, trans-*activating CRISPR RNA.*

the opposite DNA strands at a distance sufficient for dimerization of the FokI catalytic domains. Dimerized FokI introduces a double-strand break into DNA. Like ZFNs, TALENs induce targeted DSBs that activate DNA damage response pathways and enable custom alterations (Kim and Kim, 2014; Gaj et al., 2013). TALENs have been successfully used to target mutagenesis in horticultural

Table 2.2 List of Applications of Genome Editing Technologies in Horticultural Crops

Plant Species	Method	Target Genes	References
–	ZFNs	–	–
Tomato	TALENs	*PROCERA* (*PRO*), *VInv gene*	Lor et al. (2014) and Clasen et al. (2016)
Potato		*St SSR2*	Sawai et al. (2014)
Tomato	CRISPR/Cas system	*Solyc07g021170*, *Solyc12g044760*, *SlAGO7*, *mGFP5*, *eGFP*, *RIN*, *ANT1*	Brooks et al. (2014), Ron et al. (2014), Cermak et al. (2015), and Ito et al. (2015)
Potato		*StIAA2*, *StALS1*	Butler et al. (2015) and Wang et al. (2015)
Sweet orange		*CsPDS gene*	Jia and Wang (2014)

CRISPR/Cas, *Clustered regulatory interspaced short palindromic repeats/Cas;* TALENs, *transcription activator-like effector nucleases;* ZFNs, *zinc-finger nucleases.*

crops, for example, potato (*Solanum tuberosum*) and tomato (*Solanum lycopersicum*) (Clasen et al., 2016; Lor et al., 2014; Sawai et al., 2014).

A study has identified the St SSR2-knockout potato with a lower level of somatic genome alterations (SGAs) by targeted genome editing through TALENs (Table 2.2). The SSR2 is an enzyme encoding oxidases or glycosyl transferases and is responsible for the initial reaction from C-24 alkyl sterol biosynthesis to cholesterol biosynthesis on the pathway to the production of toxic SGAs in Solanaceous plants (Sawai et al., 2014). Similarly, TALEN pairs pTAL423/4 and pTAL425/6 were designed to target the negative regulator of the gibberellin (GA) signaling PROCERA (PRO) gene for targeted mutagenesis in tomato, which resulted in increased GA response, with mutants showing tall, slender, and light green vegetation (Lor et al., 2014).

6.3 CRISPR/CAS (CRISPR-ASSOCIATED) SYSTEMS

Clustered regulatory interspaced short palindromic repeats or CRISPR are loci that contain multiple short direct repeats, and provide acquired immunity against invading foreign DNA through RNA-guided DNA cleavage to bacteria and archaea. CRISPR systems rely on CRISPR RNA (crRNA) and *trans*-activating crRNAs (tracrRNA) for sequence-specific silencing and cleavage of pathogenic foreign DNA by Cas proteins. Three types of CRISPR/Cas systems exist: in the type II system, Cas9 serves as an RNA-guided DNA endonuclease that cleaves DNA upon crRNA–tracrRNA target recognition (Wiedenheft et al., 2012; Gaj et al., 2013). Two components are needed for CRISPR genome editing: the Cas9 endonuclease and a synthetic RNA chimera [single guide RNA (sgRNA); Jinek et al., 2012]. The sgRNA contains a guide sequence of 19–22 bp (also known as the protospacer), which matches with the target DNA sequence to be mutated (Khatodia et al., 2016). Work has shown that target recognition by the Cas9 requires the presence of the protospacer-associated motif (PAM) NGG trinucleotide sequence upstream of the crRNA-binding region. Thus the target region follows the consensus (N)19-22NGG (Ron et al., 2014). RNA-guided genome editing using the bacterial type II CRISPR/Cas9 system has emerged as an efficient tool for genome editing but reports of the usage and efficiency of CRISPR/Cas9 system-mediated plant genome engineering are still limited.

Applications of the CRISPR/Cas9 system in plants were successfully used to modify traits such as amendment of the plant color to white by modifying the OsSPD gene in rice (Shan et al., 2013) and modification of the tiller angle by editing the LAZY1 gene in rice (Miao et al., 2013). The successful transient of the CRISPR/Cas9 system in horticultural crops was first reported in tomato by Brooks et al. (2014). They designed a CRISPR/Cas9 construct to target the tomato homolog of the *Arabidopsis* ARGONAUTE7 (SlAGO7) sequence that contained two sgRNAs with requisite binding region, or PAM, for Cas9 cleavage, which is 59-NGG immediately after the 20-bp target DNA (Brooks et al., 2014). In addition, Ron et al., (2014) reported the transient expression of CRISPR/Cas9 in tomato roots and exhibited that hairy root transformation could be used as a means to interrogate gene function using CRISPR-mediated gene mutation. Similarly, Wang et al. (2015) generated CRISPR/Cas9 gene knockouts in double-haploid dry matter potato by targeting the second exon of the StIAA2 gene, which encodes an AUX/IAA protein (Table 2.2). Likewise, CRISPR/Cas9 systems were employed in sweet orange (*Citrus sinensis*) by targeting the CsPDS gene (Jia and Wang, 2014). These results demonstrated that the CRISPR/Cas9 system is a highly efficient tool for targeted mutations in transgenic potato and tomato plants. There has been a continuous increase in the number of publications related to the use of the CRISPR/Cas9 technique in animal and plant systems (Nemudryi et al., 2014; Khatodia and Khurana, 2014; Khatodia et al., 2016), identifying that the CRISPR/Cas9 system is an effective tool to promote functional studies on uncharacterized genes in horticultural crops.

7. FUTURE CHALLENGES

Horticulture, the science and practice of growing, processing, and marketing fruits, flowers, vegetables, spices, plantation crops, and medicinal and aromatic plants, is widely acknowledged in many aspects of innovation, production, and quality maintenance for uplifting the economic condition of farmers and entrepreneurs, and providing nutritional security to the public. Therefore developing fruits and vegetables with superior traits is a key issue for the plant scientist to increase crop quality to overcome the hidden hunger or micronutrient deficiency. However, current advances in the genetic engineering of horticultural crops have enabled the production of plants with superior characteristics such as improved quality and yield, biotic and abiotic stress tolerance, accumulation of a wide range of vitamins, amino acids, pharmaceutical products, vaccines, antibodies, etc. It is likely that in the near future, transgenic technology can overcome the incompatibility barriers between species for integrating foreign genes into target plant genomes, or even introducing synthetic artificial genes to generate new varieties with desired traits (Silva Dias and Ortiz, 2014).

Against the background of a dwindling natural resource base and growing demand for agricultural products, genetically engineered crops could contribute significantly to food and nutrition security, poverty reduction, and sustainable agricultural development at the global level. Similarly, it also enables scientists to identify and transfer entire biochemical pathways from one species to another and incorporate them into new hosts for the benefit of human health and for medical and agricultural purposes. Crop plants could be developed by using transgenic technology in countries with arid climates, and plants with abiotic stress tolerances could be irrigated with salty water or farmed in salt-damaged farmlands. Plants are suitable for the production of eukaryotic proteins, which may require posttranslational modification and oligomerization, and are also helpful in the production of pharmaceutical antibodies

and vaccines (Sharma et al., 2004; Tarafdar et al., 2014). For instance, recombinant human insulin has been successfully expressed and produced in oilseeds of the plant *A. thaliana* (Nykiforuk et al., 2006), and a human therapeutic protein was successfully produced in tobacco (Staub et al., 2000) using transgenic technology. In addition, successes have been achieved in the incorporation of the HEV2 gene in tomato (Ma et al., 2003), *Streptococcus* surface antigen in tobacco (Robinette et al., 2011), herpes simplex virus in soybean (Zeitlin et al., 1998), and hepatitis B surface antigen in tobacco (Ramirez et al., 2002). Similarly, expression of the plague antigens in the tomato fruit allowed production of an oral vaccine candidate without purification of protein and with minimal processing. These achievements suggest that the genetic engineering have changed the paradigm of plant as a food source or so-called plant bioreactor, offers a low cost technology as well as effective system for large-scale vaccination programs in developing countries. However, the future of edible plant-based vaccines using transgenic approaches will depend upon producing them safely in sufficient quantities (Silva Dias and Ortiz, 2014). There are various examples of the development of new drugs from plant sources (Harvey, 2008), depicting ample opportunities available with transgenic technologies for the generation of more horticultural crops with higher (advanced) medicinal value. Thus transgenic technology has a high potential to help develop the crops that farmers will need to meet future demands for food as the global population grows and for crops adapted to future conditions as the climate changes.

Several horticultural crops contain a high content of plant secondary metabolites, which are beneficial to human health, such as apigenin from parsley, curcumin from turmeric, fisetin from apples, kaempferol from broccoli, and anthocyanins from different plants (Tanaka et al., 2008), which are reported to inhibit several types of cancers (Wang et al., 2012). Apigenin is a flavonoid present in vegetables such as parsley, celery, and chamomile (Hoensch and Ortel, 2011). It induces apoptosis in human colon cancer cells (Turktekin et al., 2011), affects the leptin/leptin receptor pathway, and induces cell apoptosis in the lung adenocarcinoma cell line (Bruno et al., 2011). The nontoxic natural compound curcumin from turmeric exerts antiproliferative, antimigratory, and antiinvasive properties against malignant gliomas (Senft et al., 2010) through regulation of multiple cell signaling pathways including the cell proliferation pathway (cyclin D1, cmyc), cell survival pathway (Bcl2, Bclx, cFLIP, XIAP, cIAP1), caspase activation pathway (caspase 8, 3, 9), tumor suppressor pathway (p53, p21), death receptor pathway (DR4, DR5), mitochondrial pathways, and protein kinase pathway (JNK, Akt, and AMPK) (Ravindran et al., 2009). Cyanidins from grapes are reported to inhibit cell proliferation and iNOS and COX2 gene expression in colon cancer cells (Kim et al., 2008). Therefore targeted modification of some of these mentioned genes, through genome editing technologies, could be applied for the generation of new crop varieties with anticancer phytochemicals. In addition, editing of these genes with CRISPR/Cas9 may give new bits of knowledge in the prevention of cancer.

Many projects were started for the genome sequencing of horticultural crops, for instance, the tomato genome sequencing project (www.sgn.cornell.edu/about/tomato), pepper genome database (http://peppersequence.genomics.cn/page/species/index.jsp), potato genome sequencing consortium (www.potatogenome.net), papaya genome sequencing project (www.asgpb.mhpcc.hawaii.edu/papaya/), grape genome sequencing project (www.vitaceae.org), floral genome sequencing project (www.fgp.bio.psu.edu/), and many more. Such huge sequence data of several species would facilitate understanding of the molecular mechanisms involved in various developmental processes as well as comparative genomics studies in plants (Sonah et al., 2011). The information from these projects/sequence data will surely help the identification of precise target gene sequences for genome editing to

elucidate the molecular control of agronomic traits in horticultural crops. CRISPR/Cas9 technology may be used to prevent, control, or cure viral diseases in plants by targeting viral genes essential for replication or virulence. Furthermore, genome editing technologies with nanotechnology tools will play a significant role in developing new bioenergy crops that can withstand the changes arising from global warming and will be able to keep pace with the increasing demand for food.

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CHAPTER

GENE SILENCING IN HORTICULTURAL TRANSGENIC CROPS

3

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1. INTRODUCTION

India is endowed with diverse soil and agroclimatic conditions, which favor the cultivation of an array of horticultural crops, namely, fruits, vegetables, ornamentals, spices, plantation crops, tuberous crops, and medicinal and aromatic plants in different parts of the country. In the past few years, thanks to favorable policy initiatives, the horticulture sector has emerged as one of the potential enterprises in augmenting the growth of the Indian economy (Anon., 2001). India has emerged as the second largest producer of fruits and vegetables in the world after China and horticulture contributes nearly 28% to gross domestic product in agriculture (Mittal, 2007). The horticulture sector offers tremendous scope for crop diversification, food and nutritional security, income and employment generation, and the development of a processing industry. Additional advantages offered by horticultural crops as compared to field crops include their potential in the sustainable utilization of degraded wastelands, suitability to harsh and fragile ecosystems such as dry lands and cold deserts, less water requirement, higher biomass production through the efficient use of natural resources, highly remunerative nature, greater scope for value addition, and earning of foreign exchange through exports (Anon., 2001). Horticulture is also an integral component of any landscape planning, which ensures beautification of our surroundings, supports biodiversity conservation, maintains soil health, enables sustained provisioning of ecosystem services, and improves environmental sustainability (Festus, 2014).

In spite of its well-known potential to strengthen people's livelihoods and environmental sustainability, there are many current and emerging challenges that, if left unaddressed, could nullify the rich dividends in the offing. Besides well-known constraints such as lower productivity, inferior produce quality, high postharvest losses, and lack of infrastructure facilities (Mittal, 2007), some new challenges appear to create stumbling blocks in the smooth and rapid development of the horticulture industry in India. Such emerging constraints include climate change/variability being experienced in different parts of the country, land degradation, land fragmentation, labor shortages, and the overexploitation of natural resources. According to one report, unabated degradation and continued shrinkage of land and water resources has put key food production systems around the world at risk. Coupled with this, global climate change endangers global food production and developing countries such as India are likely to be the most affected (FAO, 2011). This state of affairs highlights an urgent need to design adaptation strategies that could stabilize horticultural production under adverse agro-edaphic conditions.

Genetic Engineering of Horticultural Crops. http://dx.doi.org/10.1016/B978-0-12-810439-2.00003-9

Crop improvement is one of the aspects of agriculture that satisfies human demand. Many crops have been domesticated to fulfill the requirement of food and esthetic value including horticultural crops such as fruits, vegetables, and ornamental plants. Crop variety originated through selection during early human civilization. Other technologies, such as various forms of selection, hybridization, mutation, transgenic techniques, etc., have also been invented and applied to crop breeding over the past centuries (Xiong et al., 2015). Gene silencing processes have been widely exploited as versatile experimental and biotechnological tools for functional gene studies and transgenic approaches to crop improvement, disease resistance, and metabolic engineering. Despite the fact that the first commercialized transgenic food crop was the Flavr Savr tomato, horticultural crops lag as compared to other crops. Development of transgenic crops during the 1990s was an imperative milestone in the history of crop improvement. The discovery of mechanisms that suppress gene activity in plants has extended the horizon for research on control of gene expression (Mansoor et al., 2006). Gene silencing describes the epigenetic regulation of a gene, at the level of transcription or translation, to prevent gene expression (Waterhouse et al., 2001; Wassenegger, 2002). Currently, there are several routes of gene silencing identified in plants, such as RNA interference (RNAi), transcriptional gene silencing, virus-induced gene silencing, and microRNAs, which comprise a series of mechanisms capable of suppressing gene expression in plants (Souza et al., 2007).

2. POST-TRANSCRIPTIONAL GENE SILENCING

Post-transcriptional gene silencing (PTGS) is defined as the silencing of an endogenous gene caused by the introduction of a homologous double-stranded RNA (dsRNA), transgene, or virus. In PTGS, the transcript of the silenced gene is synthesized but does not accumulate because it is rapidly degraded. This is a more general term than RNAi, since it can be triggered by several different means. PTGS as a consequence of the introduction of dsRNA molecules (e.g., viral RNA, transgenes, etc.) has been found to occur in a number of species. Discovered by accident and initially considered a strange phenomenon confined to some plant species, PTGS has received a great deal of attention: first, the finding that post-transcriptional mechanisms are ubiquitous in both the animal and plant kingdoms and are responsible for many important biological functions, such as genome defense against transposons and viruses, and second, the promising applications of PTGS in the developing field of functional genomics as a tool for the knocking out of gene expression.

The advances in PTGS mechanisms were well documented in 1990 (Napoli et al., 1990; Smith et al., 1990; Van der Krol et al., 1990) and demonstrated that the introduction of RNA sequences or transcribed sense transgenes could downregulate the expression of homologous endogenous genes; this phenomenon was called cosuppression and resulted in the degradation of endogenous gene and transgene RNA after transcription (Fagard and Vaucheret, 2000). Because post-transcriptional RNA degradation can affect a wide range of transgenes expressing plant, bacterial, or viral sequences, it was more generally renamed PTGS. PTGS can be classified according to the nature of the silencing source introduced either by transgenes or viruses, which can be a sense transgene, an antisense transgene, simultaneously expressed sense/antisense transgenes, or viruses.

2.1 PTGS MEDIATED BY SENSE TRANSGENES

This is further subcategorized into strongly transcribed sense transgenes and very weakly transcribed or untranscribed sense transgenes.

In strongly transcribed sense transgenes, once they are initiated against the RNA of a given transgene, PTGS leads to the degradation of homologous RNA from either endogenous genes (cosuppression), transgenes (*trans*-inactivation), or RNA viruses (RNA-mediated virus resistance). In RNA-mediated virus resistance, transgenic plants can be either immune, i.e., virus resistance is established prior to the infection, or can recover from the virus infection. A single transgene copy driven by a strong promoter appears to be sufficient to trigger this type of PTGS. Vaucheret (1993) reported that the transcriptional silencing of 35S-driven transgenes impedes cosuppression of homologous endogenous genes as well as resistance against homologous RNA viruses. The transgene-induced PTGS mechanism affects expression of the transgenes and of endogenous genes with which they share a considerable degree of sequence identity. The latter case is also known as cosuppression because the endogenous genes and the transgenes are silenced. Cosuppression was first observed with genes involved in petunia flower pigmentation and in tomato fruit ripening. Because PTGS depends on active transcription of the transgene itself, it is unlikely that aberrant RNA [RNA that is qualitatively distinct from the regular messenger RNA (mRNA) is called aberrant RNA, which is thought to be required for activating the PTGS mechanism] is directly produced by read-through transcription from neighboring transgenes beyond their terminators, or from transcription from neighboring endogenous promoters. However, such unintended transcription events could interfere with regular transcription of transgenes, leading to the production of aberrant RNA instead of regular RNA, or could produce antisense RNA that could interact with regular mRNA to form aberrant (partial) dsRNA. Alternatively, transgenes could produce directly single-stranded aberrant RNA because they are methylated. Since de novo methylation can be triggered in sequence-specific transgenes by introduction of homologous viroid RNA, an RNA signal is suggested to trigger transgene methylation and subsequently trigger PTGS (Wassenegger and Pelissier, 1998).

Grafting experiments revealed that PTGS-silenced plants produce a sequence-specific systemic silencing signal that propagates long distances from cell to cell and triggers PTGS in nonsilenced graft-connected tissues of the plant. Because of its sequence specificity and its mobility, this signal is assumed to be (part of) a transgene product, probably the putative aberrant RNA hypothesized earlier, that could migrate alone or within a ribonucleoprotein complex. In very weakly transcribed or untranscribed sense transgenes the analysis of plants showed cosuppression of endogenous chalcone synthase (CHS) genes by sense transgenes that are not transcribed at a high level despite the presence of a 35S promoter, or by promoterless transgenes. All plants of this type showed complex transgene arrangements, which contain at least one inverted repeat and are methylated. These observations suggest that such structures could efficiently pair with homologous endogenous genes, thereby impairing the regular production of RNA. Alternatively, this type of structure could be as efficient as a strongly transcribed single transgene to produce the amount of aberrant RNA that is hypothesized to activate the RNA degradation machinery.

2.2 PTGS MEDIATED BY ANTISENSE TRANSGENES

This phenomenon is subcategorized into transcribed antisense transgenes and untranscribed antisense transgenes. Before the discovery of cosuppression by sense transgenes, downregulation of endogenous genes was usually achieved using antisense transgenes. Hence PTGS could result from the unintended

production of antisense RNA by those sense transgene loci that trigger PTGS, leading to antisense-like inhibition. Although antisense inhibition is efficient against endogenous genes and foreign transgenes, patterns of silencing produced by antisense transgenes are usually different from those produced by sense transgenes.

Expression of antisense RNA from integrated transgenes was the first, and until recently the most widespread, method of initiating PTGS in transgenic plants. Antisense constructs usually consist of an inverted gene coding sequence driven by a strong constitutive promoter such as CaMV 35S with ~5%–20% of transformed individuals displaying a reduction in target mRNA accumulation (Wesley et al., 2001). Silencing is highly dependent upon antisense RNA:mRNA homology, whereas nuclear run-on assays have demonstrated that antisense-silenced lines are generally not deficient in transcription of the target mRNA, indicating that silencing is a post-transcriptional effect. The presence of abundant double-stranded small interfering RNA (siRNA) in antisense-silenced plants strongly supports the long-standing hypothesis that homologous pairing of sense and antisense RNAs produces dsRNA in vivo, and that this dsRNA is the primary initiator of silencing. Although sense:antisense RNA duplex formation is likely the primary mechanism of dsRNA production in antisensed plants, Stam et al. (2000) have reported that dsRNA can also arise from the read-through transcription of antisense transgene constructs integrated as inverted repeats, resulting in production of self-complementary RNA molecules.

Scientific knowledge still requires the determination of any common steps between sense and antisense inhibition or that they clearly exhibit distinct steps. The identification of mutants impaired in antisense inhibition and the analysis of PTGS in such mutants will help to identify possible common steps. In the case of untranscribed antisense transgenes the silencing could result from an actual pairing of the transgene locus with the homologous endogenous genes and their subsequent modification, leading directly to the production of degradable endogenous RNA. Alternatively, aberrant sense RNA could be produced that cannot be distinguished by run-on assays from that produced by the endogenous genes (Stam et al., 1998).

2.3 PTGS MEDIATED BY SENSE/ANTISENSE TRANSGENES

The production of dsRNA is required to trigger PTGS, and that RNA-dependent RNA polymerase (RdRp) could be involved in such production. Whether the events of cosuppression, *trans*-inactivation, or virus resistance mediated by sense or antisense transgenes rely on the same mechanism as PTGS mediated by sense transgenes alone awaits the analysis of methylation, graft transmissibility, and release by viruses that counteract PTGS mediated by sense transgenes. Nevertheless, simultaneous expression of sense p35S-GUS and antisense p35S-aGUS transgenes triggers silencing in *sgs* mutants (Beclin and Vaucheret, unpublished data), which suggests that at least the three steps controlled by SGS genes are specific to PTGS mediated by sense transgenes, and are not involved in sense- or antisense-mediated silencing.

2.4 PTGS MEDIATED BY DNA AND RNA VIRUSES

RNAi and PTGS are natural mechanisms that work against viral infection in both plants and animals. PTGS mediated by viruses can occur with DNA viruses at the nucleus or with RNA viruses at the cytoplasm. Infection of nontransgenic *Brassica napus* plants by CaMV leads to recovery by a PTGS-like

mechanism, i.e., 19S and 35S RNA encoded by CaMV are degraded, while CaMV DNA is still replicating in the nucleus (Al-Kaff et al., 1998). Infection of transgenic *B. napus* plants expressing a p35S-GUS transgene with a 35S terminator by CaMV leads to recovery from CaMV infection. CaMV is primarily a target of the cellular silencing machinery since the 19S and 35S RNA are degraded. However, CaMV can also be considered as a source (or at least an inducer) of PTGS for transgenes sharing homology with the virus within their transcribed regions because it activates the cellular RNA degradation machinery against them.

3. MECHANISM OF GENE SILENCING

RNA-based silencing mechanisms, which are effective at the genome level and in the cytoplasm, are able to combat against an RNA genome (RNA viruses) or a dsRNA replication intermediate. The ability of dsRNA molecules to trigger degradation of homologous RNAs was discovered some time ago in plants. Unifying studies showed that silencing is accompanied by the accumulation of small RNAs (21–25 nucleotides) of both sense and antisense orientation that are homologous to the silenced locus through a highly specific mechanism involved in sequence-specific RNA degradation. First reports on other post-transcriptional gene silencing phenomena, also called RNA silencing phenomena, came about in the early 1990s, when Napoli et al. (1990) described the silencing of viral transgenes and their homologous endogenous genes in transgenic plants at a post-transcriptional level. At this time, the phenomenon was called "cosuppression." Similar findings were also described in *Neurospora crassa*, where the phenomenon was termed "quelling." Silencing can be triggered locally and then spread through the organism in plants via a silencing signal. Many mechanisms of gene silencing in plants have been suggested (Wei et al., 2001; Zhang et al., 2016); however, the common features of these various mechanisms include the synthesis and amplification of dsRNA, unwinding of dsRNA, and targeting of mRNAs after binding to the ribosome.

Many well-known phenomena of PTGS, such as "cosuppression" in plants and "quelling" in fungi, share common features with RNAi. Although it is not known whether those phenomena use identical mechanisms, there is strong evidence that the mechanism of dsRNA-induced RNA silencing is at least very similar in different species. In fact, genetic studies in PTGS-deficient mutants revealed several genes involved in quelling, cosuppression, and RNAi, including members of the helicase family, RNAse III-related nucleases, members of the Argonaute family, and RdRp. A common characteristic of all RNA-silencing pathways initiated by dsRNA in nematodes, trypanosomes, flies, and mammals is the cleavage of long dsRNA by a double-strand-specific RNAse called "Dicer." Dicer cleaves dsRNA into so-called siRNA duplexes encompassing a length of 21–25 nucleotides and containing two nucleotide single-stranded overhangs at the 3′ end, which are called small dsRNAs, later termed siRNAs; these are the active components of RNA silencing. The siRNAs and some proteins are suggested to form a ribonucleotide protein complex, which promotes unwinding of the RNA duplex presumably in an ATP-dependent manner, leading to the final activation of the RNA-induced silencing complex (RISC). Eventually, this complex presents the antisense strand of the siRNA to the target mRNA and guides mRNA degradation.

The RdRp is required for transgene-induced PTGS where one possible function is the synthesis of complementary RNA from aberrant RNA templates, leading to the formation of dsRNA. Mutations in the RdRp gene prevent the effect of post-transcriptional gene silencing by dsRNA. Two novel

opposing models have been presented. The first model proposed the requirement of an RdRp. RdRps are enzymes characteristically involved in RNA virus replication by synthesizing complementary RNA molecules using RNA as a template. In cells displaying RNAi, RdRp is assumed to convert the single-stranded target mRNA to dsRNA using the antisense strands of primary siRNAs as primers. After Dicer-mediated cleavage of dsRNA, the resulting primary siRNAs are proposed to bind to their complementary target mRNA and to be extended by nucleotide addition in a target-dependent manner to form dsRNA. The newly generated dsRNA is supposed to be eventually cleaved by Dicer to form newly generated, so-called secondary siRNAs. Since RdRp should be capable of transforming all targeted mRNAs to dsRNA, the nuclease activity of Dicer would be sufficient to completely cleave the trigger dsRNA and also the target mRNA. Such a mechanism would not necessarily include RISC as an additional nuclease. RNAse III family members are known as dsRNA-specific nucleases or Dicer and contain two RNAse III domains, a dsRNA binding motif, an amino-terminal DexH/DEAH RNA helicase/ATPase domain, and a motif called "PAZ domain." This type III RNAse showed sequence-independent production of uniformly sized small RNA fragments derived from long dsRNA, a property that led to its name Dicer, which is an ATP- and Mg^{++}-dependent process. The molecular mechanism of Dicer-mediated yet to be elucidated, the nuclear binding, storage, degradation or release of RNAs to understand the bigger picture and why and when a particular transcript enters in a specific pathway.

An important result that has emerged from a number of studies is that single-stranded antisense RNA ranging from 19 to 29 nucleotides, also known as siRNA, can also enter the RNAi pathway; however, it has less efficiency than the double-stranded siRNA. The RISC complex from *Drosophila* is a ~500-kDa complex bound to ribosomes, and contains siRNAs and proteins, mainly Argonaute-2. Like Dicer, Argonaute-2 contains a PAZ domain and appears to be essential for the nuclease activity of RISC. In plants, two kinds of transgenes can act to initiate PTGS. The first corresponds to high transcription of single transgene copies. When transcription of the transgene is blocked, no PTGS occurs and inversely PTGS is most strongly triggered when plants are homozygous for the transgene or when strong promoters are used. These findings suggest that the efficacy of triggering PTGS might depend on production of a particular form of RNA above a threshold level. The second type of transgene known to induce PTGS is arranged as an inverted repeat of sense DNA. Such inverted repeats are continually transcribed at very low levels to form a hairpin-structured RNA resembling dsRNA. The pathways of PTGS triggered by single-stranded transgenes versus inverted repeats differ at least in some aspects. Possibly, there is an unknown pathway converting transgenic RNA to dsRNA, which then initiates PTGS. Investigation of genes that stimulate PTGS in plants revealed some candidate genes essential only for PTGS induced by transgene expression includes genes homologous to known RdRps.

A prominent feature of PTGS suppression by HC-Pro is the absence of the small RNAs associated with silencing. HC-Pro inhibits PTGS by an unknown mechanism even in the absence of the virus. Research has indicated that HC-Pro acts upstream of siRNA generation and does not interfere with transgene methylation, a phenomenon that is associated with PTGS in plants. Expression of HC-Pro in plants inhibits PTGS and results in activation of a cellular gene, which is termed rgsCaM (regulator of gene silencing-Calmodulin-related protein), and accumulation of its mRNA. HC-Pro suppression of PTGS possibly occurs via activation of rgsCaM and its associated unknown target proteins. In contrast to HC-Pro, the PVX p25 protein appears to suppress PTGS by targeting the mobile silencing signal. The small sense and antisense RNAs associated with silencing that derive from cleavage of dsRNA and

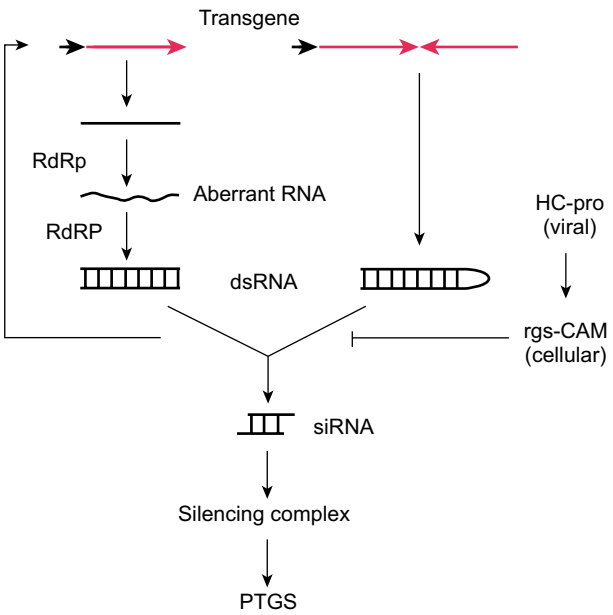

FIGURE 3.1

Post-transcriptional gene silencing (PTGS) in plants triggered by single transgene expression above a threshold level or by inverted repeat transcription. The single transgene transcript is converted into aberrant RNA and subsequently transformed into double-stranded RNA (dsRNA). The viral PTGS inhibitor acts by activation of regulator of gene silencing-Calmodulin-related protein (rgsCAM), a cellular PTGS inhibitor.

a specific ribonuclease will serve as a guide to find homologous target RNAs (Fig. 3.1). Small RNAs accumulate during both virus- and transgene-induced gene silencing, indicating the similarity in two different pathways/branches of silencing before the formation of the sequence-specific ribonuclease. This is because HC-Pro suppression of silencing interferes with accumulation of the small RNAs but does not eliminate either the production or movement of the silencing signal. Also the PVX p25 protein interferes with the mobile silencing signal, but does not affect the accumulation of small RNAs produced in the viral RdRp-dependent branch of PTGS.

4. APPLICATION OF RNAi IN TRANSGENIC PLANTS

RNAi is a promising gene regulatory approach that has significant impact on crop improvement; it permits downregulation in gene expression in a more precise manner without affecting the expression of other genes. RNAi-mediated technology has been used in the metabolic engineering of plants with respect to improvement of various traits and to target genes linked to different undesired characters. In several plants, RNAi has been used to improve their nutritional value, flavor, genetic modification of fatty acid composition, and reduce toxicity/allergenicity. Several applications of the RNAi approach are elaborated here in major consumed crops.

4.1 RNAi FOR ENHANCEMENT OF SHELF-LIFE

Postharvest losses deteriorate the quality and economic viability of many high-yielding varieties of tomato (*Lycopersicon esculentum* L.). The traditional farmers used to harvest green tomatoes, which were then sprayed with ethylene to fetch higher prices than ripe tomatoes; however, there is a substantial decrease in flavor content. The storage period of tomatoes could be addressed by a specific approach called RNA-based interference technology or RNAi. Because tomatoes are susceptible to changes in climate, they have autocatalytic activities of ethylene during their ripening period. Therefore increase in shelf-life or delay in the ripening process can be achieved by the introduction of 1-aminocyclopropane-1-carboxylate (ACC) oxidase dsRNA in tomatoes, which suppresses the expression of ethylene genes. Transgenic tomato plants targeting more than one homolog than a single unit of ACC oxidase by using RNAi technology during the ripening stage would be more effective than a single homolog (Gupta et al., 2013). The chimeric RNAi-ACS (1-aminocyclopropane-1-carboxylate synthase) construct designed to target ACS homologs effectively repressed ethylene production in tomato fruits and enhanced tomato shelf-life by 45 days. Ethylene suppression brings compositional changes to the fruits by enhancing polyamine (PA) levels. At the same time, decreased levels of ethylene in RNAi-ACS fruits lead to altered levels of various ripening-specific transcripts (i.e., upregulation of PA biosynthesis, ascorbic acid metabolism genes, and downregulation of cell wall hydrolyzing enzyme genes). The downregulation of ACS homologs using RNAi can be an effective approach for obtaining delayed ripening with longer shelf-life and an enhanced processing quality of tomato fruits, because of the reduced rate of softening. Similarly, through the RNAi technique, two ripening-specific ethylene-induced *N*-glycoprotein modifying enzymes, α-mannosidase (α-Man) and β-D-*N*-acetylhexosaminidase (β-Hex), were targeted and the shelf-life of tomato was enhanced by ≈2–2.5-fold with firmer fruits. Studies shows the role of microRNAs in tomato fruit development and ripening (Meli et al., 2010; Molesini et al., 2012; Karlova et al., 2013). MiR156 targets an important gene in fruit ripening, colorless never ripe (*CNR*), as reported by Molesini et al. (2012). The study shows that *CNR* is also negatively regulated by *APETALA2a* (a target of miR172). Although it positively regulates fruit ripening, it also negatively regulates ethylene synthesis (Karlova et al., 2013).

The silencing of a ripening-related CHS gene in strawberry fruits (*Fragaria* × *ananassa* cv. Elsanta) by an intron-hairpin-RNA construct containing the partial sense and corresponding antisense sequences of CHS was separated by an intron obtained from an *F. ananassa* quinone oxidoreductase gene. The reduced levels of CHS mRNA and enzymatic CHS activity and the levels of anthocyanins were downregulated, and precursors of the flavonoids pathway were shunted to the phenylpropanoid pathway leading to large increases in levels of (hydroxy) cinnamoyl glucose esters. This technique in combination with metabolite profiling analysis will be useful for the development and ripening of strawberry fruit (Hoffmann et al., 2006).

4.2 RNAi FOR NUTRITIONAL QUALITY IMPROVEMENT

RNAi being a promising technology has great potential to improve nutritional quality traits by modifying the expression of genes. RNAi plays a key role in the development of fruits and vegetables because of seedlessness, desired agronomic traits, and enhanced nutritional qualities such as carotenoid and flavonoids contents, both highly beneficial for human health. RNAi in combination with a fruit-specific promoter is used to suppress an endogenous photomorphogenesis gene *DET1* in tomato, a regulatory

gene involved in the repression of several light-signaling pathways. *DET1* was effectively degraded in transgenic tomato with suppressed *DET1*, with an increase in the level of flavonoids and carotenoid content (Davuluri et al., 2005). Abscisic acid (ABA) plays very significant roles at the time of fruit ripening in tomato. The SlNCED1 gene in tomato that encodes 9-*cis*-epoxycarotenoid dioxygenase, which is an important enzyme in the ABA biosynthesis, was suppressed by RNAi. The fruits showed increased accumulation of upstream compounds, chiefly lycopene and β-carotene, from these RNAi lines. Dandekar et al. (2004) reported the fruit quality of apple with regard to shelf-life using the RNAi approach by reducing the accumulating amount of a major apple allergen (Gilissen et al., 2005) and silencing the leaf sorbitol synthesis process, which affects starch accumulation, sugar–acid balance, and thereby fruit quality. RNAi technology has also been utilized to increase the carotenoid content of rapeseed (*B. napus*) by downregulating the expression of the lycopene epsilon cyclase gene (*ε-CYC*). Yu et al. (2007) highlighted the seed quality of transgenic *Brassica* having a higher content of β-carotene, violaxanthin, zeaxanthin, and lutein.

In several other crops, RNAi has been utilized to improve their quality at the nutritional level. In sweet potato (*Ipomoea batatas*), the range in amylose content is narrow (10%–25%) compared with other crops. The amylose content has been markedly increased utilizing this approach by suppressing the expression of the endogenous IbSBEII gene. The introduction of construct encoding dsRNA of SBEII into the sweet potato genome to inactivate the IbSBEII gene resulted in an increase in apparent amylose content in the sweet potato starch. The ratio of amylose to amylopectin has great influence on the physicochemical properties of starch. The improved sweet potato has new dietary and industrial applications. Similarly, in potato, through the use of the RNAi approach, carotenoids such as β-carotene and total carotenoid production have been increased through silencing of the β-carotene hydroxylase gene. Potato tuber contains low levels of carotenoids, which are mainly composed of xanthophylls lutein and viloxanthin. However, none of these compounds has provitamin A activity. β-Carotene is the main precursor of vitamin A.

A bivalent RNAi plant-transformation vector was constructed to silence both the flavanone 3-hydroxylase (F3H) gene and the flavone synthase II (GmFNSII) gene in soybean (*Glycine max*), which effectively regulate the flavone and isoflavone production in hairy roots. The bivalent RNAi vector had a significantly higher effect for increasing in isoflavone production compared with the two unit RNAi vectors. The study highlighted molecular methods that could be used to enhance isoflavone production in soybean (Jiang et al., 2013). In opium poppy (*Papaver somniferum*), through the use of this technology, the morphine line has been replaced with nonnarcotic alkaloids. RNAi was used to interfere with multiple steps of a complex biological pathway. A heterogeneous nuclear RNA construct was designed to silence the multigene codeine reductase (COR) gene family. The transgenic plant accumulated (*S*)-recticuline, a precursor nonnarcotic alkaloid that occurs seven enzymatic steps upstream from codeinone in the pathway. It occurs at the expense of morphine, codeine, opium, and baine (Allen et al., 2004). An efficient siRNA-mediated gene silencing was used to silence the omega-3 fatty acid desaturase (FAD3) gene family, which contributes mostly to the instability of soybean (*Glycine max*) and other seed oils. Therefore a significant reduction of this fatty acid will increase the stability of the seed oil, enhancing the seeds' agronomical value. The transgene-produced siRNA caused silencing of FAD3, which reduces this fatty acid significantly and suggests a role and great potential for the siRNA strategy in silencing gene families in a complex genome.

4.3 USE OF RNAi FOR SEEDLESS FRUIT (PARTHENOCARPY)

In tomato plants, RNAi enables repression of gibberellic acid and auxin signal pathways after a reduction in the level of the SlARF7 transcript, which is responsible for pollination and fertilization. It bypasses the auxin signaling–fertilization pathway in tomato that leads to the development of parthenocarpic fruits, which have great commercial value in the current agricultural market, and higher yields can be achieved even in environmental conditions undesirable for pollination and fertilization. By downregulating a CHS, a gene involved in flavonoid biosynthesis, seedless fruits have been achieved in tomato. Phytohormones such as auxin and gibberellins are closely associated with parthenocarpy in tomato, which in turn is regulated by many microRNAs (Molesini et al., 2012). Thus manipulating the level of phytohormones by controlling activities of microRNAs or their targets could prove to be an effective approach to obtain parthenocarpic fruits. Parthenocarpic fruits were also observed in tomato, in which genes of the AUCSIA family coding for 53-amino-acid-long (protein or peptide) were functionally suppressed by RNAi technology (Molesini et al., 2009).

4.4 RNAi FOR FLOWER COLOR MODIFICATION

Chrysanthemum is one of the most important cut flowers and ornamental plants used all over the world. It exhibits a range of colors but lacks bright red and blue flowers. An effort has been made in this direction to increase the brightness of petals through RNAi. Two chrysanthemum cultivars, *Chrysanthemum*×*morifolium* "LPi" and *C.*×*morifolium* "LPu", that only accumulate flavonoids in their ligulae flowers were used to isolate seven anthocyanin biosynthesis genes, i.e., *CmCHS*, CmF3H, CmF3′H, CmDFR, CmANS, *CmCHI*, and *Cm3GT* in these cultivars. Reverse transcription polymerase chain reaction (RT-PCR) and quantitative RT-PCR analyses showed that CmF3′H was the most important enzyme required for cyanidin biosynthesis. To rebuild the delphinidin pathway, the downregulation of *CmF3′H* using RNAi and overexpression of the *Senecio cruentus F3′5′H* (*PCFH*) gene in chrysanthemum demonstrated a significantly increased content of cyanidin and brighter red flower petals but did not accumulate delphinidin. These results indicated that *CmF3′H* in chrysanthemum is important for anthocyanin accumulation, and *Senecio cruentus* F3′5′H only exhibited F3′H activity in chrysanthemum but did not rebuild the delphinidin pathway to form blue flower chrysanthemum as reported by He et al. (2013).

4.5 VIRUS RESISTANCE THROUGH RNAi

RNAi-based technology has been used to engineer plants to impart virus resistance. In this case, an RNAi vector carrying viral target sequence in transgenic plants produced dsRNA that eventually silenced viruses multiplying in the cell. Nora et al. (2009) reported that virus-resistant transgenic tomato plants produced dsRNA against potato spindle tuber viroid (PSTVd) sequences and showed resistance to PSTVd infection in tomato plant. A similar strategy was used to successfully engineer resistance in cassava plants against *African cassava mosaic virus* as reported by Vanderschuren et al. (2009). Virus resistance has been engineered successfully by targeting the coat protein (CP) gene through RNAi in many horticultural plants. Transgenic tobacco expressing the CP gene of *Tobacco mosaic virus* (TMV) was resistant to TMV and the resistance was caused by the expressed CP as shown by Powell-Abel et al. (1986). Subsequently, this strategy was applied to generate resistance against several different viruses such as potato resistant to *Potato virus Y* by Missiou et al. (2004), *Cucumis* cv.

melo resistant to *Papaya ringspot virus* type W, and *Prunus domestica* resistant to *Plum pox virus* (Krubphachaya et al., 2007; Hily et al., 2007). RNA silencing strategy is not limited to RNA viruses but can successfully be applied to DNA viruses to engineer resistance. Black gram plants were recovered efficiently from geminivirus *Vigna mungo yellow mosaic virus* (VMYMV) infection when inoculated with hairpin RNA construct containing the promoter sequence of VMYMV under the control of the 35S promoter (Pooggin et al., 2003). The RNAi method has been used to generate common bean resistant to geminivirus *Bean golden mosaic virus* by Bonfim et al. (2007). Bucher et al. (2006) reported that a wide spectrum of resistance has been developed against tospoviruses by targeting multiple regions of a viral gene in tomato plants.

4.6 RNAi FOR INSECT RESISTANCE

Colorado potato beetles are a dreaded pest of potatoes all over the world. Since Colorado potato beetles do not have natural enemies in most potato-producing regions, farmers try to control them with pesticides. However, this strategy is often ineffective because the pest has developed resistance against nearly all insecticides. Now, scientists from the Max Planck Institute of Molecular Plant Physiology in Germany have shown that potato plants can be protected from herbivory using RNAi. They genetically modified plants to enable their chloroplasts to accumulate dsRNAs targeted against essential beetle genes.

4.7 REDUCED ALLERGENICITY AND TOXICITY THROUGH RNAi

RNAi has vital and potent technology to engineer plants with reduced allergenicity and toxicity. To silence the specific allergens and toxic metabolites, RNAi is highly efficient because of its sequence specificity to particular allergens without hampering the essential cellular compound. In apple (*Malus domestica*), an allergen known as mal d1 expression has been reduced through the RNAi approach. The mal d1 crossreaction antibody is IgE, which causes adverse reaction in patients. To build the RNAi construct to effectively silence the mal d1 gene, an intron containing the mal d1 gene was isolated from cultivars known as Gala. The results indicate that a wild-type plantlet had significantly ($P < .05$) higher allergenicity than five of the transform plants. The reduction of expression of mal d1 was confirmed by immunoblotting and skin prick test of apple leaflet (Gilissen et al., 2005). In another study, the expression of allergen Lyc e 3, which encodes a nonspecific lipid transfer protein in tomato plants, was reduced through a specific dsRNAi construct of LTPG1 and LTPG2 (Le et al., 2006).

4.8 RNAi FOR ABIOTIC STRESS TOLERANCE

Abiotic stress is a serious hazard for life on earth, particularly for plants whose growth and yield are affected negatively. Plants have adapted numerous physiological, biochemical, and metabolic approaches for the purpose of encountering abiotic stresses. Normally, it is tricky to envisage the complicated pathways of signaling that are stimulated and turned off in response to different abiotic stresses (Chawla et al., 2011). Current findings show that RNAi is playing an imperative role in abiotic stress stimulation in different crops. The functions of microRNAs in relation to abiotic stress such as oxidative stress, cold, drought, and salinity were reported by Sunkar and Zhu (2004). Additionally, miR402, miR319c, miR397b, and miR389a were controlled by abiotic stress under varying levels in *Arabidopsis* (Jagtap et al., 2011).

Table 3.1 Transgenic Research in Horticultural Crops (Xiong et al., 2015)

Crops	Varieties	Traits
Papaya	Rainbow, SunUp	Disease resistance
	Huanong No. 1	
Apple	Golden Delicious	Modified product quality
	Granny Smith	Nonbrowning phenotype
Plum	C-5	Disease resistance
Tomato	Da Dong No. 9, Huafan No. 1, FLAVR SAVR	Modified product quality
Brinjal	Bt Brinjal Event EE1	Insect resistance
Potato	Lugovskoi plus, Atlantic NewLeaf2 potato	Insect resistance
	Starch potato, Innate2 Russet Burbank potato	Modified product quality
	New Leaf2 Y Russet Burbank potato, New Leaf2 Plus Russet Burbank potato	Insect + disease resistance
Sweet pepper	PK-SP01	Disease resistance
	New Leaf2 Plus Russet Burbank potato	
Rose	WKS82/130-4-1	Modified product quality
Carnation	Moondust	Modified product quality
	Moonshadow	Herbicide tolerance 1 modified product quality
	Moonshade	
	Moonlite	
	Moonaqua	
	Moonvista	
	Moonique	
	Moonpearl	
	Moonberry	
	Moonvelvet	
Petunia	Petunia-CHS	Modified product quality
Creeping Bentgrass	Roundup Ready Creeping Bentgrass	Herbicide tolerance

Gene-silencing processes have been widely exploited as versatile experimental and biotechnological tools for functional gene studies and transgenic approaches to crop improvement, disease resistance, and metabolic engineering. A number of horticulture transgenic crops have been developed against several biotic and abiotic stress tolerances (Table 3.1).

5. CONCLUSION

RNAi has become a highly effective experimental tool in functional genomics for silencing genes for both basic and applied biological studies in various organisms including plants. RNAi deploys small RNAs, mainly siRNAs, to mediate the degradation of mRNA for regulating gene expression in plants.

However, RNAi stability in plants is critical, but the RNAi-mediated gene suppression approach opens new avenues for the development of eco-friendly biotech approaches for crop improvement. By way of knocking out of the specific genes for better stress tolerance and integrating novel traits in different plant species for insect/pest/pathogen resistance and enhanced nutritional status become more convenient rather than convectional practices. This technology having revolutionary capabilities could be further exploited for functional analysis of target genes and regulation of gene expression for crop improvement.

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CHAPTER

TRANSGENIC RESEARCH IN FRUIT CROPS

4

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1. INTRODUCTION

Fruits are an essential part of our daily diet because they contain essential vitamins, minerals, fiber, phytochemicals, etc. Consumption of fruits is proven to be associated with reduced risk of chronic diseases such as cardiovascular disease, diabetes, and cancer as well as gastric health. With the rising world population, demand for increased fruit production is on the rise to meet the per capita requirement. Because of their sessile and perennial nature, fruit trees experience several biotic and abiotic stresses during their life cycle resulting in considerable yield loss. Since most fruits are consumed fresh, pesticides used to control insects, diseases, and weeds pose health concerns besides environmental safety issues. Therefore development of varieties with resistance to different biotic and abiotic stresses as well as with enhanced quality is the focus of research in fruit crops worldwide. Conventional methods of breeding face several limitations with respect to fruit crops because of their perennial nature, long juvenile phase, high levels of heterozygosity, and self-incompatibility. Advances in biotechnology, especially transgenic research, have helped in overcoming several of these limitations. The transgenic approach involves transfer and integration of a specific target gene to an otherwise elite cultivar, thus resulting in a resistant cultivar without altering other quality traits. Tremendous progress has been made during the last two decades in transgenic research in fruit crops, which have started bearing fruits in the form of approval by regulating agencies for either release or sale of transgenic fruit varieties.

2. GENETIC IMPROVEMENT OF FRUIT CROPS THROUGH GENETIC ENGINEERING

2.1 TRAITS USED FOR TRANSGENIC RESEARCH

2.1.1 Disease and Insect Resistance

Diseases and insect pests are the most important factors limiting yield and also affecting the quality of fruit crops. Therefore research has mainly focused on the use of a wide range of genes to develop transgenic plants with improved resistance to fungal, bacterial, and viral diseases. Apple scab caused by the fungus *Venturia inaequalis* and fire blight caused by *Erwinia amylovora* in apple; powdery mildew caused by *Erysiphe necator*, Pierce's disease caused by *Xylella fastidiosa*, and crown gall in grape; wilt caused by *Fusarium* and *Xanthomonas* and bunchy top disease caused by the banana bunchy top virus in banana; citrus canker caused by *Xanthomonas* spp. and *Citrus tristeza* virus in citrus; *Botrytis* fruit

Genetic Engineering of Horticultural Crops. http://dx.doi.org/10.1016/B978-0-12-810439-2.00004-0

rot and powdery mildew in strawberry; and fire blight resistance in pear are the major diseases of focus for transgenic research. In the case of plum, resistance to plum pox virus (PPV) has been the focus of attention and a transgenic plum variety HoneySweet has been approved by the United States Department of Agriculture (USDA) for commercial cultivation. Table 4.1 gives details of genes used as target genes for imparting resistance to fungal and virus diseases and insect pests in these fruit crops.

2.1.2 Resistance to Abiotic Stress

Because of their sessile nature, plants experience several abiotic stresses during their life cycle. Abiotic stresses, such as drought, soil and water salinity, and extreme temperature, cause considerable loss. The perennial nature of the majority of fruit crops aggravates these losses further. Therefore resistance to different abiotic stresses has received considerable attention by researchers worldwide. Response to abiotic stress in plants is regulated by multiple signaling pathways and different stresses elicit many common genes and pathways. Among different classes of genes, transcription factor genes are most frequently used either for imparting tolerance to abiotic stress or for molecular understanding of stress response. In apple, expression of the *OsMyb4* gene (Pasquali et al., 2008) and *CBF* genes (Wisniewski et al., 2011; Artlip et al., 2014) improved response to cold stress and growth parameters. Expression of transcription factors *AtDREB1b* (Jin et al., 2009) and *VvCBF4* (Tillett et al., 2012) enhanced cold and freezing tolerance, respectively, in grapes. In banana, much emphasis has been given to enhance tolerance to salt, moisture, and cold stress, and genes belonging to transcription factors (Shekhawat et al., 2011a; Sreedharan et al., 2012; Dou et al., 2016; Negi et al., 2016), aquaporin (Sreedharan et al., 2015), and stress-related proteins (Shekhawat et al., 2011b; Rustagi et al., 2015) have been used to impart improved tolerance to transgenic plants. In *Citrus*, genes for increased accumulation of osmoprotectants such as proline (de Campos et al., 2011) and glycine betaine (Fu et al., 2011) imparted enhanced tolerance to salinity and drought, respectively. Overexpression of the spermidine synthase gene in pear has been extensively used to confer tolerance to multiple abiotic stresses (He et al., 2008; Wen et al., 2008, 2009, 2011). Expression of *RdrebB1BI* under the control of stress promoter rd29A enhanced cold tolerance (Fei et al., 2014) and overexpression of acidic dehydrin *WCOR410* improved freezing tolerance in strawberry (Houde et al., 2004). Salt stress tolerance in strawberry was improved by overexpression of tobacco osmotin genes (Husaini and Abdin, 2008).

2.1.3 Improvement of Fruit Quality and Shelf-Life

Fruits are important sources of nutrients such as vitamins, minerals, antioxidants, and secondary metabolites with antioxidant activities such as anthocyanins, stilbenes, flavonoids, etc. The stilbene synthase gene, which catalyzes the conversion of one molecule of coumaroyl-CoA and three molecules of malonyl-CoA into 3,4′,5-trihydroxystilbene, commonly known as resveratrol, has been used for enhanced resveratrol synthesis in apple (Rhmann et al., 2006) and grape (Fan et al., 2008; Cheng et al., 2016; Dai et al., 2016). Antisense expression of the *PPO* gene resulted in no browning of apple fruits (Armstrong and Lane, 2013) and transgenic "Arctic apple" has been approved by the USDA and Food and Drug Administration for sale. Apple allergy is caused by IgE antibodies against Mal d1 in birch pollen endemic areas. This allergen was silenced by using the RNA interference (RNAi) approach (Gilissen et al., 2005). Longer storage shelf-life is desirable to minimize losses caused by decay. Suppressed expression of the *MADS8/9* gene delayed ripening in transgenic apple (Schaffer et al., 2013) and improved shelf-life. The shelf-life of strawberry is reduced because of loss of firm texture. Fruit softening in strawberry was delayed by antisense expression of pectate lyase (Jiménez-Bermúdez et al., 2002) and the β-galactosidase gene *FaβGal4* (Paniagua et al., 2016).

Table 4.1 Details of Genes Used for Imparting Resistance to Fungal and Virus Diseases and Insect Pests in Different Fruit Crops

Crop	Trait	Gene/s, Source of Gene	References
Apple	Apple scab	Chitinases, *Trichoderma* spp.	Bolar et al. (2000), Faize et al. (2003), and Schfer et al. (2012)
		HcrVf2, wild apple	Belfanti et al. (2004) and Szankowski et al. (2009)
		Puroindoline-b (*PinB*), wheat	Faize et al. (2004)
		Lc, maize	Flachowsky et al. (2010)
		Hordothionin, barley	Krens et al. (2011)
		Chalcone 3-hydroxylase, *Cosmos sulphureus*	Hutabarat et al. (2016)
	Fire blight	Attacin, *Hyalophora cecropia*	Ko et al. (2000)
		MpNPR1, apple	Malnoy et al. (2007)
		EPS-depolymerase, *Erwinia amylovora* phage phi-Ea1h	Flachowsky et al. (2008)
		Lc, maize	Flachowsky et al. (2010)
		Chalcone 3-hydroxylase, *C. sulphureus*	Hutabarat et al. (2016)
	Powdery mildew	*MhNPR1*, apple	Chen et al. (2012)
	Light brown apple moth	Avidin/streptavidin, potato	Markwick et al. (2003)
		Proteinase inhibitor, tobacco	Maheswaran et al. (2007)
Grapes	Powdery mildew	Chitinase, rice	Yamamoto et al. (2000)
		Endochitinases (*ech42 and ech33*) *and* N-acetyl-β-D-hexosaminidase (*nag70*), *Trichoderma* spp.	Rubio et al. (2015)
		Magainins, *Xenopus laevis*	Vidal et al. (2006b)
		Thaumatin-like protein *vvtl-1,* grape	Dhekney et al. (2011)
		Glyoxal oxidase (*VpGLOX*), *Vitis pseudoreticulata*	Guan et al. (2011)
		Pathogenesis-related protein gene *VpPR4-1*, *V. pseudoreticulata*	Dai et al. (2016)
		DUF642 gene*, Vitis quinquangularis*	Xie and Wang (2016)
	Downy mildew	Chitinase, β-1,3-glucanase genes	Nookaraju and Agrawal (2012)
	Botrytis cinerea	Endochitinases (*ech42 and ech33*) *and* N-acetyl-β-D-hexosaminidase (*nag70*), *Trichoderma* spp.	Rubio et al. (2015)
		DUF642 gene, *V. quinquangularis*	Xie and Wang (2016)
		Stilbene synthase gene (*Vst1*), grape	Dabauza et al. (2015)
	Pierce's disease	*rpfF*, *Xylella fastidiosa*	Lindow et al. (2014)
		Antimicrobial lytic peptide *LIMA-A,* synthetic	Li et al. (2015)
	Root-knot nematode	*16D10*, siRNA	Yang et al. (2013)

Continued

Table 4.1 Details of Genes Used for Imparting Resistance to Fungal and Virus Diseases and Insect Pests in Different Fruit Crops—cont'd

Crop	Trait	Gene/s, Source of Gene	References
Banana	*Fusarium* wilt	*MSI-99*, synthetic	Chakrabarti et al. (2003)
		Pflp, sweet pepper	Yip et al. (2011)
		Defensin gene (*Sm-AMP-D1*), *Stellaria media*	Ghag et al. (2014c)
		MusaBAG1, banana	Ghag et al. (2014b)
		VELVET and FTF1 siRNA	Ghag et al. (2014a)
		Thaumatin-like protein *(tlp)* gene, rice	Mahdavi et al. (2012)
		chit42, *Trichoderma harzianum*	Hu et al. (2013)
	Sigatoka disease	*MSI-99*, synthetic	Chakrabarti et al. (2003)
		ThEn-42, *T. harzianum* + *StSy*, grape + *Cu, Zn-SOD*, tobacco	Vishnevetsky et al. (2011)
	Black sigatoka disease	Chitinase, rice	Kovács et al. (2013)
	Banana *Xanthomonas* wilt	*Hrap*, sweet pepper	Tripathi et al. (2010)
		Pflp, sweet pepper	Namukwaya et al. (2012)
	Nematode resistance	Cystatin, maize + synthetic peptide gene	Roderick et al. (2012)
	Virus resistance	Viral replication initiation protein *(Rep) siRNA*	Shekhawat et al. (2012)
Citrus	Citrus canker	*hrpN* gene, *E. amylovora*	Barbosa-Mendes et al. (2009)
		Attacin A, *H. cecropia*	Cardoso et al. (2010)
		Xa21, rice	Mendes et al. (2010) and Li et al. (2014)
		NPR1, *Arabidopsis*	Zhang et al. (2010)
		Cecropin B *and* Shiva A, synthetic	He et al. (2011)
		pthA-nls, *X. axonopodis pv. citri*	Yang et al. (2011)
		CtNH1, *Citrus*	Chen et al. (2013)
		CsMAPK1, *Citrus*	de Oliveira et al. (2013)
		Dermaseptin, *Phyllomedusa spp.*	Furman et al. (2013)
		FLS2, *Nicotiana benthamiana*	Hao et al. (2016a)
		Modified theonin, *Citrus*	Hao et al. (2016b)
	Citrus scab	Attacin E (*attE*), *H. cecropia*	Mondal et al. (2012)
	Citrus greening	*NPR1*, *Arabidopsis*	Dutt et al. (2015)
		Modified theonin, *Citrus*	Hao et al. (2016b)
	Citrus Triesteza virus	*CTV*-392 *and CTV*-393, *Citrus Triesteza virus*	Olivares-Fuster et al. (2003)
		3DF1scFv, monoclonal antibodies	Cervera et al. (2010)
		CTV-CP, *Citrus Triesteza virus*	Muniz et al. (2012)
	Citrus psorosis virus	*ihpCP*, *siRNA*	Reyes et al. (2011)
Strawberry	Powdery mildew	Chitinase, rice	Asao et al. (1997)
		PpMlo1, peach	Jiwan et al. (2013)

Table 4.1 Details of Genes Used for Imparting Resistance to Fungal and Virus Diseases and Insect Pests in Different Fruit Crops—cont'd

Crop	Trait	Gene/s, Source of Gene	References
	B. cinerea	Thaumatin *II*	Schestibratov and Dolgov (2005)
		Chitinase gene *Ch5B, Phaseolus vulgaris*	Vellicce et al. (2006)
	Crown rot disease	β-1,3-glucanase gene *bgn13.1*, *T. harzianum*	Mercado et al. (2015)
	Broad-spectrum disease resistance	*NPR1*, *Arabidopsis thaliana*	Silva et al. (2015)
	Tolerance against weevil larvae	Trypsin inhibitor *CpTi,* cowpea	Graham et al. (1995, 1997, 2002)
Pear	Fire blight	Cecropin genes *SB-37* and Shiva-1 and Attacin E	Chevreau et al. (1999)
		Attacin E gene, *H. cecropia*	Reynoird et al. (1999)
		EPS-depolymerase, virus	Malnoy et al. (2005)
Plum	Plum pox virus	Coat protein, plum pox virus	Ravelonandro et al. (2000), Scorza et al. (1995), and Malinowski et al. (2006)
		Antifungal protein gene, *Gastrodia*	Nagel et al. (2008)

2.1.4 Vegetative Traits

Genetic improvement of fruit crops is often hampered by a long juvenile phase. Thus induction of early flowering and alteration of other vegetative characters such as plant height are desirable. Early flowering transgenic apple plants have been obtained by antisense expression of *MdTFL1* (Kotoda et al., 2006), overexpression of *BpMADS4* (Flachowsky et al., 2007), heat-induced expression of *FLOWERING LOCUS T* (Wenzel et al., 2013), and overexpression of *FT* genes (Tanaka et al., 2014). Transgenic apple with reduced plant size has been developed by overexpression of *Arabidopsis gai* (Zhu et al., 2008 and maize *Lc* genes (Flachowsky et al., 2010). Similarly, in citrus, constitutive expression of the citrus *FT* (*CiFT*) gene in trifoliate orange (*Poncirus trifoliata*) (Endo et al., 2005) and constitutive expression of *Arabidopsis LEAFY* or *APETALA1* genes (Pena et al., 2001) induced early flowering, thus reducing generation time. In plum, plant architecture, dormancy requirement, and flowering were altered by transformation with poplar *FT1* (Srinivasan et al., 2012). Expression of the citrus *FLOWERING LOCUS T* (*CiFT*) gene induced early flowering in pear and such transgenic plants flowered within 10 months (Matsuda et al., 2009). RNAi silencing of PCTFL-1 and PcTFL-2 resulted in early flowering in transgenic pear (Freiman et al., 2012). Transgenic pear containing the rolC gene exhibited reduced height, number of nodes, and leaf area (Bell et al., 1999).

2.2 TRANSGENIC RESEARCH IN FRUIT CROPS

2.2.1 Apple

Apple is one of the most important fruit crops worldwide. Commercial apple production is restricted to a few commercial cultivars such as Golden Delicious, Fuji, Gala, etc., which are sensitive to many fungal and bacterial diseases. Considering this, transgenic research has received much attention in

apple. In fact, apple was the first genetically transformed fruit tree using a disarmed Ti-binary vector (James et al., 1989), and stable integration and segregation of marker genes in progenies of these transgenic clones was reported (James et al., 1994, 1995). James et al. (1989) used the cultivar Greensleeves, and since then transformation of large numbers of commercial cultivars and rootstocks has been reported. Cultivars such as M26, Delicious, Royal Gala, Golden Delicious, New Jonagold, Marshall McIntosh, Elstar, and Fuji have been transformed with genes imparting resistance to several biotic and abiotic stresses.

Rootstocks, widely used for the cultivation of fruit trees, influence the growth of scion cultivar, fruit quality, and tolerate adverse conditions such as soil salinity and soilborne pathogens such as nematode. Transgenic rootstocks in combination with nontransgenic cultivars offer excellent prospects to overcome food safety issues associated with transgenic produce (Smolka et al., 2010). Therefore equal emphasis has been given to develop transgenic apple rootstocks with desirable properties. The *Agrobacterium rhizogenes* gene *rolB* is a proven root-stimulating gene and has been used in several plant species to stimulate adventitious root formulation. The *rolB* gene was used to transform dwarfing apple rootstock, and transgenic rootstocks showed increased rooting ability both in vitro and ex vitro (Zhu and Welander, 1999; Zhu et al., 2001). Zhu and Welander (1999) demonstrated that under nonlimiting nutrient conditions, the growth rate of transgenic rootstock was comparable to the untransformed rootstocks. Smolka et al. (2010) studied the effect of transgenic rootstock on the growth and development of a nontransgenic scion variety and observed that transgenic rootstocks reduced vegetative growth parameters as well as flowering and fruiting. However, fruit quality was not affected by transgenic rootstock.

Efficient regeneration is one of the main factors that affects rate and success of genetic transformation. Yepes and Aldwinekle (1994) examined several factors affecting organogenesis in leaf explants of six apple cultivars and reported basal medium, explant origin, explant orientation, and photosynthetic photon flux to be the main factors affecting morphogenesis. Though leaf segments are the most widely used explants, leaf thin cell layers (Dobránszki and Teixeira da Silva, 2011) and cotyledons (Dai et al., 2014) are successfully used for obtaining transgenic plants.

Constitutive promoters CaMV and nopaline synthase (nos) are the most frequently used promoters for transgene expression. However, these promoters lack tissue specificity and temporal and spatial regulation and therefore several attempts have been made to develop tissue-specific promoters. The heterologous Rubisco small subunit gene from tomato and soybean (Gittins et al., 2000) and *Brassica napus* extension (*ext*) A (Gittins et al., 2001) promoters directed transgene expression in vegetative tissues of transgenic apples. Specific promoters are also useful to direct expression of transgenes in response to a pathogen. Malnoy et al. (2006) demonstrated activation of pathogen-inducible *Gst1* (glutathione *S*-transferase) promoter after elicitation by fungal pathogens in transgenic apple.

The presence of antibiotic resistance genes as a selection method is probably the main concern of opposition to genetically modified crops by the populace. Therefore researchers have given much emphasis on the development of alternate selection systems for transgenic apple. Selection systems involving *daoI* gene conferring resistance to D-amino acids (Hättasch et al., 2009), the *Vr-ERE* gene imparting resistance to benzaldehyde (Chevreau et al., 2011), acetolactate synthase conferring herbicide resistance (Yao et al., 2013), as well as positive selection systems using the phosphomannose-isomerase (*pmi*)/mannose gene (Degenhardt et al., 2006) have been developed. Kortstee et al. (2011) used mutant *MYB10*, an anthocyanin production-inducing gene from apple, as a visual selection marker. However, as with antibiotic

resistance genes, in alternate systems marker genes remain inserted in the transgenic plant. Strategies to eliminate marker genes such as heat-mediated elimination (Herzog et al., 2012; Würdig et al., 2013) and chemically inducible R/Rs recombinase (Righetti et al., 2014) have been used in apple.

The cisgenic approach where the target gene is taken from a crossable wild relative provides another alternative for developing commercial cultivars with better acceptance by the public. The first report of cisgenic apple was by Vanblaere et al. (2011). They obtained a cisgenic apple by inserting the endogenous apple scab resistance gene *HcrVf2* under the control of its own regulatory sequences into the scab-susceptible apple cultivar Gala. The marker genes in these transformed lines were eliminated by chemically induced recombinase. Subsequently, development of cisgenic apple was reported by several workers (Kost et al., 2015; Krens et al., 2015; Würdig et al., 2015). Würdig et al. (2015) obtained cisgenic apple plants of two different cultivars by transferring the scab resistance gene *Rvi6* using the *Flp/FRT* recombinase system (Würdig et al., 2013). Krens et al. (2015) used a marker-free approach and marker gene elimination by chemically induced recombinase. The performance of such transformed apple lines was evaluated in the field and the introduced cisgene *Rvi6* was found to be stable in phenotype over multiple years and performed similar to the gene in a natural configuration with respect to resistance level, resistance spectrum, and plant organs. Similarly, a fire blight-resistant cisgenic apple line was regenerated using the cisgene *FB_MR5* from wild apple *Malus* × *robusta* (Kost et al., 2015).

2.2.2 Banana

Bananas and plantains belong to the genus *Musa* and their fruits are one of the most important foods. Conventional methods of crop improvement often have limited success because of various levels of ploidy, limited genetic variability, and low fertility. Genetic transformation thus offers enormous potential for improvement of commercial varieties. Sagi et al. (1995) first reported the genetic transformation of banana by particle bombardment. May et al. (1995) reported the generation of transgenic banana plants through *Agrobacterium*-mediated transformation using apical meristem and corm tissues. *Agrobacterium*-mediated transformation of embryogenic cells of variety Rasthali (Ganapathi et al., 2001), plantain cultivar Gonja manjaya (*Musa* spp. AAB) (Tripathi et al., 2012), and *Musa acuminata* cv. Matti (AA) (Rustagi et al., 2015) have been successfully achieved. *Agrobacterium* strains also affect transformation efficiency as demonstrated by Yip et al. (2011), who used two strains of *Agrobacterium*, namely, C58C1 and EHA105, to transform bud slices of cultivars and Gros Michel belonging to two subgroups of genotype AAA. However, strain EHA105 was more effective in Pei Chiao, and the C58C1 strain resulted in higher transformation frequency in Gros Michel.

Besides target tissue and *Agrobacterium* strains, several other factors were established to affect transformation efficiency. Chong-Pérez et al. (2012b) studied the effect of incubation time and concentration of acetosyringone and spermidine and reported the highest transformation efficiency when embryogenic cell suspensions were infected for 6 h in medium supplemented with 200 μM acetosyringone and 1.0 mM spermidine. As compared to solid medium, selection of liquid media improved transformation efficiency of Furenzhi (*Musa* spp. AA group) (Hu et al., 2013). A protocol for high-throughput regeneration and transformation of embryogenic cell suspensions of three varieties Cavendish Williams, Gros Michel, and Sukali Ndiizi reported by Tripathi et al. (2015) involved cocultivation of embryogenic cell suspensions with *Agrobacterium* followed by transfer of agro-infected embryogenic cells on embryo development medium without antibiotic selection for 1 week and then transfer to selective medium supplemented with 100 mg/L kanamycin. Shoots obtained on selective medium were shifted to proliferation medium for

multiplication and maintenance. With this modification, 20–70 independent transgenic events per mL settled cell volume of embryogenic cell suspensions were regenerated on selective medium.

For obtaining tissue-specific expression of transgenes, tissue-specific promoters have been analyzed. Ghosh et al. (2012) analyzed banana fruit-specific promoters using transient expression in embryogenic cells of banana cultivar Robusta and reported glucanase promoters useful for fruit-specific expression of target genes. Similarly, generation of marker-free transgenic banana using different techniques has been achieved. Chong-Pérez et al. (2012a) reported successful use of heat shock-induced excision of selectable marker genes by the *Cre-lox* site-specific recombination system to obtain marker-free transgenic plants of cultivar Grand Naine. Heat shock promoters pGmHSP17.6-L and pHSP18.2, from soybean and *Arabidopsis*, respectively, were used and showed more than 40% excision efficiency (Onyango et al., 2016).

2.2.3 Grape

Grape (*Vitis vinifera* L.) is grown worldwide under varied climatic conditions for fresh consumption and is processed for wine and raisin production and for obtaining secondary metabolites. The commercial grape varieties are susceptible to several diseases, pests, and abiotic stresses. Several wild species of *Vitis* are known to carry genes for tolerance to different biotic and abiotic stresses and during the last several decades attempts have been made to introgress these traits into commercial varieties. However, since wine aroma and flavor are cultivar specific, bred varieties often lack the wine characteristics and hence are not easily accepted by consumers. Genetic transformation thus offers a good opportunity to transfer only a specific gene in other elite popular cultivars of wine grape. The first report of successful transformation of grapevine was by Mullins et al. (1990) who used *Agrobacterium*-mediated transformation to obtain transgenic plants of *Vitis rupestris* Scheele using the hypocotyl of mature somatic embryos as explant. Nakano et al. (1994) obtained transgenic grapevine plants via somatic embryogenesis after cocultivation of embryogenic calli with an engineered *A. rhizogenes* strain followed by selection of secondary embryos for kanamycin resistance. A biolistics method was used to transform embryogenic cell suspensions of var. Chancellor and transgenic plants were obtained (Kikkert et al., 1996). Transformation by the biolistics method was further improved by the use of gold particles for bombardment using stepwise selection on kanamycin medium and embryo induction at 27°C. This method was used to cotransform *nptII* and antimicrobial peptide genes to Chardonnay (Vidal et al., 2003). After these initial reports, several researchers reported protocols for different genotypes (Bornhoff et al., 2005; Li et al., 2006; Dhekney et al., 2008; López-Pérez et al., 2008). While several of these protocols were genotype specific, Iocco et al. (2001) developed a protocol suitable for genetic transformation of major wine grape cultivars. Transgenic plants of var. Thompson Seedless were also obtained through regeneration via organogenesis (Mezzetti et al., 2002). They used slices of meristematic bulk with a capacity to differentiate adventitious shoots for *Agrobacterium*-mediated transformation. Dutt et al. (2007) used apical shoot meristem to obtain transgenic plants of Thompson Seedless using *Agrobacterium*-mediated transformation. The meristems were subjected to a dark growth phase before wounding to obtain transformant plants. Fujita et al. (2009) demonstrated *in planta* transformation for grapevine using dormant buds, while Ben-Amar et al. (2013) used nondetached leaves for *in planta* transformation.

Several factors affect transformation efficiency and have been studied by several workers in grape. Some of the important factors were concentration of kanamycin in selection medium (Wang et al., 2005), cell density, cocultivation time, etc. The production of transgenic embryo lines on different

culture media was genotype dependent, and similarly the effect of cotyledon excision of germinated embryos on the recovery of transgenic plants varied with variety (Dhekney et al., 2009). Better plant recovery was obtained in *V. vinifera* and *Vitis riparia* as compared to other species or interspecies hybrids. Dabauza and Velasco (2012) described the development of variety-specific highly efficient genetic transformation protocols for table grape varieties Sugraone and Crimson Seedless. In this study, kanamycin concentration in selection media, *Agrobacterium* density used to infect embryogenic calli, and amount of calli varied between two varieties. Genotype specificity and choice of selection antibiotic for improved transformation efficiency were also observed by Xie et al. (2016) while using meristematic bulk. While transforming embryogenic callus, proembryonic masses, and somatic embryos using *Agrobacterium*-mediated transformation, Zhou et al. (2014) found addition of kanamycin at 4 weeks after cocultivation and alternating culture on two media, one containing 0.2 mg/L benzyladenine and the other 0.25 mg/L kinetin, to be useful for the recovery of transformed plants.

Several approaches have been used to develop transgenic grape plants without selection markers and vector sequences. Minimal gene cassette (MC) technology, which uses only the promoter, open reading frame, and terminator and lacks a vector backbone sequence, has been used for biolistics transformation of grapevine (Vidal et al., 2006a; Sanjurjo et al., 2013). No significant differences were found in transgene transcription between lines from MC and circular plasmid transformation (Vidal et al., 2006a), and 3′ end cassette protection was found effective for successful protein expression using the MC technology (Sanjurjo et al., 2013). A chemically inducible site-specific *cre/loxP* recombination system (Dalla Costa et al., 2009) and heat shock-inducible recombinase (Dalla Costa et al., 2016) were found effective in removal of selectable marker genes in transgenic grapevines. As an alternative to antibiotic-based selection genes, Li et al. (2011) developed an anthocyanin-based quantitative reporter system using the *VvMybA1* gene of grapevine. When compared with other reporter genes such as *GFP* and *GUS*, no difference in transient gene expression frequencies was recorded between the *MybA1* gene and *GUS* (Kandel et al., 2016). However, visual levels of stable gene expression were higher in GFP- and GUS-expressing cultures compared to MybA1-expressing cultures.

Field safety assessment of the transgenic grapevine-expressing coat protein gene of grapevine fanleaf virus (GFLV) indicated that such transgenic plants in the field neither affect the molecular diversity of indigenous GFLV populations nor assist the emergence of viable GFLV recombinants to detectable levels (Vigne et al., 2004).

2.2.4 Citrus

Genus *Citrus* includes commercial fruits such as oranges, lemons, grapefruit, and several other species that are used as rootstocks. The fruits belonging to *Citrus* are rich in many nutrients especially vitamin C and hence constitute an important part of the daily diet. However, the citrus industry suffers because of several diseases such as citrus canker, Huanglongbing, and citrus tristeza. Kobayashi and Uchimiya (1989) used citrus protoplast by direct DNA transfer to develop genetically modified plants; however, the first report of transgenic citrus plant was by Vardi et al. (1990) through polyethylene glycol-mediated genetic transformation of protoplast. Subsequently, *Agrobacterium*-mediated transformation was obtained by several workers (Moore et al., 1992; Kaneyoshi et al., 1994; Peña et al., 1995a,b; Bond and Roose, 1998).

Different types of explants, i.e., internodal segments, epicotyls, embryogenic suspension cultures, embryogenic calli, axillary buds, vegetative tissues, and leaf segments, were used for genetic transformation study in citrus (Almeida et al., 2003a,b; Ahmad and Mirza, 2005; Molinari et al., 2004;

Dutt and Grosser, 2010; Al Bachchu et al., 2011; He et al., 2011; Fávero et al., 2012; Khan et al., 2012). In a comprehensive study, Dutt and Grosser (2009) evaluated several parameters affecting *Agrobacterium*-mediated transformation of juvenile explants of Carrizo (*Citrus sinensis* Osb. × *Poncirus trifoliata* L. Raf.), Duncan (*Citrus paradise* Macf.), Hamlin [*C. sinensis* (L.) Osbeck], and Mexican lime (*Citrus aurantifolia* Swingle) cultivars. They observed that a short preincubation of explants in hormone-rich liquid medium, cocultivation duration, addition of acetosyringone to cocultivation medium, and cell density of the *Agrobacterium* suspension played an important role in transformation efficiency. Transformation efficiency and regeneration potential also showed a relation with the season and was highest when explants were collected early in spring (Orbović et al., 2011).

Besides *Agrobacterium*-mediated transformation, direct gene transfer using particle bombardment was also frequently used for obtaining transgenic citrus plants. The first report of successful transformation of citrus embryogenic cells resulting in stable transgenic embryos and plantlets using particle bombardment was by Yao et al. (1996). In this study, pretreatment of cells with high osmotic potential (0.3 M sorbitol + 0.3 M mannitol) was found to enhance both transient and stable transformation. Bespalhok Filho et al. (2003) studied different parameters for optimum transformation of epicotyl explants of Carrizo citrange and observed that high osmolarity medium, type of particle, helium pressure, and distance between explant and DNA/particle holder affected transformation efficiency. Boscariol et al. (2003) developed the transgenic citrus plants with a pmi/mannose selection system. Furthermore, Ballester et al. (2007) used the MAT system (Multi-Auto-Transformation) which combines the isopentenyl transferase (*ipt*) gene for positive selection. Dutt et al. (2010) used the recombinase system R/RS for removal of marker genes from transgenic plants.

2.2.5 Strawberry

Strawberry (*Fragaria* × *ananassa* Duch.) is one of the most popular fruit crops because of its unique flavor and nutritious properties. Most of the commercial varieties are octoploid and heterozygous in nature, thus proving difficult when using conventional methods of improvement. Genetic transformation methods provide tremendous opportunities for the transfer of desirable genes to a cultivar. The first successful report of genetic transformation of strawberry was by James et al. (1990). They used two disarmed Ti-binary vectors in *Agrobacterium* to transform leaf and petiole explants and obtained viable fertile transgenic strawberry plants. The transgenes *nptII* segregated in a 3:1 ratio in T1 progenies. Nehra et al. (1990) developed an efficient genetic transformation protocol for strawberry cv. Redcoat using *Agrobacterium tumefaciens*, which relied on high regeneration from leaf discs. Subsequently, *Agrobacterium*-mediated transformation has been used to obtain transgenic plants of different cultivars (El Mansouri et al., 1996; du Plessis et al., 1997; Haymes and Davis, 1998). The technique of *Agrobacterium*-mediated transformation has been modified by several workers to improve transformation efficiency. Mesa et al. (2000) combined *A. tumefaciens* and biolistics bombardment to transform strawberry cv. Chandler. In the biolistics bombardment method, gold particles were coated with *Agrobacterium* cells and used to bombard leaf explants, which resulted in enhanced transformation frequency. Hanhineva and Kärenlampi (2007) reported the development of transgenic strawberry in temporary immersion bioreactors by *Agrobacterium*-mediated gene transfer. Pantazis et al. (2013) developed an efficient *Agrobacterium*-mediated transformation method for *Fragaria vesca* by using an Ac/Ds transposon tagging construct and high-throughput paromomycin spray assay to identify the transformants.

Several factors affecting transformation efficiency in strawberry have been determined. Barceló et al. (1998) reported that the leaf discs from in vitro cultures proliferating in the presence of 2.21 μM kinetin were the best explant for transformation. Alsheikh et al. (2002) observed that the transformation efficiency for antibiotic-sensitive *F. vesca* and *Fragaria semperflorens* could be improved by using antibiotic carbenicillin for selection and suitable *Agrobacterium* strain. They achieved optimal transformation efficiency (15%) by the appropriate use of explant type and age, leaf-disc orientation, inoculation time, and phenolic compounds for bacterial virulence induction. Incubation of *A. tumefaciens* with acetosyringone and indole acetic acid (Gruchała et al., 2004), age of explant (Oosumi et al., 2006), preculture, and preselection on antibiotic-containing medium (Husaini, 2010; Haddadi et al., 2015) were the other key factors to affect the transformation efficiency in strawberry.

Tissue-specific promoters have been characterized for their use in the genetic transformation of strawberry. Zhao et al. (2004) used a vector with an expression cassette bearing the GUS reporter genes under the control of *Arabidopsis* sucrose-H+ symporter gene (*AtSUC2*) promoter, a phloem-specific promoter. This promoter regulated the expression of transgenes specifically in phloem of leaves, petioles, and roots of transgenic strawberry plants. Similarly, Schaart et al. (2011) used the strawberry fruit-specific promoter and terminator of strawberry expansin gene (*FaExp2*) along with a recombinase-based system for elimination of marker genes in transformed plants. They demonstrated tissue-specific expression of strawberry polygalacturonase inhibiting the protein gene (*FaPGIP*) in transgenic plants.

2.2.6 Plum

Plum (*Prunus domestica*) is one of the major commercial stone fruits. Mante et al. (1991) reported first the protocol of genetic transformation of plum by cocultivation of hypocotyl explant with *Agrobacterium* harboring a plasmid containing genes for *nptII* and *GUS*. Yancheva et al. (2002) transformed the leaf explants with *A. tumefaciens* strains EHA105 and LBA4401 harboring vector pBIN19 with *GFP* and *nptII* with CaMV 35s promoter. An improved protocol for the transformation of leaf explant of plum cv. Startovaya was developed by Mikhailov and Dolgov (2007). They reported high rate of transformation (80%) with *A. tumefaciens* strain CBE21 carrying vector pNOV35S-GFP containing the synthetic codon optimized *gfp* gene and *pmi* gene for positive selection. Hygromycin as a selectable marker was also used by Tian et al. (2009) for *Agrobacterium*-mediated transformation of embryonic axes of mature seeds. *A. tumefaciens*-mediated transformation of two cultivars of Japanese plum (*Prunus salicina*) was reported by Urtubia et al. (2008). Various factors such as *Agrobacterium* strain, doses of antibiotic, hormone concentration, and composition of cocultivation media were involved in the success of the genetic transformation (Gonzalez Padilla et al., 2003; Song et al., 2011; Petri et al., 2008).

In an attempt to develop transgenic plants without an antibiotic resistance gene as selection marker, a *pmi*/mannose system as positive selection method was successfully developed by several workers (Mikhailov and Dolgov, 2007; Wang et al., 2013; Sidorova et al., 2016). In addition, Petri et al. (2011) used an intron-hairpin-RNA-E10′ marker-free construct carrying the PPV coat protein (*ppv-cp*) for transformation to produce marker-free transgenic plum plants. Field evaluation of transgenic plum plants established effectiveness of transgenes in imparting stable resistance against PPV. Malinowski et al. (2006) evaluated clones of plum transformed with the coat protein gene of PPV under high infection pressure at two sites differing in climate conditions and virus isolates. Transgenic plants showed high resistance to PPV at both the sites even after 7–8 years of test. Safety assessment of transgenic plum plants was found not to have any negative impact on the environment as reported by Scorza et al. (2013).

2.2.7 Pear

Pear (*Pyrus* spp.) is one of the most widespread fruits with considerable economic and health values. Mourgues et al. (1996) established an efficient and reproducible method for genetic transformation of pear using *A. tumefaciens* using leaves of in vitro-raised plants. Up to 42% of inoculated leaves produced transformed buds or bud clusters. The transgenic plants could be successfully acclimatized in the glasshouse. Subsequently, several researchers successfully transformed different explants of pear and determined critical factors affecting the transformation. Kaneyoshi et al. (2001) reported a method for *A. tumefaciens*-mediated transformation of cotyledons of Asian pea pear *Pyrus betulaefolia*, which is used as rootstock. Among the various *Agrobacterium* strains tested, only strain AKE10 was found suitable for transformation. Gao et al. (2002) developed a method of transformation of cotyledonary explants. High transformation efficiency (82.7%) was obtained when cocultivation was in the dark for 7 days. Matsuda et al. (2005) developed a transformation method suitable for cultivars with low ability to regenerate by using leaf disc and axillary shoot meristem. In this study, selection in 5 mg/L kanamycin and 375 mg/L carbenicillin resulted in production of transformed shoots at a frequency of 4.8%. Furthermore, Sun et al. (2011) reported a fivefold increase in transformation frequency of leaf segments by cocultivation of *Agrobacterium* in leaf segments on a liquid induction medium. Transformation of nodal cuttings using *A. rhizogenes* was reported by Bosselut et al. (2011). They used a vector containing reporter gene *Egfp*. The agro-inoculated cuttings were placed in rooting medium. The rooted cuttings were successfully used to produce transgenic composite plants and hairy roots. Nakajima et al. (2013) obtained transformed adventitious shoots in Japanese pear by cocultivating cotyledonary explant with *A. tumefaciens*.

3. FUTURE PROSPECTS

Though considerable progress has been made in transgenic research in many fruit crops, a lot needs to be done to make the transgenic approach the preferred method of fruit improvement. Major concerns such as consumer acceptance and environmental safety need to be addressed. However, advancements in the field of omics technologies such as genomics, transcriptomics, and metabolomics have opened up several new prospects. These approaches will be useful to identify new and better candidate genes for different traits within the same species and/or genus, which could be used for cisgenic plant production. The advent of genome editing techniques such as CRISPR/CAS9 will enable precise manipulation of target genes and is expected to bring much needed impetus to fruit improvement programs.

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CHAPTER

GENETIC ENGINEERING OF TEMPERATE FRUIT CROPS

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1. INTRODUCTION

Genetic transformation is one of the novel approaches in plant breeding that can serve as a boon for improvement of perennials such as fruit crops wherein crop improvement is often difficult because of their long juvenile period, heterozygosity, and reproductive barriers (cross- and self-incompatibility). Moreover, since most of the adapted cultivars lack only one or a few characters, transferring the gene of interest can enhance the efficiency of the cultivar and preserve its original identity. Genetic engineering is occasionally referred to as transgenic biotechnology and involves the transfer of a single or a few genes between unrelated species, making use of recombinant DNA technology. Among various methods of genetic transformation, namely, *Agrobacterium* mediated, particle bombardment, protoplast transformation, polyethylene mediated, electroporation, microinjection, physical abrasives, laser beams, in planta transformation, and vacuum infiltration (Senior, 1998), *Agrobacterium*- and particle bombardment-mediated gene transfer are the two most commonly employed methods of generating transgenic plants. However, *Agrobacterium*-mediated gene transfer is the most preferred method used by many researchers. Successful genetic transformation depends upon the in vitro regeneration potential of the plant. However, hitherto conceded fruit crops are highly recalcitrant for in vitro culture (Gomez-Lim and Litz, 2004). In the past few decades, notable works have been done for developing efficient regeneration systems in perennial fruit and nut crops (Mante et al., 1989; Scorza et al., 1994; Srinivasan et al., 2012a,b; Petri et al., 2012a,b; Wang et al., 2013a,b) but the development of an efficient regeneration system that could allow the consistent production of many regenerated plants is still being researched. The regeneration capacity of individual species, which determines the success of genetic transformation, is in turn governed by various factors, most significantly by cultivar or genotype, type of explant, and the degree of determination in tissue (Litz and Padilla, 2012). Several explants have been tried in different temperate crops for transformation. Use of leaf explants for generation of adventitious shoots has been documented in apple since 1983 (Liu et al., 1983a,b). Somatic embryos have been reported as the most suitable explant for transformation in walnut (Escobar et al., 2000; Tang et al., 2000). Similarly, hypocotyl segments in plum and in vitro-derived leaves from shoot apices in apricot and peach can be quoted as some of the prominent examples (Yancheva et al., 2006; Gentile et al., 2002; Petri et al., 2008a,b). In addition to explants, optimization of tissue culture conditions specifically suited to each species and sometimes cultivars are paramount in achieving transformation with increased rate of efficacy. The two methods of tissue culture that have generally been employed for regeneration of transgenic

Genetic Engineering of Horticultural Crops. http://dx.doi.org/10.1016/B978-0-12-810439-2.00005-2

plants are organogenesis and somatic embryogenesis. In organogenesis, regeneration of plant occurs by formation of organs such as shoots or roots on explants, whereas somatic embryogenesis involves the formation of bipolar embryos from cells other than gametes or products of gametic fusion (Pena and Seguin, 2001; Rai et al., 2010). However, because of advantages such as higher rate multiplication and potential for scale-up, somatic embryogenesis has been recommended as the most suitable target for gene transfer (Merkle and Dean, 2000).

2. GENETIC IMPROVEMENT OF TEMPERATE FRUITS

Fruits are often termed protective foods because they are the major sources of micronutrients such as vitamins and minerals and phytonutrients such as antioxidants, and are rich sources of dietary fibers, which form major constituents of a healthy diet for a healthy and sound body (Klee, 2010). Among fruits, temperate fruit crops such as apple, pear, peach, plum, apricot, walnut, cherry, etc. are excellent sources of phytonutrients. However, the efficient utilization of this valuable gift of nature could be achieved only by helping them to realize their full potential. Temperate fruit crops are confronted with several problems such as diseases, insect pests, and early frost, and others such as self-incompatibility, short shelf-life, uncontrolled ripening, etc. Hence human intervention for the improvement of agronomically and horticulturally desirable traits has become inevitable. Genes that confer resistance to biotic and abiotic stresses or alter other traits are generally introduced into adapted varieties with minor changes, or in local germplasm or other breeding lines including wild species or genera (Varshney et al., 2011). However, conventional breeding approaches are not always feasible because of the limitations associated with them. Genetic transformation offers a new tool for the development of plants suited to the specific needs of humankind. In the past two decades, genetic engineering in fruit crops has mainly focused on developing disease resistance traits, increasing tolerance to abiotic stresses such as salt, drought, and frost, and modifying plant growth habit and quality parameters. Several genes from diverse sources have been used for incorporating the desired character into the target plant species. The insecticidal protein gene isolated from *Bacillus thurengiensis* effective against lepidopterans, dipterans, and coleopteran insects, and cowpea trypsin inhibitor gene isolated from cowpea that inhibits insect feeding, are the most widely used genes for conferring resistance against insect pests in plants (Aronson et al., 1986; Herrnstadt et al., 1986; Hilder et al., 1989). Although the use of these genes has mostly been validated in field crops such as cotton and maize (Christou et al., 2006), their use in temperate fruit crops can help in alleviating the problems of insect pests and need extensive research. Lytic peptide genes, naturally occurring (*cecropin, attacin E*) and synthetic analogs of cecropin (*SB-37 and Shiva 1*), have widely been used in apple to develop cultivars resistant to apple scab (Liu et al., 2001; Ko et al., 2002). Control of viral diseases using the *virus coat protein (CP)* gene has been most extensively researched because control of viral disease is not possible efficiently using other plant protection measures. "HoneySweet" plum, a plum pox virus (PPV)-resistant transgenic plum transformed with *CP* gene of PPV via *Agrobacterium tumefaciens*-mediated transformation, has recently been validated for cultivation in the United States (Scorza et al., 2013). Among the abiotic stresses, resistance is mostly coveted for cold, drought, and salt and in some cases metal toxicity. Under drought or salinity stress, plants respond primarily in two ways, either as osmotic stress resulting in disruption of homeostasis and ion distribution in the cell or as oxidative stress, which may lead to denaturation of functional and structural proteins (Munns and Tester, 2008; Jewell et al., 2010).

Stress-specific genes used in transgenic production can be classified on the basis of the type of control they exert and can be grouped broadly as: (1) genes involved in signal transduction pathways and transcriptional control such as mitogen-activated protein kinase (MAPK), calcineurin B-like-interacting protein kinase, son of sevenless kinase, and transcription factors such as AP2/ERF, bZIP, MYB, MYC, NAC, Cys2His2 zinc finger, and WRKY; (2) genes involved in the protection of membranes and proteins such as heat shock proteins and late embryogenesis abundant (LEA) proteins, synthesis of osmoprotectants such as proline, betaine, sugars and sugar alcohol, and polyamines, and detoxification or elimination of reactive oxygen species; and (3) genes involved in water and ion uptake and transport such as aquaporins and ion transporters (Wang et al., 2003; Bhatnagar-Mathur et al., 2008; Ashraf and Akram, 2009; Jewell et al., 2010). Several genes such as *Osmyb, MdNHX1, MdCIPK6L, DREB1b*, etc. have been employed for developing tolerance/resistance against abiotic stress such as cold, salt, and drought in temperate crops such as apple and grapevine (Jin et al., 2009; Wang et al., 2012; Li et al., 2010; Pasquali et al., 2008). Other than incorporating resistance, improving the keeping quality and yield has also been attempted using the transgenic approach. Short shelf-life is a major problem in climacteric fruits such as apple, peach, pear, etc., which reduces their marketability and economic value. Strategies that can delay fruit ripening could be very useful in increasing the shelf-life of these fruits. Many biotechnological approaches such as antisense RNA technology have been applied to reduce autocatalytic ethylene production by suppression of *ACC synthase* or *ACC oxidase (ACO)* genes, which are involved in ethylene biosynthesis (Matas et al., 2009). Several fruit crops have been transformed using *ACC* or *ACO* genes in antisense orientation to extend their shelf-life (Callahan and Scorza, 2007; Bapat et al., 2010; Litz and Padilla, 2012). Although the transgenic approach provides a lucrative opportunity to enhance crop productivity and profitability by addressing specific problems in crop production, and considerable success has been achieved herein, the field evaluation and commercialization of these transgenic plants still have a long way to go.

3. GENETIC ENGINEERING IN TEMPERATE FRUITS

3.1 APPLE

Apple (*Malus* spp.), belonging to the family Rosaceae and subfamily Pomoideae, is the most important temperate fruit crop in the world. The cultivated apple (*Malus* × *domestica* Borkh.) is considered to be an amphidiploid with a basic haploid chromosome number of $n = 17$, which is common among other members of the same subfamily, namely, pear and quince. Hybridization between two remote ancestors, Prunoideae ($n = 8$) and Spiroideae ($n = 9$), has been suggested to be the probable reason for the origin of the basic haploid chromosome of $n = 17$ (Challice and Westwood, 1973). Apple is one of the highly nutritious fruits and its significance in human nutrition can be judged by the long known adage "An apple a day keeps the doctor away." Apart from being a crowned fruit crop, apple has gained the status of a model crop for genomic research in woody perennials because of its small genome size (750 Mb/haploid), availability over 300,000 expressed sequence tags, bacterial artificial chromosome libraries, linkage map, first-draft physical map, and a successful genetic transformation and regeneration system (Tatum et al., 2005; Newcomb et al., 2006; Han et al., 2007; Keller-Przybyłkowicz and Korbin, 2013). Velasco et al. (2010) reported a high-quality draft genome sequence using Golden Delicious and showed genome-wide duplication had resulted in the transition from nine ancestral chromosomes to 17 chromosomes in the Pyreae. Although more than 10,000 cultivars of apple have been

documented worldwide, cultivation is dependent mainly on a few adapted cultivars with superior characters and is often hindered by the incidence of pests and diseases and other natural constraints such as spring frosts.

Therefore breeding of apple cultivars and rootstocks suited for answering the different needs of growers, namely, dwarf tree stature resistance to biotic and abiotic stresses, regular bearing, and improved nutritional qualities, remains the major challenge of fruit breeders. Undoubtedly, myriad works have been done in apple exploiting the conventional breeding methods and their success deserves to be acknowledged; nevertheless, these methods are slow and lack full efficacy. Releasing a new cultivar using conventional methods could take about 10 years, and furthermore it could take almost four decades to introduce and establish it in the market (Korban and Chen, 1992; Brown, 1992). Improvement of this adapted cultivar for just one or two characters will again necessitate crossing with suitable donors, wherein, if improvement occurs, it will be at the cost of original characters for which it was bred in the first place. Hypothesizing such a case, introducing the trait of interest in an elite variety, and conserving all the other characters through genetic engineering, by far appears to be the perfect choice for such crops (Borejsza-Wysocka et al., 2010). The possibility of producing transgenic apple was for the first time reported by James et al. (1989) when they succeeded in transforming the apple cultivar "Greensleeves" using leaf disc as explant, and disarmed Ti-binary vector plasmid pBIN6 in an LBA4404 strain of *A. tumefaciens* followed by kanamycin selection; however, the efficiency of transformation was rather low ranging between 0.1% and 0.5% on a per explants basis. The GUS gene in transformed Greensleeves plants showed stable expression in the fruits and segregated in normal Mendelian fashion in the progeny (James et al., 1995, 1996). Ever since this first report of transformation, several works have been done to transform different apple cultivars such as Delicious (Sriskandarajah et al., 1994), Royal Gala (Yao et al., 1995), Golden Delicious, Gala, Elstar (Puite and Schaart, 1996), Jonagold (De Bondt et al., 1996), McIntosh Wijcik (Song et al., 2000), Orin (Kanamaru et al., 2004), Fuji (Seong et al., 2005), Pinova (Hutabarat et al., 2016), etc. for their improvement, and many modifications have been reported in the techniques of transformation because all the genotypes do not respond to the same protocols in a similar manner. Since many of the important cultivars of apple are susceptible to bacterial and fungal pathogens, most studies on genetic transformation in apples have focused on improving their resistance to these pathogens.

Fire blight, caused by a Gram-negative necrogenic endobacterium *Erwinia amylovora*, has been known as one of the most destructive diseases in apple and pear for more than two centuries (Baker, 1971), and incidentally is the first plant disease reported to be of bacterial origin (Norelli et al., 2003). Different genes, including the *lysozyme* gene from bacteriophage T4 and the *attacin E* gene from *Hyalophora cecropia*, have been used to confer resistance against fire blight in apple (Ko et al., 1997). *Lysozyme* gene from bacteriophage *T4* transferred to Pinova apple could increase the resistance of some of the transgenic lines under greenhouse conditions (Hanke et al., 2000). *T4 lysozyme* and *attacin E* gene were reported to increase the resistance of transgenic Galaxy apple against fire blight in preliminary growth chamber tests (Ko et al., 2002). The transgenic lines containing both attacin E and *T4 lysozyme* genes were not found to be more resistant than the transgenic lines possessing either of these genes, suggesting that there is no in plant synergy between these two genes with regard to resistance against *E. amylovora* (Ko et al., 2002). Transgenic lines of "Royal Gala" obtained with hen egg white *lysozyme (HEWL)* genes exhibited resistance to fire blight in both field and greenhouse conditions (Norelli et al., 1999). The effector protein harpin produced by *E. amylovora* is reported to increase resistance against fire blight by inducing the natural plant defense (Reddy et al., 2000). Transgenic

M.26 lines containing a *harpin* gene *(hrpN)* driven by the gst1 pathogen-inducible promoter displayed increased fire blight resistance in growth chamber and in preliminary field tests (Norelli et al., 2003). Cecropins include a family of naturally occurring lytic peptides present in the giant silk moth (*Hyalophora cecropia*) (Boman and Hultmark, 1987). Diploid and tetraploid transgenics of Royal Gala apple transformed by *MB39*, a modified cecropin *SB37* gene placed under the control of a wound-inducible osmotin promoter from tobacco, exhibited significant resistance against *E. amylovora* compared to nontransformed Royal Gala lines (Liu et al., 2001).

Apart from fire blight, apple scab caused by the fungus *Venturia inaequalis* is the most widespread fungal disease of apple in most of the apple-growing regions receiving high spring and summer rainfall. The disease alone causes severe losses in apple production and up to 15 fungicidal spray treatments can be required to produce scab-free apple. Apple resistance contributing 17 genes have been identified and their global positions have been located (Bus et al., 2011). Two genes, namely, *Rvi15 (Vr2)* and *Rvi6* (*Vf*, formerly *HcrVf2*), have been found to be more useful in transformation of susceptible cultivars (*Vf*: Belfanti et al., 2004; *Vr2*: Schouten et al., 2014; Joshi et al., 2011; Würdig et al., 2015). A *Vf* locus conferring resistance against scab was identified in the wild apple species *Malus floribunda* and has been widely used to breed cultivars resistant to this malady (Korban, 1998). Apple cultivars with the *Rvi6 (Vf)* gene have been developed using marker-assisted selection and are commercialized, e.g., Aori 25′ (Kudo et al., 2013). However, because breakdown of resistance conferred by a single gene has been observed at several experimental farms (Bénaouf and Parisi, 2000; Parisi et al., 2006), accumulation of multiple resistance genes has become an essential strategy. Belfanti et al. (2004) showed that *A. tumefaciens*-mediated transformation of scab-susceptible apple cv. Gala with the *HcrVf2* gene under the control of constitutive promoter CaMV35S resulted in development of resistance against *V. inaequalis* in the susceptible cultivar. However, this resistance was effective only toward races-1–5, and not toward race-6 (Silfverberg-Dilworth et al., 2005). Similar results were shown by Malnoy et al. (2007) when the *Vfa2* gene was transferred in Galaxy and McIntosh apple, under the control of its own promoter. Sixteen single-nucleotide polymorphism (SNP) markers for resistance selection have been developed and have mapped *Rvi12 (Vb)* on *LG12* of *Malus baccata* Hansen's *baccata #2* (Padmarasu et al., 2014).

To circumvent issues of suggested or perceived risks of genetic modification regarding the presence of foreign gene(s) in the genome, cisgenics techniques have been utilized in some transgenic apples including scab resistance (Vanblaere et al., 2011; Krens et al., 2015; Würdig et al., 2015; Chizzali et al., 2016; Igarashi et al., 2016). Cisgenesis is the genetic modification of a recipient plant that uses all parts of the transgenes including the promoter and terminator, derived from sexually compatible plants (Holme et al., 2013). The resistance of apple to heat and low temperature was increased by overexpression of the cytosolic ascorbate peroxidase gene (Artlip et al., 2006). Resistance to freezing injury was 1–3°C greater and that to heat stress up to 7°C greater in the transformed lines (Artlip et al., 2006). Enzymatic browning caused by polyphenol oxidase is one of the major problems in apple and an apple cultivar with reduced browning can be very useful for the food industries. Murata et al. (2001) attempted to develop a transgenic apple lacking polyphenol activity through *Agrobacterium*-mediated transformation using the antisense *PPO* gene. Transgenic callus showed repressed *PPO* expression and lower browning potential than the nontransformed control (Murata et al., 2001). *MdAAT*, *MdCXE*, and *MdLOX* genes in the expression of apple flavor volatiles using SNPs have been identified (Kumar et al., 2015).

Dwarfing rootstocks are predominantly used in apple cultivation for increasing productivity; however, the most important constraint in vegetative propagation of these dwarf rootstocks is their poor

rooting ability (Welander et al., 1998). Efforts to identify genes that can improve the dwarfing capacity without reducing rooting have been made resulting in identification of *rol* rooting (A, B, C, and D) genes from *Agrobacterium rhizogenes* (Jasik et al., 1997). Among these, *rol B* gene is most effective in promoting root formation and has been successfully used to transform apple rootstock M.26 (Welander et al., 1998), M.9/29 (Zhu et al., 2001), and Jork9 (Sedira et al., 2005), resulting in transgenic lines with enhanced rooting per explants. Holefors et al. (1998) in their study observed altered phenotype and growth capacity of transgenic M.26 lines transformed with *rol A* gene. The transgenic lines showed wrinkled leaves and reduced internodal length, leaf area, and dry weight (Holefors et al., 1998). Elevated levels of the *RGL (DELLA)* gene expression played a role in the reduced growth in RG/T166 trees and T166 trees (Artlip et al., 2016).

To reduce the breeding cycle, reduction in juvenility is paramount. However, woody perennials such as fruit crops have a long juvenile phase lasting for several years, during which flowering cannot be induced under natural conditions (Hackett, 1985). Several genes controlling flowering time such as *LEAFY (LFY)*, *APETALA 1 (AP1)*, *TERMINAL FLOWER 1 (TFL1)*, and *FLOWERING LOCUS (FT)* have been isolated from *Arabidopsis* (Weigel et al., 1992; Mandel et al., 1992; Ohshima et al., 1997; Kardailsky et al., 1999). Overexpression of genes *LFY, AP1*, or *FT* has been shown to reduce the juvenile phase in transgenic *Arabidopsis* leading to early flowering (Mandel et al., 1992; Weigel and Nilsson, 1995; Kardailsky et al., 1999). Apple orthologs of genes *AFL1, AFL2, MdAP1 (MdMADS5)*, and *MdTFL1* have already been isolated and characterized (Kotoda et al., 2000, 2002; Kotoda and Wada, 2005). Kotoda et al. (2006) produced transgenic Orin cultivar expressing *MdTFL1* antisense RNA. Transgenic lines showed precocity in flowering and one of them flowered 8 months after transferring to the greenhouse with normal flowers, resulting in normal fruit with many seeds. Some flowers of transgenic trees were observed to develop without undergoing dormancy (Kotoda et al., 2006). In a different study, Flachowsky et al. (2007) used the approach of overexpression of genes involved in floral meristem formation, such as *LFY, AP1*, or *FT*, to induce flowering and transformed apple cultivar Pinova with the *BpMADS4* gene of silver birch having a similar function as the *FT* gene. Some transgenic lines were reported to flower 13 weeks after transformation under in vitro conditions, most of the flowers being normal (Flachowsky et al., 2007). The use of transgenic lines with reduced juvenile phase will prove to be highly useful in reducing the breeding cycle in apple, thus making conventional breeding approaches more efficient.

3.2 PEAR

Pear (*Pyrus communis*) is one of the most important temperate fruit crops of the family Rosaceae and subfamily Pomoideae following apple. Most cultivated pears are practically diploids ($2n=34$), although a few triploids and tetraploids have also been reported (Morgan et al., 1994). In various pear improvement programs, resistance to insect pests and diseases has been one of the major objectives. Pear production is often confronted by various diseases and pests, some of them causing severe damage such as fire blight (*E. amylovora*), pear scab (*Venturia pirina*), and pear psylla (*Cacopsylla pyricola*), vector of pear decline, etc. Although direct use of gene transfer in the breeding of pear hitherto seems to be a distant dream, the myriad possibilities offered by the transgenic breeding approach can without doubt be used for designing a pear crop of our choice. The first report of successful gene transfer in pear was in 1996 (Mourgues et al., 1996) and since then various pear cultivars have been transformed such as Beurre Bosec (Bell et al., 1999), Passe Crassane (Reynoird et al., 1999), and La France (Gao et al., 2007). Transformation of pear

cultivar Passe Crassane using the gene *attacin E* and synthetic analogs of *cecropin (SB-37 and Shiva 1)* under the control of inducible *Pin2* and constitutive promoter CaMV35S to increase the resistance against *E. amylovora* has been reported (Reynoird et al., 1999). The transgenic lines showed significant reduction in the symptoms as compared to the susceptible control (Reynoird et al., 1999). Hairpin N_{Ea} induces systemic acquired resistance in plants. To develop resistance against *E. amylovora*, pear lines were transformed with elicitor hairpin N_{Ea} *E. amylovora* with constitutive CaMV35S promoter (Malnoy et al., 2005). The transgenic lines showed significant increase in resistance against fire blight under in vitro conditions (Malnoy et al., 2005). Flachowsky et al. (2008) transferred a gene encoding for an extracellular polysaccharide-depolymerase (*dpo*) from the *E. amylovora* phage phi-Ea1h driven by the constitutive promoter CaMV35S into apple scion cv. "Pinova" through *A. tumefaciens*-mediated leaf disc transformation using a binary vector and strain EHA105 or LBA4404. A positive correlation was observed between depolymerase activity and resistance to fire blight in vitro and one of the transgenic lines showing highest depolymerase activity exhibited the least susceptibility to fire blight in vitro as well as under greenhouse conditions (Flachowsky et al., 2008).

Pear psylla, *C. pyricola* Foerster, is another major pest of pear feeding primarily on the phloem tissues of pear and is specific in its host range, confining to certain *Pyrus* spp. (Bell and Stuart, 1990). Plants transformed with genes against one pathogen may show resistance against another pathogen. Such a case was observed because pear psylla adults showed preference toward transgenic Bartlette pear lines carrying a synthetic antimicrobial gene, *D5C1*, to settle and oviposit, and nymphs feeding on transgenic pear developed faster than on nontransgenic pear in long-term psylla colony development, produced fewer eggs, and significantly reduced nymphal hatch on the transgenic pear (Puterka et al., 2002). These results where a fourfold decrease in psylla population was recorded suggested that such a transgenic line can prove to be of added advantage for integrated pest management programs in pear (Puterka et al., 2002).

As in the case of apple, the use of the *rol B* gene in inducing dwarfing in rootstocks has also been reported in pear. Pear rootstock BP10030, which has many desirable properties such as dwarfism, frost hardiness, and compatibility with most pear varieties, is highly difficult to propagate because of its poor rooting ability. BP10030 lines transformed with the *rol B* gene showed 67%–100% rooting without auxin, while the untransformed control completely failed to produce roots on hormone-free medium (Zhu et al., 2003). Vigorous pear scion grafted on transgenic rootstock showed reduced stem height and stem diameter (Zhu et al., 2007). Similar types of results were reported for *rol C* genes integrated in pear cultivar Beurre Bose where transgenic lines showed reduced stem height, number of nodes, and leaf area under greenhouse conditions (Bell et al., 1999).

For induction of early flowering, pear cultivars "La France" and "Ballade" were transformed with the *Citrus FLOWERING LOCUS T (CiFt)* gene where the transgenic lines exhibited early flowering when grown in micropropagation media (Matsuda et al., 2009). To investigate the inheritance of the transgene, seedling progenies were obtained by crossing wild type with transgenic line. Out of seven seedlings that expressed the *CiFt* gene, five were reported to flower within 10 months after being transferred to a greenhouse, thus confirming the inheritance of an early flowering phenotype in the progenies (Matsuda et al., 2009). Freima et al. (2012) successfully developed an early flowering transgenic line of pear cultivar "Spadona," named Early Flowering-Spadona, by RNA interference (*RNAi*)-mediated silencing of native pear genes *PcTFL1-1* and *PcTFL1-2*. The transgenic lines produced solitary flowers from apical or lateral buds and showed reduced vegetative vigor. This transgenic line can prove to be a potential tool for accelerating the pear breeding cycle (Freima et al., 2012).

Preservation of fruit quality and extension of shelf-life are two of the prime objectives when breeding cultivars in climacteric fruits. Ethylene is the plant growth regulator that plays a major role in triggering events leading to fruit ripening and softening; an inhibition of ethylene biosynthesis hence can improve fruit quality preservation and shelf-life. Gao et al. (2007) reported transformation of pear cultivar "La France" with sense and antisense cDNA that encodes an *ACO*. Gene cosuppression was found in almost all antisense lines including one sense line, while overexpression was observed in other sense lines with a reduction of 85% in ethylene production in in vitro shoots in antisense lines. Transgenic lines showed in vitro flowering at a remarkably higher frequency, while it was seldom observed in nontransgenic lines, suggesting that a reduction of ethylene production might be responsible for induction of flowering (Gao et al., 2007). Many genes have been employed for the development of transgenic fruit plants resistant to biotic and abiotic stresses. In most cases, increased resistance is associated with increased antioxidant capacity of the tissue or accumulation of compatible solute through control of genes involved in these processes (Rai and Shekhawat, 2014). A transgenic pear line (cv. Ballad) overexpressing apple spermidine synthase (*MdSPDS1*), when subjected to heavy metal stress using CdCl2, PbCl2, ZnCl2, and a combination of these, exhibited reduced accumulation of heavy metals as compared to the wild type (Wen et al., 2010). These results indicate the role of spermidine in imparting long-term multiheavy metal tolerance possibly caused by its antioxidant activities along with metal chelating properties (Wen et al., 2010). This is in accordance with the earlier reports of increased tolerance against aluminum metal stress in the transgenic pear expressing *SPDS1* (Wen et al., 2009). Enhancement in salt tolerance by integration of the *SAMDC2* gene encoding for *S*-adenosylmethionine decarboxylase, wherein the transgenic lines showed increased expression of polyamines, has also been reported (He et al., 2008). Stilbene production was reported in the transgenic pear line "Spadona" containing grapevine cDNA encoding stilbene synthase, responsible for the synthesis of resveratrol, a health-promoting compound with antiaging, antiinflammatory, antiplatelet, and anticarcinogenic activities (Flaishman et al., 2005). Developing cultivars with improved functional food properties is an attractive approach for achieving nutritional security, and transgenics can be a potential tool for introducing health beneficial genes in fruit crops.

3.3 PEACH

Peach (*Prunus persica* L. Batsch.) is one of the most important stone fruit crops of the Rosaceae family, widely grown in temperate zones of the world. Peach is highly recalcitrant to regeneration, making crop improvement through genetic transformation very difficult to achieve (Carrasco et al., 2013). Although different explants for shoot regeneration such as proximal region of cotyledons, immature embryos, hypocotyl segments, longitudinal sections of mature embryos (Mante et al., 1989; Pérez-Clemente et al., 2004), and leaves from shoots cultured in vitro (Gentile et al., 2002) have been tried, reports of transgenic peach regeneration have been sporadic so far. The biolistics method provides an opportunity for meristem transformation in difficult-to-regenerate fruit tree cultivars and can facilitate regeneration. *Agrobacterium*-mediated transformation using explants such as leaf segments, immature embryos, and embryogenic callus has been reported in peach (Scorza et al., 1990). Ye et al. (1994) optimized the physical and biological parameters that affect the efficiency of biolistic transformation in peach and reported successful integration and stable expression of foreign genes in peach embryo callus, affirming that transformation using the particle bombardment method

is feasible for developing stable transgenic peach lines. Developing cultivars with compact growth habit is an important objective in peach breeding programs (Chalmers et al., 1978). Compact plants contain higher levels of endogenous cytokinin because it inhibits apical dominance and promotes development of axillary buds along with reduction in stem length, leaf area, and root system (Medford et al., 1989; Smigocki, 1995; Smigocki and Owens, 1989). The shooty mutant strain of *A. tumefaciens* carries an octopine type Ti plasmid containing a functional bacterial cytokinin biosynthesis (ipt) gene and a Tn5 transposon-inactivated auxin biosynthesis (iaaM) gene (Garfinkel et al., 1981; Schell, 1987). Such a strain was used to transform immature embryos of "Red Heaven" peach where the transformed regenerants contained 50-fold higher cytokinin content than nontransformed regenerants and exhibited increased branching and root development under in vitro conditions with reduced height 2 weeks after being transferred to a greenhouse (Smigocki and Hammerschlag, 1991). Further studies proved that transgenic lines containing the *ipt* gene exhibit altered growth habit with significantly higher branching and axillary growth along with delayed senescence, suggesting that introduction of the *ipt* gene can be useful for generating commercially viable peach cultivars with compact growth habit (Hammerschlag and Smigocki, 1998). The most devastating viral disease of peach and other stone fruits is sharka, caused by PPV. Genetic engineering appears to be a tool to deal with this major problem. In the last two decades, the analysis in both screen house and endemic areas throughout Eastern Europe have clearly demonstrated the effectiveness of genetic engineering approaches to benefit stable and durable sharka resistance in plum (Scorza et al., 2016). Fast track, a recent technology for rapid cycle breeding developed in another species (apple, plum, etc.), is a breeding system that uses a genetic engineering tree expressing a flowering pathway gene, such as *FT* gene orthologs, to acquire fruiting trees in less than 1 year (Flachowsky et al., 2011; Srinivasan et al., 2012a,b). This technology would accelerate the conventional breeding procedures for the introgression of sharka resistance from peach-related species. Also the trees produced would not be genetically engineered unless the PPV resistance gene is a transgene. Peach was transformed with an efficiency of 3.6% using the *Rhizobium radiobacter* strain C58, and transgenic plants were regenerated on medium supplemented with 7.5 μM thidiazuron and 2.4 μM indolacetic acid (Pérez-Clemente et al., 2004). Padilla et al. (2006) evaluated different explants, *R. radiobacter* strains, vectors, and promoters for their efficacy in causing transformation wherein the highest transformation rate (56.8%) was obtained using epicotyl internodes.

3.4 PLUM

Plums comprise a wide range of sexually compatible species possessing both desirable as well as undesirable traits (Ramming and Cociu, 1991). For crop improvement in plums, breeding should include diverse germplasm for introgression of desired traits in the existing cultivars; however, often these introgressions, by means of conventional breeding, transfer with them unwanted traits by the phenomenon of what is called linkage drag. Under such circumstances where the trait could not be transferred from the native germplasm because of its absence or complexity of inheritance, genetic engineering can come to our rescue. One such trait for which improvement is highly coveted in plums is resistance to sharka disease. Sharka (pox in Bulgarian) or plum pox disease caused by PPV is one of the most disastrous diseases of stone fruits, causing huge economic losses in apricot, peach, and plum (Dunez and Sutic, 1988; Németh, 1994; García et al., 2014). The virus is nonpersistently transmitted by aphids that carry the virus on their stylus (Kunze and Krczal, 1971;

Labonne et al., 1995). For the first time, the disease was reported in Bulgaria on plums and apricots around 1917 and 1933, respectively (Atanasoff, 1932, 1935). Since then the virus spread throughout the important *Prunus* growing area of the world, except Australia, New Zealand, and South Africa (Barba et al., 2011; Šubr and Glasa, 2013).

Conventional breeding for sharka resistance has been undertaken in the past; however, scarcity of resistant sources and the polygenic nature of the resistance trait remained the main constraints to success (Dosba et al., 1992; Badenes et al., 1996). This paved the way for the search for alternative methods for inducing resistance against this dreadful disease and led to research for evaluating the efficacy of genetic engineering approaches such as pathogen-derived resistance to impart resistance against PPV. For diseases of viral origin, viral genome and proteins including CP, movement proteins, and proteins involved in genome replications are the potential targets for a genetically modified resistance strategy (Collinge et al., 2010). CP-mediated resistance is an efficient method for providing PPV resistance to herbaceous as well as woody PPV hosts (López-Moya et al., 2000). The resistance conferred by the CP gene is reported to be mediated by RNA via posttranscriptional gene silencing (PTGS) (Rai and Shekhawat, 2014). Genetic transformation in European plum (*Prunus domestica* L.) has been undertaken through regeneration of the cotyledon and hypocotyl sections of mature embryos (Mante et al., 1989; Scorza et al., 1994), immature embryos, and embryonic axes (Tian et al., 2007a,b; Srinivasan et al., 2012a,b; Petri et al., 2012a,b; Wang et al., 2013a,b).

A. tumefaciens-mediated transformation of plum with the *CP* gene of PPV yielded both low and high PPV CP-expressing transgenic plum lines (Scorza et al., 1994). Five lines obtained by initial transformation were bud grafted on rootstocks and inoculated with PPV strain D using aphids (Ravelonandro et al., 1997) and graft inoculated with PPV strain M (Ravelonandro et al., 1997; Scorza et al., 2001) to evaluate the resistance capacity of these transgenic lines. These lines were observed for 3–4 years in the greenhouse and none of the transgenic C5 lines were reported to show symptoms of PPV. The high level of resistance exhibited by clone C5 made it a potential source for transferring resistance against PPV to hybrids and hence C5 plants were cross-hybridized with transgenic plum expressing papaya ringspot virus CP (PRSV CP), which was susceptible to PPV (Scorza et al., 1995). Transgenic seedlings containing either both virus CP genes (PPV and PRSV) or PPV-CP alone showed a highly resistant phenotype; however, seedlings containing only the PRSV insert were susceptible (Scorza et al., 1998). Evaluation of stability of "C5" under field conditions in different countries affirmed that it can survive several years without being infected (Scorza et al., 2001). A European plum cultivar, "HoneySweet," resistant to PPV developed from the C5 transgenic line is the first transgenic commercial *Prunus* released showing heritable and stable virus resistance traits (Carrasco et al., 2013). Confined trials performed using C5 clones micrografted on the rootstock Adesoto 101 (*Prunus insititia* L.) infected with PPV local isolates showed that even after 3 years of growth in a greenhouse, only mild symptoms were observed on leaves of some plants, warranting the significance of long-term trials (Wong et al., 2010). Plum cv. HoneySweet presents an important step in the utilization of genetic engineering by helping to solve major virus disease problems of stone fruit trees (Polák et al., 2017). Field tests of over 15 years in the European Union have demonstrated the effectiveness and safety of plum cv. HoneySweet. Srinivasan et al. (2012a,b) succeeded in transforming European plum with the flowering locus *T1* (*PtFT1*) gene from *Populus trichocarpa*. The transgenic lines of European plum exhibited a continuous reproductive phenotype (flower and fruit) under greenhouse and infield conditions during autumn and spring (Srinivasan et al., 2012a,b).

3.5 CHERRY

Following the same pattern as the majority of woody fruit species, improvement in cherry through genetic engineering is limited only to a few cultivars (Petri and Burgos, 2005). The year 1995 marked the development of the first cherry rootstock, "Rosa" (Laimer da Câmara Machado et al., 1995), and thenceforth genetic transformation has been reported for several commercial genotypes, consisting of sour cherry, black cherry (*Prunus serotina* Ehrh), chokecherry (*Prunus virginiana* L.), and cherry rootstocks such as Gisela 6, Gisela 7 (*Prunus cerasus*×*Prunus canescens*), Colt (*Prunus avium*×*Prunus pseudocerasus*), Inmil (*Prunus incise*×*Prunus serrula*), and Damil (*Prunus dawyckensis* Sealy) (Laimer da Câmara Machado et al., 1995; Druart et al., 1998; Gutiérrez et al., 1998; Dolgov and Firsov, 1999; Song and Sink, 2005, 2006; Liu and Pijut, 2010; Song et al., 2013). However, most of the cherry species, together with sweet cherry, are hitherto considered as recalcitrant to transformation mainly because of their recalcitrance to in vitro regeneration. Different regeneration and transformation protocols using leaves and internodes from in vitro grown plants and slices of hypocotyls have been tried for *P. cerasus* (Mante et al., 1989; Tang et al., 2002; Song and Sink, 2005, 2006) and *P. avium* (Hammatt and Grant, 1998; Tang et al., 2002; Matt and Jehle, 2005; Feeney et al., 2007) with varied degrees of success. Stable transformation with an efficiency of 3.1% was obtained in cv. "Montmorency" using 6-benzylaminopurine 0.5 mg/L and indolebutyric acid 0.05 mg/L in QL medium (Song and Sink, 2006).

Plants of cherry rootstock "Colt" transformed with rol A, B, and C genes of the nondisarmed *A. rhizogenes* pRi1855 TDNA showed enhancement in rooting capacity, shortened internodes, and wrinkled leaves phenotype (Gutiérrez et al., 1998; Gutérrez and Rugini, 2004; Dolgov and Firsov, 1999). Transgenic lines of "Gisela 6" and "Gisela 72" cherry rootstocks exhibiting resistance against *Prunus* necrotic ringspot virus through RNAi-mediated silencing have been reported, showing the possibility of using genetic transformation for improvement in cherry production and fruit quality by control of this disease (Song et al., 2013). A stable genetic transformation system has been developed for "Gisela 6" rootstock with identification of a total of 12 *MAPK* genes designated *PcMPKs* (Zong et al., 2016). Four *PcMPKs* (i.e., *PcMPKs* 4-1, *PcMPKs* 4-2, *PcMPKs* 6, and *PcMPKs* 18) exhibited differential expression, suggesting their potential roles in plants responding to various stresses.

3.6 APRICOT

Apricot (*Prunus armeniaca*) is an important stone fruit of the Rosaceae family originating in China and Central Asia from Tien Shan to Kashmir (Vavilov, 1951). Cultivated apricot is referred to as the most polymorphic among all cultivated fruit and nut species having diverse genetic origin (Byrne and Littleton, 1989; Martínez-Gómez et al., 2003). However, in almost all the apricot-growing areas, cultivation basically thrives on a few cultivars, and to increase the production and area under this crop-elaborated breeding program is paramount. Although the conventional breeding programs that mainly utilize controlled crosses to breed new cultivars are going on at various centers round the world, to achieve the basic breeding objectives such as developing varieties resistance to PPV, introducing self-compatibility, and increased fruit quality (Fideghelli and Della Strada, 2010), the conventional breeding methods should go hand in hand with the novel biotechnological approaches, one such being genetic transformation. For efficient utilization of genetic transformation, it is highly desirable that the plant under question should be amenable to a reliable and efficient regeneration system (Canli and Tian, 2009; Petri and Burgos, 2005). As before, akin to other members of the *Prunus* genera, apricot has also been found to be a recalcitrant species for shoot regeneration and consequently genetic transformation

(Wang et al., 2013a,b). Adventitious shoot regeneration in apricot has been achieved from immature cotyledons (Goffreda et al., 1995; Laimer da Câmara Machado et al., 1992; Lane and Cossio, 1986; Pieterse, 1989), mature seed-derived hypocotyls (Wang et al., 2011), and leaves of "H.152," "H.146" (Escalettes and Dosba, 1993), "Bulida," "Helena," and "Canino" (Burgos and Alburquerque, 2003; Pérez-Tornero et al., 2000). However, the report of regeneration of transgenic apricot is scanty and until now "Helena" remains the only genetically modified commercial cultivar of apricot (López-Noguera et al., 2009; Petri et al., 2008a,b). Regeneration of transgenic lines of apricot cultivar "Moniquí" from mature seed hypocotyl segments with an efficiency of $3.8 \pm 1.4\%$ utilizing an herbicide selection strategy by means of a selective bar gene has been reported (Petri et al., 2015).

PPV causing sharka disease is an important threat to apricot plants. Transgenic apricot lines expressing the PPV *CP* gene have been developed using seed-derived tissue imparting CP-mediated resistance against PPV (Laimer da Câmara Machado et al., 1992). The introduction of self-compatibility is one of the breeding objectives in *Prunus* sp., which is hard to achieve via conventional breeding. S-locus is involved in the self-incompatibility reaction in apricot. Liu et al. (2016) succeeded in cloning an S-haplotype-specific F-box (SFB) protein gene from the "Xiaobaixing" apricot and constructed three SFB expression vectors, which they were able to transfer into *A. tumefaciens* strain LBA4404. The successful construction and transfer of these expression vectors can provide a foundation for transforming "Xiaobaixing" apricot for generating self-compatible *Prunus* cultivars (Liu et al., 2016).

3.7 STRAWBERRY

The dessert strawberry (*Fragaria* × *ananassa* Duch.) of the Rosaceae family is one of the most important fruit crops showing adaptability to various environmental conditions across the world (Hancock, 1999). The polyploid nature of the crop ($2n = 8x = 56$) makes the traditional breeding system very cumbersome for developing varieties with desirable agronomical traits (Marta et al., 2004). Under such conditions, genetic engineering offers a realistic approach for creating varieties of choice by selectively targeting a single gene or a few heterologous traits for introducing into the strawberry genome. Considerable progress has been made in genetic transformation of strawberry ever since the first transgenic strawberry plant was reported in 1990 (Nehra et al., 1990a,b). Successful *Agrobacterium*-mediated transformation in strawberry relies on the availability of an efficient regeneration system (Debnath and Teixeira-da-Silva, 2007) as well as selection and recovery of transformed cells after organogenesis. Different genotypes of strawberry perform differently to the transformation and regeneration protocols. Transformation in strawberry has been reported for biotic and abiotic stress resistance as well as improved fruit qualities (Sesmero et al., 2007; Khammuang et al., 2005; Mercado et al., 2007).

Transgenic strawberry lines constitutively expressing the *cowpea protease trypsin inhibitor* (*CpTi*) gene exhibited tolerance against vine weevil (*Otiorhynchus sulcatus*) and were less preferred for feeding by weevil under greenhouse and field conditions (James et al., 1992; Graham et al., 1995, 1997, 2001, 2002). Gray mold (*Botrytis cinerea*) and anthracnose (*Colletotrichum* fungi) are the two most destructive fungal diseases of strawberry, causing severe damage to strawberry plantations (Sutton et al., 1988; Sutton, 1990; Horowitz et al., 2002; Legard et al., 2003; Cesar et al., 2006). Chitinase, an endo-type enzyme that hydrolyzes chitin, is an important component of preexisting plant defense responses against fungal pathogens (Caesar and Ignacimuthu, 2012). Transgenic strawberry lines expressing enhanced chitinase levels showed reduced levels of damage by powdery mildew fungi

(Asao et al., 1997, 2003). Strawberry cultivar "Joliette" transformed with *chitinase* gene (*pcht28*) from *Lycopersicon chilense* using *Agrobacterium*-mediated transformation exhibited significantly higher resistance against *Verticillium dahlia* (Chalavi et al., 2003). Similar reports are available for transgenic strawberry lines expressing thaumatin-like proteins wherein the integration of a thaumatin II cDNA into the plant genome increased the resistance of plants against gray mold fungus (Schestibratov and Dolgov, 2005). Susceptibility to abiotic stresses such as salinity is one of the most important limiting factors in the cultivation of strawberry (Orsini et al., 2012). Several efforts have been made to breed strawberry cultivars with increased abiotic stress tolerance (Husaini and Abdin, 2008; Christou et al., 2013, 2014). Genetic engineering strategies for abiotic stress tolerance rely mostly on the expression of genes that are involved in the stress-mediated response (Galli et al., 2015). Several genes involved in stress tolerance have been identified, namely, *betaine aldehyde dehydrogenase (BADH)* (Weretilnyk and Hanson, 1987, 1990), LEAs (Wise, 2003), cold-induced transcription factor (CBF1) (Gilmour et al., 1998), antifreeze protein (AFP) (Georges et al., 1990), and osmotin (Husaini and Abdin, 2008). Transgenic strawberry plants expressing these proteins exhibited constitutive activation of stress-responsive genes and enhanced salt (Liu et al., 1997; Wang et al., 2004; Husaini and Abdin, 2008) and freezing tolerance (Firsov and Dolgov, 1999; Owens et al., 2002, 2003; Khammuang et al., 2005). Low-temperature injury is another important constraint in the perennial strawberry cultivation system. Dehydration responsive element binding protein (DREB)/C-repeat binding factor (*CBF*) genes are among the low-temperature-induced genes that encode key transcription factors in the major transcriptional cascade responding to low temperature (Shinozaki and Yamaguchi-Shinozaki, 2000). Wang et al. (2014) succeeded in transferring the *RdreB1BI* gene under the control of the rd29A promoter (rd29A:RdreBIB1), which is reported to harbor a response element for cold, abscisic acid, and salt stress (Msanne et al., 2011). Transcriptome analysis of rd29A:RdreB1BI transgenic strawberry using Illumina/Solexa sequencing technology revealed that DREB transcription factor RdreB1BI is the central regulator of low-temperature stress responses and tolerance in plants along with other transcription factor s, and coordinates the expression of stress-responsive and anthocyanin biosynthesis genes (Gu et al., 2015). Strawberry is a very delicate fruit with a short shelf-life. Use of antisense technology for preventing fruit softening is a very useful tool for increasing the durability of fruit without altering fruit quality (Woolley et al., 2001; Jiménez-Bermúdez et al., 2002; Palomer et al., 2006; Sesmero et al., 2007). Pectate lyase (PL), an extracellular enzyme, plays an important role in cell wall disassembly and maceration during fruit ripening (Jiménez-Bermúdez et al., 2002). Strawberry plants transformed with an antisense sequence of a strawberry *PL* gene were reported to produce fruits with increased firmness and extended postharvest shelf-life without showing any significant negative effect on other fruit attributes such as color, size, shape, and weight during fruit ripening (Jiménez-Bermúdez et al., 2002; Sesmero et al., 2007).

3.8 GRAPE

Cultivated grapes (*Vitis vinefera*) belonging to the family Vitaceae are globally the most important fruit species in terms of economic returns because of the myriad uses of its fruit, from fresh table grapes to juice, wine, raisins, and organic compounds (Aigrain, 1999). The first report of production of transformed grapevine roots was by Gribaudo and Schubert (1990). They were successful in transforming grape cultivars using 8196 *A. rhizogenes* strain where the transgenic lines showed hairy root phenotype and produced opine (Gribaudo and Schubert, 1990). Although Baribault et al. (1989,

1990) were the first to obtain transformed cells of grape cultivar Cabernet Sauvignon expressing *npt*II, Mullins et al. (1990) succeeded for the first time in achieving regeneration of transgenic grapevine plants. Among the various selectable markers used, nptII has proved to be the most efficient selectable marker and is being used in transforming various grape cultivars and hybrids using kanamycin (Mullins et al., 1990; Motioike et al., 2002) or paromomycin (Wang et al., 2005) as antibiotic. Among the major problems in grape cultivation, grapevine fanleaf virus (GFLV), transmitted through nematodes, poses a serious threat to the grape industries. Soil disinfection, which is commonly used to check nematode population, is not very effective and eventually leads to pollution of soil and groundwater. Mauro et al (2005) attempted the transformation of grape vine rootstocks 4IB, S04, and cultivar Chardonnay plants through cocultivation of embryogenic cell suspensions with an engineered *A. tumefaciens* strain to integrate the chimeric *GFLV CP* gene to develop resistance against GFLV. Developing resistance in grapevines against fungal diseases, namely, powdery mildew and downy mildew, is one of the most important breeding objectives in grapevines. To improve the antifungal potential, *rice chitinase* gene was transferred to grape cultivars under a maize-ubiquitin promoter via *Agrobacterium*-mediated transformation (Nirala et al., 2010). The transgenic plants showed delayed onset of the disease and smaller lesions after in vitro inoculation of powdery mildew (*Uncinula necator*), and were well adapted under greenhouse conditions without any apparent change in phenotypic expression (Nirala et al., 2010). Several other genes have been introduced into grape plant genome for developing resistance against powdery mildew, downy mildew, and GFLV (Jardak-Jamoussi et al., 2009; Xu et al., 2010; Nookaraju and Agrawal, 2012). Transgenic "Thompson Seedless" and "Chardonnay" expressing the pear fruit polygalacturonase-inhibiting protein (PGIP)-encoding gene (*pPGIP*) under the control of the CaMV35S promoter were reported to show increased tolerance to Pierce's disease and *Botrytis* (Agüero et al., 2005).

Although attempts to develop transgenic grape plants with improved fruit quality traits such as decreased browning of raisins and seedlessness in table grapes have been made (Thomas et al., 2000; Perl et al., 2003), any significant breakthrough in this field is yet to be achieved.

3.9 NUTS

3.9.1 Walnut

The Persian walnut (*Juglans regia* L.), native to central Asia, is one of the most important temperate nut crops belonging to the family Juglandaceae. Walnut is the richest source of fat (52%–70%) and contains several vitamins such as vitamins A and E, niacin, riboflavin, thiamine, along with magnesium, phosphorus, and potassium (Prasad, 2003). Improvement through transgenics is in its infancy in walnut; however, it is being perceived as a potential tool for developing cultivars with improved characters such as resistance against cherry leaf roll virus, a major viral disease that spreads by pollen (Massalski and Cooper, 1984), and *Phytophthora*, the most significant root disease that spreads through the irrigation water and winter flooding waters (Mircetich and Matheron, 1983) of walnuts in California. Other breeding objectives include lateral bud fruitfulness, delayed bud break and flowering, homogamy, reduced chilling requirement, winter hardiness, and high fruit and kernel quality (Germain, 1992). Walnut was among the foremost woody perennials for which *A. tumefaciens*-mediated transformation and regeneration were demonstrated (Dandekar et al., 1988; McGranahan et al., 1988). Young proliferating somatic embryos of walnut are suitable explants for *A. tumefaciens*-mediated transformation

(McGranahan et al., 1988). A single transformed embryo can give rise to several independent transgenic lines indicating the presence of multiple infection sites on the surface of walnut embryo and renders the walnut transformation system very efficient (McGranahan et al., 1990). However, the major hurdle in generating transgenic lines by using somatic embryo transformation systems is difficulty in the selection and identification of transformants in culture. Selectable marker genes, especially the kanamycin resistance gene *nptII,* are widely employed for selection of transformed cells, but survival of nontransformed tissue is prevalent in somatic embryo cultures (McGranahan et al., 1990; El Euch et al., 1998). Green fluorescent protein (GFP) has been found to be an effective scorable marker in walnut somatic embryo culture, which does not affect walnut development in culture or in the greenhouse (Escobar et al., 2000). Wang et al. (2009) developed an *Agrobacterium*-mediated genetic transformation and regeneration protocol from shoot explants of Paradox (*Junglans hindsii* × *Junglans regia*) walnut rootstock "Vlach." Crown gall disease and the root lesion nematodes (RLNs) (*Pratylenchus vulnus*) are among the major problems being faced by the California walnut industry leading to reduced productivity and increased cost of cultivation. Walawage et al. (2013) developed a resistance walnut against these two maladies by using gene stacking technology. They used *A. tumefaciens*, carrying self-complementary *iaaM* and *ipt* transgenes, and *A. rhizogenes*, carrying a self-complimentary Pv010 gene from *P. vulnus*, as cotransformation vectors to transform the walnut roots. Silencing genes encoding *iaaM, ipt*, and Pv010 resulted in decreased crown gall formation and RLN populations in walnut (Walawage et al., 2013). Coddling moth is another major problem in walnut cultivation wherein the larval stage is a damage-causing stage leading to severe economic loss as it feeds on the walnut meat. Efforts have been made to develop a transgenic walnut possessing resistance against coddling moth by transferring the *cryIA(c)* gene, expressing a *Bacillus thuringiensis* (*Bt*) insecticidal crystal protein, into walnut somatic embryo (Dandekar et al., 1998). Aflatoxins are among the antinutritional compounds having carcinogenic and teratogenic properties that can contaminate walnut kernels rendering them unfit for consumption. Such kernels also lose their marketability and overall economic value. Gallic acid present in the pellicle of walnut has inhibitory effects on the generation of aflatoxins. Transgenic lines of the cultivar Chandler overexpressing the *shikimate dehydrogenase (SDH)* gene encoding for *SDH*, the enzyme responsible for gallic acid production, showed increased inhibition of aflatoxins (Muir, 2005).

3.9.2 Almond

Almond (*Prunus dulcis*) is highly heterozygous and highly polymorphic because of its strong self-incompatibility system and predominance of open pollination. Several breeding programs have focused on the development of self-compatibility and delayed blooming because almond is often affected by the spring frost because of its early blooming tendency restricting its cultivation to sites with low risk of spring frost. Blooming time is often considered as a quantitatively inherited trait and a similar inheritance pattern has also been shown for almond (Dicenta et al., 1993; Kester and Gradziel, 1996). In the case of almond, the blooming time is regulated by a major gene, *LATEBLOOMING* (*Lb*), and late blooming is dominant over early blooming (Oliveria et al., 2008). The other breeding objectives include developing cultivars exhibiting resistance against diseases and insect pests. *Prunus* necrotic ringspot virus and prune dwarf virus are among the major viral diseases posing a threat to almond production because they are spread very rapidly across the orchard even after good management practices. Genetic engineering is one of the most efficient approaches for

control of virus propagation wherein viral genes such as *CP* genes or genes coding for movement proteins are used to transform plants for developing resistance against the virus (Yadav et al., 2005). Efforts are being made for engineering resistance against prune dwarf virus in almond by introducing the *CP* gene in the plant genome via genetic transformation methods (Raquel et al., 2005). Reports of transformation in almond are hitherto fewer and most of the studies with the exception of the use of rol genes are concerned with the use of marker genes. Almond tissues show susceptibility to *Agrobacterium*, making *Agrobacterium*-mediated transformation a desirable method for generating transgenic lines in almond (De Cleene and De Ley, 1976). Ramesh et al. (2006) succeeded in transforming the almond cultivar NePlus Ultra via *Agrobacterium*-mediated transformation using a positive selection strategy based on the use of the *phosphomannose isomerase* structural (*pmi*) gene as a selectable marker with mannose as a selective agent through a positive selection mechanism. Optimum rooting is desirable for propagating almond through cutting or in vitro micropropagation; however, almond shows a difficult-to-root tendency. Almond cultivar Supernova infected with *A. rhizogenes*, strain 1855 NCPPB, at the basal portion of microcutting exhibited 96.8% rooting under in vitro conditions (Damiano et al., 1995). Genetic engineering is one of the most efficient approaches for control of virus propagation wherein the viral genes such as *CP* genes or genes coding for movement proteins are used to transform plants for developing resistance against the virus (Yadav et al., 2005). Efforts are being made for engineering resistance against prune dwarf virus in almond by introducing the *CP* gene in the plant genome via genetic transformation methods (Raquel et al., 2005). Although successful stories of transformation in almond are fewer, it hopefully provides a scope for developing almond cultivar suited to a present-day cultivation scenario.

4. GENE SILENCING IN TEMPERATE FRUIT CROPS

Once a transgenic plant is regenerated and established in greenhouse or field conditions, it is expected that the gene for which it has been transformed will act as a dominant Mendelian trait. However, in many cases significant deviations have been noticed in expected phenotypes. Gene silencing can be observed in different situations such as single transgene only, transgene–transgene, or transgene–endogenous gene interactions and may arise because of either transcriptional or posttranscriptional mechanisms (Senior, 1998). Several factors influence plant gene silencing such as position of T-DNA insertion into the genome and environmental conditions or it may be a result of transcriptional silencing, posttranscriptional silencing, antisense silencing, or chromatin-mediated silencing expression (Senior, 1998). The phenomenon of silencing the expression of homologous (chromosomal) loci by some transgene was first reported in plants (Vaucheret et al., 1998). Homology-dependent *trans*-silencing has been classified into two types depending on the nature of the effect on the target. In the first type, although transcription of the target locus remains unaffected, the half-life of target RNAs is significantly decreased (De Carvalho, 1992; Cogoni, 1996; Ngô et al., 1998; Montgomery et al., 1998). This is referred to as "PTGS". In the second category, homology-triggered processes primarily affect the chromatin template, and are termed transcriptional gene silencing (Matzke, 1989). Fitzgeralda et al. (2004) reported simultaneous silencing of multiple genes, GFP transgene, and trihydroxynaphthalene reductase involved in melanin biosynthesis in the apple scab fungus *V. inaequalis* by RNA-mediated gene silencing at a frequency of 51%. They claim that such silencing method will facilitate the development of high-throughput screening for functional genomics. Virus-induced

gene silencing (VIGS) is another efficient technique for analyzing gene functions in plants. Sasaki et al. (2011) reported an easy and rapid VIGS system capable of efficiently inducing reliable VIGS of endogenous genes in the seedlings of apple, pear, and Japanese pear using apple latent spherical virus vectors. This could prove to be a potential tool for functional genomic studies in Rosaceae fruit trees (Sasaki et al., 2011).

5. FUTURE PROSPECTS

Genetic engineering has opened up new avenues for designing plant varieties suited to our specific needs. Trait-specific improvement in crops has minimized, to a greater extent, the risk of undesirable gene combination and linkage drags most frequently observed in the conventional breeding programs involving inter- or intraspecific hybridization. Although the transformation and regeneration rates in fruit and nut crops are hitherto low, the researchers are incessantly making efforts for developing protocols for successful transformation and efficient regeneration of transformed plants thus obtained. In fruit crops where the long breeding cycle and reproductive barriers hinder crop improvement via conventional means, transgenics is an attractive approach for improving various agronomically desirable traits such as biotic and abiotic stress tolerance, extended shelf-life, improved fruit qualities, as well as various neutraceutical properties that can help in attaining food and nutritional security. However, development of transgenic plants and their commercialization has to pass through a stringent regulatory system because of various biosafety and ethical issues related to them. Selectable marker genes encoding for antibiotic or herbicide resistance are mostly used for the selection of transformed plants. These selectable marker genes are no longer needed once the transgenic plants have been established and hence they can be removed from the plant genome because they are not desirable from consumer and biosafety points of consideration (Tuteja et al., 2012). Several reports have claimed regeneration of marker-free transgenic plants using binary vectors devoid of selectable marker genes in different temperate fruits (Malnoy et al., 2007, 2010; Herzog et al., 2012; Petri et al., 2012a,b). In developed countries, consumers show aversion toward transgenic crops because they consider that the use of genes from unrelated or alien species, including microbes and weeds, in transforming the plants poses a threat to human health and environmental safety and integrity. This repugnance could be overcome by new concepts in genetic engineering, which have been proposed as cisgenic or cisgenesis (Schouten et al., 2006; Molesini et al., 2012; Vanblaere et al., 2011) and intragenesis (Rommens et al., 2007; Jacobsen and Schouten, 2009; Molesini et al., 2012). The two approaches differ in the composition of the genetic construct. While the former refers to plants transformed with one or more genes isolated from either the same or a sexually compatible species, in the latter case, the intragene is not a perfect copy of a natural gene and is composed of regulatory and coding sequences derived from the same or sexually compatible species (Molesini et al., 2012). Cisgenic approaches have been successfully used to breed apple and grapevine cultivars (Vanblaere et al., 2011; Krens et al., 2015). With the increase in genomic studies and identification and cloning of various genes as well as developments made in the field of in vitro propagation, genetic engineering will emerge as a potential approach to crop improvement in the near future. Nonetheless standardization and utilization of novel concepts such as cisgenic and intragenic along with selectable marker-free transformation technology, which possesses the capability of transforming the perception of consumers toward genetically modified crops making them more acceptable, will indubitably be the major concern of the future line of research.

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CHAPTER

TRANSGENIC RESEARCH IN FLORICULTURAL CROPS

6

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1. INTRODUCTION

Progress in the development and commercialization of transgenic floricultural plants has significantly lagged that of the major food crops. While millions of hectares of maize, cotton, canola, and soybean are now grown around the world, as well as transgenic varieties of other foods, only a few transgenic floricultural products are on the market. The first of these varieties, color-modified carnation, was launched in 1997 in Australia, indicating a very slow uptake of genetic modification technology by the floriculture industry. The floricultural industry is a subset of the horticultural industry and, as the name implies, floriculture focuses on flowers. The floriculture industry includes cut flowers, potted flowering plants (including hanging baskets), bedding plants, flowering shrubs, and trees for landscaping and gardens.

The most important floricultural plants are listed in Table 6.1 (Chandler and Sanchez, 2012). Table 6.1 represents a qualitative "top 10" of species of importance within each floricultural subgroup and as such they account for the bulk of market value within the floriculture industry. The value of the floricultural market is difficult to estimate because reliable statistics are not available for all countries. Estimations also depend on whether calculations are made at the breeder, grower, distributor, or retailer level. Van Rijswick (2015) has estimated the annual value of international exports in the floricultural industry to be US$20 billion. Because of the multiplier effect on value as a product is moved through the distribution channel and because exports exclude intracountry trade in floricultural products, this underestimates the value of floriculture at the consumer level, which is typically three times the value at distribution. Chandler and Sanchez (2012) estimated that the ornamentals sector of the horticulture industry had a global economic value of US$250–400 billion. Floricultural products are traded internationally and are very important in the rural economies of some countries. For example, there is a very significant air freight trade in cut flowers grown in parts of South America and Africa to the United States and Europe, and cuttings and plants grown in Central America and Africa are also exported to Europe and the United States. Within Europe and between Russia, the Middle East, and Europe, flowers and plants are traded on a daily basis. Japan is a major destination for produce grown in some Asian countries and Europe, and with increasing disposable income in China and India, imports and exports of floricultural products to these two economies are also increasing.

New technologies, such as micropropagation, embryo rescue, and exploitation of somaclonal variation, have contributed to the improvement of floricultural crops. In addition, genetic modification

Genetic Engineering of Horticultural Crops. http://dx.doi.org/10.1016/B978-0-12-810439-2.00006-4

Table 6.1 Important Key Floricultural Crops

Group	Most Important Species
Cut flowers	Rose (*Rosa*×*hybrida*), carnation (*Dianthus caryophyllus*), chrysanthemum (*Chrysanthemum morifolium*), tulip (*Tulipa* spp.), lily (*Lilium longiflorum*), gerbera (*Gerbera jamesonii*), baby's breath (*Gypsophila paniculata*), freesia (*Freesia* spp.), lisianthus (*Eustoma grandiflorum*), dendrobium (*Dendrobium* spp.)
Potted and bedding plants	Rose (*R.*×*hybrida*), petunia (*Petunia*×*hybrida*), pansy (*Viola bicolor*), impatiens (*Impatiens hawkeri* and *Impatiens walleriana*), begonia (*Begonia*×*hybrida*), African violet (*Saintpaulia* spp.), geranium (*Pelargonium* spp.), cyclamen (*Cyclamen* spp.), anthurium (*Anthurium* spp.), chrysanthemum (*C. morifolium*)
Flowering shrubs and trees	Rose (*R.*×*hybrida*), rhododendron (*Rhododendron* spp. and hybrids), azalea (*Rhododendron occidentale* and *Rhododendron arborescens)*, camellia (*Camellia japonica* and *Camellia sasanqua*), fuchsias (*Fuchsia* spp.), magnolia (*Magnolia* spp. and hybrids), hydrangea (*Hydrangea macrophylla*), lavender (*Lavandula angustifolia*), hibiscus (*Hibiscus* spp.), viburnum (*Viburnum carlesii*, *Viburnum* spp., and hybrids)

technology provides an opportunity to create varieties with new flower colors, enhanced vase life, and improved fragrance. Use of genetic engineering to realize this opportunity has been achieved in several species, including the most important cut-flower species rose, chrysanthemum, and carnation. This chapter highlights the current status of transgenic research in floricultural plants, with a focus on the status of commercialization.

2. GENETIC TRANSFORMATION

A primary enabling technology that needs to be developed for a floricultural species before a genetic modification strategy can be considered is the transformation technique. Genetic transformation has been reported in many ornamental species, including important floricultural species, as overviewed by Brand (2006), Shibata (2008), Nishihara and Nakatsuka (2011), and Milošević et al. (2015). The key constraints to transformation of a floricultural plant are similar to those with other plant species, namely, the ease with which regeneration and stable gene transfer can be achieved. High regeneration efficiency and transformation success largely depend on plant species and choice of explants. As with many plant species, regeneration capacity and susceptibility to infection in floricultural plants also varies depending on genotype (variety). As a principle it is more difficult to transform woody plants than herbaceous floricultural crops because woody plants have mature phase tissues that may overproduce polyphenols and are difficult to induce regeneration. Floricultural monocot species, which are less amenable to infection by *Agrobacterium*, are more typically transformed using biolistics methods. An important part of the development of transformation vectors suitable for use in transformation protocols is the choice of promoter for both genes of interest and selectable marker genes (Smirnova et al., 2015). For a selectable marker gene, strong constitutive expression is required in the cells of the explant used in the transformation protocol. In contrast, selection of a promoter with a suitable temporal and tissue specificity profile (Dutt et al., 2014; Spitzer-Rimon et al., 2010) is important in targeting expression to specific tissues, such as flowers.

3. TARGET TRAITS FOR GENETIC MODIFICATION

An important target trait for genetic modification of floricultural plant species is flower color (Holton et al., 1993). This is a reflection of the fact that floricultural plants are primarily grown for show or harvest of their flowers and that novel flower color is important in determining the value of a new variety. In a related area, flower fluorescence and/or luminescence are also traits that are under investigation at the research level (Sasaki et al., 2014). Other "consumer traits" that could be the target of genetic modification in floricultural plants include the introduction of fragrance (Dudareva and Pichersky, 2008; Oliva et al., 2015; Saxena et al., 2007; Zvi et al., 2012), alteration of plant form (Gion et al., 2012; Ohtsubo, 2011; Sun et al., 2011), and improvement of flower longevity and vase life (Chandler, 2007; Milbus et al., 2009; Olsen et al., 2015). Vase life can be modified by manipulation of either ethylene biosynthesis or reception of ethylene, and demonstration of the efficacy of genes directed toward these functions has been demonstrated in carnation (Chandler, 2007) and other floricultural species (Olsen et al., 2015).

From the growers' viewpoint, improvement of floricultural varieties by genetic engineering would be beneficial if productivity (Shulga et al., 2011), disease resistance (Debener and Byrne, 2014; Jiang et al., 2016), and/or pest resistance (Birkett and Pickett, 2014; Vieira et al., 2015) could be improved. Improvements in vase life would also be of benefit to growers (Chandler, 2007).

4. GENE ISOLATION AND CURRENT GENE AVAILABILITY

Genes that have been isolated from floricultural plants are primarily those involved in pigment biosynthesis, with focus on the pigments accumulated in flowers. Key genes on the pathway to pigment biosynthesis have been identified and isolated (Tanaka and Brugliera, 2014; Yuan et al., 2013) as well as genes for transcription factors involved in the control of biosynthetic pathways (Huang et al., 2016; Kee et al., 2016). The anthocyanins, carotenoids, and betalains are alone or in combination responsible for the variation in flower color seen in all flowering plants, aside from cases where chlorophyll contributes a green hue. Isolated genes include all the key genes on the anthocyanin biosynthesis pathway (Holton et al., 1993; Tanaka and Brugliera, 2013, 2014), genes on the carotenoid biosynthesis pathway (Zhu et al., 2010), and genes involved in betalain biosynthesis (Polturak et al., 2016; Zheng et al., 2016). Furthermore, in flowers accumulating anthocyanins, genes responsible for modulation of flower color through either modification of the iron profile of the vascular environment in which pigments are accumulated (Shoji et al., 2010), elevating vacuolar pH (Fukada-Tanaka et al., 2000), or modification of the secondary structure of the anthocyanins (Fujiwara et al., 1998; Nakamura et al., 2015) have been isolated.

Genes involved in the biosynthesis of compounds associated with fragrance in some floricultural species have been isolated from petunia (Verdonk et al., 2005), as have transcription factors modulating the biosynthetic pathway associated with fragrance (Zvi et al., 2012). As outlined previously, there are many flower color modification genes that have been isolated and these have been used to both complement biosynthetic pathways and downregulate endogenous pathways in transgenic floricultural plants. Other types of gene, for both consumer and producer traits, are also available (Azadi et al., 2016; Chandler and Brugliera, 2011; Chandler and Sanchez, 2012).

The selectable marker genes used in other plant species, such as antibiotic resistance and herbicide resistance, can also be used in floricultural plants (Chandler and Brugliera, 2011; Sundar and Sakthivel, 2008). In the carnation and rose varieties that have been commercialized to date,

both these types of selectable marker have been used (Chandler et al., 2013; Katsumoto et al., 2007). In summary, gene availability is currently not a limiting factor for the development of new transgenic floricultural varieties and is unlikely to be in the future, given the advent of the release of genome sequence for more ornamental species and the introduction of artificial gene technology (Yu et al., 2016).

5. GENETIC MODIFICATION: STATUS BY SPECIES AND TRAITS

Space does not allow us to itemize every report in which a trait has been introduced into a floricultural plant by genetic engineering methods. For reference, Noman et al. (2017) have compiled a summary grid in which modified trait is collated against target ornamental crop, including some floricultural crops. For an additional overview, readers are directed to reviews by Azadi et al. (2016), Chandler and Sanchez (2012), Lutken et al. (2012), Noman et al. (2017), and Tanaka and Brugliera (2014).

By species, there are reports of modification for different traits in the important cut-flower species chrysanthemum (Brugliera et al., 2013; He et al., 2013; Noda et al., 2013; Shinoyama et al., 2015), rose (Nakamura et al., 2015), gentian (Nishihara et al., 2015), *Dendrobium* spp. (Teixeira da Silva et al., 2016), and in the important pot plant torenia (Nakamura et al., 2010; Nishihara et al., 2013; Sasaki et al., 2016).

The trait that has been most commonly modified in floricultural plant species is flower color, and this has been reviewed by Chandler and Tanaka (2017), Nishihara and Nakatsuka (2011), Sasaki and Nakayama (2015), Tanaka et al. (2010); Tanaka and Brugliera (2013, 2014), Zhang et al. (2014), and Zhao and Tao (2015). Flower color modification in petunia was one of the first genetically modified traits reported (Meyer et al., 1987). Color modification has been achieved by modification of anthocyanin, carotenoid, and betalain profiles (Chandler and Tanaka, 2017; Nakatsuka et al., 2013; Ono et al., 2006). In a significant breakthrough, Nakatsuka et al. (2013) achieved the introduction of the betalain biosynthetic pathway in cultured cells of the anthocyanin-producing plants tobacco and *Arabidopsis*. Color modification in flowers has been described in carnation (Chandler et al., 2013; Yagi, 2015), chrysanthemum (Brugliera et al., 2013), *Phalaenopsis* (Chen et al., 2011; Mii, 2012), iris (Jeknic et al., 2014), *Tricyrtis* (Kamiishi et al., 2011), *Lotus japonica* (Suzuki et al., 2007), gentian (Nakatsuka et al., 2011), *Dahlia* (Mii, 2012), and rose (Katsumoto et al., 2007; Nakamura et al., 2015). Genetic modification has been used to both complement and downregulate pigment biosynthetic pathways in transgenic floricultural plants. In the commercially available transgenic varieties of carnation and rose, genetic modification has been used to add a key gene in the biosynthetic pathway absent from these species in nature, leading to the accumulation of anthocyanins not found naturally in these species [for details refer to Tanaka and Brugliera (2013) and Chandler and Tanaka (2017)]. The same strategy has been used to modify flower color in chrysanthemum (Brugliera et al., 2013; Noda et al., 2013). Downregulation of endogenous gene function to redirect pigment biosynthetic pathways and so modify pigment profile in flowers of transgenic plants has been achieved using RNA interference (RNAi) or cosuppression technology in *Phalaenopsis* (Chen et al., 2011), chrysanthemum (He et al., 2013), *Tricyrtis* (Kamiishi et al., 2011), and torenia (Nakamura et al., 2011a). To modify flower color, gene editing technology such as clustered regularly interspaced short palindromic repeat (CRISPR)/Cas9 can be used to knock out a specific gene on a biosynthetic pathway.

In developments toward the modification of flower and plant forms using genetic modification, multipetal cyclamens with possible commercial value have been developed using a chimeric repressor

of a transcriptional factor (Tanaka et al., 2013). Various morphologically modified plants, including rose (Gion et al., 2012), are shown at FioreDB (http://www.cres-t.org/fiore/public_db/f_contact.shtml).

Table 6.2 summarizes the current status of genetic modification by trait and species in floricultural crops that are at or close to commercialization.

Table 6.2 Current Status of Genetic Modification by Trait and Species in Floricultural Crops

Species	Modified Trait	Inserted Gene	Description of Modified Phenotype	References
Rose (*Rosa×hybrida*)	Flower color	Flavonoid-3′5-hydroxylase	Production of novel anthocyanins in petals	Katsumoto et al. (2007) and Nakamura et al. (2015)
Rose (*R.×hybrida*)	Disease resistance	Ace-SAMP1 or down-regulation of RhMLO1	Decreased fungal infection	Li et al. (2003) and Qiu et al. (2015)
Rose (*R.×hybrida*)	Fragrance	*PAP1* transcription factor	Modified terpenoid profile	Zvi et al. (2012)
Carnation (*Dianthus caryophyllus*)	Improved vase life	Downregulation of ACC synthase, insertion of mutant ethylene receptor gene	Reduced ethylene production or reduced ethylene sensitivity	Chandler (2007) and Bovy et al. (1999)
Carnation (*D. caryophyllus*)	Flower color	Flavonoid-3′5-hydroxylase	Production of novel anthocyanins in petals	Tanaka et al. (2010) and Tanaka and Brugliera (2013)
Chrysanthemum (*Chrysanthemum morifolium*)	Flower color	Flavonoid-3′5- hydroxylase, downregulation of flavonoid-3′-hydroxylase	Production of novel anthocyanins in petals	Brugliera et al. (2013), He et al. (2013), Kee et al. (2016), Noda et al. (2013), and Zhang et al. (2014)
Chrysanthemum (*C. morifolium*)	Flowering time	*AP-1* genes	Early flowering	Shulga et al. (2011)
Chrysanthemum (*C. morifolium*)	Pest resistance	*Bacillus thuringiensis* cry1Ab or *N*-methyltransferases	Resistance to lepidopteran larvae or armyworm	Kim et al. (2011) and Shinoyama et al. (2015)
Gerbera (*Gerbera jamesonii*)	Flower color	Downregulation of ACC synthase	Altered anthocyanin profiles in flowers	Elomaa et al. (1996) and Ainasoja et al. (2008)
African violet (*Saintpaulia* spp.)	Disease resistance	Chitinase/glucanase	Fungus resistance	Ram and Mohandas (2003)
Torenia	Flower color	Downregulation of flavonoid 3′-hydroxylase and flavonoid 3′,5′-hydroxylase; dihydroflavonol 4-reductase expression	Altered anthocyanin profiles	Nakamura et al. (2010) and Nishihara et al. (2013)

ACC synthase, *1-Aminocyclopropane-1-carboxylate synthase.*

6. TRANSFORMATION OF FLORICULTURAL CROPS

For reference, reviews of the transformation of ornamental and floricultural plants have been published (Azadi et al., 2016; Milošević et al., 2015; Singh et al., 2016) as well as papers dealing with the specific floricultural plants dendrobium (Teixeira da Silva et al., 2016), torenia (Nishihara et al., 2013), and anthurium (Teixeira da Silva et al., 2015). Of the most important cut flowers, transformation protocols have been reported for rose (Katsumoto et al., 2007), carnation (Iantcheva, 2016), *Phalaenopsis* (Hsing et al., 2016; Mii, 2012), gypsophila (Zvi et al., 2008), gerbera (Chung et al., 2016), *Dahlia* (Mii, 2012), and chrysanthemum (Noda et al., 2013). Protocols are also available for the pot plants listed in Table 6.1, which are largely herbaceous plants amenable to transformation. Though woody plants are more difficult to transform (Guan et al., 2016), transformation protocols have been published for several of the trees and shrubs listed in Table 6.1.

As the most important cut-flower species, transformation protocols for rose, carnation, and chrysanthemum are the most commercially relevant and are summarized here.

Carnation transformation was reported in the early 1990s and reliable protocols for transformation across multiple varieties were reported by Lu et al. (1991) and van Altvorst et al. (1996) at that time. Adventitious regeneration from leaf or shoot tip explants in combination with *Agrobacterium* cocultivation is very effective in this species (Lu et al., 1991) and this general process has been optimized (Arici and Koc, 2009; Nontaswatsri et al., 2004; Prasad et al., 2016). Like carnation, chrysanthemum is both amenable to adventitious shoot regeneration from leaf or stem material and susceptible to *Agrobacterium* infection. Cocultivation protocols, which must be tailored with suitable promoters for both selectable marker gene and gene of interest, have been reported by Brugliera et al. (2013), Naing et al. (2016), and Noda et al. (2013).

Rose is a woody plant and though susceptible to *Agrobacterium* infection is more difficult to transform through adventitious shoot protocols. Though regeneration from vegetative tissue is possible (Robinson and Firoozabady, 1993) excessive production of polyphenols and flavonoids often results in poor regeneration. Accordingly, transformation protocols have been developed that use regeneration of transformed cells via somatic embryogenesis (Katsumoto et al., 2007; Lee et al., 2013; Zakizadeh et al., 2008, 2013; Shen et al., 2016).

7. THE REGULATION OF GENETICALLY MODIFIED FLORICULTURAL PLANTS

Virtually every country in the world has enacted legislation to control laboratory experimentation, field release, and marketing/general release of genetically modified organisms. There is also an international treaty (the Cartagena biosafety protocol within the convention on biological diversity; https://www.cbd.int/doc/legal/cartagena-protocol-en.pdf) concerning international trade on what are termed living modified organisms (for all practical purposes, living modified organism are the same as genetically modified organisms). Within the biosafety protocol a biosafety clearing house has been established (https://bch.cbd.int/). This hosts a searchable database of genes, genetically modified traits, and genetically modified organisms.

Genetically modified floricultural varieties fall under the rules and regulations of the territory(ies) in which the genetically modified organism is grown and/or imported. In the absence of any legislation covering multiple countries (with the single exception of the European Union) it is therefore likely to be necessary to obtain regulatory clearance in multiple countries because many floricultural products are routinely traded internationally (van Rijswick, 2015).

8. OVERVIEW OF REGULATIONS WORLDWIDE

For the purpose of an overview it is important to recognize that floricultural species are nonfood plants. There are instances where flowers are utilized as garnishes, or may be utilized in herbal teas, but the primary purpose of floricultural plants is for their amenity and decorative value. This is a very important point, because nonfood genetically modified organisms are subject to less regulation than genetically modified organisms used in food. In some countries, additional regulatory scrutiny is required if pest or pathogen resistance is intended to be exploited in the genetically modified plant, even if the product is not a food. A consistent feature of regulation of genetically modified plants is that assessment is undertaken on the basis of risk assessment. Different regulatory frameworks are in place across the world; however, because different guidelines are in place, the details of the application process and the risk assessment process vary between countries. Because they are minor crops, the consensus documents available for the major food crops have not been prepared for floricultural products, aside from carnation (http://www.ogtr.gov.au/internet/ogtr/publishing.nsf/Content/biologycarnation-toc). In practice, for the same genetically modified product different authorities require different information in differing amounts of detail.

Though some countries regulate on the basis of process, others regulate on the basis of phenotype or genotype. Accordingly, it is possible in some countries to manage the regulatory dossiers on the basis of multiple events made with one construct (Beker et al., 2016). In other countries, each event must be submitted in a separate application, leading to duplication of baseline information. The need to apply on an event basis is largely driven by the need to provide detailed bioinformatic information on an event basis (Ladics et al., 2015). Even where it is possible to apply for multiple events it is necessary and advisable to provide a unique identification number for each event. Readers are directed to details of the regulatory work associated with the commercial release of rose (Nakamura et al., 2011a,b) and carnation (Chandler et al., 2013) as a guide to the regulatory work associated with these two vegetatively propagated, nonfood floricultural plants.

A critical consideration in the research planning process is to identify where a crop is to be produced and where the final product (a cut flower or flowering pot plant, for example) is to be utilized. For example, a regulatory assessment will determine a different level of potential risk if a cut-flower species is to be grown within a particular jurisdiction than if the final product (imported harvested cut flowers, for example) is the subject of regulation. Because many ornamental plants have established as weeds, and are invasive in some parts of the world (Dehnen-Schmutz et al., 2007; Li et al., 2004; Parrella et al., 2015), the potential for gene flow will be a primary consideration for regulators, particularly if wild relatives are present in the country where it is intended to release the genetically modified floricultural product.

9. COST OF REGULATION

The cost of regulatory compliance depends on three main factors. The first factor is the amount of trial work required to generate the information necessary to comply with the regulatory requirements of the country in which the genetically modified floricultural product is to be grown and/or sold. This in turn will depend on whether there are related wild species in the country chosen for production (if so, there is likely to be a need to evaluate the potential for gene flow) and the data requirements dictated by the legislation of that chosen country. In most cases it will be at least necessary to measure any differences in

morphological character between the genetically modified event and the variety used for transformation (Gomez-Galera et al., 2012). A second factor is the molecular characterization requirements of the country in which regulatory approval is required. The extent of characterization can simply be a description of the transformation vector and southern information on insert number. However in some countries, more complete information is required such as the complete sequence of the vector and insertions, sequence of the genomic DNA flanking the inserts, and a validated identification protocol. If an event is to be traded in the international marketplace the amount of molecular characterization will need to be generated to meet the needs of the authority requiring the information (Schnell et al., 2015). To reduce the cost of compliance and simplify the risk assessment process it is advisable to select for commercialization single insert events in which no extra T-DNA vector elements have been incorporated into the transgenic event. A third cost center for compliance is the administrative cost associated with working internationally with genetically modified plant varieties. Some countries require that application is made through local representatives (an institutional biosafety committee and/or legal representative) and it is sometimes the case that applications can only be made in the local language, requiring translation costs even if there is no intention to carry out trials. Fees are required to lodge applications for market approval in some countries and a significant fee is required for validation of unique identification protocols in the European Union.

The cost of regulatory compliance for a genetically modified organism has been estimated in millions of US dollars for a food crop (Kalaitzandonakes et al., 2007) and has for many years been recognized as a barrier to market entry for minor crops (Alston et al., 2006; Strauss, 2011). This estimation is not to be expected for a floricultural product. However, when all costs are considered (trial costs, molecular analysis, administration, monitoring, travel, consultant costs, translation, etc.) it is reasonable that a budget of several hundred thousand US dollars should be allocated to the regulatory process when considering the commercial release of a genetically modified floricultural product.

10. INTERNATIONAL TRADE CONSTRAINTS

As mentioned earlier, many floricultural products are traded internationally. However, aside from within the European Union, there is no international agreement that allows for a regulatory approval issued by one country to be adopted by another. This is a constraint to trade because multiple regulatory approvals are required for the same product, adding costs to the regulatory approval process overall. Datasets generated for one authority may be used in regulatory applications in another country, but the need for multiple approvals is potentially a frustrating obstacle to the commercial development of a genetically modified floricultural variety because it may be necessary to carry out duplicate trials to obtain the same information if a product is to be grown in several countries.

Currently, regulation of genetically modified organisms for market release in the United Kingdom is covered by EU legislation. Potentially, this will no longer be the case after the United Kingdom exits the European Union (Mitchell, 2016).

11. NEW BREEDING TECHNOLOGIES

Questions have been raised regarding the way in which plants developed using new breeding technologies (Abdallah et al., 2015; Cardi and Varshney, 2016; Lozano-Juste and Cutler, 2014), such as genome editing, CRISPR/Cas9 (Barakate and Stephens, 2016; Khatodia et al., 2016; Ma and Liu, 2016; Samanta

et al., 2016), RNAi (Cascuberta et al., 2015; Ramon et al., 2014), and transcription activator-like effector nuclease technologies will be regulated in the future (Caplan et al., 2015; Jones, 2015). This is because the development of these technologies has outpaced legislation, which was developed before this technology was available and before the importance of epigenetics was as well understood as it is now (Alvarez-Venegas and De-la-Pena, 2016). Several countries are working on redefining genetic modification or are revising legislation to adapt to the potential outcomes from the use of the new technologies. The potential for synthetic genes (Yu et al., 2016) to be included in future genetically modified plants will also require adaptation of existing regulations (Puchta, 2016; Tiwari et al., 2014).

12. STATUS OF COMMERCIALIZATION OF GENETICALLY MODIFIED FLORICULTURAL PLANTS

As at the end of 2016, the only genetically modified varieties found in the floricultural marketplace were eight color-modified varieties of carnation and one rose variety (www.florigene.com). The genetically modified carnation is grown in Ecuador, Colombia, and Australia and cut flowers are imported into the Unite States, Japan, Australia, and Europe. The genetically modified "Applause" rose is currently grown and sold in Japan. On the basis of trials that have been carried out in Colombia, the scientific literature, and public information such as that posted by the US government (https://www.aphis.usda.gov/aphis/ourfocus/biotechnology/am-i-regulated), it is a reasonable assessment to say that the only genetically modified floricultural products likely to be released in the near future will also be color-modified cut-flower varieties of rose, chrysanthemum, and gypsophila. We are unaware of genetically modified varieties of any floricultural pot plants, bedding plants, shrubs, or trees that are close to commercial release.

13. FUTURE PROSPECTS

There continues to be significant research on genetic modification of various floricultural plants and there also continues to be an interest in more exotic applications of the technology such as flowers that "glow in the dark" (https://www.aphis.usda.gov/aphis/ourfocus/biotechnology/am-i-regulated). From the experience of the products already on the market, public perception is not a significant factor determining the uptake of the technology (Anderson and Walker, 2013). Rather, it is the cost of introduction of genetically modified varieties compared to conventionally bred varieties that is a deterrence to market introduction. Part of this cost is related to the cost of research but a significant cost burden, unique to the introduction of genetically modified plant varieties, is regulatory compliance.

The cost of regulatory compliance for genetically modified varieties goes a very long way to explaining the lack of commercialization of the technology for "consumer traits." We do not feel it is likely that floricultural plants with producer-oriented traits will be commercialized because of the cost of regulatory compliance combined with the difficulties associated with cost recovery by the developer (Chandler, 2007).

For the floricultural industry, a significant barrier to commercialization could be removed if international regulations could be moved to a phenotype, rather than process based regulatory regime, which could then be harmonized internationally (Ramessar et al., 2009; Roberts et al., 2015). As many floricultural plants are vegetatively propagated, regulation on a construct basis (Beker et al., 2016), rather than on an event basis, would reduce regulatory costs.

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CHAPTER

GENETIC ENGINEERING IN PAPAYA

7

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1. INTRODUCTION

Papaya is the common name of the genus *Carica* and is represented by only one species, *Carica papaya*, in the family Caricaceae. The genus name *Carica* is derived from the Latin name for a kind of fig that the leaves and fruits of this crop resemble, while the species name *papaya* comes from the common name of the fruit (Du Puy and Telford, 1993). It is one of the few tree crops that produce ripe fruits as quickly as 9 months from planting. *Papaya*, with a world production of 6.8 million metric tons in 2005 (FAO, 2006), is a major economic crop in many tropical countries. The fruit is a valuable export commodity, which generates hard currency earnings in many developing countries. It is a perennial crop that fruits throughout the year. In the revised taxonomy of the family Caricaceae, some species that were earlier associated with the genus *Carica* have now been classified as another genus, *Vasconcellea*, within the family (Badillo, 2002). Hence, according to the latest classification, the genus *Carica* is monotypic.

1.1 GENETICS, SYSTEMATICS, AND GENOMICS

C. papaya L. is a dicotyledonous polygamous diploid species having male, female, or hermaphrodite flowers on the same plant. In nature, plants are dioecious with male and female flowers found on different plants (Da La Cruz Medina et al., 2002). However, the commercial papaya cultivars grown in different parts of the world are inbred gynodioecious, which means the plants have bisexual or hermaphrodite flowers on some plants and only female flowers on other plants of the same species. Some taxonomists reported an outcrossing dioecious character in papaya (Kim et al., 2002), but monoecious plants having both male and female flowers on the same plants have also been reported. Systematically, according to updated classification, *C. papaya* is the only species of the genus *Carica* in the family Caricaceae. The family has a total of 31 species belonging to five genera, namely, *Carica*, *Jacaratia*, *Jarilla*, *Horovitzia*, and *Vasconcellea* (Badillo, 2000). The genus *Vasconcellea* is the most contiguous relative of *C. papaya.* These two species can crossbreed, hence they are being used in hybrid breeding programs (Van Droogenbroeck et al., 2004). In general, papaya is a cross-pollinated species but its genetic makeup is such that even self-fertilization does not result in inbreeding depression. This characteristic of papaya has an advantage in breeding programs to fix useful genetic characteristics in both gynodioecious and dioecious lines (Aquilizen, 1987).

Genetic Engineering of Horticultural Crops. http://dx.doi.org/10.1016/B978-0-12-810439-2.00007-6

Papaya genome sequencing started as an integrative multiinstitutional consortium that was constituted in the University of Hawaii in 2004 and the sequencing was completed in 2007. Papaya is diploid with nine pairs of chromosomes having a small genome of 372 Mbp/1C (Arumuganathan and Earle, 1991; Bennett and Leitch, 2005). There are considerable phenotypic variations available within the genus *C. papaya*; it has been crossed with different species of *Vasconcellea* such as *C. papaya* × *Vasconcellea cauliflora*, *C. papaya* × *Vasconcellea quercifolia*, and *C. papaya* × *Vasconcellea pubescens* to obtain resistant interspecific hybrids (Drew, 2005). However, because of genome incompatibility such crosses are not very successful except for *C. papaya* × *V. quercifolia*, which shows some level of fertility and thus proved to be helpful in backcrossing programs (Magdalita et al., 1997). Sajise et al. (2004) revealed cross-compatibility of *C. papaya* × *V. quercifolia* of local inbred selections from the Philippines but the same cross-compatibility lacked in between two species from other countries of the world. The aim of the papaya genome project was to map and clone *Papaya* genes related to the improvement of economic value and efficiency of cultivation. The insight from the results of the papaya genome project has been used to develop *Papaya* varieties resistant against various biotic and abiotic stress factors.

2. GENETIC TRANSFORMATION STUDY IN PAPAYA

Plant tissue culture regeneration systems have been used as important tools for successful genetic transformations in papaya. Immature zygotic embryos or young seedling tissues have been found most successful in regeneration of papaya plants for genetic transformations (Fitch and Manshardt, 1990; Fitch, 2005). The transformation protocol was developed on papaya using both biological and physical methods by Fitch et al. (1990) and Fitch (1993). The modified versions of these two protocols have been used and both have produced good results (Fitch, 2005). A biolistics system using somatic embryos as target tissue ensured 41% successful transformation against kanamycin resistance and it took 6 months to get a genetically transformed plantlet of papaya (Mahon et al., 1996). The main drawback of biolistics transformation technology in papaya is that it cannot be used directly to an elite female or hermaphrodite cultivar, only to progeny (Drew, 2005).

2.1 BIOTIC AND ABIOTIC STRESS

To adapt abiotic and biotic stresses in their natural habitats, plants have developed immense mechanisms (Agarwal et al., 2011). Major focus has been given to gene regulation at the transcriptional level to find the responses of plants to their environments. Transcription factors (TFs) are basically the proteins capable of activating or restraining the transcription of downstream target genes. They do this by binding directly to promoters of target genes in a sequence-specific mode (Pape et al., 2010). The *WRKY* TFs are known to have a dual role by acting as both positive and negative regulators in the responses to abiotic and biotic stresses in plants and also form one of the largest families (Agarwal et al., 2011). The response of plants to abiotic and biotic stresses is more likely to be a polygenic cooperative defense response induced by cold, drought, salt, wounding, and disease rather than the single response of a single gene. Therefore in improving the stress tolerance of plants through molecular breeding, the traditional transformative method that applies a single resistance gene has limitations. TFs that regulate the expression of multiple genes are more promising candidates for manipulation. TFs regulate gene expression in response to various external and internal cues by activating or suppressing

downstream genes in the pathway. Zinc-finger proteins (ZFPs) are a group of TFs that are expressed in different stressful conditions. *C2H2* ZFP TFs are induced by various stresses and are the important candidate genes that make resistant plants. Ming et al. (2008) compared gene numbers between the TF tribe and related tribes of *Arabidopsis* and *Papaya* and also reported that most TFs were represented by fewer genes in *Papaya* than in *Arabidopsis*. Jiang and Pan (2012) showed that *ZF30,912.1* could be an important TF that mediates responses to abiotic and biotic stresses in *Papaya*. Pan and Jiang (2014) reported the identification and expression of the *WRKY* TFs of *C. papaya* in response to abiotic and biotic stresses.

Papaya ringspot virus (PRSV) is the most significant problem for papaya all over the world, which should be solved using genetic transformation technologies. The significance of PRSV on papaya crops can be estimated from the fact that the first trait used in genetic transformation experiments for *Papaya* was PRSV. The first genetically modified crop was used for human consumption in Hawaii and South-East Asia (Gonsalves, 1998). Two PRSV virus-resisting cultivars named "SunUp" (red fleshed) and "Rainbow" (yellow fleshed) were successfully developed in Hawaii (Gonsalves and Manshardt, 1996). In these two virus-resistant cultivars a concept of parasite-derived resistance was used in which the PRSV coat protein (CP) expresses through a posttranscriptional gene silencing method (Gaskill et al., 2002).

As a typical climacteric fruit, papaya fruit undergoes massive problems such as rapid ripening and susceptibility to biotic or abiotic stresses, which usually result in a high percentage of production loss. A better understanding of the postharvest physiology and molecular biology of papaya fruit would be helpful to overcome these problems. Gómez et al. (2009) found that ripening in papaya fruit is altered by 1-aminocyclopropane-1-carboxylate (ACC) oxidase cosuppression and found a sharp reduction in ethylene and CO_2 production was detected along with altered softening pattern and peel color development.

Papaya has been genetically modified to delay fruit ripening by downregulating the ACC synthase enzyme, which is an intermediate enzyme in the biosynthesis of ethylene. Another modification has been done in the genetics of *Papaya* for delayed fruit ripening by a change in the pathway responsible for perception of ethylene (Fitch, 2005). Overall, transgenic *Papaya* showed a delayed fruit-ripening rate. A reduction in messenger RNA level for ACC oxidase in transgenic fruit was clearly detectable by Northern blot. Li et al. (2013) isolated and characterized the ethylene response factor family genes during development, ethylene regulation, and stress treatments in papaya fruit. The results support the role for *Papaya* ethylene response factors in the transcriptional regulation of ripening-related or stress response genes. Papaya production is currently limited to tropical and subtropical regions and the species is extremely sensitive to frost. Papaya has been genetically transformed to improve traits that are difficult to incorporate through conventional breeding such as resistance to PRSV (Fitch et al., 1993; Gonsalves, 1998; Yeh and Gonsalves, 1984; Ying et al., 1999), extended shelf-life (Magdalita et al., 2002), and insect resistance (McCafferty et al., 2003). Fitch et al. (1990) obtained transgenic plants after culturing embryogenic mass on induction media containing 150 mg/L kanamycin sulfate for 1 year; however, abnormal plants were regenerated because of long exposure of the cultures to 2,4-D. Fitch et al. (1993) obtained two transgenic lines from embryogenic cultures. Cheng et al. (1996) and Cai et al. (1999) achieved 15 and 83 transgenic lines, respectively. These differences might be attributed to a cultivar-specific embryogenesis response. *Agrobacterium*-mediated genetic transformation and plant regeneration were also achieved using embryogenic culture with CBF1 and CBF3 genes. Only a few transgenic plant lines have been recovered compared to the amount of embryogenic tissue that was cocultivated. This could be caused by low transformation efficiency and/or loss of embryogenic potential of the tissue because of prolonged exposure to 2,4-D.

The CBF1 sequence was used as a probe to detect the presence of both *CBF1* and *CBF3* transgenes in transgenic plant lines because their sequences share 88% homology (Gilmour, personal communication). The probe detected a single copy of the transgene in tissues obtained from plants transformed with *CBF3*, whereas three copies of the transgene were detected in tissues obtained from plants transformed with *CBF1*. Genetic transformation can involve the integration of multiple copies (Jorgensen et al., 1996), which can cause transcriptional or posttranscriptional gene silencing (Matzke et al., 1994). Thus the effect of the integration of multiple *CBF1* transgenes on *trans*-protein synthesis in transgenic plants can be determined using Western blot hybridization. *CBF* proteins are synthesized in response to low temperatures (Jaglo-Ottosen et al., 1998) and bind to the Crepeat/DREDNA regulatory element in the promoter region of the *COR* genes and activate these genes (Stockinger et al., 1997). *CBF1* overexpression induces *COR* gene expression without a low-temperature stimulus. Thus the actual increase in freezing tolerance is brought about by expression of the *COR* genes, and the *CBF* genes simply activate the transcription of these *COR* genes. The *CBF3* gene activates multiple components of the cold acclimation process in response to low temperatures (Gilmour et al., 2000). For *CBF* genes to be functional in papaya there must be a cold acclimation signaling mechanism and downstream genes in the signaling pathway, which are activated in response to the *CBF* protein.

Papaya was transformed via *Agrobacterium*-mediated transformation with four constructs containing either the unmodified or modified CP gene of Florida isolate H1K of PRSV. The CP genes were in the sense orientation (S-CP), antisense orientation (AS-CP), sense orientation with a frameshift mutation (FS-CP), or sense orientation mutated with three-in-frame stop codons (SC-CP). Two hundred and fifty-six putative transgenic lines with the CP constructs were inoculated mechanically with PRSV H1K. None of the lines was immune to PRSV; however, highly resistant lines were found in each CP transgene group. For breeding purposes, 21 PRSV-resistant lines representing the four transgene constructs were selected and crossed with six papaya genotypes. The lines from the FS-CP and SC-CP transgene groups were highly fertile, but those from the S-CP and AS-CP transgene groups were practically infertile. Plants derived from 54 crosses and representing 17 transgenic lines were planted in the field. After 1 year in the field trials, 293 of the 1258 the plants (23.3%) became naturally infected with PRSV, whereas 29 of 30 of the nontransgenic control plants (96.7%) became infected. The incidence of PRSV infection varied in the R_1 progeny depending on both the transgenic line and the nontransgenic parent.

The microprojectile is one of the preferred methods of transformation in papaya (Fitch et al., 1992; Gonsalves et al., 2004). The microprojectile or biolistics method consists of transporting biologically active DNA into cells by using metal particles with high velocity. Klein et al. (1987) found that tungsten particles could be used to introduce macromolecules such as RNA and DNA into epidermal cells of onion with subsequent transient expression of enzymes encoded by these compounds. Christou et al. (1988) demonstrated that the process could be used to deliver biologically active DNA into living cells, which results in the recovery of stable transformants. Microprojectile offers no biological limitation to the actual DNA delivery process, therefore it can be used across the genotypes. It is a substitute for difficult tissue culture processes, which are observed in *Agrobacterium*-mediated transformation. The microprojectile system was refined using soybean and rice as model plants (Christou and Swain, 1990; McCabe et al., 1988; Christou et al., 1991), which proves that this system can work across all dicotyledonous and monocotyledonous species.

The concept of microprojectile has been described in detail by Sanford (1988). He developed the first acceleration device, which accelerated tungsten particles coated with biologically active DNA to high velocities (328–656 m/s), which were able to penetrate cell wall and membranes and enter cells.

Several recalcitrant crops were genetically engineered with ease using this technology. However, there are flip sides to this technology too. It is difficult to transform organized structures using microprojectile (shoot tips, meristems). Optimizing pressure of compressed gas is very crucial otherwise soft tissues can be killed. Sometimes transgenic plants developed through microprojectile have been reported to have multiple copy numbers. Papaya was transformed using the microprojectile technique (Fitch et al., 1992; Cai et al., 1999). The biolistics method for transforming *C. papaya* L. was developed that targeted a thin layer of embryogenic tissue. The key factors in this protocol include:

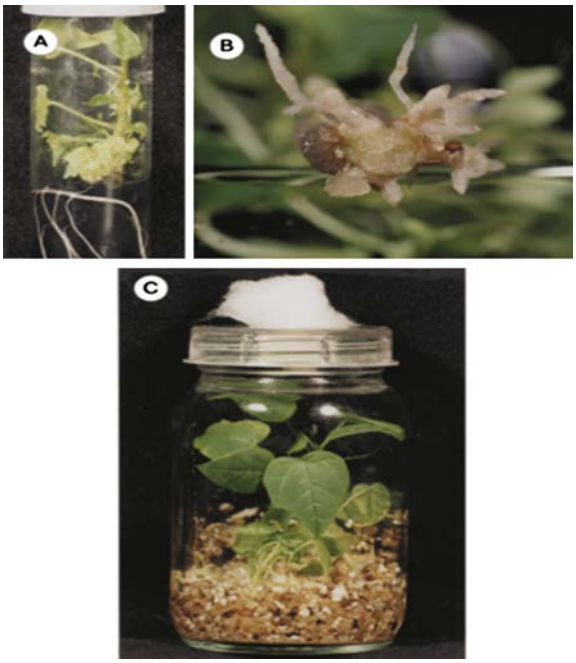
1. Spreading of young somatic embryo tissue that arose directly from excised immature zygotic embryos, followed by another spreading of the actively growing embryogenic tissue 3 days before biolistic transformation.
2. Removal of kanamycin selection from all subsequent steps after kanamycin-resistant clusters had been isolated from induction media containing kanamycin.
3. Transfer of embryos with finger-like extensions to maturation medium.
4. Transferring explants from germination to the root development medium only after the explants had elongating root initials, with at least two true green leaves about 0.5–1.0 cm long.

Total of 83 transgenic papaya lines expressing the nontranslatable CP gene of PRSV were obtained from somatic embryo clusters that originated from 63 immature zygotic embryos. Transformation efficiency was very high: 100% of the bombarded plates produced transgenic plants (Cai et al., 1999). Christou (1992) identified three critical variables, namely, physical, environmental, and biological, which require careful optimization.

2.2 REGENERATION OF TRANSFORMED PLANTS

C. papaya L. is an important crop in many subtropical and tropical countries. Many problem areas still exist with the commercial in vitro propagation of papaya. These include leaf senescence, stunted plants produced as a result of cytokinin toxicity, reduced rooting ability, and poor root quality. Methods for regeneration of in vitro papaya plants have been described using callus cultures (Debruijne et al., 1974; Litz and Conover, 1977; Arora and Singh, 1978; Jordan et al., 1983; Pandey and Rajeevan, 1983; Fitch, 1993), somatic embryos (Cheng et al., 1996; Ernawati et al., 1997; Castillo et al., 1998), and apical and axillary bud explants (Medhi and Hogan, 1976; Drew and Smith, 1986; Rajeevan and Pandey, 1986; Drew, 1992; Singh et al., 1997). The addition of activated charcoal to plant growth media is known to have many beneficial effects. These include the release of naturally present or previously adsorbed compounds into the media and the creation of a dark environment, thus facilitating the accumulation of photosensitive auxins or cofactors and encouraging a rooting response (Pan and Staden, 1998). Other beneficial effects include the adsorption of inhibitory compounds such as phenolics (Weatherhead et al., 1978) and plant growth regulators such as cytokines, auxins, and ethylene (Ebert et al., 1993). The multiplication rate for plantlets grown on gelrite with activated charcoal was significantly reduced when 3 g/L activated charcoal was used. The multiplication rate for plantlets grown on 1.5 g/L activated charcoal and gelrite was significantly higher than when grown on agar alone and almost equaled the multiplication rate of those grown on gelrite alone. However, the addition of 1.5 g/L activated charcoal to the media was not sufficient to eliminate hyperhydricity completely. Drew et al. (1993) also demonstrated that 1 g/L activated charcoal added to the media gave finer roots and smaller shoots compared to those produced on a riboflavin treatment. The addition of activated charcoal to growth media is known

to have various effects on plants such as the adsorption of inhibitory compounds such as phenolics (Weatherhead et al., 1978) and plant growth regulators such as cytokines, auxins, and ethylene (Ebert et al. 1993). It is possible that activated charcoal played a role in reducing hyperhydricity in papaya by the adsorption of such inhibitory substances.



(A) Papaya planted with smooth roots formed on medium supplemented with 2 g/L charcoal; (B) papaya roots formed on medium in vitro; (C) papaya plantlet grown on medium containing a cotton-wool plug (McCubbin and Stadden, 2003).

Callus formation is an undesirable feature during micropropagation, which leads to genetic variability of regenerated plants (D'Amato, 1977). In the present investigation, we have taken epicotyl segments of an Indian papaya cultivar Co7 as explants, and an efficient multiple shoot induction was achieved via direct organogenesis from in vitro grown papaya seedling plants. We are assured that this procedure generates only true to type plantlets, which could be extended for other papaya cultivars. Shoot growth induction explants exposed to thidiazuron were transferred to shoot proliferation medium [Murashige and Skoog (MS) medium containing B5 vitamins supplemented with blood agar plates (1.0–10 a.m.) and kinetin (1.0–10 μM) in combination with NAA at two levels (0.05 and 0.10 μM)]. After 4–5 weeks, a percentage of explants produced multiple shoots and numbers of shoots per explants were recorded. After 4–6 weeks of induction culture and 4–5 weeks of shoot proliferation, the explants with regenerated shoots were transferred onto the shoot elongation medium containing half strength MS salts, B5 vitamins, and L-glutamine (400 mg-L) supplemented with gibberellic acid at different concentrations (0.5–5.0 μM). The cultures were maintained for 2 weeks in the foregoing medium without subculturing and then transferred to half strength MS medium for elongation of shoots and also to drain the excess hormone to the medium.

3. MARKER-FREE TECHNOLOGY

For removing or eliminating selection marker genes in transgenic plants there are a few major approaches in the literature that offer this possibility (Hare and Chua, 2002; Ebinuma et al., 2001; Puchta, 2000; Yoder and Goldsbrough, 1994), and these include the following:

1. Selection marker gene excision;
2. Intragenomic relocation of transgenes via transposable elements.

This was the first reported genetic system where removal of a marker gene event was successfully attempted. Here it involves the incorporation of transgenes within the *Ds* elements and *Ac* transposon and then inserting this within the T-DNA (Goldsbrough et al., 1993). The autonomous *Ac* element possesses two essential characteristics for transposition that can be physically separated: a transposase coding gene (*Ac*) and the repeat termini (*Ds* elements). In the absence of *Ac*, the *Ds* elements lack the transposase function and are stable, but these can be activated by the introduction of *Ac* transposase coding sequences in *trans*. Very important is the fact that sequences can also be mobilized to new genomic locations that are cloned between the inverted repeats of a *Ds* element in the presence of a transposase gene (Lassner et al., 1989; Masterson et al., 1989). There were different observations made that led to the development of a series of novel transformation vectors that incorporated transposable elements to eliminate selectable marker genes and other ancillary sequences from transformed plants (Goldsbrough et al., 1993; Fedoroff, 1989). For transgene elimination, two types of vector systems have been developed. In the first type, between the *Ds* inverted repeats the gene of interest is inserted. By relocating the gene of interest, different levels of expression, both qualitative and quantitative, can be achieved; this is the advantage of this system. The genomic location that can be reflected by this change in expression pattern, at which the element is inserted, is referred as a the "position effect" (Yoder and Goldsbrough, 1994). In the second type, the *Ds* repeats flanked the selectable marker gene. The *Ds* element containing the marker gene will transpose to a new genomic location in the presence of an active transposase, which can be introduced into the plant as an additional component of the T-DNA, or by crossing to a plant containing transposase, or by a secondary transformation (Belzile et al., 1989). Intragenomic relocalization of the transgene mediated by transposon provides a very useful alternative to carrying out multiple independent transformations to achieve the optimal transgene expression (Cotsaftis et al., 2002) (Fig. 7.1).

For the production of marker-free transgenic plants a new vector system was developed. It is named Multi-Auto-Transformation (MAT) because it can be used for repetitive transformation without sexual crossing as an added advantage (Ebinuma et al., 1997).

3.1 USES OF SITE-SPECIFIC RECOMBINATION

Site-specific recombination has been put to a variety of uses in prokaryotic organisms. A commonly used one is in bacteriophage λ cDNA cloning vectors (Fig. 7.2). Its objective is to excise the cDNA clone from the λ genome and thus this maintains the clone as a smaller and more easily handled plasmid. These vectors are comprised of bacteriophage λ that have a high copy plasmid DNA inserted into its genome. At the boundaries between the plasmid DNA and the λ DNA are recombination sites, typically loxP. The cDNA is cloned into restriction sites contained within the plasmid DNA prior to packaging into viral particles. Upon infection of the appropriate host strain

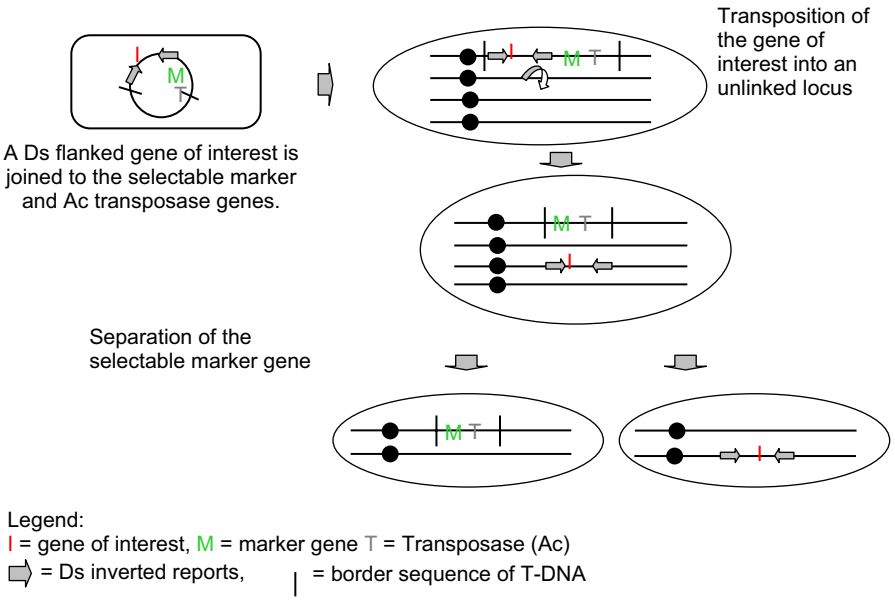


FIGURE 7.1

A *Ds* flanked gene of interest is joined to the selectable marker and *Ac* transposase genes. After transposition of the gene of interest into an unlinked locus, it is segregated from the selectable marker gene by sexual crossing.

expressing the recombinase, by site-specific recombination the plasmid is excised from the λ genome, thus allowing the cDNA-containing plasmid to be replicated autonomously. The use of site-specific recombination also generates molecular diversity for phage display systems. Fusion of the coding sequences for either random peptides or mutated proteins to a phage CP gene is involved by phage display technology; this results in the display of the expressed peptide or protein on the surface of the virus.

In this manner, larger libraries displaying different peptides or proteins have been constructed and used for selecting molecules that bind to a given target. The power of phage display technology in part results from the ability to construct vast libraries displaying differing molecules from which to select. For instance, an alternative to the traditional animal immunization route for generating antibodies has been provided by the development of phage-displayed antibody libraries. One of the constraints on this technology is creating sufficient diversity to form different pairwise combinations of heavy and light variable domains. While for shuffling heavy and light chains in vitro there are methods available; there is an inherent limitation on the size of library that can be built imposed by the efficiency of electroporation of DNA into cells. Libraries with complexities of 108–109 can be constructed using electroporation methods. Using the Cre-lox system a method for constructing libraries of much higher complexity was demonstrated (Waterhouse et al., 1993). The strategy outlined in Fig. 7.2 relies on in vivo site-specific recombination to combinatorially create different pairwise combinations of heavy and light chains. Additionally, there is a VL gene cassette immediately upstream, which is then bounded by lox sites. One site is wild-type loxP and the other one is

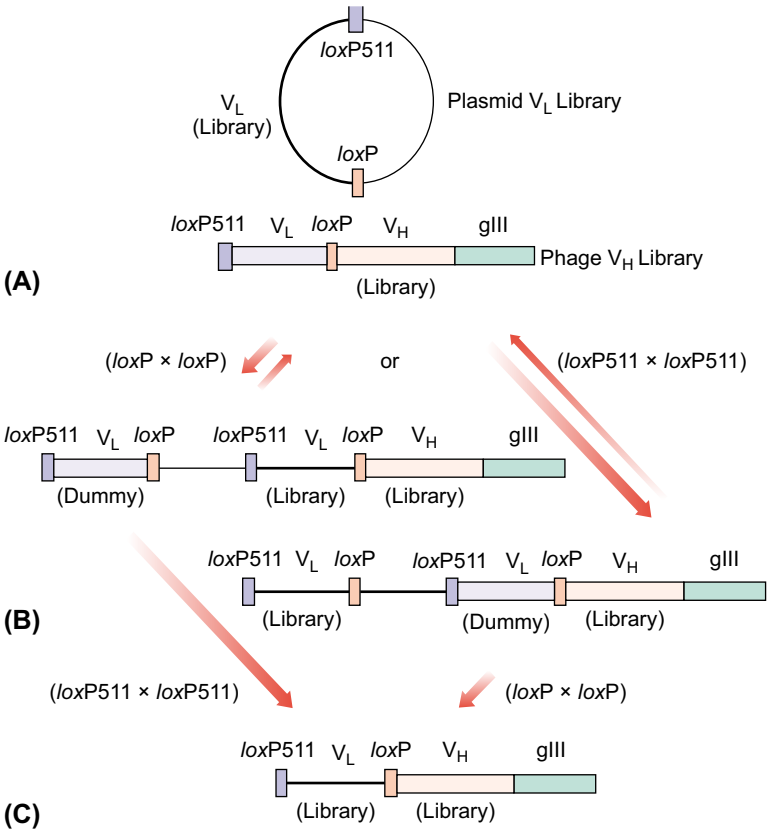


FIGURE 7.2

Increasing molecular diversity using site-specific recombination. (A) Phage fd containing a library of VH genes fused to the minor coat protein gIII are used to infect cells with plasmids containing a library of VL chains. (B) In the presence of Cre recombinase the phage and plasmid undergo recombination via the loxP or loxP511 sites to yield the two possible cointegrate structures shown. If the identical lox sites used to form the cointegrates recombine a second time the starting structures shown in (A) will result. (C) If the second set of sites in the cointegrate structures is recombined, the structure pairing both libraries will be formed (Ronald et al., 1978).

loxP511, which contains a base substitution mutation. The two lox sites are unable to recombine because of the base substitution with one another but are still capable of recombining with a like partner, i.e., loxP511×loxP511. The phage library is used to infect cells harboring plasmids and this contains a library of VL genes bounded by loxP and loxP511. These cells also contain a source of the Cre recombinase. Upon infection, the phage undergoes Cre-mediated recombination with the plasmid forming two possible cointegrate structures depending on which set of lox sites recombines (Fig. 7.2). In the presence of Cre the resulting cointegrate structures are unstable and then undergo a second round of recombination. Two possible outcomes can occur. One is to form the cointegrate, the two sites that originally recombined can undergo recombination again, yielding the starting structures.

The second event, which is equally possible, is that the two sites not originally involved in recombination will recombine to form the product shown (Fig. 7.2). This final product has the net effect of swapping the VL gene from the infecting phage with the VL gene on the resident plasmid. Since the phage infection is far more efficient at introducing DNA into cells than electroporation, libraries with complexities of 1013–1014 have been constructed.

The finding that prokaryotic site-specific recombination systems work in yeast and mammalian cells has fostered multiple uses in eukaryotic cells. For instance, one of the problems encountered in the construction of gene therapy vectors is avoiding the incorporation of viral genes that have deleterious effects on the host cells.

3.2 INTRACHROMOSOMAL HOMOLOGOUS RECOMBINATION

Intrachromosomal homology recombination (ICR) between two homologous sequences is a less complicated approach of inducing DNA deletions. Although by stimulation of repair systems ICR can be enhanced, the frequencies are currently too low for an efficient application of this system to produce deletions of transgene regions. For example, in tobacco, on average, among all cells of a 6-week-old plant, fewer than 10 ICR events were detectable (Puchta et al., 1995). However, if recombination substrates provide a more efficient target for the recombination machinery, the low ICR frequencies could be increased.

A novel ICR strategy was developed that was based on the recombination of the *attP* region (for *att*achment phage site) of bacteriophage l to generate deletions; this was followed by the identification of ICR products among a relatively small number of transformants, thus providing a feasible procedure to remove undesirable transgene regions (Zubko et al., 2000). This system was used to delete a region that is 5.9 kb from a recombinant vector, which in tobacco had been inserted into two different genomic regions. This plant transformation vector is called p*attP*-ICR, which contained *nptII*, *gfp*, and *tms2* genes inserted between two 352 bp *attP* regions. The transformation booster sequence was positioned next to the left of the *attP* site, which enhances homologous and illegitimate recombination. An *oryzacystatin-I* gene served as an example of the gene that will be transferred into the tobacco genome by the *attP* system. On some of the independent transgenic tobacco, molecular analysis has been carried out, plants lines (showing a mixture of white and green shoots), with primers specific for *nptII* and *oryzacystatin* genes and the region is flanking the *attpII* dimer cassette. This showed that the *oryzacystatin* gene had been retained while there had loss of the *nptII* gene, and that the region between the two *attP* fragments had been reduced by about 6 kb (as expected if the two *attP* sites had recombined). However, the *nptII*/*tms2* region had been lost by most plantlets, and also lost transgene regions outside the *attP* cassette. This clearly indicated that the ICR system is not always associated with precise homologous recombination between the *attP* regions, and thus it can generate larger deletions; this is probably because of illegitimate recombination.

4. ADVANTAGES OF EACH STRATEGY

There are factors that need to be considered: the frequency and timeframe in which marker-free plants are produced; the ease with which the transgenes can be cloned into vectors; for sequential transformation, the number of genes that can be introduced into plants at one time; and the number of times a given system could be used. However, in the transposon system, the excision frequency is

very high (>80%) and it does not correlate with the percent of unlinked loci, especially when some of the excised genes can be integrate back into the genome at unpredictable loci. The cre/lox site-specific recombination as well as its transposable element system requires the cloning of flanking sequences, and an enzyme that is encoded by an additional gene must be active in the target plant. Retransformation or sexual crossing is also required by the system with the recombinase cregene. This results in an increase in the production time for marker-free plants. We use the transposable element system to remove a selectable marker. It could also allow the recovery of many insertion events from one transformation event, and the study of positional effects on expression of the gene of interest, provided such genes are then flanked by the *Ds* inverted repeats (Yoder and Goldsbrough, 1994). Such an arrangement, however, would preclude a second round of transformation using this system, since the position of the *Ds*-flanked genes of interest from the first event would be destabilized. If the marker gene is inserted between the *Ds* repeats, then these *Ds* sequences will be removed with that marker, thus allowing marker-free plants produced by this method to be retransformable using the same system.

There are several advantages to using various types of site-specific recombination systems. Recombination takes place only between specific sequences, usually several dozen base pairs in length, and makes the system highly specific. The frequency is also remarkably high (Sugita et al., 1999, 2000; Endo et al., 2002a,b), unlike that of homologous recombination, which is extremely low (Ebinuma et al., 1997). However, it has also been postulated that undesirable secondary effects in plants may be caused by recombinase and transposase proteins (Zubko et al., 2000). Without sexual crossing, the MAT-based site-specific recombinase systems also have the advantage of producing marker-free transgenic plants; hence they can be potentially used for any crop. *Agrobacterium* cotransformation is also a method for marker removal because it does not require any development or application of additional selectable marker genes or DNA excision, but whether the one or two *Agrobacterium* strains provides a higher frequency of marker-free plant recovery is still not clear. This comparison of single and double strain methods using the same species, similar plasmids, and transformation protocols showed higher cotransformation frequencies but a similar derived strain was used. Similar data revealed that here the progeny from about half of the cotransformants exhibited independent transgene segregation using the octopine-derived strain (Daley et al., 1998). The genotyping of the additional progeny could also increase the percentage of lines classified as segregating independently for both transgenes, especially in cotransformed lines with multiple insertions of one or both genes (Daley et al., 1998). However, use of the two plasmids in one octopine-derived strain has several desirable attributes and advantages, and these include simplicity, easy cloning ability, high cotransformation efficiency, high frequency of unlinked integration, reasonable production time line, and unlimited use of retransformation.

5. ACHIEVEMENTS IN TRANSGENIC PAPAYA IN HAWAII

Papaya is widely grown in the tropics and has been grown in Hawaii for over a century (Gonsalves, 1998). The major production constraint for papaya worldwide is PRSV; this is a virus that is rapidly transmitted by a number of aphid species in a nonpersistent manner (Gonsalves and Ishii, 1980). PRSV was first reported in Hawaii in the middle of the 1940s, but it did not become a major problem to the Hawaiian papaya industry until the 1950s. At that time, the Hawaiian papaya industry was located on

Oahu island, and PRSV had severely affected papaya production on Oahu by the late 1950s and early 1960s. Fortunately, the industry was relocated to the Puna district on Hawaii island. Puna had many advantages for raising papaya: (1) this area had an abundance of rather inexpensive land to lease; (2) the area had plentiful sunshine and rainfall; (3) the excellent "Kapoho" papaya cultivar was uniquely adapted to this region; and (4) PRSV was not present in this region. Consequently, Hawaii's papaya industry was expanded and prospered; by the 1970s, about 95% of Hawaii's approximately 2500 acres of papaya were located in the district of Puna. However, when PRSV was discovered in Puna in May 1992 things changed (Gonsalves, 1998). By late 1994, the virus spread very rapidly and the papaya industry was in a critical situation.

During 1986, the researchers had cloned the CP gene of PRSV, and in 1988, using the biolistics approach, they started on the transformation of embryogenic cultures of papaya. Researchers have worked on transforming commercial cultivars of "Sunrise", its sib "Sunset", and "Kapoho". These cultivars were excellent candidates because they bred true to type, were commercial, and the cultivar grown almost exclusively in Puna was "Kapoho." A pioneering challenge was transformation of papaya, because no one had yet reported its successful transformation (Fitch et al., 1990). Using the biolistics approach to transform embryogenic papaya cultures, 17 independently transformed plants were obtained. A test was performed on the resistance of these lines at the R_0 stage by propagating each line and then inoculating clones with PRSV HA, a severe strain from Hawaii (Fitch et al., 1992; Tennant et al., 1994, 2001).

Fortunately, in 1991 a single line was identified (designated *line 55-1*), which was resistant to PRSV HA in greenhouse inoculations (Fitch et al., 1992). The resistant line was the red-fleshed "Sunset," which was much less desirable than the yellow-fleshed "Kapoho." The purpose of this initial field trial was to observe resistance under field conditions and to determine whether the single resistant line had the desirable horticultural properties (Lius et al., 1997). A major benefit of the 1992 R_0 field trial that was held in Waimanalo was that it gave a head start in developing cultivars, which might be useful for growers in Puna. Then, two new transgenic cultivars were developed: "SunUp" and "Rainbow." "SunUp" is a transgenic red-fleshed Sunset and is homozygous for the CP gene. "Rainbow" is a yellow-fleshed F_1 hybrid developed by crossing "SunUp" and nontransgenic yellow-fleshed "Kapoho" (Manshardt, 1998). As noted earlier, "Kapoho" was the almost exclusive variety grown in Puna. Thus the 1992 trial confirmed the field resistance of the transgenic line and helped to speed up the development of the transgenic cultivars.

6. FUTURE PROSPECTS

Breeding strategies such as backcrossing, and probes, promoters, and plasmid vectors can all be used to promote variability enhancement in papaya, besides allowing targeted improvements. A combination of techniques of genetic engineering in improving and enhancing the quality as well as quantity of crop production per unit area proved to be beneficial for papaya. On these lines, use of molecular markers and backcrossing techniques for introgression of genes to obtain appropriate recovery of recurrent genome can be permitted in the improvement of papaya. All the molecular techniques associated with the conventional procedures accelerate this process and allow different targeted improvements. For this, a microsatellite can be used to perform genetic–molecular characterization of papaya genotypes. There are only a limited number of papaya varieties available and this reflects the narrow genetic base

of this species. However, there are very few close relatives of papaya available for inbreeding crop improvement programs, hence a lot of challenges still need to be overcome to get the desired quality and quantity of papaya.

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CHAPTER

8 TRANSGENIC RESEARCH IN VEGETABLE CROPS WITH SPECIAL REFERENCE TO BRINJAL

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1. INTRODUCTION

India is mainly an agricultural country, has around 168 million hectares of arable land area, and has various climatic regions, mainly tropical. A variety of vegetables, cereals, oil seeds, pulses, and horticultural crops has been cultivated in these agroclimatic zones. India has achieved self-sufficiency in food grain productions; however, productivity in all crops is needed to meet future challenges (Paroda, 1999). Major crop productivity in India, which has increased considerably using plant breeding programs, is still far below the global average, largely because of persisting diseases caused by pests. Some important pests such as the fruit and shoot borer of brinjal, yellow stem borer in rice, stem borers of sorghum and maize, the fruit borer of tomato, and the diamondback moth of cruciferous crops (cabbage and cauliflower) are perennial and persistently affect these economically important crop varieties. Farmers generally spend millions of dollars on pesticides to control these pests to desired levels. Out of US$630 million, about US$380 million for the control of bollworms and sucking pests of cotton and US$250 million for pesticides are utilized per year for agriculture in India (Reddy and Zehr, 2004). Increased pest problems and the indiscriminate use of pesticides are responsible for environmental problems and ecological imbalance (Zadoks and Waibel, 2000). In this scenario, yield losses because of insect pests are found to be the important obstacles. Assessment of such losses has become inevitable to develop a strategy to overcome them. One possible alternate strategy is to develop genetically engineered crops. Crop biotechnology has produced transgenic plants with desired genetic traits. In India, insect-resistant crops/transgenic *Bacillus thuringiensis* (*Bt*) crops based on toxins (Cry proteins) from naturally occurring, Gram-positive, spore-forming soil bacterium—*Bacillus thuringiensis*—are being vigorously experimented to test whether they can be utilized in cultivation and biosafety. Regulatory/biosafety guidelines are laid down that provide a framework to control the activities of genetic engineering in plants.

1.1 WORLD SCENARIO OF GENETICALLY MODIFIED CROP PRODUCTION

Biotechnology crops covered an area of 1.7 million ha in 1996 and 179.7 million ha (444 million acres) in 2015, which is an unmatched 105.7-fold increase, and is the fastest evolving crop technology in

Genetic Engineering of Horticultural Crops. http://dx.doi.org/10.1016/B978-0-12-810439-2.00008-8

modern agriculture. About 45.5% of biotech crops in the world are grown in North America with the United States as the top producer. Brazil is the main producer in developing countries, planting 44.2 million ha of biotech crops in 2015 (http://isaaa.org/resources/publications/briefs/51/executivesummary/default.asp). Maize extends over almost 30% of the global transgenic crop area and *Bt* crops are the second most commercially cultivated transgenic crops with nearly 13% of the global transgenic crops area being used to grow *Bt* maize.

About 67,000 pest species are associated with production losses in the major crops (52% loss in wheat, 58% loss in soybean, 59% loss in maize, 70% loss in brinjal, 74% loss in potato, 83% loss in rice, and 84% loss in cotton) (Oerke et al., 1994), of which approximately 9000 species are insects and mites (Ross and Lembi, 1985). Insects cause losses both in quantity and quality of agricultural production (Kumar et al., 2006). Dichlorodiphenyltrichloroethane, which was used first in the 1940s as the modern insecticide, has been extensively used to protect crops, forests, and also eradicate insects, vectors of human diseases. Farmers who have adapted to green revolution technology use huge quantities of chemical fertilizers and pesticides on high-yielding varieties to increase productivity, but these substances have created a complex group of problems such as alkalization of land, decreasing soil fertility, gene erosion, harmful effects on human health, environmental pollution, killing of nontarget beneficial insects, and generation of insect populations resistant to chemical insecticides. Because of the need to produce more food to feed the burgeoning population, biotechnologists are continuously searching for and developing alternative methods for insect pest control to avoid environmental pollution caused by heavy use of chemical pesticides. The most important challenge for the ever-growing global population is to provide ample food at affordable prices and promote sustainable development of natural resources. Biopesticides and entomopathogenic microorganisms are some of the best alternatives to biological control agents. However, management technology also needs to be developed, especially soil irrigation, water conservation, biodiversity conservation techniques, integrated pest management, etc. to counteract the destruction of the agro-ecosystem caused by ongoing unplanned agriculture. The use of *Bt* transgenic crops has successfully increased productivity and provided benefits to farmers, consumers, and the environment. Successful *Bt* crop production is the backbone of modern and significantly safer crop biotechnology implementation, whose products are being distributed in the world market to bring about sustainable agricultural development.

1.2 POPULATION GROWTH AND FOOD SECURITY ISSUES

The current average annual global population change is estimated at around 83 million people, although the global population growth rate (1.18% per year) is slowing down, and most of this increase is found in the developing countries. World food supply will have to expand by more than 60% from the 2005–07 baseline to 2050 to ensure enough food stock for the world population, which is expected to increase to 9.1 billion by the year 2050 (https://esa.un.org/unpd/wpp/) (Nikos Alexandratos and Bruinsma, 2012). A large number of people all over the world also suffer from significant health problems related to deficiencies of micronutrients such as iron, zinc, and vitamin A. The agricultural land area is being gradually decreased because of urbanization (49% of the world population) and land degradation, which is more pronounced in the developing countries than in the developed countries. Developing countries such as India, Bangladesh, Pakistan, Indonesia, Egypt, Ethiopia, Nigeria, and Mexico will need three times more food grain imports by 2025 or shortly thereafter (Engelman and LeRoy, 1995).

These decreases in available agricultural land area and increase in human population will have a major impact on food availability over the next two to three decades. Total food grain production between 1950 and 1980 increased remarkably, but these consistent increments were marginal during 1980–96 (Myers, 1999). Genetic engineering opens an avenue for plant breeders to exploit the wide range of novel genes that can be inserted through an event into high-yielding and locally adapted cultivars.

1.3 PRESENT STATUS OF BRINJAL

Eggplant, popularly known as the poor man's crop, aubergine, guinea squash, and brinjal (Gaur and Chaudhary, 2009), is distributed throughout the world, and economically low-income consumers in South Asia, especially in Bangladesh, India, Nepal, and Sri Lanka, consume it in their daily meal. It is an important vegetable crop that is produced in high quantities by more than 1.4 million small, marginal, and resource-poor farmers. India is the second largest producer of eggplant after China and produces 13.44 million tons with 0.72 million ha of cultivated area (2013), but it lags behind many countries concerning productivity (18.62 tons/ha) (http://nhb.gov.in). West Bengal (30% production share), Orissa (20%), and Gujarat and Bihar (around 10% each) are the main brinjal-producing states (http://www.isaaa.org), and Tripura, Uttar Pradesh, Karnataka, Himachal Pradesh, etc. are successively ranked in productivity (http://nhb.gov.in). The popular varieties of brinjal in India include Ritu Raj, Pusa Purple Cluster, Hybrid-6, ARBH-1, ABH-1, Pusa Purple Long, Pusa Ankur, Arka Navneet, etc. It contributes to ayurvedic medicine for diabetes and liver problems by providing important ingredients (http://www.moef.nic.in). The nutritional quality of brinjal is also high compared to other regular crops available in India (Table 8.1).

Because *Bt*-brinjal is currently commercialized only by Bangladesh, no such production statistics of *Bt*-brinjal are available. However, more than 90% of brinjal is produced by Asia, mainly China and India (Fig. 8.1). Brinjal is very much prone to infection caused by insects, pests, fungi, and microbes, and production is constantly and mainly hampered by brinjal fruit and shoot borer (BFSB; *Leucinodes orbonalis*) and fruit borer (*Helicoverpa armegera*) insects (Purohit and Khatri, 1973; Kuppuswamy and Balasubramanian, 1980; Allam et al., 1982), which cause 31%–86% fruit losses in Bangladesh, and 37%–63% and 50%–70% losses in India and Pakistan, respectively. The distribution of BFSB clearly shows that the pest is well adapted to major brinjal growing countries such as Asia and Africa (Waterhouse, 1998; Bhagirath and Kadambini, 2009). Asian countries in most cases depend solely on insecticides to manage the pest. In India, transgenic overexpression of δ-endotoxins (*Bt* Cry proteins) of *B. thuringiensis* in several plant species has been extensively examined to study their effectiveness against lepidopteran insect pests (*L. orbonalis*) (Singh et al., 2005).

Table 8.1 Nutritional Facts of Brinjal With Standard Serving Size of 548 g

Water (g)	Fiber (g)	Protein (g)	Fat (g)	Carbohydrate (g)	Vitamin (mg)				Mineral (mg)							Total Calories
					C	B9	A	K	Ca	K	Fe	Mg	P	Na	Zn	
505.8	16.4	4.37	0.99	32.22	12.1	121	126	19.2	49	1255	1.26	77	132	11	0.88	136

Courtesy: USDA-ARS National Nutrient Database for Standard Reference.

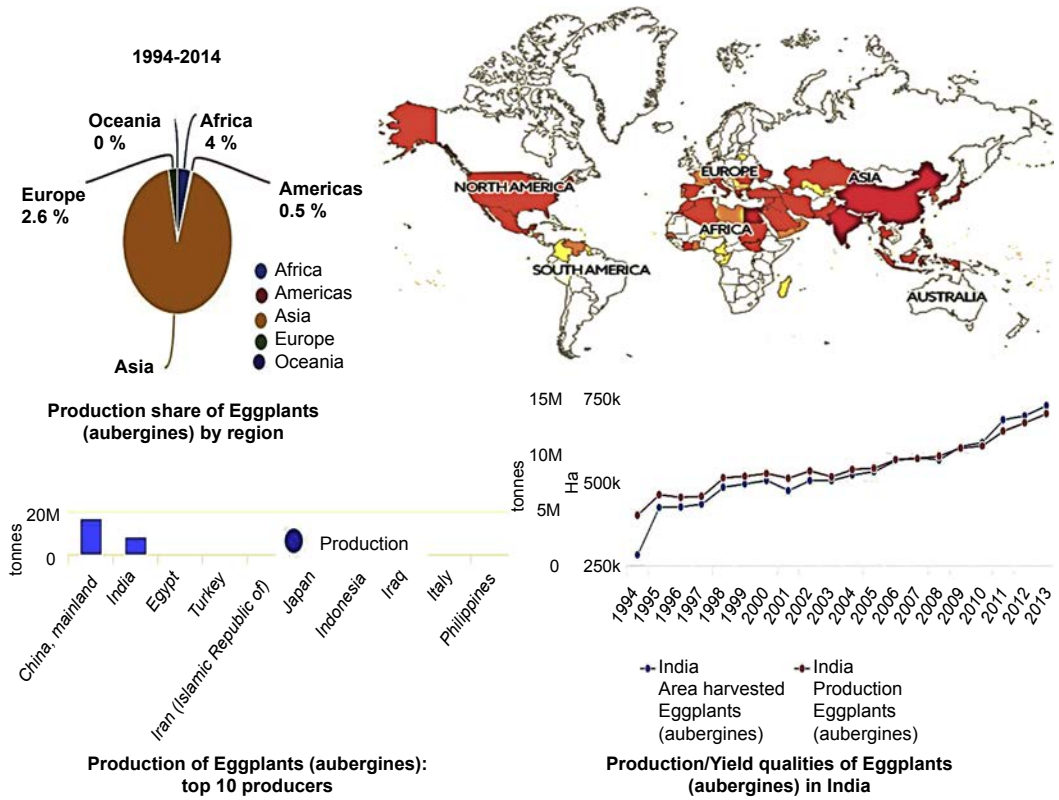

FIGURE 8.1

Worldwide production statistics of brinjal from 1994 to 2014, with emphasis on India.

Data source: http://faostat.fao.org/beta/en/#data.

2. SHOOT, STEM, AND FRUIT BORER IN BRINJAL

Resistance to BSFB in cultivated varieties has brought about restricted gains because of sexual incompatibilities in the source species. Generally, BSFB occurs in two ways. First, young shoots are infected during the vegetative phase, which limits the potential of a plant to produce healthy fruits. Second, it shift into fruits during the reproductive phase and the larvae remain concealed because of the cryptic nature of the pest rendering insecticide applications ineffective, which contributes to the next generation of infections. It has been reported that third- and fourth-instar larvae of BSFB are the main causative agents of infection of eggplant (Sandanayake and Edirisinghe, 1992). All the naturally occurring *Solanum* species are susceptible to BSFB, which has become a major obstacle to developing BSFB-resistant cultivars. BSFB-resistant transgenic eggplant was formed by genetic engineering using insecticidal crystal protein (*cry*) genes from *B. thuringiensis* (Vaeck et al., 1987; Kumar et al., 1998). Different cry genes have been reportedly used in transgenic crop engineering to provide various forms of insect resistance (Pal et al., 2009), e.g., sugarcane with the cry1Aa3 gene (Kalunke et al., 2009), field

corn with the cry1Ab and cry1F genes (Hardke et al., 2011), and chickpea carrying the *cry1Ac* gene (Mehrotra et al., 2011). In India, the success story of *Bt* cotton (*Gossypium hirsutum*) reflects genetic engineering potential. Net income was increased by 37% because of higher yields and 41% fewer pesticide sprays were used compared to the wild cotton variety (Subramanian and Qaim, 2010).

BFSB successfully maintains its high population because of potent reproduction and short generation times. Disease symptoms show small darkened holes surrounded with brownish areas on the fruit surface or fruit stalk, and wilted shoots and fruit eventually become hollow and filled with the excrement of insect larvae. As a result the fruit loses its market value and is refused by customers (Tewari and Krishnamoorthy, 1984; Tewari and Sardana, 1987). In untreated conditions, BFSB becomes most destructive and unmanageable, which accounts for up to 70% yield loss (Krishnaiah, 1980; Islam and Karim, 1991).

3. EFFICACY OF TRANSGENIC *BT*-BRINJAL

Early in 1995, transgenic brinjal-expressing insecticidal protein (Cry1Ab) was conducted in field trials at the Indian Agricultural Research Institute farm, which performed limited protection against BSFB. Afterward, *Bt*-brinjal was developed through a recombinant DNA containing the *cry1Ac*

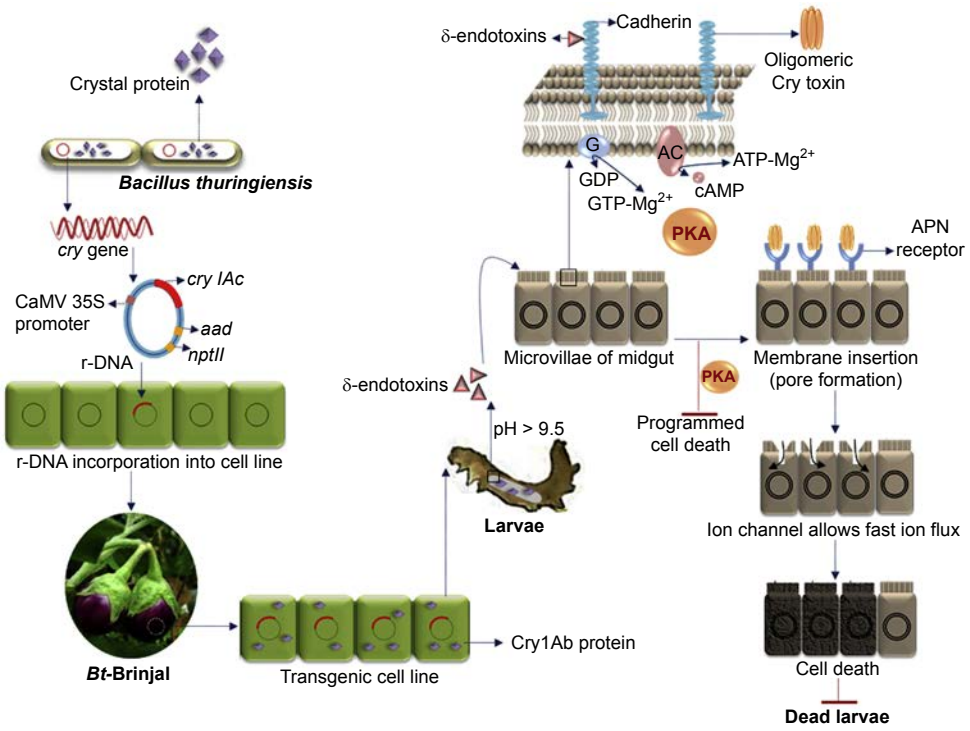

FIGURE 8.2

Flow chart of the transgenic development of brinjal.

gene, a CaMV 35S promoter, and the selectable marker genes *nptII* and *aad* (Fig. 8.2) to transform young plant cotyledons by the Maharashtra Hybrid Seeds Company (Mahyco) (http://www.mahyco.com/). In Mahyco's breeding program, from several attempts to make transgenics, EE-1 has been chosen as the successful transgenic line. The EE-1 was backcrossed with open-pollinated brinjal varieties and significantly reduced the count of BSFB larvae (0–20) on *Bt*-brinjal as compared to around 3.5–80 larvae on the control. Mahyco gifted the *Bt*-brinjal technology to the Tamil Nadu Agricultural University, Coimbatore, and University of Agricultural Sciences, Bangalore, public research institutions in the Philippines and Bangladesh. Mahyco conducted multilocation and large-scale trials from 2004 to 2008 and the Indian Council for Agricultural Research independently trialed and confirmed that on average 80% less insecticide was required for *Bt*-brinjal hybrids than for the non-*Bt* counterpart (Bandopadhyay et al., 2012). Scientists estimated that *Bt*-brinjal cultivation in India would provide a net economic benefit ranging from Rs 16,299 (US$330) to Rs 19,744 (US$397) along with national benefits exceeding $400 million at one acre per year (http://isaaa.org/resources/publications/pocketk/48/default.asp).

3.1 MECHANISM OF *CRY* GENE ACTION

Toxicity of δ-endotoxins is derived from its N-terminal half, which is composed of seven antiparallel α-helices. Insects solubilize the crystalline inclusions (δ-endotoxins) in their midgut at pH>9.5 (Hofmann et al., 1988a,b) after ingestion and it exhibits a highly specific insecticidal activity upon proteolytic activation by binding to specific receptors on the terminal brush border of microvillae of its midgut (Hofmann et al., 1988a,b). Meanwhile, antiparallel α-helices penetrate the membrane to form an ion channel in the apical brush border membrane (Knowles and Dow, 1993) and may allow fast ion flux (Sacchi et al., 1986; Wolfersberger, 1989). This collapse of gut integrity leads to starvation and/or septicemia in the insects and they eventually die. Highly insoluble δ-endotoxins in normal *Bt* also induce certain cytolytic (Cyt) proteins and vegetative insecticidal proteins (VIPs), which can cooperatively increase the insecticidal activity of Cry proteins. This *cyt* gene of *B. thuringiensis* subsp. *israelensis* (more than eight) has been cloned and sequenced (Agrawal and Bhatnagar, 2003) and it differs from the Cry protein in its smaller size and high cytolytic activity against a wide range of cell types including those of vertebrates (Drobniewski and Ellar, 1988). *Bt* during its vegetative growth produces VIPs in a very small amount and leaches out into the medium. A VIP3A protein containing a 60 to 70 amino acid-long motif, called "death domain" reported by Estruch et al. (2001), is found to be involved in protein–protein interaction and induces apoptosis in insect cells by binding to receptors, which is different from the receptor of δ-endotoxins in the midgut, resulting in the formation of ion channels (Fig. 8.2).

3.2 PATENTING TREND OF TRANSGENIC *BT*-BRINJAL

Using the patent analysis tool Relecura (Fig. 8.3), it was observed that document distribution wise USA is heading *Bt*-brinjal research followed by Canada, Australia, India, and China. Also over the last decade, although the maximum number of patents were filed in the year 2013, the maximum number of patents were published in 2015. The top assigning companies throughout the world are Bayer, followed by BASF and Monsanto. From India, IARI and Mahyco are the leading companies in *Bt*-brinjal research. Mahyco is also the first company in India that developed *Bt*-brinjal (Fig. 8.3).

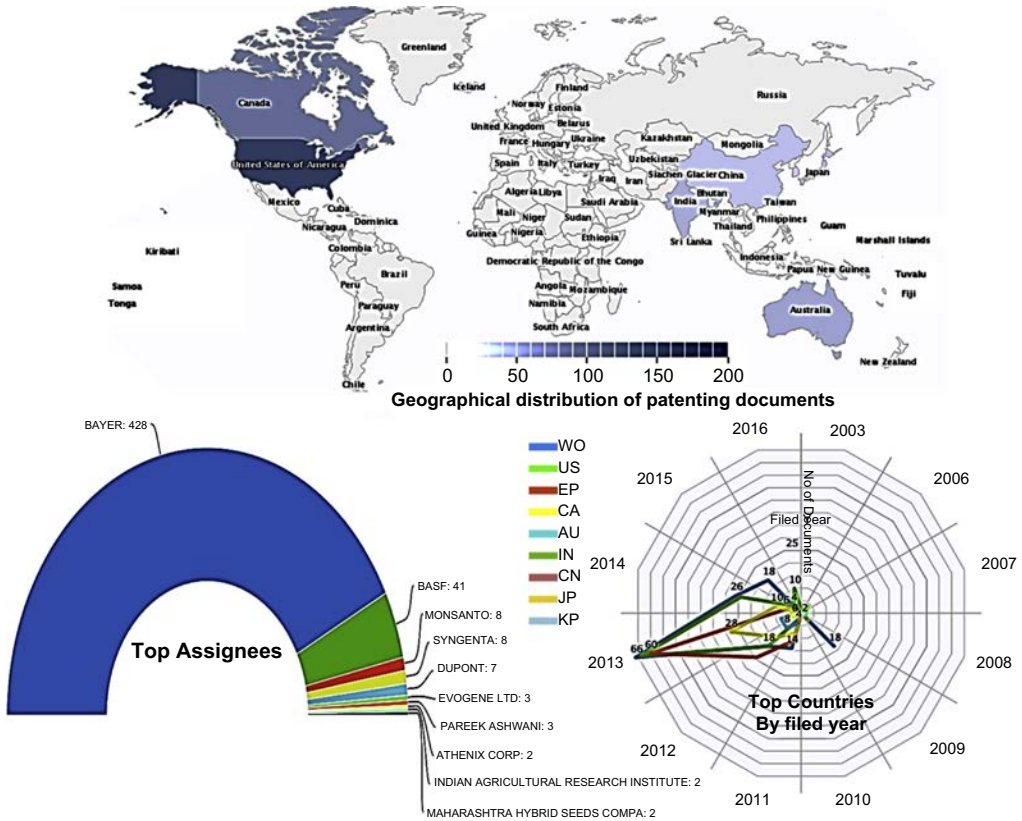

FIGURE 8.3

Geographical information on patenting documents.

4. FLOW OF TRANSGENE: IS IT A REAL RISK?

4.1 GENERAL METHOD OF MAKING GENETICALLY MODIFIED CROPS

Genetic engineering allows specific genes to be transferred across species. Like other genetically modified (GM) crops, the introduction of *Bt*-brinjal involves two steps. First, production of the primary transformant: the *Bt* gene is inserted and integrated into a chromosome of a target crop variety because of its high cellular acceptance. Second, introduction of the commercially viable GM hybrid is performed by transferring the *Bt* gene from the primary transformant into a hybrid variety by conventional breeding techniques. In nature, a gene that can accidentally enter into a related cell is immediately degraded, except for the DNA of parasitic bacteria and viruses. Based on this phenomenon, a transgene is attached to such a mobile microbial DNA-producing recombinant, which is transferred to host cells by suitable protocols. Thus Mahyco, by incorporating the *Bt* gene into a bacterial plasmid DNA (pMON10518), produced primary transformant *Bt*-brinjal and transferred this r-DNA into a brinjal variety by the common *Agrobacterium*-mediated transformation technology. This transformant was

usually bred with several brinjal hybrids (MHB 4, 9, 10, 80, 99, etc.) to produce the *Bt* MHB lines for commercialization. Mahyco uses a slightly modified technique in which the plasmid contains antibiotic resistance markers (*npt II* and *aad*) and the 35S CaMV promoter. At present, *Bt* gene expression is nontissue specific in the commercially released *Bt* crops. Tissue-specific promoters for transgene expression in plants, especially in susceptible tissues (root), have also been focused on (Bandopadhyay et al., 2012).

4.2 TARGETED INTEGRATION OF DESIRED GENES

Over last two decades, plant molecular biology has been revolutionized in crop improvement (Moose and Mumm, 2008). The groundbreaking discovery of sequencing makes it easy to produce a gene pool library of all the living organisms, which makes it possible to use beneficial genes for making transgenic desired crop varieties (Kumar et al., 1996). *Agrobacterium*-mediated transformation and particle bombardment are the standard plant genetic transformation techniques (Christou, 1996). In both of these approaches, illegitimate recombination occurs in the genome by random transgene integration, resulting in predictable changes in transgene expression across different integration events and undesirable mutations with unpredictable phenotypes in the host (Deineko et al., 2007; Gelvin and Kim, 2007). Gene targeting is a potent technique based on homologous recombination that makes transgenic plants with predictable transgene expression (Hanin and Paszkowski, 2003; Reiss, 2003; Puchta and Hohn, 2005). This process has been successfully applied to modify the gene expression of bacteria (Weller et al., 2002), fungi (Pafiques and Haber, 1999), and eukaryotes (Offringa et al., 1993) where the gene targeting frequency is about 10-2 (Doetschman et al., 1987; Thomas and Capecchi, 1987) and only 10-6 to 10-3 have been reported in plants (Lee et al., 1990; Miao and Lam, 1995; Terada et al., 2002). Different strategies have been taken to extend the homology length in targeting vectors and enrich the targeted events through strong positive–negative selection (Thykjaer et al., 1997; Gallego et al., 1999). Brinjal, an important vegetable crop, is very badly infested by the insect BFSB. It was proved experimentally that the *cry1F* gene acts against BFSB and it was selected after introduction in brinjal to sort out the random T-DNA integration events in the primary stages of plant development. Out of a total 954 random gene delivery events, only 2 targeted gene integrations have been successful (Terada et al., 2004).

4.3 RISK ASSESSMENT OF *BT*-BRINJAL

All the Cry proteins in *Bt* crops seem to be powerful allergens because of their amino acid similarity to known allergens. There is an assumption that the integrated desired gene in GM food could be transferred into the human body cells during digestion. Gilles-Eric Seralini, President of the Committee for Research and Independent Information on Genetic Engineering, pointed out that *Bt*-brinjal supplies 15% less energy and poor alkaloid content as compared to wild varieties (Miller, 2007; Padmanaban, 2009). Some adverse effects and changes of natural activity were observed when it was fed to animals, for example, weight gain, increased fibrous fodder consumption and milk production by 10%–14% in cows, diarrhea, increased water consumption, liver weight loss, etc. Furthermore, no such significant tests were conducted to see the effect of *Bt*-brinjal on the subsequent crop variety. Similarly, the feeding effect of *Bt*-brinjal to open grazing animals was not considered a valid experimental setup.

5. PRESENT SCENARIO OF TRANSGENIC BRINJAL IN INDIA AND ITS COMMERCIALIZATION CONTROVERSY

It is true that food problems can only be solved by GM technology, but the *Bt*-brinjal issue divided the Indian cabinet. It is developed by Mahyco in cooperation with Monsanto, the St. Louis-based seed company. The Genetic Engineering Approval Committee (GEAC), India's official regulatory body for registering GM organisms, permitted seven *Bt* crops for large-scale field trials during 2007–09. On October 14, 2009, GEAC approved the release of transgenic brinjal and opened the door for commercial release of many other GM crops. On February 9, 2010, the government of India officially announced that more time is required before releasing it, and Indian Environment Minister Jairam Ramesh also said that there is no hurry to introduce *Bt*-brinjal in India (http://news.bbc.co.uk/2/hi/south_asia/8506047.stm; http://timesofindia.indiatimes.com/india/Govt-says-no-to-Bt-brinjal-for-now/articleshow/5552403.cms?referral=PM). This decision split the cabinet and the prime minister of India, Dr. Monmohan Singh, immediately consulted with senior government officials. Chavali Kameswara Rao, Secretary of the Bangalore-based Foundation for Biotechnology Awareness and Education, believed that the environment minister gave this decision under heavy lobbying pressure from activists. It was feared that the resultant delay in commercialization would keep secret the cultivation of *Bt*-brinjal—similar to the early stages of *Bt*-cotton cultivation in India. On February 24, 2010, Dr. M. Singh and senior cabinet members gave the national biotech regulatory authority permission to supervise registration of transgenic organisms and requested the GEAC to settle outstanding safety measures related to *Bt*-brinjal. Activists and nongovernmental organizations are frequently raising opinions on GM crops suggesting that efficient techniques are important to monitor field trials, so that proper safety measurements on the environment or human health are expressed. A decade ago the court of India apparently banned *Bt*-brinjal including field trials based on a petition by anti-GM activists. A regulatory bill for this crop that failed to gain approval in parliament in 2013 is now being reviewed. It would take two or more years to be passed by parliament, said Sunkeswari Raghavendra Rao, an adviser to the government's Department of Biotechnology. Now India's government is taking the commercial cultivation of transgenic crops seriously. In the meantime, farmers in neighboring Bangladesh have begun to cultivate *Bt*-brinjal as of October 30, 2013. However, the government still seems to be reluctant to organize an open debate regarding the use of GM biotechnology in India.

6. PROBABLE STRATEGY TO COMBAT THE BIOSAFETY ISSUE AND EXTRANUCLEAR TRANSFORMATION

A new technology would be exciting if it brings up betterment of mankind and biosafety. *Bt*-brinjal is a highly pest-resistant crop and reduces the cost of chemical insecticide, but unfortunately it is still struggling to fulfill the requirements of the bio-safety issue. Therefore we need to wait some time before biosafe *Bt*-brinjal crops are produced. We have to look forward to developing an alternative in GM technology. Confinement of *Bt* toxin production only to susceptible tissues such as roots rather than every part of the plant can be achieved with the help of new techniques. Transplastomic GM crops enhanced the production of transgenes because a plant cell contains only one nucleus and many chloroplasts (Singh et al., 2010), which are associated with chloroplast transformation and also minimize the chances

of gene contamination. Tissue-specific regulation of the *Bt* Cry1A(b) gene with the promoter derived from phosphoenolpyruvate carboxylase (PEPC) has been used to maintain highly regulated expression in plant leaves (Hudspeth and Grula, 1989), while the promoter taken from the calcium-dependent protein kinase (CDPK) gene is pollen specific (Estruch et al., 1994). A combination of PEPC and CDPK promoter genes specific to green tissue and pollen-specific tissue, respectively, can regulate effective CryA(b) gene expression in leaves and pollen to control the European corn borer (*Ostrinia nubilalis*).

7. CONCLUSION

In India, the Maharashtra Hybrid Seeds Company (Mahyco) first developed *Bt*-brinjal and conducted field trials from 2002 to 2006; but because a suspension was issued in 2009, the government of India banned its implementation in 2010. An estimated $400 million per year economic benefit can be delivered to farmers if *Bt*-brinjal were to be legalized. Bangladesh is the first small Asian country to commercialize *Bt*-brinjal. On October 30, 2013, the Bangladesh Agricultural Research Institute obtained permission from the ministries of Environment and Forests and Agriculture for commercial cultivation of four *Bt*-brinjal varieties: *Bt*-Uttara, *Bt*-Kajla, *Bt*-Nayantara, and *Bt*-ISD006. In 2015, the Philippines government also upheld the ban on *Bt*-brinjal. This is a satisfactory sign that different countries are accepting the technology in different ways. Because the major loss of brinjal can be combatted through commercializing *Bt*-brinjal, extranuclear transformation might be a probable strategy to solve farmers' and different government and religious sentiment issues.

ACKNOWLEDGMENTS

The authors are thankful to UGC-Centre of Advanced Study, Department of Botany, the University of Burdwan for pursuing research activities and a patent analysis tool named Relecura. Raju Biswas is thankful to CSIR for financial assistance as JRF [File No:09/025(0216)/2015-EMR-I]. Aparna Banerjee is thankful to SRF (state funded) for financial assistance [Fc (Sc.)/RS/SF/BOT./2014-15/103(3)].

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CHAPTER

GENETIC ENGINEERING OF OIL PALM

9

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1. INTRODUCTION

Oil palm, a perennial oil yielding crop, produces 5–8 metric tons of crude palm oil from mesocarp and 0.4–0.8 metric tons of palm kernel oil from kernels per hectare per annum, which is higher than any other oil seed crop. Oil palm cultivation has been expanded rapidly in recent years and is now second only to soybean as a major source of the world supply of oils and fats. Indonesia followed by Malaysia, Thailand, and Nigeria are the leading countries in the area and production of oil palm. Major exporters of palm oil in the world are Indonesia and Malaysia. The major palm oil-importing countries are India, China, Netherlands, and Pakistan. With the increase in area of the crop under diverse environments, it has become essential to develop oil palm varieties/hybrids with high yield, compactness/dwarfness, high oil extraction ratio, and tolerance to low-moisture regimes. Some of the research trials in Malaysia have reported as high as 10–12 metric tons of oil/ha/year.

2. ORIGIN AND DISTRIBUTION

The history of palm oil can be traced back to the days of the Egyptian pharaohs (5000 years BC). Over 5000 years ago, it was initially traded for culinary purposes (Zeven, 1972). The center of origin and diversity of cultivated species of *Elaeis guineensis*, (known as **African oil palm**) appears to be concentrated in the tropical rainforests of West and Central Africa, consisting of Angola, Cameroon, Congo, Ghana, Ivory Coast, Nigeria, and Zaire (Chevalier, 1934; Zeven, 1964). Pollen dating methods (Zeven, 1968) as well as genetic diversity studies using molecular markers such as restriction fragment length polymorphism (RFLP) indicate that Nigeria may well be the center of diversity of the African oil palm (Maizura et al., 2006). Based on recent findings, as per the highest allelic diversity, Nigeria may be the center of origin of oil palm in Africa (Bakoume et al., 2014).

The genus *Elaeis* consists of two species, namely, *E. guineensis* Jacq. and *Elaeis oleifera* (HBK) Cortes (Uhl and Dransfield, 1987). Both species have 16 numbers of haploid chromosomes. *E. guineensis* is found in wild, semiwild, and cultivated forms in the tropics within 10° latitude of the equator in South East Asia, and South and Central America. This species is endemic to the tropical lowlands of West and Central Africa spreading from 16°N in Senegal to 15°S in Angola (Hartley, 1988). *E. oleifera*, also known as **American oil palm**, is native to South and Central

Genetic Engineering of Horticultural Crops. http://dx.doi.org/10.1016/B978-0-12-810439-2.00009-X

America and is found scattered in wild palm groves in Brazil, Ecuador, El Salvador, Peru, the Amazonian belt, etc (Rajanaidu, 1986). This species is underutilized, is considered to have tolerance to several biotic and abiotic stresses, and has high oil quality, compact canopy, and dwarfness (Hayati et al., 2004). *E. guineensis* when crossed with *oleifera* shows homoeologous pairing reflecting similarity between the two species. Both species are monoecious and produce male and female inflorescences separately on the same palm.

3. TAXONOMY

The oil palm (*Elaeis* spp.) belongs to the family Arecaceae. The genus of oil palm *Elaeis* consists of two taxonomically well-defined species, i.e., one is African oil palm (*E. guineensis*) and second is American oil palm (*E. oleifera*). It is monoecious with male and female inflorescences produced separately on the same palm. Rarely, hermaphrodite inflorescences are also seen. It is a cross-pollinated crop with the female and male inflorescences being produced in alternate cycles (Hartley, 1988). Artificial pollination is resorted to when specific hybrids are to be produced. Fruits ripen within 6 months after pollination. The fruit of oil palm is a drupe. It consists of a pericarp, made up of exocarp (skin), mesocarp (husk), and endocarp (shell) surrounding the kernel. The kernel has a testa (skin), a solid endosperm, and an embryo. Shell thickness is of direct relevance to breeding and its presence or absence classifies cultivated species (*E. guineensis*) into three fruit forms. This is controlled by a single gene (Beirnaert and Vanderweyan, 1941). The homozygote ***pisifera*** ($Sh^{-}Sh^{-}$) is shell-less. Generally, they are sterile, though some palms set fruit, and varying degrees of sterility are observed. The other homozygote ***dura*** ($sh^{+}sh^{+}$) has a thick shell. The heterozygote ***tenera*** ($Sh^{+}Sh^{-}$) has a thin shell surrounded by a ring of fibers in the mesocarp. *Tenera* is the only form used for commercial planting because of higher mesocarp content. Characteristic features of three fruit forms are mentioned in Table 9.1.

Table 9.1 Fruit Form Types in Oil Palm

S. No.	Character	Dura	Pisifera	Tenera
1	Shell thickness (mm)	2–8	–	0.5–4
2	Shell thickness (%)	25–65	–	1–30
3	Mesocarp content (%)	33–55	96–100	60–95
4	Kernel (%)	5–25	0–4	2–15
5	Presence of ring of fiber	No	Yes	Yes
6	Seed/nut size (cm)	2–3	–	<2
7	Seed/nut weight (g)	4–13	–	2
8	Utility	Mother palm	Pollen parent (mostly female sterile)	Commercial planting material (Cross between Dura × Pisifera)

3.1 WORLD GERMPLASM COLLECTION

The success of any crop improvement program necessitates the sound presence of a wider spectrum of genetic variability (germplasm) in the species, especially in respect of crops like oil palm, where a narrow genetic base is the major constraint in achieving genetic progress through breeding. Present-day oil palm breeding mainly depends on the *Deli* Duras derived from four seedlings planted in the botanical gardens at Bogor, Java, Indonesia, in 1848 as a source of females (Rajanaidu and Jalani, 1999). Furthermore, the source of *pisifera* (males) in oil palm breeding is limited to a few palms.

3.1.1 African Centres

Realizing the importance of genetic resources, many countries started prospection programs and the earliest and most important was that carried out in **Congo** in the 1920s. After World War II, prospection in Congo was done in estates planted with Yangambi material, among palms of local origin in estates and in grove areas. Stringent selection was practiced, which resulted in the selected palms having 92.5% mesocarp per fruit and more than 32% oil per bunch against the Yangambi average of 70% and 22%, respectively. In **Nigeria**, collection and exploitation of oil palm (Calabar, Aba, Nkwele/Umuabi, and later Ufuma natural groves of eastern Nigeria) was started in 1912. The Nigerian Institute for Oil Palm Research (NIFOR) was established in 1939. Realizing the superiority of Deli *duras*, an aggressive exchange program was pursued with other countries to collect Deli dura from as many sources as possible. Further prospection was carried out in the marginal regions of Nigeria and also through exchange with other African countries; a diverse oil palm germplasm was established by 1962. In **Ivory Coast** a systematic prospection was taken up in 1969, where the groves were in general less dense and more scattered than those in Nigeria. Prospection in **Cameroon** was taken up by Institut de Recherches pour les Huiles et Oleagineux (IRHO) now CIRAD. In the 1970s a major collection program was organized jointly by NIFOR and the Malaysian Agricultural Research and Development Institute (MARDI) [later the Palm Oil Research Institute of Malaysia (PORIM) and now the Malaysian Palm Oil Board (MPOB)]. In 1991, NIFOR made another prospection in marginal areas on the northern edge of the oil palm belt in Nigeria.

3.1.1.1 Malaysia

PORIM, designated as having "Primary core collection of oil palm germplasm," has assembled the largest oil palm (*E. guineensis* and *E. oleifera*) germplasm collection in the world. It was collected worldwide from primary centers of diversity through bilateral collaborations or with the Food and Agriculture Organization and/or other organizations. For example, collections were obtained from Nigeria (more than 52% of total collection), Zaire (23%), Cameroon, Tanzania, Madagascar, Angola, Senegal, Gambia, Sierra Leone, and Guinea. *E. oleifera* genetic materials (about 7%) were collected in Honduras, Nicaragua, Costa Rica, Panama, Colombia, and Surinam.

Some of the collections from Nigeria upon evaluation resulted in 10–12 metric tons of oil/ha/year. In addition, these palms are short with a height increment of 20–25 cm/year as compared to 45–75 cm/year with the present planting material. The selected Nigerian *duras* and *teneras* are being utilized for broadening the variability of current duras and teneras and initiating a new foundation breeding program.

3.1.1.2 Indonesia

The earliest record of the introduction of oil palms into South East Asia was of four seedlings planted in the Buitenzorg, now Bogor, Indonesia, Botanical Gardens in 1848 in Java in the then Dutch East Indies. The seeds from these four palms were distributed wisely and originally used as ornamentals, but experimental plots were established as early as 1860; one of them was at **Deli** in Sumatra from which the name (***Deli dura***) is widely in use for all descendants. About seven distinct subpopulations of Deli dura together with African teneras including AVROS, Yangambi, La Mé, and NIFOR through reciprocal recurrent selection were developed (Lubis et al., 1990). The CIRAD program developed in Ivory Coast was replicated in Sumatra in collaboration with PT Socfindo (Durand-Gasselin et al., 2006) and a program of testing 450 D×P crosses was established at three sites in Sumatra along with the parents of the crosses from Agricultural Services & Development (ASD) Costa Rica (available as 225 dura selfings and 60 pisifera clones belonging to Yangambi, AVROS, Calabar, La Mé, and Ekona) (Breure, 1998). Kushairi et al. (2010) gave a detailed account of nine other programs in Indonesia and stated that there was production of 250 million seeds in 2009.

3.1.1.3 Brazil

Oil palm was introduced to Brazil by the Portuguese during the 15th century, but it did not flourish. In 1963 two crosses selected by IRHO were introduced for hybrid seed production locally. During the 1970s introductions were made from MARDI and from IRHO. Some collections were made from oil palm groves of the Brazilian state of Bahia, close to Valencia, Taperoa, and Nazare districts, which are mostly *dura* and believed to be brought by African slaves. *E. oleifera* was collected from the Brazilian Amazon.

Although **Colombia** started to grow oil palm on a large scale in the 1980s, today it is the world's fourth biggest exporter of palm oil and other oil palm products after Malaysia, Indonesia, and Nigeria. The first oil palm seeds introduced in Central America arrived in Guatemala from Sierra Leone around 1920. Later, in 1926 and 1927, oil palms from South East Asia were introduced in Panama and in the Botanical Gardens of Lancetilla, Honduras, which was created by the United Fruit Company (UFC) to maintain a collection of tropical crop germplasms. The first oil palm plants in Central America were planted by ASD (formerly UFC) in 1926 and 1929 using seeds brought from Malaysia, Indonesia, and Sierra Leone (Richardson, 1995). From 1967 onward, through international germplasm exchange programs, ASD consolidated one of the broadest collections of *E. guineensis* in the world. This gene bank includes all the major breeding populations of restricted origin (BPRO), as well as wild introductions from specific environments. *E. oleifera* lines from Brazil, Surinam, Colombia, Panama, Costa Rica, and Nicaragua are also present in the collection (http://www.asd-cr.com/ASD-Pub/Bol24/B24-1ing.htm).

3.2 *ELAEIS OLEIFERA* COLLECTION

Useful collections of *E. oleifera* have been established in Malaysia, Ivory Coast, Costa Rica, Brazil, etc. Though many accessions are available, most of the crosses studied in Malaysia were derived from a single palm, Kuala Lumpur Melanococca (the earlier name of *oleifera* being *melanococca*).

3.2.1 Classification and Varieties

The commercial oil palm is a hybrid between *Dura* × *Pisifera* called tenera. Hence most of the varieties have been developed with the population of *dura* and *pisifera*. The germplasm of these materials offers the parental materials for hybrid seed production. Four seedlings introduced into Indonesia in 1848 show homogeneous vegetative characteristics and bunch performance (Hartley, 1988). Nevertheless, several differentiations resulting from breeding programs were developed independently by a number of private companies. Currently, there are several Deli subpopulations/breeding materials available worldwide. Soh et al. (2003) have described the following breeding populations used in major breeding programs.

1. **Deli**: This is thick-shelled Dura derived from the original four Bogor palms in Java. Distribution of subsequent progenies to other countries, followed by local selection, led to the development of the Elmina, Serdang, Avenue, and Ulu Remis Deli Dura subpopulation/selection in Malaysia and the Dabou and Le Mé Dura subpopulation/selection in Ivory Coast. The rather high-yielding uniform populations led to speculation of common progenitors for the four Bogor palms. Deli dura provides the mother palms for all major palm commercial hybrid seed production programs. The Dumpy and Gunung Melayu palms are short variants of the Deli.
2. **AVROS**: These are *pisiferas* obtained from the seed from Eala Botanical Garden (Jardin Botanique d'Eala) in Zaire (presently the Democratic Republic of Congo) by AVROS in 1923 at Sangai Panchur. These pisiferas are well known as SP540. These are noted for vigorous growth, precocious bearing, thin shell, thick mesocarp, and high yield-conferring traits. Major seed production programs in Indonesia, Malaysia, Colombia, Papua New Guinea, and Costa Rica are based on Deli (dura) × AVROS (pisifera).
3. **Yangambi**: The breeding program started at INEAC, Yangambi, Democratic Republic of Congo (erstwhile Zaire) with open pollinated seeds of Dejongo palm and tenera of Yawenda and developed Yangambi population. This population is characterized by excessive vigor, bigger fruit, and high oil content.
4. **La Mé**: IRHO developed La Mé populations from 21 tenera palms from seeds collected from wild groves of Ivory Coast. It is used in seed production in West Africa and Indonesia. La Mé progenies (pisiferas) and teneras are characteristically smaller palms with smaller bunches and fruits, but they appear to be more tolerant to suboptimal growing conditions.
5. **Binga**: This subpopulation (pisifera) was derived from F_2 and F_3 of Yangambi progenies planted in the Binga plantation, Yangambi, Zaire. Palms Ybi 69MAB and Bg 312/3 are the parent palms of breeding interest.
6. **Ekona**: The Ekona population was derived from wild palms of the Ekona area of Cameroon and bred further at the Unilever plantation of Crown Estate, Ndian Estate, and Lobe Estate. It is noted for its high bunch yield, good oil content, and wilt resistance. Pisiferas from this population are of breeding interest.
7. **Calabar**: The breeding population of NIFOR is a much broader based collection from Aba, Calabar, Ufuma, and Umuabi. Pisiferas are used in various seed production programs.

The varieties developed by ASD are normally offered for use on commercial plantations after being progeny tested for a period of at least 8 years. The first variety produced by ASD is Deli × AVROS, which was first planted on a commercial scale in 1976. Later, Deli × Calabar was released in 1989,

Deli × Ekona in 1991, Deli × La Mé in 1995, Deli × Nigeria in 1997, and Deli × Yangambi in 1998. Three other cold-tolerant varieties, Tanzania × AVROS, Tanzania × Ekona, and Bamena × AVROS, have been supplied for small projects in the highlands of several African countries since 1985.

4. GENETICS AND BREEDING

Until the 1950s, oil palm breeding was confined to the thick-shelled *dura* variety. Efforts to improve wild genes in Africa as well as the work in Malaysia and Indonesia on the progenies of the four famous Bogor palms resulted in various breeding populations of restricted origin. Until the 1930s ***dura*** was cultivated on a commercial scale in Asia as well as in African countries. The superior oil content of ***tenera*** led to the use of T × T seed for commercial planting from the 1930s. It was later (in 1938) found that about 25% palms are sterile (***pisifera***) in the T × T population resulting in great loss to the farmers as one-quater of the plantation was not yielding. Tenera selfing for obtaining planting material was found to yield ***dura***, ***tenera***, and ***pisifera*** in a 1:2:1 ratio because the shell character is controlled by a single gene. Beirnaert and Vanderweyan (1941) found the hybrid nature of tenera and advocated use of hybrid seed (from D × P). Consequently, in Congo (late 1940s), Sumatra (1953), and Malaysia (1956) large-scale planting of tenera was undertaken because of superior oil content. Thus the hybrid seed from selected mother palms (***dura***) and pollen parents (***pisifera***) became the commercial planting material.

The main emphasis of breeders is to evolve varieties with high fresh fruit bunches (FFB) yields and better mesocarp content, thereby increasing palm oil productivity. Reduced height increment, drought tolerance, superior oil quality, pest and disease tolerance, as well as precocity are also important considerations.

The four African *E. guineensis* palms brought over by the Dutch in 1848 and planted in Buitenzorg Botanical Garden (now Bogor), Indonesia, laid the foundation for the oil palm industry in Malaysia and Indonesia. From these, the Deli *dura* palms with unique and favorable fruit qualities were developed. The Deli *dura* population is widely utilized for seed production and in genetic improvement programs in Malaysia and Indonesia. The most cultivated high-yielding oil palm variety, the thin-shelled *tenera* [oil: bunch > 20%] is produced when the thick-shelled *dura* (O/B ~ 17%) crosses with the shell-less *pisifera*. The *pisifera*, which is mostly female sterile, is used as the pollen parent.

The major breeding objectives of oil palm are:

- Higher oil productivity from higher yield of FFB with better mesocarp content and bigger kernels.
- Dwarfness (low annual height increment) and desirable tree structure (compactness) enabling high-density planting (180 palms per hectare) and easy harvest.
- Superior oil quality with a high level of unsaturation and nutritive value (high iodine value, higher levels of vitamin E and carotenoids).
- Development of location-specific hybrids.
- Hybrids with early vegetative growth, higher bunch index, big kernels, and longer stalks.
- Development of hybrids resistant to diseases such as bud rot, *Ganoderma*, stem wet rot, etc.).

5. GENETIC ENGINEERING

The commercial exploitation of oil palm in Malaysia was started from four Bogor palms, which were further planted in Deli and Sumatra, and formed the basic breeding stock (known as Deli dura) of all the presently available germplasms of the world (Ooi and Rajanaidu, 1979). A major breakthrough that revolutionized the oil palm industry was started with the invention of single gene inheritance for shell thickness (*Sh* gene) by the plant breeders at Yangambi Research Station, the Democratic Republic of Congo, Africa, during the 1920s. The *SHELL* gene is responsible for identification of oil palm fruit forms, namely, dura, pisifera, and tenera (Beirnaert and Vanderweyen, 1941). The dura genotype has a thick shell consisting of a dominant *Sh* allele (*Sh/Sh*), and contributes 15% of oil, whereas pisifera genotypes are shell-less, consisting of recessive shell alleles (*sh/sh*), usually female sterile (Corley and Tinker, 2003), and contributing 25% of oil. However, the tenera genotypes are considered as hybrids, which have heterozygous Sh alleles (*Sh/sh*) derived from the cross between dura and pisifera. The tenera genotypes had 30% more mesocarp than dura genotypes and produce significantly more oil (36%).

Although traditional plant breeding based on phenotypic selection is very effective, it has suffered from several limitations for complex traits. Unlike morphological and biochemical markers, DNA markers are basically unlimited in number and are not affected by environmental factors and/or the developmental stage of the plant (Winter and Kahl, 1995). The molecular markers were transformed from the earlier RFLP markers to the highly variable and effective single nucleotide polymorphism (SNP) markers. The most widely used markers in recent times are simple sequence repeat (SSR) and SNP markers for several purposes such as genetic diversity, linkage maps, and for genome-wide association studies.

The advances in omics technologies are that comprehensive and integrated genomic, transcriptomic, and proteomic analyses can elucidate the genetic architecture of plant genomes and the relationships between genotype and phenotype. The rapid advances in DNA sequencing technology have made whole-genome sequencing (WGS) both technically and economically feasible. More than 25 economically important plants' genomes have been sequenced (Hamilton and Buell, 2012). Next-generation sequencing technologies are used not only for WGS but also to allow applications related to target region deep sequencing, epigenetics, transcriptome sequencing (RNA-seq), megagenomics, and genotyping. Oil palm is a diploid ($2n=32$) with an estimated genome size of 1.8 Gb. The full draft genome sequence of 1.535 Gb of *E. guineensis* has been published (Singh et al., 2013a,b) and is freely available.

5.1 GENETIC DIVERSITY ANALYSIS OF OIL PALM GERMPLASM

Since only four Bogor palms laid the basis for the development of the present industrialized development of oil palm, there exists a narrow genetic diversity among the oil palm germplasm. However, against that a considerable amount of variation also exists among the different sources of oil palm germplasm. The development of modern plant breeding techniques has greatly facilitated the wider use of a wealth of diversity from many sources including landraces. A comprehensive exploration of potential genetic resources and exploitation of natural genetic variations are proven sources of useful genomic information. A rich diversity of germplasms can be explored for their desirable traits such as yield and

can be further utilized to develop new varieties through molecular plant breeding approaches. Molecular marker techniques have revolutionized tree genomics and the understanding of structure and behavior of palm genomes. This will pave the way for the detection of novel and superior genotypes.

Initially, several genetic diversity works were based on using random amplified polymorphic DNA (RAPD), RFLP, and amplified fragment length polymorphism (AFLP) molecular markers. However, because of certain drawbacks these markers were replaced by SSR and SNP markers. Use of RAPD for the study of genetic diversity of oil palm was reported for the first time by Shah et al. (1994). Oil palm germplasm accessions collected from Africa (Cameroon, Tanzania, Nigeria, and Zaire) were studied using 20 primes and recorded high levels of genetic variation among the accessions. Rival et al. (1998) studied the suitability of RAPD markers for detection of soma clonal variants in oil palm. The results from the 387 arbitrary primers showed no intraclonal variability and no difference between mother and regenerated palms. The authors opined that the RAPD approach is not suitable for the detection of the mantled variant phenotype. Later, Mayes et al. (2000) used RFLP markers (40 probes covering 60% oil palm genome) to assess genetic diversity within 54 palms of a specific oil palm breeding program. Diversity studies using isozyme and AFLP markers reported their consequences for oil palm breeding indicating that crosses between the Africa subpopulation may be more interesting than the African and Deli cross-population (Purba et al., 2000). Barcelos et al. (2002) studied genetic diversity and relationship of American and African germplasms using AFLP and RFLP markers. Both markers revealed that genetic divergence between the two species is of the same magnitude as that among provenances of *E. oleifera*. Sathish and Mohankumar (2007) used RAPD markers for determining DNA polymorphism among the oil palm (*E. guineensis*) varieties *dura*, *pisifera* and *tenera*, and monitored the specificity of the primers for identifying each genotype. The three varieties were evaluated using 30, 10-mer primers. Of the 30 primers, 26 yielded significant polymorphic DNA bands. Jayanthi et al. (2008) studied the genetic diversity of oil palm accessions using RAPD markers.

Later, genetic diversity was focused on trait-specific diversity of oil palm accessions using different molecular markers. Arias et al. (2015) studied genetic and phenotypic diversity of natural American oil palm germplasm. The results from SSR markers and agro-morphological traits showed that analyses of variance for yield and bunch components demonstrated statistically significant differences among countries and geographical regions for several of the traits evaluated. SSR marker analyses revealed high genetic diversity (HT = 0.797) and the presence of specific alleles by each country of origin from *E. oleifera*. Okoye et al. (2016) studied the genetic relationship between elite oil palms from Nigeria and Malaysia using SSR markers. A comprehensive list of a few genetic diversity studies is given in Table 9.2.

5.2 FUNCTIONAL MARKERS AND THEIR USE FOR CHARACTERIZATION

SSR markers offer several advantages such as high polymorphic ability, codominant inheritance, polyallelic nature, integrating the genetic, physical, and sequence-based physical maps in plant species, and simultaneously having provided molecular breeders with an efficient tool to link phenotypic and genotypic variation. However, the construction of SSRs is often tedious, and cloning and enrichment procedures required for their generation are costly (Zane et al., 2002; Squirrell et al., 2003; Weising et al., 2005). The expressed sequence tag (EST) databases have become particularly attractive resources for such in silico mining, as was demonstrated in, e.g., citrus (Chen et al., 2006), coffee (Poncet et al., 2006), and particularly in cereals (Yu et al., 2004). The SSRs developed from ESTs, popularly known

Table 9.2 List of Genetic Diversity Studies Conducted in Oil Palm

S. No	Marker Used	Population	References
1	Isozymes	Pollen of seven accessions of *Elaeis oleifera* and hybrid between *E. oleifera* and *Elaeis guineensis*	Ataga and Fatokun (1989)
2	SSRs	194 oil palms from 49 populations	Bakoume et al. (2014)
3	RFLP	11 oil palm germplasm collections, namely, Nigeria, Cameroon, Democratic Republic of Congo, Tanzania, Angola, Senegal, Sierra Leone, Guinea, Ghana, Madagascar, and Gambia	Maizura and Rajanaidu (2001)
4	AFLP	687 accessions belonging to 11 African countries and Deli *dura*	Kularatne et al. (2001)
5	AFLP and RFLP	Within oil palm germplasm (both *E. oleifera* and *E. guineensis*)	Barcelos et al. (2002)
6	RAPD	*E. oleifera* accession collected from the Amazon forest	Moretzsohn et al. (2002)
7	RAPD	Five *dura* germplasm accessions	Mandal et al. (2004)
8	SSR	Elite oil palms from Nigeria and selected breeding and germplasm materials from Malaysia	Okoye et al., 2016
9	SSR	*E. oleifera* and *E. guineensis*	Zaki et al. (2012)
10	SSR	NIFOR oil palm main breeding parent genotypes	Okoye et al. (2016)
11	SSR	121 breeding plants from three different populations in Thailand	Taeprayoon et al. (2015)

AFLP, *Amplified fragment length polymorphism;* RAPD, *random amplified polymorphic DNA;* RFLP, *restriction fragment length polymorphism;* SSR, *simple sequence repeat.*

as EST-SSRs or genic microsatellites, represent functional molecular markers because a putative function for a majority of such markers can be deduced by database searches and other in silico approaches. Furthermore, they represent genic regions of the genome. In oil palm a considerable amount of EST sequences (nearly 40,979) were available until June 2016 on the National Center for Biotechnology Information (NCBI) website. Few reports were available on the in silico identification of EST-SSRs and their use in the characterization of oil palm germplasm. The first report of a systematic study of genes expressed by means of EST analysis in oil palm was done by Jouannic et al. (2005). A total of 2411 valid EST sequences were thus obtained from five different cDNA libraries generated from male and female inflorescences, shoot apices, and zygotic embryos. Mining of oil palm EST sequences from dbEST of the NCBI resulted in 1180 SNP sites and 137 indel polymorphisms with a frequency 1.36 SNPs/100 bp (Riju et al., 2007). Among the six tissues from which the EST libraries had been generated, mesocarp had a high frequency of 2.91 SNPs and indels per 100 bp, whereas zygotic embryos had the lowest frequency of 0.15 per 100 bp. Tranbarger et al. (2011) tested 289 EST-SSRs to detect polymorphisms in elite breeding parents and their crosses; 230 of these were amplified polymerase chain reaction (PCR) products, 88 of which were polymorphic within the breeding material tested. Detailed analysis and annotation of the EST-SSRs revealed that they were mostly related to transcriptional and posttranscriptional regulation. Ting et al. (2010) performed SSR mining in the EST sequences of 19,243 *E. guineensis*, which were available on that date. They found that dinucleotide repeats formed the largest group (45.6%) consisting of 66.9% AG/CT motifs. This was followed by trinucleotide repeats,

which are the second most abundant repeat types (34.5%) consisting of AAG/CTT (23.3%). Singh et al. (2008) exploited the EST database of oil palm for assessment of genetic diversity. A total of 5521 EST sequences were mined and 145 SSRs were developed.

5.3 CONSTRUCTION OF LINKAGE MAPS AND QTL MAPPING

The basic requirement for marker-assisted selection (MAS) is identification of markers associated with the trait being targeted and development of a linkage map. By screening a large population of sibs of many different markers, pairs or groups of markers that are linked, and tend to be inherited together, can be identified. Such groups are expected to be on the same chromosome, and the closeness of the linkage, calculated statistically, shows the relative position of the markers along the chromosome (Corley and Tinker, 2003). In many important agronomic plant species, a large number of DNA markers and linkage maps have been developed. Many quantitative trait loci (QTLs) for important traits have been mapped on whole genomes, setting up the basis for rapid genetic improvement through MAS.

The first AFLP marker-based genetic linkage map from a mapping population generated by the selfing of an important breeding material was segregated for shell thickness character (Mayes et al., 1996). The closest RFLP marker (pOPgSP1282) was located at a distance of 9.8 cM from the *Sh* locus. The map was updated by Jack et al. (1998) where they reported tagging of two genes, namely, shell thickness (Sh) and virescence (Vr).

QTL mapping using 153 RFLP markers in combination with phenotypic data from an F_2 population to estimate the position and effects of QTLs for traits including yield of fruit and its components as well as measures of vegetative growth was developed by Rance et al. (2001). A high-density linkage map using microsatellite markers from a tenera palm in La Mé population (LM2T) and a dura palm from the Deli population (DA10D) was developed with a set of 255 microsatellites; 688 AFLPs and an identified locus of the Sh gene near an AFLP marker E-Agg/M-CAA132 were mapped at 4.7 cM from the Sh locus (Billottte et al., 2005). A map was constructed using AFLP, RFLP, and SSR markers for an interspecific cross involving a Colombian *E. oleifera* (UP 1026) and a Nigerian *E. guineensis* (T128), and at a 5% genome-wide significance threshold level, QTLs associated with iodine value, myristic acid (C14:0), palmitic acid (C16:0), palmitoleic acid (18:0), oleic acid (18:1), and linoleic acid (18:2) content were detected (Singh et al., 2009). Significant QTLs for C14:0, 16:1, 18:0, and 18:1 content were detected around the same locus on group 15, thus revealing another major locus influencing fatty acid composition in oil palm.

A consensus linkage map of oil palm using codominant markers (i.e., microsatellite and SNPs) and two F_1 breeding populations generated by crossing dura and pisifera individuals identified a major QTL for stem height (Lee et al., 2015). Four hundred and forty-four microsatellites and 36 SNPs were mapped onto 16 linkage groups with a total coverage of 1565.6 cM, with an average marker space of 3.72 cM. They mapped a major QTL for stem height on linkage group 5. *VIRESCENS* (*VIR*) gene, which controls fruit exocarp color and is an indicator of ripeness, has been identified (Singh et al., 2014). Pootakham et al. (2015) reported genome-wide SNP discovery and identification of QTLs associated with agronomic traits in oil palm using genotyping-by-sequencing; out of 3417 fully informative SNP markers, they were able to place 1085 on a linkage map, which spanned 1429.6 cM and had an average of one marker every 1.26 cM. Besides the foregoing reports, several linkage map and QTL mapping studies were performed, which are given in Table 9.3.

Gan (2014) carried out marker development studies in oil palm for genetic linkage mapping and QTL analysis for use in MAS. Use of the AFLP method identified 29 primer pairs that yielded 49

putative shell thickness-related polymorphic bands. The use of a relatively new Diversity Array Technology "Genotyping-by-Sequencing (DArTSeq) platform, through genotyping of two closely related tenera self-pollinated F_2 populations, generated a total of 11,675 DArTSeq polymorphic markers of good quality. These markers were used in the construction of the first reported DArTSeq-based high-density maps for oil palm. Saturation of the shell thickness (Sh) region with all available DArTSeq markers as well as map integration around the Sh regions for both populations resulted in identification of 32 SNPs and DArT markers mapped within a 5 cM flanking region of the *Sh* gene. Homology search of the DArTSeq marker sequence tag (64 bp) against the published oil palm genome assembly confirmed that 23 out of the 32 (72%) DArTSeq markers were located on the p5_sc00060 scaffold in which the *SHELL* gene was identified.

Table 9.3 List of Quantitative Trait Locus (QTL) Mapping and Linkage Analysis Studies Conducted in Oil Palm

S. No	Marker Used	Population	Number of Palms	Trait(s) for QTL Mapping	Salient Findings	References
1	SSRs and gene-based markers	A controlled cross-progeny population established from a cross between female Topi Deli dura and male Yangambi pisifera parents	52	Oil yield-related traits such as fresh fruit bunch (FFB) yield, ratio of oil per fruit (OF), oil per bunch (OB), etc.	16 putative QTLs on seven linkage groups affecting important oil yield-related traits such as fresh fruit bunch (FFB) yield, ratio of oil per fruit (OF), oil per bunch (OB), etc.	Jeennor and Volkaert (2014)
2	SSRs	The *Elaeis guineensis* cross LM2T×DA10D	116 full-sibs	Palm oil fatty acid composition	16 QTLs affecting palm oil fatty acid proportions and iodine value were identified	Montoya et al. (2014)
3	SSR and SNPs	*Elaeis*-interspecific pseudo-backcross of first generation (*E. oleifera*×*E. guineensis*)×*E. guineensis*	134 full-sibs	Palm oil fatty acid composition	19 QTLs associated to the palm oil fatty acid composition were evidenced	Montoya et al. (2013)
4	SSRs, EST-SSRs, AFLP	From a cross between two tenera palms	208	Sex ratio and related traits	Eight QTLs across six linkage groups associated with sex ratio and related traits	Ukoskit et al. (2014)

Continued

Table 9.3 List of Quantitative Trait Locus (QTL) Mapping and Linkage Analysis Studies Conducted in Oil Palm—cont'd

S. No	Marker Used	Population	Number of Palms	Trait(s) for QTL Mapping	Salient Findings	References
5	SNP, RFLP, and SSR markers	From the self-pollination of the tenera palm, T128	240 palms	VIRESCENS gene	Five independent mutant alleles of VIR	Singh et al. (2014)
6	SNPs and 252 SSRs	On two mapping populations, an intraspecific cross with 87 palms and an interspecific cross with 108 palms	–	Linkage map	Integrated map with 1331 markers spanning 1867 cM	Ting et al. (2014)
7	SSR, AFLP, and RFLP	The mapping population is a high-yielding dura × pisifera cross	–	Linkage map	Integrated map was 2247.5 cM long and included 479 markers	Seng et al. (2011)
8	SSR	From a cross between a tree (D1) from the Deli population and a tree (L1) from La Mé population	–	Lipase gene	Identified the lipase and its gene cosegregates with the low/high lipase trait	Morcillo et al. (2015)
9	AFLP, RFLP, and SSR	An interspecific cross involving a Colombian *Elaeis oleifera* (UP1026) and a Nigerian *E. guineensis* (T128)	118	Fatty acid composition	Significant QTL for C14:0, C16:1, C18:0, and C18:1 content was detected around the locus on group 15	Singh et al. (2009)

5.4 GENOME-WIDE ASSOCIATION MAPPING STUDIES FOR QTL IDENTIFICATION

Most of the agro-morphological characters are controlled by multiple QTLs (i.e., complex traits). Genetic mapping of these functional loci facilitates marker-assisted breeding. Two of the most commonly used tools for dissecting complex traits are linkage analysis and association mapping (Bodmer, 1986). Association mapping offers several advantages when the population is subjected to natural population along with other important prerequisites such as high-resolution mapping of the QTLs, more allele numbers, less time, and broader reference population (Mott et al., 2000). Association mapping in oil palm, however, is not reported that often. Teh et al. (2016) performed GWAS for oil-to-dry-mesocarp content on 2045 genotyped *tenera* palms using 200K SNPs and found that 80 loci were significantly associated with oil-to-dry mesocarp yield ($P \leq 10-4$), and three key signals were found. They reported the most comprehensive use of high-density SNP genotyping with the use of a GWAS approach to identify SNP variants associated with differences in the key oil-to-dry mesocarp yield trait, and confirmation of their action in an independent cross.

5.5 MOLECULAR MARKER APPLICATIONS AND MARKER-ASSISTED SELECTION IN OIL PALM

In addition to the use in breeding for specific traits through MAS, molecular markers are also useful for the diagnosis and characterization of diseases, determining legitimacy of genotypes/progenies, protection of intellectual property rights, etc. Oil palm in South East Asia is badly affected by basal stem rot (BSR) disease caused by *Ganoderma boninense*. BSR disease causes serious problems in oil palm production. Breeding for resistance is an obvious approach and a long-term solution for ganoderma disease. Ali et al. (2015) identified SSR markers for BSR disease. In their study, 58 SSR markers were utilized with three progeny types, namely, KA4G1, KA4G8, and KA14G8, to perform a comparative molecular mapping for association with BSR. Mandal et al. (2014) developed a PCR-based early detection of *Ganoderma* sp. in India. Thonghawee et al. (2010) used microsatellites for parentage analysis in an oil palm breeding population. They reported that a combination of four SSR loci was sufficient to reach a nonexclusion level below 1% for the detection of planting errors. To detect pollination errors confidently, seven or eight SSR loci were necessary. Ten monomorphic SSR markers and two half sib families were used for detection of illegitimacy in oil palm as reported by Hama-Ali et al. (2015). Illegitimate off-spring IDs 97 and 180 were detected by three monomorphic loci, mEgCIR0425, mEgCIR3769, and mEgCIR3902, in family-1 and family-2. In addition, five loci detected one illegitimate off-spring, ID180. Mayes et al. (1996) reported DNA finger printing of 111 elite breeding palms using a comparatively limited number of highly variable RFLP probes. Using this method all but eight of the palms could be distinguished uniquely. Mandal et al. (2014) attempted PCR-based detection of *Ganoderma* sp. causing BSR of oil palm in India.

5.6 GENOME SEQUENCING, ITS IMPLICATIONS FOR COMPARATIVE MAPPING

The first draft genome sequence of African oil palm *E. guineensis*, the predominant source of worldwide oil production, was published by Singh et al. (2013a,b) using a combination of Roche/454 GS FLX Titanium (Roche/454) and Sanger bacterial artificial chromosome end sequencing. They reported a total of 1.535 Gb of assembled sequences, which was predicted to have 34,802 genes including transcriptional regulators present in the kernel. The guanine–cytosine content of the *E. guineensis* genome (37%) is similar to that of other plant genomes. Analysis of conserved gene order revealed that the duplications were retained in *E. oleifera*, so that segmental duplications predated the divergence of the African and South American oil palms. The genome sequence of oil palm will be a rich resource for oil palm breeders, geneticists, and evolutionary biologists alike. The genome sequence of this tropical plantation crop is an important achievement for identification of genes for various agro-morphological traits. With the availability of genome sequencing of oil palm, it can now act as reference genome for closely related crops. Since there is a conservation of gene sequences within the same plant family, comparative genomics plays an important role by utilizing the synteny among the conserved regions of crops belonging to the same family.

5.7 IMPROVEMENT OF OIL PALM THROUGH IN VITRO CULTURE

In oil palm, vegetative propagation is made possible only via tissue culture. Oil palm tissue culture is unique, undergoing callusing and embryogenesis processes, which had been rigorously attempted between the 1960s and 1970s. The early successes of plantlet production were seen in the 1970s (Jones, 1974; Rabéchault and Martin, 1976), inspiring various organizations to delve into in vitro propagation.

Most laboratories have established their own improved culture media and protocols for cloning and conducted field trials. Clonal plantlets derived from selected ortets were reportedly more uniform and in many cases yielded at least 20% more than the seed-derived D×P standards. Because of the slow process in oil palm in vitro propagation, which takes 2–5 years from explants to nursery seedlings, oil palm clones cannot meet the entire demand for improved planting materials in the near future. Modifications of the culture media and protocols with reduced abnormality have somehow limited large-scale propagation.

In Malaysia, there are 12 tissue culture laboratories, including the MPOB, producing clonal oil palm ramets. In 1995, there were only three major oil palm seed producers in Indonesia, which produced 61 million seeds. Then, IOPRI was the dominant seed producer at 50 million seeds. Oil palm D×P seed production in Malaysia increased marginally from 50 million in 1995 to 65 million in 2007 and 88 million in 2008. Most tissue culture labs, such as Advanced Agriecological Research Sdn. Bhd (AAR) and Felda are equipped with advanced facilities. AAR and Felda are poised to produce about 1 million ramets per year. The mean oil yield of AAR clones is 7.5 t/ha/year compared to 6.5 t/ha/year of its D×P hybrid seeds. De Touchet et al. (1991) studied plant regeneration from embryonic suspension cultures of oil palm. They found that 80 or 100 mg/L 2,4-dichlorophenoxyacetic acid (2,4-D) and 1 g/L activated charcoal from calli produced embryogenic cells and protruding proembryos. Thuzar et al. (2011) developed an efficient and rapid plant regeneration system through somatic embryogenesis, which was developed using 13-week-old zygotic embryos of oil palm (*E. guineensis* Jacq.) cv. "Tenera." Zygotic embryos were cultured on MS and N6 media supplemented with 2.0 mg/L picloram, 2,4-D, and dicamba. The highest embryogenic callus formation (32%) was observed on N6 medium with 2,4-D after 3-month culture on callus induction medium. A high-frequency plantlet regeneration protocol was developed for oil palm (*E. guineensis* Jacq. Var. tenera) through secondary somatic embryogenesis (Te-Chato and Hilae, 2007).

The advantages of semiclonal and biclonal seeds over the conventional D×P seeds include: greater degree of uniformity because the crossings are confined to a limited number of parental combinations; low cost of seed production (lower than tissue culture plantlets); low risk of clonal abnormality (because of limited plantlets production from each parent); small tissue culture set up to clone the parents and limited number of plantlets production per ortet; and an oil yield gain expectation of of 15% compared to conventional D×P hybrid seeds. Results from genetic marker and genome-wide methylation studies indicated that the tissue culture abnormalities in oil palm arise from an interplay of genetic and epigenetic mechanisms. Various efforts are geared toward developing diagnostic tools for predicting genetic predisposition to abnormality. These include global gene expression analysis via DNA microarray, genetic mapping, and the candidate gene approach. It is anticipated that an effective screening process, preferably at the ortet stage, will provide greater confidence to the industry in producing and utilizing clones (Cheah, 2003).

5.8 GENETIC ENGINEERING OF OIL PALM

Genetic engineering could be the best method to overcome the limitations of conventional breeding and/or to achieve objectives that would be difficult or impossible by traditional means. Attempts are in progress for transforming traits such as disease or pest resistance and quality traits such as oil composition. Genetic engineering could be applied to produce transgenic oil palms with high value-added fatty acids (oleic acid) and novel products to ensure the sustainability of the palm oil industry.

Establishment of a reliable transformation and regeneration system is essential for genetic engineering. Particle bombardment was the most successful method of transformation in monocots like oil palm. *Agrobacterium*-mediated and green fluorescent methods were also tried to improve oil palm through genetic engineering. Upon the development of a reliable transformation system, a number of useful targets are being projected for oil palm improvement. Among these targets are high-oleate and high-stearate oils, and the production of industrial feedstock such as biodegradable plastics. MPOB first initiated genetic engineering to produce high-oleate palms for the industrial feedstock and liquid oil market. The estimated value for high-oleate palms is US$1500/ha/year if the oleic acid content is >65%. More recent targets in genetic manipulation include high-stearate palms such as cocoa butter substitute, nutraceutical oils enriched in palmitoleic acid and lycopene, and biopolymers for industrial applications (Sambanthamurthi et al., 2002). Masani et al. (2014) developed novel transformation protocols based on polyethylene glycol-mediated transfection and DNA microinjection showing that protoplasts are suitable as a target for oil palm genetic engineering. They successfully expressed a reporter gene encoding green fluorescent protein (GFP) allowing the rapid and efficient generation of nonchimeric transgenic calli without the use of standard selectable markers. They observed that 5 mL of DNA (at a concentration of 100 ng/mL) injected into the cytoplasm of protoplasts embedded in an alginate layer was identified as the optimal platform for the transformation of oil palm protoplasts. This resulted in approximately 14% of the injected protoplasts developing into microcalli that continued to express GFP.

5.9 TRANSGENIC OIL PALM FOR HIGH OLEIC ACID CONTENT

In Malaysia, the main goal for genetic engineering of oil palm is to increase oleic acid content at the expense of palmitic acid. Besides increasing oleic acid, modification of fatty acid composition (unsaturation vs. saturation) and production of novel high-value products have also been targeted. Two main targets are oil palm producing high-stearic acid and oil palm synthesizing biodegradable plastics [polyhydroxybutyrate (PHB)]. Based on the fatty acid composition of palm oil and the fatty acid biosynthesis pathway common to all plants, the following postulation was made: (1) β-ketoacyl-acyl carrier protein (ACP) synthase I1 (KAS I1) activity is rate limiting in the oil palm mesocarp, resulting in a "bottleneck" of palmitic acid; (2) thioesterase activity toward palmitoyl-ACP is very high, resulting in release of palmitic acid; and (3) oil palm mesocarp contains an active stearoyl-ACP desaturase, thus most of the stearoyl-ACP formed is effectively desaturated to oleic acid. Therefore two approaches were considered for channeling palmitic acid further along the pathway to produce more oleic acid: i.e., increase KAS I1 activity and reduce thioesterase activity toward palmitoyl-ACP. Transformation of oil palm with a sense copy of KAS I1 and an antisense copy of palmitoyl-ACP thioesterase under the control of a mesocarp-specific promoter will be carried out once the genes are made available (Parveez et al., 2000). Putative transformed plants with genes for high-oleic acid, high-stearic acid, and high-ricinoleic acid are being screened in a biosafety greenhouse (Parveez et al., 2011). Transformed cultures with genes governing carotene composition and palmitoleic acid are also in progress.

Abdullah et al. (2005) used immature embryo (IE) for oil palm transformation studies. They found that transient transformation frequencies were comparable to other plant systems reported, with as high as 97.4% recorded for biolistic and 64.4% for *Agrobacterium*-mediated gene transfer. IEs were also more responsive to in vitro manipulations as compared to other explants such as leaf and root tissues. Lee et al.

(2006) reported expression of *Bacillus thuringiensis* insecticidal protein gene in transgenic oil palm. They found that pretreatment of target tissues with phytohormones is essential for increasing transformation efficiency. This finding could enable higher transformation rates in oil palm that were previously difficult to transform. PCR analysis further confirmed the presence of the *CryIA(b)* in the transformed tissues. Expression of *CryIA(b)* from PCR-positive samples was further confirmed using a rapid gene expression detection system. Masli et al. (2009) reported transformation of oil palm using *Agrobacterium tumefaciens*. The calli were transformed with an *Agrobacterium* strain, LBA4404, harboring the plasmid *pUBA*, which carries a selectable marker gene (bar) for resistance to glufosinate-ammonium and is driven by a maize ubiquitin promoter. Transgenic oil palm was selected and regenerated on a medium containing glufosinate-ammonium. This is the first report of a successful transformation of oil palm using *A. tumefaciens* for selection against glufosinate-ammonium. The development of transgenic oil palm is still in its infancy. For example, it is still unclear whether biolistics or *Agrobacterium*-mediated gene transfer will be the gene delivery method of choice (Izawati et al., 2012). Regeneration from isolated protoplasts as described by Masani et al. (2013) could also be a choice for genetic engineering in oil palm.

The oil palm fruit mesocarp contains high lipase activity that increases free fatty acids and necessitates postharvest inactivation by heat treatment of fruit bunches. Even before heat treatment the mesocarp lipase activity causes consequential oil losses and requires costly measures to limit free fatty acid quantities. Morcillo et al. (2013) reported elite low-lipase lines that yield oil with substantially less free fatty acids than standard genotypes, allowing more flexibility for postharvest fruit processing and extended ripening for increased yields. Genes for lipase and its cosegregates with the low-/high-lipase trait provide breeders with a marker to rapidly identify potent elite genitors and introgress the trait into major cultivars. Similarly, enzymes responsible for fruit abscission were well studied (Henderson et al., 2001). Tranbarger et al. (2011) identified a gene in the ethylene synthesis pathway (*ACC synthase*), which is expressed in fruit mesocarp 160 days after anthesis, when fruit abscission is starting, and Roongsattham et al. (2012) found several polygalacturonase genes in oil palm fruit. Based on these developments, genetic engineering for developing transgenics with low lipase content and nonshedding fruits (altered enzymes involved in fruit abscission) could soon be a reality.

Making of a dioecious oil palm (date palm model) through conventional breeding coupled with genetic engineering [of sex determination genes as described by Adam et al. (2011)] and use of female palms (100% sex ratio) along with 1%–2% of supermacho males producing only male inflorescence could be possible through biotechnological tools. Bhore and Shah (2012) studied the genetic transformation of American oil palm using IEs for the antisense Palmitoyl-Acyl Carrier Protein Thioesterase (PATE) gene by the particle bombardment method. The PATE gene is one of the key genes involved in plastidial fatty acid biosynthesis and is known to regulate the accumulation of C16:0. They hypothesized that posttranscriptional PATE gene silencing in *E. oleifera* fruit mesocarp tissue may increase the level of palmitoleic (C16:1), stearic (C18:0), and oleic (C18:1) acids to some extent at the expense of C16:0.

5.10 TRANSGENIC OIL PALM FOR HIGH-STEARIC ACID CONTENT

High-stearic acid is the second most important oil quality trait for genetic engineering studies. Downregulation of genes responsible for conversion of stearoyl-ACP to oleoyl-ACP will result in increasing the stearic acid content, which was first reported in rapeseed (Knutzon et al., 1992). In oil palm also, silencing the activity of stearoyl-ACP desaturase in the mesocarp could be an effective strategy to increase the stearic acid content. Parveez et al. (2015) designed a construct consisting of stearoyl-ACP desaturase gene and palmitoyl-ACP thioesterase gene under the control of a mesocarp-specific promoter.

5.11 TRANSGENIC OIL PALM FOR PRODUCING BIODEGRADABLE THERMOPLASTICS

Biodegradable plastics such as PHB and polyhydroxyalkanoate are produced by bacteria as storage materials (Senior and Dawes, 1973). In oil palm, Yunus et al. (2008) and Masani et al. (2009) made efforts to synthesize PHB in the mesocarp and leaf using three genes from bacteria. Two different strategies were used by different workers for producing the biodegradable plastics. The first is introduction of an antisense ACCase gene (Sambanthamurthi et al., 2002; Omar et al., 2008) into oil palm to inhibit the oil synthesis in the mesocarp, and as a result the entire acetyl-CoA pool will be diverted into the synthesis of biodegradable plastics. In the second strategy, the bacterial ketothiolase gene will be replaced with an oil palm ketothiolase gene (Teen et al., 2008) because the native gene may be more effective in utilizing acetyl-CoA to synthesize biodegradable plastics.

5.12 GENOME EDITING: FUTURE PERSPECTIVES

Genome editing has been a widely used technology in animals and to some extent in plants for manipulation of any gene or cell of any living organism (Gaj et al., 2013). This could be effectively used in oil palm for editing desirable genes using customized nucleases carrying sequence-specific DNA-binding domains to target specific DNA sequences. In soybean, transcription activator-like effector nucleases were used to downregulate two fatty acid desaturase 2 genes (*FAD2-1A* and *FAD2-1B*), resulting in an increase in oleic acid content caused by the reduction in linoleic content (Haun et al., 2014). Since it is an emerging tool with a number of advantages, it can be applied to oil palm for more effective regulation of targeted genes or for replacing an unwanted gene. This has become easier with the availability of oil palm genome sequencing.

6. FUTURE PERSPECTIVES AND CONCLUSIONS

Genetic transformation and production of transgenic palm with desirable genes would be the ultimate aim of all biotechnologists. Work, although initiated in countries such as Malaysia with the aim of altering oil composition, still has a long way to go. Various constraints such as time required, clonal abnormalities, transformation protocols, etc. preclude the application of biotechnological tools for genetic engineering. Nevertheless, transgenics through genetic engineering is the need of the hour in oil palm. The technology would be promising in developing drought-resistant varieties, dwarf palms, etc. In addition to analysis of genetic diversity and specific character tagging, strategic application needs to be adopted to resolve some of the practical issues such as detecting the most identical progeny (for further use in breeding and seed production) from the selfed population of parental palms where the variation is limited. There is an urgent requirement of molecular markers for testing clonal fidelity and detecting abnormalities (mantled flowering), which were reported to be caused by epigenetic change (hypomethylation) in a transposable element called *Karma* (Ong-Abdullah et al., 2015).

Construction of linkage maps (with focus on ESTs, DArTSeq) may be continued until a fully saturated map is developed and is accessible for common use. Since there are a few important issues such as development of mapping population, long generation time of the crop, heterozygous nature of the palms, lengthy time and cost involvements associated with the development of a linkage map, joint ventures by different beneficiary countries and reputed laboratories need to be joined together for this purpose.

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CHAPTER

GENETIC IMPROVEMENT OF VEGETABLES USING TRANSGENIC TECHNOLOGY

10

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1. INTRODUCTION

Population explosion, global warming, depleting natural resources such as land, water, etc., coupled with biotic and abiotic stresses have resulted in severe problems for the global population, which is increasing alarmingly and is projected to reach 8.5 billion by 2025. To fulfill the growing demand of food from the limited natural resources, it is imperative to adopt improved sustainable agricultural methods (Ashraf and Akram, 2009). Agricultural biotechnology has opened up new opportunities and novel possibilities to enhance the qualitative and quantitative traits of crop plants (Brookes and Barfoot, 2015). Agricultural biotechnology has also become a sustainable approach to combat the shortage of food and malnutrition by increasing the productivity of different crops as well as enhancing or altering proteins, carbohydrates, lipids, vitamins, and micronutrient composition (Brookes and Barfoot, 2015). Since the 1990s, the major emphasis of agricultural biotechnology has been on traits for improvement in crops related to pests, diseases, and herbicide resistance, nutritional quality, longer shelf-life, and tolerance to abiotic stresses such as temperature, moisture, and salt stress. All these traits involve a number of genes and these genes may not be available in the gene pool of the crop varieties and their related landraces. Therefore crop improvement through conventional breeding techniques has resulted in limited success. To overcome these limitations, genetic engineering or transgenic technology has been developed. In this technique, transgenic plants have been developed by transferring gene(s) from the same crop gene pool or unrelated organism and then integrating it into the genome of the target plant with the desired expression of the trait (Ashraf and Akram, 2009).

Vegetables are considered vital components in achieving nutritional security because they have a shorter maturity cycle, higher productivity, nutritional richness, economic viability, and also provide a valuable source of income, leading to improved livelihood. Thus growth in the vegetable sector has played an important role in India's food and nutritional security, health, and economic development. Vegetables contain valuable food ingredients that can be successfully utilized to build up and repair the body. They are rich in carbohydrate, vitamin, mineral, and fiber content. Vegetables make up a significant proportion of the human diet and their production plays a significant role in ensuring the nutritional security of human beings. Vegetable production and consumption offers new market opportunities for farmers, consumers, and the agro-industry and thereby generates income for producers and takes care of the health and wellbeing of consumers.

Genetic Engineering of Horticultural Crops. http://dx.doi.org/10.1016/B978-0-12-810439-2.00010-6

The genetic improvement of vegetables should address and suit the needs of both consumers and farmers (Dias and Ryder, 2011). The general objectives for farmers are quality and uniform produce, higher yield, and tolerance to diseases, pests, and abiotic stresses, whereas the needs of consumers are quality, appearance, shelf-life, taste, and nutritional value (Dias and Ryder, 2011). However, few vegetable cultivars are resistant to diseases, pests, and abiotic stresses. Resistance may also be unstable because of the insurgence of pathogens and pests. Furthermore, insects, including aphids, whiteflies, thrips, and leafhoppers, are also very important for limiting vegetable production because they are vectors of many viruses. Viruses can significantly reduce production and quality and are becoming more and more problematic worldwide because of the lack of control measures as well as absence of virus-resistant germplasms for many important vegetable crops (Zitter et al., 1996; Gonsalves, 1998). Improvement of postharvest traits, mainly transport quality, shelf-life, and pleasing appearance, is of increasing importance in vegetables (Dias and Ryder, 2011). Because vegetables are rich in vitamins, minerals, and other micronutrients and therefore are vital for health, breeding objectives should include improving their nutritional value (Dias and Ryder, 2011). Enhanced nutritional content would add value for poor, malnourished populations. Breeding for provitamin A, carotenoids, iron, and zinc is of keen interest as a biofortification strategy to alleviate nutrient deficiencies in developing world (Khush, 2002; King, 2002; Hotz and McClafferty, 2007).

In the past, conventional plant breeding approaches were considered the backbone of vegetable genetic improvement strategies, but they have a number of limitations. The power of plant transformation, coupled with the vast available information about genes and their products, has attracted plant biotechnologists to develop transgenic vegetables to address some of the most challenging biotic and abiotic constraints faced by farmers worldwide, challenges that are not easily addressed through conventional vegetable breeding alone (Tarafdar et al., 2014).

2. NATIONAL AND INTERNATIONAL STATUS OF VEGETABLE PRODUCTION

Vegetables are grown worldwide on different lands and in climates in both small and large scales. Global fruit and vegetable production has experienced a remarkable increase at an annual rate of about 3% over the last decade (FAO, 2013). As per the Food and Agriculture Organization (FAO) statistics, the global production of vegetables in 2011 reached 1 billion tons (FAO, 2013). Asia produced 671 million tons of vegetables from 52.7 million ha of land and has a share of 74.7% of the world's vegetable production. China has emerged as the world's largest vegetable producer, with global output shares of more than 50% (FAO, 2013). India is the second largest producer of vegetables in the world, but represents a sixfold lower level than China. Global arable land devoted to vegetables is expanding at 2.8% annually, higher than fruits (1.75%), oil crops (1.47%), root crops (0.44%), and pulses (0.39%), and at the expense of cereals (−0.45%) and fiber crops (1.82%) (FAO, 2009). India has made impressive strides on the agricultural front during the last three decades. In India, vegetable production shares 59.45% of total horticultural production (Mamta, 2014). Production of vegetables has increased from 58,532,000 to 168,300,000 tons from 1991–92 to 2014–15 (Sexena, 2015). India, being blessed with a diverse climate and distinct seasons, grows more than a hundred types of vegetables. Total potato production is recorded at 44,893,000 tons from 2,060,000 ha. Potato ranks first (26.67%) in total production of vegetables followed by other important vegetables such as onion (11.24%), tomato (10.34%), and brinjal (7.55%). Cauliflower and cabbage are the most preferred winter vegetables and

their total share in the country's vegetable production is 4.69% and 5.11%, respectively. Other important vegetables are okra, peas, and a good range of cucurbits. India also holds a unique position in the production and export of fresh vegetables. Out of a total of 68 million tons of India's exports of fresh vegetables, over 75% went to Asian countries, mainly to Nepal (23%) followed by UAE and Pakistan (20%). Tomato contributes to a maximum of 44% of total export of all vegetables followed by chilli (27%), garlic (19%), pea (3%), and cucumber (1%) (Sexena, 2015). To meet the projected demand by the year 2020, India must attain a per hectare yield of 22.3 tons for potato, 25.7 tons for vegetables, and 24.1 tons for fruits (Sexena, 2015).

3. CONSTRAINTS IN VEGETABLE PRODUCTION

Global climate change will increase the surface air temperature by 1.8–4.0°C by the end of the century, which will increase the frequency of extreme climatic events such as heat, cold waves, droughts, and floods. This will noticeably change various horticultural crops and their impact on nutritional security and sustainable farm income. So, it is imperative to understand and promote adaptation strategies/measures to minimize the adverse effects of climate change. Adaptive mechanisms such as adjustment of crop growing periods, the growing of tolerant varieties, crop rotation, and efficient and sustainable use of water will help to reduce these negative impacts. The challenges could be effectively addressed by identifying the gene(s) and quantitative trait loci for tolerance to high temperature, submergence, drought, salinity, and their utilization for the development of climate-resilient cultivars having high nutrient and water use efficiency and tolerance to biotic and abiotic stresses. It is also important to analyze the physiology of plant growth and its inherent mechanism for mitigating climate change. For mitigating the bad effects of climate change, there is an urgent need to identify the research gaps and socioeconomic issues, and to strengthen ongoing focused research programs.

4. NEED OF THE TRANSGENIC VEGETABLE

Plants are usually infested with pests and diseases. Fungi, bacteria, viruses, and nematodes frequently cause diseases in plants and reduce the yield drastically. More than 70,000 species of pest exist in the world, of which 10% are considered serious pests (Pimentel, 1997). Chemical pesticides have been used since 1945, and have been extremely successful in reducing crop losses to some pest insects, plant pathogens, and weeds, and in increasing crop yields (Pimentel, 1997). One estimate suggests that without pesticides, crop losses to pests might increase by 30%. In spite of the widespread use of pesticides, insects, pathogens, and weeds continue to reduce crop production, which is approaching 40% (Oerke et al., 1994; Pimentel, 1997; Tarafdar et al., 2014). Preharvest losses of 15% by insect pests, 13% by diseases, and 12% by weeds have been recorded (Pimentel, 1997). Vegetables, because of their cultivation intensity, disease and pest loads are alarming and complex compared to field crops. The main method for controlling pathogens, pests, and weeds is the use of pesticides. Because vegetables are often consumed in fresh or cooked form, pesticide residue and biological contamination are serious issues. Vegetables account for the major share of the global pesticide market. Almost 25 kg/ha of active pesticide substances are used on average in vegetable production in the European Union

(OECD, 1997). Although vegetable production accounts for less than 1% of the US crop area, it accounts for 14% of total pesticide use (Osteen, 2003). Nearly 20% of worldwide annual pesticide expenditures, valued at US$8.1 billion, are applied to vegetables (Krattiger, 1997). Natural enemies play a major role in keeping the insect populations under control. However, indiscriminate use of pesticides also eliminates the natural predators and parasites that are responsible for the control of herbivorous pest populations in both natural and agro-ecosystems. Pesticide residues can affect the health of growers and consumers and contaminate the environment. Pesticide residues in vegetables can exceed tolerance limits both in developing and developed countries (Mansour, 2004; Ferreira, 2009). In India, a survey of pesticide residues in vegetable crops taken at the farm gate and in markets from 1999 to 2003 confirmed that of the 3043 samples, 9% contained residues above acceptable levels (Choudhary and Gaur, 2009).

Climate change, increasing population, and stagnant production have resulted in serious threats to populations. It is projected that the world population will reach 8.5 billion by 2025. To feed the growing population from limited land, water, and other natural resources is a big challenge. Thus producing crop varieties having higher productivity and tolerance to biotic and abiotic stresses is imperative to feed the growing population, which could be attained using conventional selection and breeding or through genetic engineering (Ashraf and Akram, 2009). Through conventional breeding programs a lot has been achieved in developing new crop varieties, but it is a very slow process, taking 8–10 years or longer. The time needed to transfer a desired gene into a crop plant depends on the source and nature of the gene and recipient parent (Jauhar, 2006). Wild crop species and landraces forming the secondary or even tertiary gene pool are rich reservoirs of genes for agronomic traits such as disease or pest resistance. However, pre- and postfertilization barriers may impede sexual hybridization between the donor and the crop species and compound the problem of alien gene transfers (Jauhar, 2006). In some cases it may not even be possible to incorporate a certain trait by conventional means because a suitable donor may not be available or, if available, there is linkage drag. These limitations have directed plant scientists toward the gene revolution as a consequence of the green revolution because of advances in the field of plant biotechnology. The gene revolution involves the direct transfer of desired genes from one species to another by using tools of genetic engineering technology. The gene of interest to be transferred may come from either closely or distantly related species or even unrelated organisms such as fungi, bacteria, and viruses. Thus this technology allows access to an unlimited gene pool without the constraint of sexual compatibility. The process of moving genes from one species to another is called transformation. Plants developed by this technique are referred as transgenic plants, genetically engineered (GE) plants, or genetically modified (GM) plants.

Over the past few decades, breeding opportunities have been broadened by genetic engineering and gene transfer technologies, as well as by gene mapping and identification of the genome sequences of model plants and crops, which has resulted in efficient transformation and generation of transgenic lines in a number of crop species (Gosal et al., 2009). Furthermore, pyramiding of desirable genes with similar effects can also be achieved by using these approaches. Now transgenic technology is emerging as an important means for crop researchers to modify traits of economic significance in crops. Redesigning crops to ameliorate biotic (pathogen and insect pests) and abiotic (herbicide, drought, salinity, salt, etc.) stresses by using genetic transformation is a better option for developing new plant varieties for enhancing agricultural production under adverse conditions (Lemaux, 2008). The advent of molecular genetic technologies has produced a clear understanding of crop stress resistance mechanisms. Moreover, the powerful combination of transgenic technology and conventional breeding

permits exploration and utilization of valuable traits encoded by transgene(s) to be introduced into commercial crops within an economically viable timeframe. During the last two and a half decades, transgenic plants having improved resistance against insect pests, pathogens, drought, and salinity have been developed that express/overexpress genes regulating osmolytes, specific proteins, antioxidants, ion homeostasis, transcription factors, and membrane composition. Thus transgenic crops should contribute to long-term goals, namely, to (1) ensure enhanced food production, (2) provide nutritional security, (3) operate sustainable agricultural technologies, (4) generate employment, (5) reduce regional imbalances in growth, and (6) minimize gender inequity. It ultimately provides solutions to solve the global problem of hunger and malnutrition (Qaim, 2009). Transgenic technology leads to more efficient production methods as well as a reduction in loss, which in turn leads to lower food prices both in the United States and abroad. Transgenic crops should be able to feed the demand of approximately 9 billion people by 2050.

5. HISTORY AND BACKGROUND OF DEVELOPMENT OF TRANSGENIC VEGETABLES

The foundation of plant biotechnology and the concept of transgenics date back to the 19th century when Haberlandt (1902) predicted cellular totipotency, i.e., the production of somatic embryos from vegetative cells. The first transgenic kanamycin-resistant tobacco plant (*Nicotiana plumbaginifolia*) was developed by Framond and his group in 1983 at Washington University (Bevan et al., 1983). Scientists at the agri-based multinational company Monsanto had developed an antibiotic kanamycin-resistant transgenic petunia plant (Fraley et al., 1983). In 1983, John Kemp and Timothy Hall of the University of Wisconsin inserted a bean gene into a sunflower plant. Development of transgenic crops during the 1990s was an important landmark in the history of crop improvement. The Flavr Savr tomato (also known as CGN-89564-2) was the first commercially grown, GE vegetable for human consumption granted a license by the US Food and Drug Administration (FDA) in 1994 (Bruening and Lyons, 2000). These tomatoes were taken off the market by 1997 because of their nonacceptable taste and aroma. In 1995, an insect-resistant *Bt*-potato crop was approved by the FDA. Just after commercialization of *Bt*-potato a few more transgenic vegetables such as virus-resistant squash, "AMFLORA potato," etc. were developed by different agri-based companies after receiving approvals for commercial cultivation. In China, the GM tomato "Huafan No. 1" (from Huzahong Agricultural University), which had long shelf-life characteristics, was approved for commercialization in 1996. The transgenic vegetable "AMFLORA potato" was accepted by the European Commission for production in the European Union (Devos et al., 2006) and the registration period was 12 years. The company BASF succeeded in developing this GM plant by suppressing genes for the production of amylase; the EH92-527-1 potatoes produce over 98% of amylopectin (James, 2010). Global biotech crop coverage and production continued to grow.

In 2014 it was reported that 18 million farmers in 28 countries planted more than 181 million ha as compared to 175 million ha in 27 countries in 2013 (James, 2014). It is noteworthy to mention here that Bangladesh approved *Bt*-brinjal/eggplant for the first time on October 30, 2013 and commercial cultivation started on January 22, 2014. In November 2014, a bruise-resistant potato (Innate potato) was approved in the United States. In 2014, different studies on 147 published biotech crops reported the significant and multiple benefits that biotech crops have generated from 1995 to 2014; on average GM

technology adoption has reduced chemical pesticide use by 37%, increased crop yields by 22%, and increased farmer profits by 68% (James, 2014). These findings corroborate earlier and consistent results from other annual global studies, which estimated increases in crop productivity valued at US$133.3 billion for the period 1996–2013.

6. FACTORS INVOLVED IN SELECTING THE TARGET CROP AND TRAIT

Transgenic manipulation is a powerful approach to crop improvement (Bhat, 2010). However, it is not a panacea for all problems. There are many biosafety concerns raised from various angles and some of the biosafety concerns associated with transgenic crops are genuine. By careful choice of crops, traits, strategies, and government policies, however, engineered crops can be developed and deployed to achieve worldwide agricultural goals within the purview of biosafety standards. The following factors should be considered during transgenic improvement of crop varieties.

6.1 CROP BIOLOGY AND ITS BREEDING BEHAVIOR

Biology of the crop plant assumes importance from the perspective of transgene movement. In the case of vegetatively propagated plants such as potato, sweet potato, pointed gourd, and colocasia the probability of transgene escape is limited. Similarly, in the case of highly self-pollinated crops such as tomato, eggplant, pea, etc. restricted transgene movement was found. In contrast, preventing transgene movement in cross-pollinated crops such as maize, pearl millet, mustard, etc. poses serious challenges. Therefore crop preference from the perspective of transgene movement will be vegetatively propagated > self-pollinated > cross-pollinated. Biotechnology offers novel ways to modify the breeding behavior of crops and thereby allows effective options to address concerns of transgene movement.

6.2 PRESENCE OF LANDRACES AND WILD RELATIVES

Escape of pollen resulting from interbreeding among crop varieties and their wild relatives is often observed. Therefore there is the likelihood of transgene escape to the wild relatives where transgenic crops are cultivated in centers of crop origin/diversity. The precise consequences of such transgene escape on biodiversity are difficult to predict, and will depend on the trait conferred by the transgenes and the environment. In view of poor consequences of transgene escape, transgenic crops are not allowed for commercial release in areas where there are wild relatives growing in nature. Because India is considered one of the major centers of crop diversity, it would be wise to take this aspect into consideration when prioritizing crops for transgenic improvement. On the other hand, ample countermeasures should be incorporated in transgenic crops to prevent transgene escape.

6.3 CONSUMPTION PATTERN OF THE CROP

Consumption pattern of the crop is also highly relevant in prioritizing crops for transgenic improvement. If the produce is not eaten, such as cotton, concerns of toxicity are minimal. Similarly, concerns of toxicity are minimal in the case of highly processed and purified products such as sugar and canola

oil. Products that are eaten either raw or cooked such as tomato, eggplant/brinjal, etc. would need the highest assurance of possession of minimal or no toxicity. Thus priority setting based on consumption patterns of the crop will be nonedible crops > fodder crops > crops subjected to industrial processing and purification > crops eaten after cooking > crops consumed raw.

6.4 TARGET TRAITS FOR MODIFICATION

At present the transgenic approach is viable for engineering qualitative traits that are controlled by one or a few major genes. Quantitative traits such as yield are not easily amenable for transgenic improvement. Similarly, traits that can be routinely modified through conventional breeding techniques should not be considered for transformation. The gene(s) controlling complex traits and closely tagged with molecular marker(s) need not be targeted for transgenic improvement. In this case, where conventional approaches are inadequate, genes controlling novel traits should be attempted for transgenic improvement. Conventional breeding techniques have not proved successful in addressing biotic stresses caused by pests, pathogens, and viruses. On the other hand, chemical control measures are harmful and environmentally nonsustainable. Viruses cause heavy crop losses and pose severe challenges to management because chemical control measures do not exist to confine their spread. At present, genes such cryo endotoxins, lectins, protease inhibitors, etc. have proved to be highly effective in the management of pests and diseases of several crops. Similarly, conventional breeding techniques are also futile in addressing complex traits such as tolerance to various abiotic stresses such as moisture stress (flood and drought), temperature stress (heat and cold), and salt stress. Basic knowledge of stress–response biochemical pathways and their key regulatory genes will be essential for tackling these problems. Genetic engineering also offers crucial ways to fortify food with vitamins and minerals, as demonstrated with rice, canola, and tomato. Nutritional enrichment via transgenics has become viable and is receiving worldwide support. The transgenic approach has proved effective for engineering traits for enhancing shelf-life and slowing ripening of the vegetables and fruits. Production of commercial hybrid seeds in crop plants bearing hermaphrodite flowers requires special genetic stocks, where sexuality (male sterility and bisexuality) of the plant can be controlled. Cytoplasmic male sterility, a trait under the control of mitochondrial–nuclear gene interactions, is used in most crops. As cytoplasmic male sterility is not readily available in all crops which limits commercial exploitation of hybrid seed production in those crops. The genetic engineering approach in creating male sterile lines in these crops is highly relevant for facilitating hybrid seed production and this approach has been successfully demonstrated in *Brassica*. Similarly, engineering male sterility can also be helpful in transgene containment where pollen is the major route through which transgenes escape to other crop varieties and landraces. Keeping transgenics in a male sterile background can significantly lower transgene spread and this approach is predominantly viable in crops such as eggplant, tomato, etc. where seed is not the commercial product. Linking genes for parthenocarpy (fruit set without pollination) and male sterility along with genes for traits such as slow ripening and biofortification will allow development of environmentally safe transgenic crops.

6.5 IMPROVEMENT STRATEGIES ADOPTED

The nature of transgenes and the strategies adopted assume importance from a biosafety and ethical perspective. In general, if the transgenes derived from nonpathogenic organisms do not code for any product, those transgenes pose less of a concern. The gene silencing approach by expressing

viral genome sequences is found very effective in controlling the corresponding viruses. Since produce from plants infected with viruses is commonly consumed by humans and animals and does not cause any harm to them, transgenic plants expressing a small piece of the viral genome would not evoke serious biosafety concerns. This also applies to extension of shelf-life of vegetables and fruits through antisense expression of gene sequences derived from the same or related plants. The antisense technique does not eliminate the normal plant metabolism pathway, but slows down precise metabolic steps in the ripening process. Antisense or gene silencing strategies are also found very effective in eliminating antinutritional compounds or allergens present in different crop species. Thus transgenic improvement using such gene sequences and strategies should be given priority.

Selection markers (antibiotic or herbicide resistance, etc.) are extensively used for the development of transgenic crop varieties. There is considerable opposition to their use because of the synthesis of the toxic antibiotic in the plant system. These marker genes have no utility in cultivation of the crop and also constitutive expression of marker gene products is a drain on plant metabolism. Fortunately, strategies are now available to eliminate the marker genes after transgenic plants have been developed. Such strategies should be effectively utilized during the transgenic variety development program to minimize biosafety concerns.

7. GENETIC TRANSFORMATION

Genetic transformation is a method of transferring the gene of interest to the host and it is a key technique for plant molecular breeding to introduce desirable traits into the existing genomes while preserving the genetic identity of plants. The basic requirement of genetic transformation is identification and isolation of the gene of interest and then construction of an "expression cassette" of genes comprising the gene of interest flanked by the promoter and terminator sequence. Promoter and terminator genes are added in the expression cassette to control desirable expression of the trait in the transformed plants. In addition, marker genes/reporter genes (antibiotic or herbicide resistance genes) are added in the gene cassette, which facilitates selection of transformed cells from nontransformed ones. The gene cassette is delivered into the host plant cell by a vector, which is a cloning vehicle that transports the gene cassette to the host plant cell. Genetic transformation is adapted either through vectorless or direct genetic transformation or through *Agrobacterium tumefaciens* or *Agrobacterium rhizogenes*.

7.1 DIRECT GENETIC TRANSFORMATION

The cellular wall acts as the natural barrier that all methods of genetic transformation have to overcome to achieve transgene insertion into the host cell. The direct method of genetic transformation originated in the 1980s for improving crops where *Agrobacterium*-mediated genetic transformation did not succeed (Paszkowski et al., 1984; Davey et al., 1989; Masson et al., 1989; Vasil, 2005; Rivera et al., 2012). These methods offer an alternative for integrating multiple copies of a desired gene with minimal cellular toxicity at random sites into the genome (Qayyum et al., 2009; Rivera et al., 2012). In these methods, the foreign gene of interest is delivered into the host plant cell without the help of a vector. Direct methods in use are electroporation (Fromm et al., 1986; Tsukada et al., 1989; Bates et al., 1990; Langridge et al., 1985; Chang and Loescher, 1991; Bates et al., 1990; Bower and Birch, 1990),

biolistics (Kikkert et al., 2004; Prakash and Varadarajan, 1992; Schulze et al., 1995; Aragão et al., 1996; Barandiaran et al., 1998; Ivo et al., 2008; Ruma et al., 2009), vacuum infiltration (Tague and Mantis, 2006), ultrasound (Sawahel, 1996), silicon carbide fibers (Kaeppler et al., 1990, 1992), microinjection (Neuhaus and Spagenberg, 1990), macroinjection (Peffley et al., 2003), laser microbeams (Weber et al., 1988), and sonication (Jiang et al., 2004). Their disadvantages involve problems with plant regeneration and a low transient expression of transgenes.

7.2 GENETIC TRANSFORMATION THROUGH *AGROBACTERIUM*

A. tumefaciens is a soil bacterium and is widely used for the genetic transformation of plant cells. For genetic transformation the suitable explants of the target plants were selected and cultured in vitro for the production of a mass of undifferentiated cells. The undifferentiated mass cells further cocultivated with *A. tumefaciens* inoculums. *Agrobacterium* genetically transforms the plant cells by transferring a well-defined transgene segment from its tumor-inducing (Ti) plasmid to the host-cell genome (Gelvin, 1998). Ti plasmids are used as gene vectors for delivering useful foreign genes into target plant cells and tissues. The native transfer DNA (T-DNA) carries a set of oncogenes (Gaudin et al., 1994) and opine catabolism genes, whose expression in plant cells leads to neoplastic growth of the transformed tissue and the production of opines, amino acid derivatives that are used by the bacteria as a nitrogen source. The two 25–28 bp direct repeat borders are the only *cis*-acting elements essential for T-DNA transfer and for this reason T-DNA boarders are required to flank the gene of interest to be transferred. The native wild-type oncogenes and opine synthase genes from the T-DNA can be replaced by genes of interest (Klee et al., 1987). As a result, any DNA placed between the borders will be transferred to the host cell. Because the T-DNA is not able to mediate its own transfer, other bacterial features need to be altered. The vir genes, residing on the virulence region of the Ti plasmid, are required for T-DNA transfer and integration. Altering their regulation (Ankebauer et al., 1991) and copy number (Rogowsky et al., 1987) proved to be useful for increasing transformation efficiency (Klee et al., 1987). Thereby the size of the T-DNA that can be mobilized into plants could be enlarged (Hamilton et al., 1996). The ability of vir genes to act in *trans* led to the development of binary and superbinary transformation vectors as a major step toward increasing the range of species that are amenable to *Agrobacterium*-mediated transformation (Lee et al., 2008; Nester et al., 2005). Many *Agrobacterium* strains, plasmids, and protocols have been developed and adapted for the genetic transformation of various plant species (Draper et al., 1988). The molecular machinery needed for T-DNA production and transport into the host plant cell comprises proteins that are encoded by the bacterial chromosomal DNA as well as Ti plasmid vir genes. In addition, various host plant proteins have been reported to participate in the *Agrobacterium*-mediated genetic transformation process (Tzfira and Citovsky, 2002; Gelvin, 2003), mostly during the later stages of the process (i.e., T-DNA intracellular transport, nuclear import, and integration). Recombinant *Agrobacterium* strains, in which the native T-DNA has been replaced with genes of interest, are the most efficient cloning vehicles for the introduction of foreign genes into plants and for the production of transgenic plant species (Draper et al., 1988). T-DNA transfer and its integration into the plant genome are controlled by various factors such as host plant genotype, type of explant, plasmid vector, bacterial strain, composition of culture medium, tissue damage, suppression, or elimination of *Agrobacterium* infection after cocultivation (Mohammad and Baghérieh-Najjar, 2009; Kavitah et al., 2010; Sood et al., 2011). Two methods are followed for genetic transformation, i.e., *Agrobacterium*-mediated

in vitro transformation and *Agrobacterium*-mediated in planta transformation. In the *Agrobacterium*-mediated in vitro method of transformation the suitable explants of plants were selected and allowed to produce a mass of undifferentiated cells. The undifferentiated cells were further cocultivated with *A. tumefaciens* inoculum for transformation and the transformed cells were screened (antibiotic selection) and allowed to regenerate as whole plant.

In planta transformation (Bechtold et al., 1993) is the alternative method, which is more advantageous than the in vitro method because it requires less labor, a shorter time period, fewer chances of contamination and somaclonal variation, etc. Approaches such as vacuum infiltration (Tague and Mantis, 2006) and floral dip are used during the in planta method of transformation. Vacuum infiltration is carried out by placing flowering parts of plants upside down in a beaker containing recombinant *A. tumefaciens* solution with 5% sucrose. Plants are placed in such a way that only inflorescences are submerged in the solution. The beaker containing *Agrobacterium* inoculums solution and inflorescence is placed in a vacuum chamber (0.05 bar) for several minutes. In the floral dip method, plants are placed in a similar way as in vacuum infiltration but no vacuum is applied. Plants are kept in the infiltration solution for several minutes before removal. The advantages of the *Agrobacterium*-mediated transformation method are simplicity and low cost, transgenes linked with the transformation marker, higher frequency of single copy insertions, stable integration and inheritance, consistent gene expression over the generations, and lower chances of transgene silencing. Reproducible and efficient protocols have been developed for many crops including vegetables. However, it requires a very sterile in vitro protocol.

8. TRAITS INVOLVED IN THE DEVELOPMENT OF GENETICALLY MODIFIED CROPS

Selection of traits for the improvement of vegetables plays a vital role in addressing the needs of both consumers and farmers (Dias and Ryder, 2011). Farmers' choices for improvements are higher yield, uniform and quality produce, and tolerance to diseases, pests, and abiotic stresses. Consumers' preferences are quality, appearance, shelf-life, taste, and nutritional value (Dias and Ryder, 2011). However, few vegetable cultivars have exhibited resistance to diseases, pests, and abiotic stresses and in due course the resistance has diminished because of insurgence of pathogens and pests. Improvement of postharvest traits, mainly transport quality, shelf-life, and pleasing appearance, is also becoming significant because of the preferences of traders and consumers (Dias and Ryder, 2011). Because vegetables are rich in vitamins, minerals, and other micronutrients and therefore are vital for health, breeding objectives should include improving their nutritional value (Dias and Ryder, 2011; Gerszberg et al., 2015). Traits for transgenic improvement will be discussed in the following categories: (1) transgenics for improved storage (shelf-life); (2) transgenic plants for increased nutritional value; (3) transgenic plants for resistance to abiotic stress, and (4) transgenic plants for resistance to biotic stress (Bhat, 2010; Tarafdar et al., 2014; Gerszberg et al., 2015).

8.1 TRANSGENIC VEGETABLES FOR IMPROVED SHELF-LIFE PERIOD

There are two approaches for improving the shelf-life of vegetable crops: employing antisense RNA technology using the gene 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase, which degrades

Table 10.1 Transgenic Vegetables With Improved Storage Period

Transgenic Crop	Gene and Gene Product	Trait/Character Acquired	References
Tomato	Antisense version of the polygalacturonase (PG) gene	Delayed fruit ripening	Kramer and Redenbaugh (1994)
Tomato	Expansin and polygalacturonase (LePG and LeExp1)	Increased juice viscosity, consistency, and pectin molecular size	Kalamaki et al. (2003) and Powell et al. (2003)
Tomato	Pectin methylesterase	Increased juice viscosity and serum viscosity; higher total solids, decreased pectin hydrolysis	Thakur et al. (1996)
Tomato	Antisense suppression of deoxyhypusine synthase	Delayed postharvest softening and senescence	Wang et al. (2005)
Tomato, pea	β-Glucuronidase	Decrease in fruit softening	Rasori et al. (2003), Hobbs et al. (1990), and Ruma et al. (2009)
Tomato, papaya	1-Aminocyclopropane-1-carboxylate (ACC) oxidase	Reduced ethylene production in tomato	Bolitho et al. (1997) and Pillai et al. (2000)
Tomato, papaya	ACC synthase	Delayed postharvest softening and senescence. Pleiotropic effects on RNA interference	Oeller et al. (1991) and Magdalita et al. (2003)
Tomato	β-Glucuronidase	Prolonged shelf-life	Moon and Callahan (2004)
Tomato	β-Galactosidase, EXP1A (expansin)	Firmness	Brummell et al. (1999) and Smith et al. (2002)

ACC to ethylene and leads to ripening, and suppression of the enzyme polygalacturonase, which occurs naturally in the cell walls and causes vegetables and fruits to soften (Parvaiz et al., 2012; Gerszberg et al., 2015). The first approval for the commercial sale of a food product was Flavr Savr tomato, developed by Calgene in Davis, California, which produced a delayed ripening trait so that the fruits stayed firm after harvest. The trait acquired by the genetic crop is presented in Table 10.1.

8.2 TRANSGENIC VEGETABLES FOR BETTER FRUIT QUALITY AND NUTRITIONAL VALUE

Most people in developing countries are suffering from micronutrient malnutrition deficiencies (iron, iodine, vitamin A, and zinc deficiencies). It is one of the most important risk factors for illness, and death of pregnant women and young children particularly. By using transgenic methods, it is now possible to develop transgenic crops having enhanced nutritional value. Transgenic modification of plant nutritional value can be achieved by adopting the following methods: (1) improving the quality, composition, and levels of nutrients such as protein, starch, and fatty acid in different crops and (2) increasing the levels of antioxidants (e.g., carotenoids and flavonoids) (Gerszberg et al., 2015). A major example of transgenic plants for improved nutrition value is golden rice developed for vitamin A deficiency. Details of cropwise development are presented in Table 10.2.

Table 10.2 Transgenic Vegetables With Nutritional Value

Transgenic Crop	Gene and Gene Product	Trait/Character Acquired	References
Tomato	Lin5 (invertase 5)	Soluble solids content	Zanor et al. (2009)
Tomato	Chalcone isomerase (CHI), chalcone synthase (CHS), flavonole hydroxylase (F3H), flavonole synthase (FLS), stilbene synthase (STS), *Arabidopsis* MYB12 (flavonol-specific transcription factor)	Flavonoid content	Adato et al. (2009), Bassolino et al. (2013), Butelli et al. (2008), Colliver et al. (2002), and Maligeppagol et al. (2013)
Tomato	S1MYB12 (*Solanum lycopersicum* transcription factor), ANT1 (gene encoding homologous R2R3)	Fruit color	Ballester et al. (2010) and Schreiber et al. (2012)
Tomato	Aconitase (SlAco3b)	Carboxylic acids	Morgan et al. (2013)
Tomato	L-Galactono-1,4-lactone dehydrogenase (L-GalLDH), GDP-D-mannose 3,5-epimerase (GME), aminodeoxychorismate synthase, chloroplastic (ADCS)	Ascorbic acid content	de la Garza et al. (2004, 2007), Garcia et al. (2009), Gilbert et al. (2009), Zhang et al. (2011), and Waller et al. (2010)
Tomato	Thaumatin, GES (geraniol synthase), carotenoid cleavage dioxygenase (LeAADC1, LeAADC2)	Flavor and aroma	Bartoszewski et al. (2003), Davidovich-Rikanati et al. (2007), Mathieu et al. (2009), Tieman et al. (2006), and Sun et al. (2006)
Tomato	Quantitative trait locus fw2.2	Size of fruit	Cong and Tanksley (2006) and Liu et al. (2003)
Tomato	*Solanum lycopersicum* auxin response factor 7 (SlARF7); *Solanum lycopersicum* indole-3-acetic acid (Sl-IAA27); auxin/indole-3-acetic acid (Aux/IAA9); auxin response factor 8 (ARF8)	Parthenocarpy	Bassa et al. (2012), de Jong et al. (2011), Goetz et al. (2007), and Wang et al. (2005)
Potato	δ1-Pyrroline-5-carboxylate synthetase (P5CS)	Tuber yield	Hmida-Sayari et al. (2005)
Potato	Strawberry D-galacturonic acid reductase (GalUR) gene	Enhanced accumulation of vitamin C in tuber	Hemavathi et al. (2009)

8.3 TRANSGENIC VEGETABLES FOR RESISTANCE TO ABIOTIC STRESS

Abiotic stress refers to the environmental conditions that reduce crop growth and yield below optimum levels. It includes heat, drought, salinity, cold, nutrient deficiency, and metal toxicity. It is estimated that the yield of crop plants may be reduced to the extent of 50% or more because of abiotic stress. Most abiotic stresses are interlinked, resulting in common consequences of cellular water deficit or osmotic stress. In response to cellular water deficit or osmotic stress, plants start synthesizing low molecular weight compounds called osmolites or osmoprotectants. These molecules lower the osmotic potential and maintain turgor in cells. Several transgenic approaches employed to improve stress tolerance in plants are the introduction of genes encoding enzymes that catalyze the conversion of a naturally occurring substrate into a product with osmoprotective properties and the introduction of genes encoding membrane-modifying enzymes, radical-scavenging enzymes, or stress-induced proteins (Holmberg and Bulow, 1998; Zhang and

Blumwald, 2001; Ashraf, 2010). Enhanced abiotic stress tolerance was achieved by the overexpression of the strawberry D-galacturonic acid reductase gene in potato, which led to the accumulation of vitamin C with enhanced abiotic stress tolerance (Hemavathi et al., 2009). Hmida-Sayari et al. (2005) reported that overexpression of the Δ1-pyrroline-5-carboxylate synthetase gene in transgenic potato plants confers salt tolerance. Similarly, transformation of tomato with the BADH gene from *Atriplex* exhibited a considerable level of salt tolerance (Jia et al., 2002). Details are presented in Table 10.3.

Table 10.3 Transgenic Vegetables Tolerant to Abiotic Stress

Transgenic Crop	Gene and Gene Product	Trait/Character Acquired	References
Bean	Pyrroline-5-carboxylate synthase (P5CS)	Drought, salt, and cold	Chen et al. (2009)
Potato	Pyrroline-5-carboxylate synthase (P5CS)	Salt	Hmida-Sayari et al. (2005)
Potato	UND/PUB/ARM repeat type gene (StPUB17)	Salt	Ni et al. (2010)
Tomato	*Arabidopsis thaliana* homeodomain-leucine zipper class I genes (ATHB-7)	Drought	Mishra et al. (2012)
Tomato	Vacuolar Na^+/H^+ antiporter (AtNHX1)	Tolerance to salt	Zhang and Blumwald (2001)
Tomato	Choline oxidase from bacterial *Arthrobacter globiformis* (codA gene)	Salt and water stress	Goel et al. (2011)
Tomato, eggplant	Bacterial mannitol-1-phosphate dehydrogenase gene (MtlD)	Drought	Khare et al. (2010) and Prabhavathi et al. (2002)
Tomato	BADH1 (betaine dehydrogenase)	Salt and drought	Jia et al. (2002)
Tomato	C_2H_2 zinc-finger transcription factor (BcZAT12)	Drought and temperature	Rai et al. (2013) and Shah et al. (2013)
Tomato	Cytosolic ascorbate peroxidase (cAPX)	Temperature	Wang et al. (2006)
Tomato	Dehydrin (TAS14)	Drought and salinity	Munoz-Mayor et al. (2012)
Tomato	Glyoxalase II genes (GlyII)	Salt	Alvarez-Viveros et al. (2013)
Tomato	H^+-Pyrophosphatase and Na^+/H^+ antiporter gene (TaNHX2)	Drought	Bhaskaran and Savithramma (2011) and Yarra et al. (2012)
Tomato	Osmotin	Cold	Patade et al. (2013)
Tomato, eggplant	*Poncirus trifoliata* arginine decarboxylase (PtADC)	Drought	Wang et al. (2011) and Prabhavathi and Rajam (2007)
Tomato	Polyphenol oxidases (ppo)	Drought	Thipyapong et al. (2004)
Tomato	*S*-Adenosyl-L-methionine decarboxylase (SAMDC)	Salt, cold, and drought	Alcazar et al. (2010)
Tomato	*N*-Acetylglutamate synthase (SINAGS1)	Improved germination and drought	Kalamaki et al. (2009)
Tomato	GDP-D-mannose-3′,5′-epimerase genes (SlGME1)	Drought	Zhang et al. (2011)
Tomato	*Solanum pimpinellifolium* mitogen-activated protein kinases (SpMPK1, SpMPK2, SpMPK3)	Drought	Li et al. (2013)
Tomato	*Malus domestica* subunit B of the V-ATPase (MdVHA-B)	Drought	Hu et al. (2012)
Tomato	*Lycopersicon esculentum* ethylene responsive factor (LeERF2/TERF2)	Freezing	Zhang et al. (2009) and Zhang and Huang (2010)

8.4 TRANSGENIC VEGETABLES FOR RESISTANCE TO BIOTIC STRESS

Biotic stress occurs as a result of damage to plants caused by other living organisms such as bacteria, viruses, fungi, parasites, insects, weeds, etc. In spite of the use of pesticides, fungicides, and herbicides, insects, pathogens, and weeds continue to destroy crop production to a tune of 40% (Oerke et al., 1994; Pimentel, 1997). Preharvest losses are globally estimated at 15% for insect pests, 13% for diseases, and about 12% for weeds (Pimentel, 1997). To minimize the loss and production of quality and safe vegetables the adoption of transgenic technology is inevitable.

8.4.1 Insect Resistance

High-yielding varieties are found naturally less resistant to pests in comparison with their progenitors. Each year, insects destroy about 25% of food crops worldwide. The introduction of plant monocultures and indiscriminate and widespread application of chemical pesticides resulted in an insurgence of pests, development of resistance, and occurrence of residual toxicity. To overcome these problems, development of resistant crop varieties through a transgenic approach will contribute to a reduction in pest attack and decrease the use of chemical insecticides (Slater et al., 2003).

The greatest achievement in the field of agriculture is the development of *Bacillus thuringiensis* (*Bt*) insect-resistant crops using plant transgenic technology. *Bt* toxin is a crystal insecticidal protein (δ-endotoxin) produced from the cryo gene of a spore-forming soil bacterium *B. thuringiensis*. It is a potent insecticide and very much toxic to lepidopterans (Cohen et al., 2000), dipterans (Andrews et al., 1987), and coleopterans (Herrnstadt et al., 1986). The *Bt* cry protein is nontoxic to humans and animals, but toxic to insects (BANR, 2000). The first *Bt* toxin gene was cloned in 1981 (Schnepf and Whiteley, 1981; Jain et al., 2007) and a field trial of transgenic tobacco expressing *Bt* toxin was performed in 1986. Subsequently, GE tomato and eggplant/brinjal were developed and tested under field conditions in different countries. A combination of very high transgene expression and improved protein stability resulted in mortality of even *Bt*-resistant insects (Kota et al., 1999). At present, other insecticidal proteins such as lectins, protease inhibitors, antibodies, wasp and spider toxins, microbial insecticides, and insect peptide hormones (Whetstone and Hammock, 2007; Van Damme, 2008) were successfully used for the development of insect-resistant vegetable crops. *Bt* eggplant (*Solanum melongena*) was developed and field tested by the collaborative research between the US-based company Monsanto and Maharashtra Hybrid Seeds Company (Mahyco), India, by incorporating a crystal gene (Cry1Ac) from *B. thuringiensis* (Krattiger, 2010; Cotter, 2011).

Serine proteinase such as trypsin and chymotrypsin enzymes are present in insect guts for digestion. Proteinase inhibitors have been found very effective in controlling the growth and development of many insects by inactivating serine proteinase enzymes of the insect (Larry and Richard, 2002). Various types of proteinase inhibitors such as potato protease inhibitors II, cowpea trypsin inhibitors(CpTi), etc. have been expressed in crop plants (Sharma et al., 2004). Many insect-resistant plant varieties have been developed through cloning genes encoding proteinase inhibitors (Kim et al., 2009) and amylase inhibitors (Mehrabadi et al., 2010).

Lectins, carbohydrate-binding proteins, occur abundantly in seeds and storage tissues of different plants and were also found to be injurious to the different insects but not to mammals (Li and Romeis, 2009). The lectin from snowdrop (*Galanthus nivalis* agglutinin) is very toxic to insects, causing about 80% mortality (Fitches et al., 2010). Transgenic potato expressing the gna gene showed reduced damage to leaves (Bell et al., 2001a). A *Bt* transgenic potato plant resistant to potato tuber moth was

developed by transferring the cry1Ab gene under a granule-bound starch synthase promoter (Kumar et al., 2010). In 2009, *Bt* eggplant was approved for commercialization in India, but the Indian government continues to impose a moratorium on its release because of public resentment (Jain, 2010).

8.4.2 Transgenic Vegetables for Disease Resistance

Plant diseases such as fungal, viral, and bacterial diseases are major problems in vegetable production and productivity. Disease-free plants are possible by the method of genetic transformation (Table 10.4).

Table 10.4 Transgenic Vegetables Resistant to Insect, Nematode, and Disease

Transgenic Crop	Inserted Gene or Gene Product	Resistance to Insect	References
Insect Resistance			
Pea, azuki bean	Alpha-amylase	*Callosobrunchus* spp.	Fatunla and Badaru (1983), Shade et al. (1994), and Ishimoto et al. (1996)
Potato	CpTi	*Lacanobia oleracea*	Bell et al. (2001b) and Gatehouse et al. (1999)
Potato	cry3a	*Leptinotarsa decemlineata*	Alyokhin et al. (2008)
Tomato, eggplant/ brinjal, cabbage, cauliflower, okra, watermelon, potato	Cry 1b, Cry1Ac, Cry 1Ab, Cry 1Fa1 (δ-endotoxin)	Lepidopteran insects	Rashid and Bal (2011), Saker et al. (2011), Kumar et al. (2010), Krattiger (2010), Cotter (2011), and Kim et al. (2016)
Tomato, cabbage	Cry 2Ab	Lepidoptera and dipteral insects	Saker et al. (2011), Jin et al. (2000), and Paul et al. (2005)
Tomato	Kchip LnvRNAi-2214	Insect resistance	Tarafdar et al. (2014)
Tomato	*Galanthus nivalis* agglutinin (GNA)	Insect resistance	Wakefield et al. (2006)
Resistance Against Nematodes			
Tomato	*Colocasia esculenta* cysteine proteinase inhibitor (CeCPI)	*Meloidogyne incognita*	Chan et al. (2010)
Tomato	Rice chitinase gene (*chi* 11)	*M. incognita*	Kalaiarasan et al. (2008)
Tomato	Cry6A	*Meloidogyne* spp.	Li et al. (2007)
Tomato	CaMi gene from *Capsium annuum* L.	*Meloidogyne* spp.	Chen et al. (2007)
Tomato	Mi-1 from *Solanum peruvianum*	*M. incognita*	Milligan et al. (1998)
Brinjal	Cry1ab	*M. incognita*	Phap et al. (2010)
Disease Resistance			
Potato	Cercosporin-melittin cationic peptide (34-aa chimeric peptide MsrA1^{+}melittin)	Broad-spectrum resistance to phytopathogens	Osusky et al. (2000)
Potato	Defensins (alfAFP)	*Verticillium dalhiae*	Gao et al. (2000)
Potato	Endochitinase gene from mycoparasitic fungi	Foliar and soilborne fungal pathogen	Lorito et al. (1998)

Continued

Table 10.4 Transgenic Vegetables Resistant to Insect, Nematode, and Disease—cont'd

Transgenic Crop	Inserted Gene or Gene Product	Resistance to Insect	References
Potato	Osmotin gene	*Phytophthora infestans*	Li et al. (1999)
Tomato, potato	Lactoferrin	Bacterial wilt (*Ralstonia solanacearum*)	Chong and Langridge (2000) and Lee et al. (2002)
Tomato	Artificial microRNAs to silence viral coat proteins AV2/AV1 (amiR-AV1-3)	Tomato leaf curl virus	Vu et al. (2013)
Tomato	Human antimicrobial peptide (hCAP18/ll-37)—cathelicidin	Bacteria	Jung (2013)
Tomato	Bs2	Bacteria (*Xanthomonas*)	Horvath et al. (2012)
Tomato, cucumber, carrot	Endochitinase	Fungal pathogens	Shah et al. (2010), Kishimoto et al. (2002), and Punja and Raharjo (1996)
Tomato	Bivalent gene chitinase and alfalfa defensin (CHIAFP)	*Botrytis cinerea* (fungus)	Chen et al. (2009)
Potato	*RB* gene from the wild species *Solanum bulbocastanum*	Late blight resistance (*P. infestans*)	Kuhl et al. (2007) and Liu et al. (2009)
Potato	*StPUB17* (*UND/PUB/ARM*) repeat type gene	Late blight resistance (*P. infestans*)	Ni et al. (2010)

8.4.2.1 Bacterial Resistance

Transgenic plants resistant against bacterial infection are made by the transfer of antibacterial proteins such as lytic peptides, lysozymes, and iron-sequestering glycoproteins. Lytic peptides are small proteins with an amphipathic α-helical structure whose effect is to form pores in bacterial membranes (e.g., cecropins, attacin, etc.). The first bacterial disease-resistant plant was tobacco resistant against *Pseudomonas syringae* pv. *tabaci* and *P. syringae* pv. *syringae* by Anzai et al. (1989). Overexpression of an endochitinase gene with a strong gene promoter in potato resulted in increased resistance to fungal attack. The plants exhibited increased resistance to the foliar and soilborne fungal pathogens.

8.4.2.2 Fungal Resistance

Genetic engineering enables new ways of managing fungal infections. Several transgenic approaches used are introducing genes coding for hydrolytic enzymes (chitinase or glucanase), introducing plant genes (phytoalexins or proteinase inhibitors) to enhance innate plant defense mechanisms, and invoking a hypersensitive reaction in which infected individual cells quickly die at the site of fungal infection and thereby restrict their spread.

8.4.2.3 Virus Resistance

Virus-resistant transgenic plants are particularly valuable if no genetic source of resistance has been identified or if host resistance is difficult to transfer into elite cultivars by conventional breeding methods because of genetic incompatibility or links to undesired traits. In such cases, the transgenic approach may be the only viable option to develop virus-resistant cultivars. Virus-resistant plants are made by two approaches: transferring the gene coding for viral coat protein, which prevents the replication of

virus, and the RNA silencing method. Transgenic vegetables engineered for the enhanced tomato mosaic virus (TMV) coat protein (CP) gene were resistant to TMV infection (Mundembe et al., 2009) and this CP-mediated resistance is widely used to protect many crops from a large number of viruses (Mundembe et al., 2009). China was the first country to commercialize virus-resistant GM crops (James, 1997), and subsequently, virus-resistant tomato, potato, squash, and watermelon plants were developed (Meeusen, 1996; James, 2008). Transgenic potato resistant against potato virus Y was developed by Monsanto through transgression of the gene coding for viral CP, which prevents replication of the virus. Similarly, transgenic tomato and cucumber varieties resistant to cucumber mosaic virus were developed by transferring viral CP. By the RNA silencing method, transgenic common bean resistant against bean golden mosaic virus was developed (Table 10.5).

Table 10.5 Transgenic Vegetables to Enhance Viral Resistance

Transgenic Plant	Source/Gene Product	Mechanism Employed	Resistance	References
Common bean, cucurbits	Replication initiator protein (rep; AC1), transactivator protein (TrAP; AC2), replication enhancer protein (REn; AC3), and movement protein (BC1)	RNA interference	Bean golden mosaic virus and other viruses	Aragao and Faria (2009) and Gaba et al. (2004)
Papaya	Papaya ringspot virus replicase gene	Replication interface	Papaya ringspot virus	Bau et al. (2004) and Kumari et al. (2015)
Papaya	Coat protein (CP)	Coat protein-mediated resistance	Papaya ringspot virus	Tennant et al. (2002), Gonsalves et al. (1998) and Davidson (2006)
Cucumber, melon	Coat protein (CP)	Coat protein-mediated resistance	Cucumber mosaic virus	Nishibayashi et al. (1996)
Squash	Coat protein (CP)	Coat protein-mediated resistance	Cucumber mosaic virus, watermelon mosaic virus 2, and zucchini yellow mosaic virus	Tricoli et al. (1995)
Tomato	Coat protein (CP)	Coat protein-mediated resistance	Cucumber mosaic virus	Fuchs et al. (1996), Tomassoli et al. (1999), and Xue et al. (1994)
Tomato	N gene	Coat protein-mediated resistance	Tomato spotted wilt virus	Goldbach et al. (2003)
Tomato	Serine acetyltransferase (Sat-S)	RNA satellites	Cucumber mosaic virus	Stommel et al. (1998)
Hot pepper	*Sat-I17N*	RNA satellites	Cucumber mosaic virus	Kim et al. (1997)
Potato	PAP (*Phytolacca americana*)	RNA satellites	RIP	Lodge et al. (1993)
Potato	ScFv antibodies	Plantibodies	Potato virus Y	Gargouri-Bouzid et al. (2006)
Tomato	ScFv antibodies	Plantibodies	Cucumber mosaic virus	Villani et al. (2005)

8.4.3 Herbicide Resistance

Herbicide-tolerant GM plants account for 71% of all transgenic crops grown worldwide (James, 2008). Three approaches have been used to create herbicide-tolerant crops: structural alteration/modification of the target enzyme so that plant sensitivity to the herbicide is inhibited engineering of the herbicide-detoxifying pathway into the plant, and overproduction of an herbicide-sensitive biochemical target (Simoens and Van Montagu, 1995). Gaines et al. (2010) developed herbicide-resistant *Amaranthus palmeri* by expressing glyphosate-insensitive herbicide target site gene 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS), which is involved in the shikimate cycle. The gene encoding EPSPS has been transferred into plants and confers plants with herbicide resistance by detoxification. In the case of the herbicide Ignite/Basta, the bar resistance gene from *Streptomyces hygroscopicus* was used to detoxify the herbicide; various transgenic plants expressing the bar gene were produced including sugar beet, tomato, and potato.

9. GLOBAL STATUS OF TRANSGENIC VEGETABLES

The first transgenic vegetable (Flavr Savr tomato) was approved in the United States in 1994 for commercial cultivation (Bruening and Lyons, 2000). Subsequently, insect-resistant *Bt* potato "AMFLORA potato" and virus-resistant squash and papaya were approved for commercial cultivation. On October 30, 2013, Bangladesh approved *Bt* brinjal/eggplant for the first time for commercial cultivation and after a short period of approval, small farmers cultivated *Bt* brinjal in January 2014. In 2015, 250small farmers successfully cultivated *Bt* brinjal in an area of 25 ha as compared with 120 farmers on 12 ha in 2014. Success with *Bt* brinjal has led Bangladesh to prioritize the field testing of a new late blight-resistant potato and intend to approve it for commercial release as early as 2018. Transgenic bruise-resistant potato (Innate potato) was approved in the United States in November 2014. Potato is the fourth most important food staple globally and can contribute to food security in Asian countries such as Bangladesh (0.5 million ha of potato), China (6 million ha), and India (2 million ha).

The adoption of transgenic crops has increased 100-fold from 1.7 million ha in 1996 to 179.7 million ha in 2015 (James, 2015). The first 20 years of commercialization of transgenic technology (1996–2015) has established that the early promise of transgenic technology has been fulfilled (Qaim, 2016). Transgenic crops have delivered significant agronomic, environmental, economic, health, and social benefits to farmers and to society at large (Areal et al., 2013; Gilbert, 2013; Qaim and Kouser, 2013). The rapid adoption of transgenic crops in this period reflects the considerable multiple benefits that have been realized by both large and small farmers in both developed and developing countries, which have permitted transgenic crops for commercial cultivation (James, 2015). At present 17–18 million farmers worldwide are growing transgenic crops successfully and it was realized that transgenic technology is the fastest adopted crop technology of recent times (James, 2015; Qaim, 2016). This inspiring adoption rate speaks for itself in terms of its sustainability, resilience, and the important benefits it delivers to both small and large farmers as well as consumers.

Klümper and Qaim (2014) studied the pros and cons of adoption of transgenic crops during the last 20 years and concluded that the average transgenic technology adoption has reduced chemical pesticide use by 37%, increased crop yields by 22%, and increased farmer profits by 68%. Yield gains and pesticide reductions are larger for insect-resistant crops than for herbicide-tolerant crops.

Yield and profit gains have been recorded higher in developing countries than in developed countries. Qaim (2016) presented a more thorough description of the impacts of current and possible future applications of transgenic technology, and their sizeable contribution to sustainable agricultural development and food security. He concluded that continued opposition to transgenic technologies that were shown to be beneficial and safe lead to unnecessary human suffering and environmental degradation.

10. ETHICAL AND BIOSAFETY ISSUES, RISKS, AND CONCERNS

There is a consistent increase in the use of GM plants for food or other essential commodities (Smale et al., 2009; James, 2015). Transgenic vegetables have proven to be a powerful tool for disease and pest management, and their use has been accompanied by dramatic economic and environmental benefits (Brookes and Barfoot, 2011, 2015). Transgenic technology has raised agricultural productivity, assisted the development of safer, more nutritious foods with a longer shelf-life, and contributed to the goal of increased food security for the poor in developing countries (Carpenter, 2010; Finger et al., 2011; Parvaiz et al., 2012). The promoters of GM foods claim that they are environment friendly, have no risk to human health, are profitable for farmers, and are well regulated (DeFrancesco, 2013; Andreasen, 2014). Furthermore, several GM crops possess antibiotic resistance genes that could be taken up by bacteria present in the body, thereby increasing bacterial resistance against antibiotics (Gilbert, 2013). Critics also argue that transgenic technology is a threat to human health and the environment, and its introduction will raise the profits of private suppliers while depriving poor producers of primary commodities access to markets and to the new varieties of seed (Bradford et al., 2005; Areal et al., 2013). They also argue that GM crops have potential negative impacts on the environment, nontarget organisms, food safety, the unintentional spread of transgenic traits into conventionally bred crops or landrace gene pools of the same species particularly in centers of crop diversity or origin, and raise questions of seed ownership (Gilbert, 2013). The domains of concern related to potential adverse health effects of consuming GM crops are: allergenicity, horizontal transfer and antibiotic resistance, consumption of foreign DNA as part of transgenic plants and changed nutrient levels (Godfrey, 2000), and adverse effects on the environment, wildlife populations, biodiversity, and gene transferring in non-GM wild herbs. The following cases strengthen these concerns: plight of the monarch butterfly, crop-to-weed gene flow, leakage of GM proteins into soil, and reductions in pesticide spraying. Thus every country needs to frame well-defined rules and regulations for the utilization of GM organisms, although many developed and some developing countries have already formulated specific regulations. Government regulatory agencies should be fully responsible for ensuring that GM crops do not harm the environment and human health (Rigaud, 2008). Crops produced through genetic engineering are formally examined to ensure that they do not possess noncongenial characteristics before field testing or commercial release.

In view of concerns raised from people of different walks of life, a number of new approaches have been developed to reduce the risks from antibiotic resistance genes in transgenic plants and resolve public issues. New approaches to the development of transgenic crops are removal of the antibiotic resistance genes before the plants are released for commercial use (Dale and Ow, 1991; Iamtham and Day, 2000; Zuo et al., 2001) and using marker genes such as green fluorescent protein or mannose (Joersbo et al., 1998) in place of antibiotic marker genes.

11. FUTURE PROSPECTS

Impact studies confirm that the average agronomic and economic benefits of GM crops are large and significant. Yield gains and pesticide reductions are larger and benefit from GM crops. Food security is an imperative inertial problem in the world. According to the FAO of the United Nations, over the past decade, the malnutrition rate has increased to around 20% and is projected to remain stable until 2022. It was reported that more than 870 million people were chronically malnourished in 2012, with almost 250 million living in India. To overcome the worldwide problem linked to half-starved people, a alteration in agriculture is urgently needed. Adaptation of transgenic crops can resolve food scarcity by expansion of transgenic technology at the ground level. However, continued opposition to transgenic technologies will entail unnecessary human suffering and environmental degradation and pose a challenge to food security.

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CHAPTER

TRANSGENIC RESEARCH IN TUBER AND ROOT CROPS: A REVIEW

11

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1. INTRODUCTION

Crop improvement and development of new varieties plays a vital role in crop production. Although traditional breeding has significantly contributed to trait improvement in crops in the last several decades and leads to a significant boost in crop productivity, classical breeding technology will only improve crops to a certain level because it relies on the endogenous or population gene pools. As an alternative, transgenic technology recruits genes from other organisms, such as algae, bacteria, or distantly related plants, to overcome species or outcrossing barriers and thus enlarge the gene pools in crops (Van Lijsebettens et al., 2013). Genetically modified (GM) crops can help us to meet the demand for high-yielding, nutritionally balanced, biotic and abiotic stress-tolerant crop varieties by the expression of native or foreign genes (Baulcombe, 2010).

Tuber and root crops are basic to the diets of millions in the tropics and subtropics where most of the world's undernourished people live. Tuber and root crops consist of a number of important major food crops such as potato (*Solanum tuberosum* L.), sweet potato [*Ipomoea batatas* (L.) Lam.], and taro [*Colocasia esculenta* (L.) Schott] and spice and flavor crops such as ginger (*Zingiber officinale* Rosc.) and turmeric (*Curcuma longa* L.). Most of them are propagated vegetatively, and this vegetative propagation is susceptible to accumulation and transmittance of pathogens and diseases. It is essential to combine tissue culture and genetic engineering techniques with conventional breeding methods to develop new varieties. The transgenic approach is an alternative way to help increase production and productivity of crops in agriculture. This chapter summarizes some achievements of GM technology in the improvement of tuber and root crops by giving some successful transgenic examples.

2. BACKGROUND OF GENETIC ENGINEERING IN TUBER AND ROOT CROPS

Food insecurity and malnutrition are currently among the most serious concerns for human health, causing the loss of countless lives in developing countries (Datta, 2013). To ensure food security for future generations, the world must produce 50%–100% more food than at present in spite of the predicted adverse environmental conditions (Baulcombe, 2010).

Particle bombardment and *Agrobacterium*-mediated transformation are two main approaches that have been utilized to obtain transgenic plants. *Agrobacterium* is perceived to have advantages over

Genetic Engineering of Horticultural Crops. http://dx.doi.org/10.1016/B978-0-12-810439-2.00011-8

biolistics because it can introduce larger segments of DNA with minimal rearrangement and with fewer copies of inserted transgenes at higher efficiencies and at lower cost. Both of them are successfully applied in the improvement of tuber and root crops. Genetic transformation holds promise for introducing novel traits to tuber and root crops in cases where no solutions by conventional breeding are available. Efforts to improve tuber and root crops by genetic transformation have concentrated on traits and for which solutions do not seem likely via conventional breeding in the near future. The major traits are focused on biotic stresses such as pests and pathogens; abiotic stresses such as drought, salinity, extreme temperatures, chemical toxicity, and oxidative stresses; and quality and yield improvement.

3. GENETIC ENGINEERING OF POTATO

Potato (*S. tuberosum* L.) ranks as the fourth most important food crop in the world following maize, wheat, and rice. Because of the need to supply dietary fiber, carbohydrates, high-quantity protein, vitamins, and minerals, the potato is usually regarded as a starchy food or a vegetable. Potatoes are grown in over 125 countries and more than a billion people worldwide consume them on a daily basis (Mullins et al., 2006). The challenges facing potato breeding have actually changed very little over the years with resistance to pests and pathogens remaining high on the agenda, together with improvements in storability, reduction in blemishes, and novelty and consistency in cooking/processing qualities (Davies et al., 2008). Genetic engineering provides a faster and more reliable means for potato crop improvement and the technique is especially applicable to the development of resistance to biotic and abiotic stresses such as pests, pathogens, drought, salt, and so on.

3.1 TRANSGENIC RESEARCH INTO PEST AND DISEASE RESISTANCE

3.1.1 Transgenic Research Into Pest Resistance

The Colorado potato beetle (CPB; *Leptinotarsa decemlineata* Say) is the major defoliator of potato throughout the northern hemisphere (Alyokhin et al., 2008). Both larvae and adults feed on plants in the Solanaceae family (*Solanales*, *Solanaceae*) including potato. The beetles can eaten almost all of the leaves of potato plants, which seriously affects photosynthesis. There are biotechnological and genetic engineering ways to improve the resistance of potatoes to the CPB (Chougule and Bonning, 2012; Bravo et al., 2013; Mi et al., 2015). Since 1996, potatoes expressing the Cry3A protein have been available commercially in the United States, which was resistant to the CPB. However, because of low sales and buyer focus on GM organisms, those potato varieties containing the *cry3a* gene were taken off the market in 2001 (Cooper et al., 2004).

Aphids are considered as the largest group of sap-sucking pests, which cause significant yield losses in agricultural crops worldwide (Wang et al., 2013a,b). *Macrosiphum euphorbiae* (Thomas) and *Myzus persicae* (Sulzer) aphid species constitute two of the major potato pests affecting the production of this crop (Pelletier and Michaud, 1995). The snowdrop lectin [*Galanthus nivalis* agglutinin (GNA)] can confer resistance to chewing and sap-sucking insects without toxicity to higher animals (Gatehouse et al., 1995). Levels of GNA accumulated in different parts of a transgenic potato showed variation and increased with maximum levels showed insect resistance (Down et al., 2001).

Table 11.1 Genes Overexpressed in Transgenic Potato Plants for Pest Resistance

Gene	Gene Product	Pest	References
Bt-cry5	Bt-cry5	*Phthorimaea operculella* (Zeller)	Mohammed et al. (2000)
HPLs	Hydroperoxide lyases	Aphid	Vancanneyt et al. (2001)
SN19	A cry1Ba/cry1Ia hybrid gene	Colorado potato beetle, potato tuber moth, and European corn borer	Naimov et al. (2003)
CryllIA	CryllIA	Colorado potato beetle	Ashouri (2004a,b)
CryIIIA	*Bacillus thuringiensis* CryIIIA toxin	Colorado potato beetle, *Leptinotarsa decemlineata* (Say)	Ashouri (2004a,b)
Cry2Aa2	Cry2Aa2 protein	*Heliothis virescens* larvae	Zaidi et al. (2005)
cry9Aa2	cry9Aa2	Potato tuber moth (*Phthorimaea operculella*)	Chakrabarti et al. (2006)
cry1Ac	*B. thuringiensis* crystalline insecticidal protein	*Tecia solanivora* Povolny (Lepidoptera: Gelechiidae)	Valderrama et al. (2007)
BBPs	The high-affinity biotin-binding proteins	*P. operculella* (Zeller)	Murray et al. (2010)
Cry3A	Cry3A	Colorado potato beetle	Zhou et al. (2012)
dsRNA	dsRNA	Colorado potato beetle	Palli (2014)
cry3A	cry3A	Colorado potato beetle	Mi et al. (2015)

Recombinant fusion proteins containing arthropod toxins have been developed as a new class of biopesticides. The recombinant fusion protein Hv1a/GNA, containing the spider venom toxin ω-ACTX-Hv1a linked to GNA, was shown to reduce survival of the peach-potato aphid *M. persicae* when delivered in artificial diet (Nakasu et al., 2014). The survival of the aphid *M. persicae* was reduced when fed on the SFI1/GNA fusion protein, comprising GNA fused to an insecticidal spider venom neurotoxin [*Segestria florentina* toxin 1 (SFI1)]. The SFI1/GNA fusion protein also slowed the development of *M. persicae*, and the reproductive capacity of the aphids fed on the SFI1/GNA fusion protein was severely reduced (Down et al., 2006). Examples of GM potatoes resistant to pest developed by transgenic approaches since 2000 are listed in Table 11.1.

3.1.2 Transgenic Research Into Disease Resistance

Viruses are a major threat causing massive yield loss and economical damage to crop production worldwide (Yeam, 2016). Resistance to virus infection has been engineered into a number of transgenic plant species against a range of individual viruses (Gottula and Fuchs, 2009). Since many crops are subject to infection by more than one virus, it will be necessary to engineer them for resistance to multiple viruses. This was done first with potato expressing the coat protein (CP) coding sequences of potato virus X (PVX) and potato virus Y (PVY) and providing resistance to these viruses (Kaniewski et al., 1990; Lawson et al., 1990). Transgenic potato plants of cultivar Vales Sovereign were generated that expressed fused, tandem, 200-bp segments derived from the capsid protein coding sequences of PVY strain O (PVY-O) and potato leafroll virus (PLRV), as well as the cylindrical inclusion body coding sequences of potato virus A (PVA), as inverted repeat double-stranded RNAs (dsRNAs), separated by

an intron. Four transgenic potato lines tested showed 100% resistance to infection by either PVY-O or PVA, but variable resistance to infection by PLRV, ranging from 72% to 96% in different lines (Chung et al., 2013).

RNA silencing technology has become the tool of choice for inducing resistance against viruses in plants. A significant discovery of this technology is that dsRNA, which is diced into small interfering RNAs, is a potent trigger for RNA silencing. Arif et al. (2012) constructed a chimeric expression vector containing three partial gene sequences derived from the *ORF2* gene of PVX, helper component protease gene of PVY, and CP gene of PLRV. Because of simultaneous RNA silencing, the expression of a partial triple-gene sequence cassette depicted that 20% of the transgenic plants are immune against all three viruses.

Potato late blight, caused by *Phytophthora infestans*, is considered to be the most serious potato disease worldwide. Many potato *R*-genes have been cloned and characterized, and some *R*-genes have been applied in potato breeding. In a 2-year replicated trial under field production conditions, the RB transgene provided effective disease resistance in various genetic backgrounds, including commercially prominent potato cultivars, without fungicides. Disease resistance was enhanced as copy numbers and transcript levels increased. Transgenic potato lines with an estimated 15 copies of the RB transgene maintain high RB transcript levels and were ranked among the most resistant of 57 lines tested (Bradeen et al., 2009). Another type of resistance, based on the loss-of-function of a susceptibility gene (*S*-gene), has been introduced. Mutations in multiple susceptibility genes can result in resistance to different pathogens, opening a new way to achieve plant disease resistance. Sun et al. (2016a) silenced *DND1* ortholog in potato using an RNA interference (RNAi) approach, which resulted in resistance to the pathogenic oomycete *P. infestans*. Sun et al. (2016b) selected 11 *Arabidopsis thaliana S*-genes and silenced orthologous genes in the potato cultivar Desiree, which is highly susceptible to late blight. The silencing of five genes resulted in complete resistance to the *P. infestans* isolate Pic99189, and the silencing of a sixth *S*-gene resulted in reduced susceptibility.

Rhizoctonia solani and *Erwinia carotovora* are important pathogens that cause serious potato diseases that occur in most regions worldwide. Almasia et al. (2008) generated the transgenic potato plants with the snakin-1 (*SN1*) gene that confers resistance to *R. solani* and *E. carotovora*.

Examples of transgenic potatoes resistant to disease developed by transgenic approaches since 1999 are listed in Table 11.2.

3.2 TRANSGENIC RESEARCH FOR ABIOTIC STRESS

Abiotic stresses, such as drought, salinity, extreme temperatures, chemical toxicity, and oxidative stresses, are serious threats to agriculture besides their deteriorative impact on the environment. Potato is regarded as a moderately salt-sensitive (Ahmad and Abdullah, 1979) and drought-sensitive crop because of its shallow root system (Bouaziz et al., 2012). Potato suffers damage at −3°C and has no ability to acclimate to cold conditions (Chen and Li, 1980). Several previous reviews have discussed enhancing drought and salinity tolerance through transgenic approaches in plants (Apse and Blumwald, 2002; Rontein et al., 2002; Wang et al., 2003; Chen and Murata, 2008; Kolodyazhnaya et al., 2009) and potato (Byun et al., 2007). It is generally accepted that drought and salinity tolerance of plants could be increased through transgenic approaches by incorporating genes involved in stress protection into plants that lack them.

Table 11.2 Genes Overexpressed in Transgenic Potato Plants for Disease Resistance

Gene	Gene Product	Disease or Pathogen	References
Hp	Harpin protein	Late blight	Li and Fan (1999)
AATP1	ATP/ADP transporter	Pathogenic fungus *Alternaria solani*	Conrath et al. (2003)
MsrA3	Temporin A	*Erwinia carotovora*	Osusky et al. (2004)
Sgt1	SGT1 protein	Late blight	Bhaskar et al. (2008)
SN1	Snakin-1	Stem cankers, damping-off, *E. carotovora*	Almasia et al. (2008)
RB	Rpiblb1	Late blight	Kramer et al. (2009)
Rpi-vnt1	Rpi-vnt1	Late blight	Foster et al. (2009)
Rpi-sto1, *Rpi-vnt1.1*, and *Rpi-blb3*	Broad-spectrum resistance	Late blight	Zhu et al. (2012)
eIF4E-1	*eIF4E-1*	Potato virus Y	Duan et al. (2012)
StSYR1 and *StSNAP33*	Syntaxin-related 1 and soluble *N*-ethylmaleimide-sensitive factor adaptor protein 33	Late blight	Eschen-Lippold et al. (2012)
StCDPK5	Calcium-dependent protein kinase	Late blight	Kobayashi et al. (2012)
pEKH2IN2CMVai	pEKH2IN2CMVai	Cucumber mosaic virus	Ntui et al. (2013)
AMPs	Antimicrobial cationic peptides	Biotic (necrotroph *Fusarium solani*) and abiotic stressors (dark-induced senescence, wounding, and temperature stress)	Goyal et al. (2013)
GSL2	Gibberellin stimulated-like 2	*Pectobacterium atrosepticum*	Mohan et al. (2014)
NahG-Rywal	Ny-1 mediated	Potato virus Y	Baebler et al. (2014)
ChiC and *WD*	*Streptomyces griseus* strain HUT 6037 and wasabi defensin	*Fusarium oxysporum* (*Fusarium* wilt) and *A. solani* (early blight)	Khan et al. (2014)
ROPs	Small GTPases	*Phytophthora infestans* Montagne de Bary	Zhang et al. (2014)
miR482	miR482	*Verticillium* wilt	Yang et al. (2015)

3.2.1 Transgenic Research for Drought Stress

Many studies have been undertaken to investigate drought stress in potato using transgenic techniques. Examples since 2000 are listed in Table 11.3. Trehalose is a nonreducing disaccharide of glucose. A plant that produces trehalose is often highly tolerant to desiccation stress. Goddijn et al. (1997) engineered trehalose biosynthesis in potato by introducing the *otsA* and *otsB* genes from *Escherichia coli*, which encode trehalose-6-phosphate synthase and trehalose-6-phosphate phosphatase, respectively. Fructan is thought to contribute to osmotic control in potato transformants. Ambard-Bretteville et al. (2003) suppressed the *FDH* gene encoding for formate dehydrogenase in transgenic potato plants increasing formate levels and resulting in accumulation of proline in response to osmotic stress.

Table 11.3 Genes Overexpressed in Transgenic Potato Plants for Drought Stress

Gene	Gene Product	Performance of Transgenic Plant	References
TPS1	Trehalose-6-phosphate synthase	Increased tolerance to drought	Yeo et al. (2000)
FDH	Formate dehydrogenase	Accumulation of proline and enhanced drought tolerance	Ambard-Bretteville et al. (2003)
SOD and *APX*	Cu/Zn superoxide dismutase and ascorbate peroxidase	Multiple stresses including drought, salinity, oxidative stress, and high temperature	Tang et al. (2006)
SST/FFT	Fructan	Proline accumulation	Knipp and Honermeier (2006)
codA	Choline oxidase	Enhanced tolerance to oxidative, salt, and drought stresses	Ahmad et al. (2008)
GLOase	L-Gulono-*c*-lactone oxidase	Enhanced tolerance to various abiotic stresses such as oxidative, salt, and drought stresses	Hemavathi et al. (2010)
SOD and *APX*	Superoxide dismutase and ascorbate peroxidase	Enhanced drought and salinity tolerance	Ahmad et al. (2010)
BADH	Betaine aldehyde dehydrogenase	Enhanced drought and salinity tolerance	Zhang et al. (2011)
StMYB1R-1	MYB-like domain transcription factor	Enhanced drought tolerance	Shin et al. (2011)
STANN1	Endogenous annexin	Enhanced drought tolerance and productivity	Szalonek et al. (2015)
CBP80/ABH1	Cap-binding complex	Enhanced drought tolerance	Wyrzykowska et al. (2016)
StnsLTP1	StnsLTP1	Enhanced heat, drought, and salinity tolerance	Gangadhar et al. (2016)

Oxidative stress is a major damaging factor for plants exposed to environmental stresses. Tang et al. (2006) obtained transgenic potato plants with increased tolerance to multiple environmental stresses that overexpressed both superoxide dismutase (SOD) and ascorbate peroxidase (APX) in chloroplasts.

Glycine betaine (GB) is one of the most important osmolytes in higher plants to cope with environmental stresses by osmotic adjustment. Many plant species can accumulate GB in response to drought and salinity. Ahmad et al. (2008) and Zhang et al. (2011) showed that transgenic potato plants overexpressing *codA* and betaine aldehyde dehydrogenase (BADH) genes for GB synthesis were more tolerant to drought stress. Glucosylglycerol (GG) is a compatible solute typically found in moderate halotolerant cyanobacteria and other bacteria. Potato plants were generated expressing the *ggpPS* gene under control of the CaMV 35S promoter or the rd29A promoter. Although both transformants accumulated GG in leaves, only the rd29A transformants accumulated GG in tubers. In greenhouse experiments, a number of lines from both transformants exhibited improved shoot growth compared to nontransformants (NTs) under drought and salinity conditions (Sievers et al., 2013).

The yucca family is known to contribute to auxin biosynthesis in plants. Drought tolerance by overexpression of *AtYUC6* in potato was evaluated in greenhouses and the transgenic plants exhibited higher water content in leaves and lower reactive oxygen species (ROS) content with increased expression of CuZnSOD compared to NTs (Kim et al., 2013a). Cho et al. (2016) subjected transgenic potato plants expressing the sweet potato orange gene (*IbOr*) for carotenoid accumulation to water-deficient conditions in the greenhouse. The transgenic plants exhibited increased tolerance to drought stress under greenhouse conditions.

There are some studies that use transcriptional factors to improve abiotic stress tolerance by regulating the expression of downstream genes. Huynh et al. (2014) evaluated for drought tolerance in vitro in the transgenic potato with the *AtDREB1A* gene from *Arabidopsis* and observed enhanced drought tolerance in the transgenic potato. Pino et al. (2007) obtained transgenic potato plants with enhanced drought tolerance using *DREB/CBF* genes driven by CaMV 35S and rd29A promoters independently. Shin et al. (2011) overexpressed an *StMYB1R-1* transgene in potato plants that improved plant tolerance to drought stress while having no significant effects on other agricultural traits. Other transcriptional factors have also been successfully transformed into potato to increase drought tolerance, such as the *CaPF1* gene from *Capsicum annuum* (pepper) (Youm et al., 2008), the ScCBF1gene from *Solanum commersonii* (Pino et al., 2013), the *IbMyb1* gene from sweet potato (Cheng et al., 2013), and the *StDREB1A* gene from potato (Bouaziz et al., 2013).

3.2.2 Transgenic Research for Salinity Stress

Potato is regarded as a moderately salt-sensitive crop (Ahmad and Abdullah, 1979). It is found in many plant species and is known as the most abundant sugar alcohol in nature. Mannitol is an osmoregulatory substance typically used in experiments. Potato plants do not naturally accumulate mannitol. The transgenic potato plants were obtained to confer salinity tolerance transformed with the mannitol-1-phosphate dehydrogenase (*mtlD*) gene from *E. coli* (Rahnama et al., 2011). Proline is known as one of the important stress responsive substances. Improvements in proline biosynthesis resulted in enhanced salinity tolerance in transgenic potato with the pyrroline-5-carboxylate synthetase (*P5CS*) gene from *Arabidopsis* (Hmida-Sayaria et al., 2005).

Jeong et al. (2001) introduced the *GPD* gene for glyceraldehydes-3-phosphate dehydrogenase from the oyster mushroom (*Pleurotus sajor-caju*) into potato and obtained transgenic potato plants with enhanced salinity tolerance. Turhan (2005) developed transgenic potato plant with higher salinity tolerance by expressing the *oxo* gene for oxalate oxidase, which catabolizes oxalic acid. The overexpression of the *DREB1A* gene for dehydration-responsive element (DRE)-binding protein from *Arabidopsis* in transgenic potato showed that salt stress tolerance was increased in proportion to its copy number in tetrasomic tetraploid potato (Behnam et al., 2006).

The alternative strategy for improving salinity tolerance is to remove Na^+ from the cytoplasm. The transgenic potato plants with increased salinity tolerance were generated with a vacuolar Na^+/H^+ antiporter (*HvNHX2*) gene from barley (Bayat et al., 2010) and with the *AtNHX1* gene from *Arabidopsis* (Wang et al., 2010).

Examples of GM potatoes resistant to salinity developed by transgenic approaches since 2000 are listed in Table 11.4.

Table 11.4 Genes Overexpressed in Transgenic Potato Plants for Salinity Stress

Gene	Gene Product	Disease or Pathogen	References
GPD	Glyceraldehydes-3-phosphate dehydrogenase	Improvement of salt tolerance	Jeong et al. (2001)
oxo	Oxalate oxidase	Higher salinity tolerance	Turhan (2005)
DREB1A	Dehydration-responsive element-binding protein	Tolerance to salt stress	Celebi-Toprak et al. (2005)
DREB1A	Dehydration-responsive element-binding protein	Highly tolerant to salinity	Behnam et al. (2006)
StEREBP1	Ethylene-responsive element binding protein 1	Tolerance to NaCl stress	Lee et al. (2007)
AtNDPK2	Nucleoside diphosphate kinase	Enhanced tolerance to salt	Tang et al. (2008)
codA	Choline oxidase	Enhanced tolerance to oxidative, salt, and drought stresses	Ahmad et al. (2008)
GLOase	L-Gulono-*c*-lactone oxidase	Enhanced tolerance to various abiotic stresses such as oxidative, salt and drought stresses	Hemavathi et al. (2010)
PR-10a	PR-10a protein	Increased salt and osmotic tolerance	El-Banna et al. (2010)
BADH	Betaine aldehyde dehydrogenase	Enhanced drought and salinity tolerance	Zhang et al. (2011)
GalUR	D-Galacturonic acid reductase	Enhanced salinity tolerance	Upadhyaya et al. (2011)
MSP	Manganese stabilizing protein	Accumulation of ascorbate, α-tocopherol, and proline	Gururani et al. (2013)

3.3 TRANSGENIC RESEARCH TO INCREASE QUALITY AND YIELD IMPROVEMENT

Potato tuber is a high-yielding food crop known for its high levels of starch accumulation, but negligible levels of other substances such as lipid and protein, and so on. Liu et al. (2016) evaluated the potential for lipid production in potato tubers by simultaneously introducing three transgenes, including WRINKLED 1 (WRI1), DIACYLGLYCEROL ACYLTRANSFERASE 1 (DGAT1), and OLEOSIN, which resulted in over a 100-fold increase in triacylglycerol accumulation to levels up to 3.3% of tuber dry weight. Phospholipids and galactolipids were also found to be significantly increased in the potato tuber. The increase of lipids in these transgenic tubers was accompanied by a significant reduction in starch content and an increase in soluble sugars. Li et al. (2016) generated three marker-free transgenic potato lines that expressed the *Arabidopsis thaliana* flavonol-specific transcriptional activator AtMYB12. Marker-free potato tubers displayed increased amounts of caffeoylquinic acids (CQAs) (3.35-fold increase on average) and flavonols (4.50-fold increase on average). Accumulation of CQAs and flavonols resulted in twofold higher antioxidant capacity compared to wild-type potatoes. Tubers from these marker-free transgenic potatoes have therefore improved antioxidant properties. Transgenic potato plants were generated, which express an RNAi construct directed against the pathogen-inducible 9-divinyl ether synthase. Efficient reduction of 9-divinyl ether synthase transcript accumulation correlated with reduced levels of colneleic and colnelenic acids (Eschen-Lippold et al., 2007).

High starch and low glucose levels are desirable traits in commercial potato tubers. Glucose levels were decreased 17%–56% and the starch content increased 23%–30% in the tubers of the transgenic potato plants by overexpressing a sucrose nonfermenting-1-related protein kinase-1 (*SnRK1*) gene under the control of a patatin (tuber-specific) promoter (McKibbin et al., 2006). β-Carotene is a kind of carotenoid, which is an essential health-protecting compound involved in human vision, immunity, embryonic development, and reproduction. Song et al. (2016) isolated a potato lycopene β-cyclase (*StLCYb*) gene overexpressed in transgenic potato, resulting in β-carotene content of transgenic potato tubers that increased 1.5–1.9 times compared with the wild-type control.

The production of marketable tubers (over 80 g) was improved in transgenic plants expressing sweet potato orange gene (*IbOr*) compared with nontransgenic plants under drought stress. These results suggest that expressing the *IbOr* transgene can lead to significant gains in drought tolerance and tuber production in potato, thereby improving these agronomically important traits (Cho et al., 2016). Waterer et al. (2010) obtained transgenic potato plants with mitochondrial *MnSOD* (*SOD3:1*) from wheat, *dehydrin 4* (*DHN 4*) from barley, a cold inducible transcriptional factor *DREB/CBF* from canola, and the stress-associated gene *ROB5* belonging to the late embryogenesis abundantgroup 3 protein from bramegrass under control of the *COR78* promoter. The overall average tuber yield taken across the 4 years is apt to extinguish the stress tolerance trait of the transgenic plants. Ahmad et al. (2016) developed transgenic potato plants expressing components of a novel cyanobacterial photorespiratory glycolate catabolism pathway by expressing a glycolate dehydrogenase I (*glcD1*) gene (referred to as synGDH), and glyoxylate carboligase (*gcl*) and tartronic semialdehyde reductase (*tsr*) genes simultaneously (referred to as synGT). Phenotypic evaluation revealed that synGDH plants accumulated 11% higher dry weight, while tuber weight was 38% and 16% higher than NT and synGT, respectively, which indicated that genetic transformation of the complete pathway in one plant held promising outcomes in terms of biomass accumulation to meet future needs for food and energy.

4. GENETIC ENGINEERING IN SWEET POTATO

Sweet potato [*Ipomoea batatas* (L.) Lam.] is clonally propagated, highly heterozygous, polyploid, and outcrossing. Combined with the low fertility found in sweet potato, even introgression of dominant single gene traits may present a challenge. Genetic improvement of sweet potato through conventional breeding methods is very difficult but transgenic technology is a suitable method for transfer of novel traits (Kreuze et al., 2009). Efforts to improve sweet potato by genetic transformation have concentrated on traits including resistance to weevils and viruses, modified starch properties, nematode resistance, herbicide resistance, stress tolerance, and improved amino acid composition.

4.1 TRANSGENIC RESEARCH INTO PEST AND DISEASE RESISTANCE

Sweet potato weevils are the most devastating insect pests of sweet potato. Early research focused on decreasing the digestibility of sweet potato for insects by transforming with proteins including a cowpea (*Vigna unguiculata*) trypsin inhibitor (CTI) and the mannose-binding snowdrop lectin (Newell et al., 1995), and a soybean (*Glycine max*) Kunitz-type trypsin inhibitor (SKTI) and a rice (*Oryza sativa*) cysteine proteinase inhibitor (OCI) (Cipriani et al., 1999, 2001; Gao et al., 2011a,b). Engineering resistance to weevil in sweet potato using *Bt* gene technology (Moar et al., 2007). Zhang et al. (2000) increased resistance to nematodes in transgenic sweet potato by transformation of *CTI* and *GNA* genes.

Table 11.5 Genes Overexpressed in Transgenic Sweet Potato Plants for Pest and Disease Resistance

Gene	Gene Product	Pest or Disease	References
Sporamin	Sporamin	Insect resistance	Chen et al. (2006)
CP	Coat protein	Feathery mottle virus	Haque et al. (2007)
swpa4	Peroxidase	Resistance to abiotic stresses and pathogen infection	Ryu et al. (2009)
Sporamin	Trypsin inhibitor	Resistance to insects and pathogens	Senthilkumar et al. (2010)
Thionin	Thionin peptide	Black rot	Muramoto et al. (2012)
SPFMV, *SPCSV*, *SPVG*, and *SPMMV*	Coat protein	Sweet potato feathery mottle virus, sweet potato chlorotic stunt virus, sweet potato virus G	Sivparsad and Gubba (2014)
Sporamin, *taro cystatin* and *chitinase*	Sporamin, taro cystatin, and chitinase	*Spodoptera litura* and *Spodoptera exigua*	Chen et al. (2014)
unc-15	Muscle protein paramyosin	Stem nematode	Fan et al. (2015a,b)
IbMIPS1	Myo-inositol-1-phosphate synthase	Enhanced salt and drought tolerance and stem nematode resistance	Zhai et al. (2016)
IbNAC1	NAC transcription factor	Resistance to mechanical wounding and herbivore attack	Chen et al. (2016)

The major viruses infecting sweet potato plants are sweet potato chlorotic stunt virus (SPCSV) and sweet potato feathery mottle virus (SPFMV) et al. Okada et al. (2002) transformed with the CP-encoding sequence of SPFMV resistant to SPFMV of sweet potato. Cipriani et al. (2001) reported increased resistance to SPFMV in sweet potato plants transformed with OCI. Kreuze et al. (2008) obtained transgenic sweet potato plants with increased resistance to SPCSV with an intron-spliced hairpin construct targeting the replicase-encoding sequences of SPCSV and SPFMV.

Examples of GM sweet potatoes resistant to pest and disease developed by transgenic approaches are listed in Table 11.5.

4.2 TRANSGENIC RESEARCH FOR ABIOTIC STRESS

Abiotic stresses are critical delimiters for the increased productivity and cultivation expansion of sweet potato. Environmental stresses, such as drought, salinity, and low temperature, frequently restrict the increased production of sweet potato in many areas of the world. New sweet potato varieties with enhanced tolerance to multiple abotic stresses are desirable. Fan et al. (2012) developed transgenic sweet potato plants expressing the *BADH* gene from *Spinacia oleracea*. The expression of the *SoBADH* gene increased BADH activity and GB synthesis in transgenic sweet potato plants, which subsequently improved their tolerance to multiple abiotic stresses (salt, oxidative, and cold stresses) by induction or activation of ROS scavenging and the accumulation of proline.

Oxidative stress is one of the major factors causing injury to plants exposed to environmental stress. The orange (*Or*) gene is responsible for the accumulation of carotenoids in plants. Kim et al. (2013b) isolated the *Or* gene (*IbOr*) from storage roots of orange-fleshed sweet potato and overexpressed in sweet

potato calli with increased tolerance to antioxidant activity and salt stress. Abiotic stresses cause accumulation of ROS in plants; CuZnSOD and APX are first-line defenses against ROS caused by oxidative stress. Transgenic sweet potato plants overexpressing both CuZnSOD and APX under the control of a stress-inducible SWPA2 promoter in chloroplasts showed increased resistance to methyl viologen-mediated oxidative stress and chilling (Lim et al., 2007) and sulfur dioxide (SO_2) (Kim et al., 2015). Yan et al. (2016) transferred *CuZnSOD* and *APX* genes into a salt-sensitive sweet potato cv. Xushu 55-2 under control of an SWPA2 promoter and obtained transgenic sweet potato with improved tolerance to salt stress.

Myo-inositol-1-phosphate synthase is a key rate-limiting enzyme in myo-inositol biosynthesis. Zhai et al. (2016) developed *IbMIPS1*-overexpressing sweet potato plants and found that *IbMIPS1* significantly enhanced salt and drought tolerance and stem nematode resistance of the transgenic plants. Liu et al. (2014a) isolated a novel maspardin gene, named *IbMas*, from salt-tolerant sweet potato, and when overexpressed in the transgenic plants exhibited significantly higher salt tolerance by regulating osmotic balance, protecting membrane integrity and photosynthesis, and increasing ROS scavenging capacity. Liu et al. (2014b) cloned the *IbP5CR* gene from sweet potato and the *IbP5CR*-overexpressing sweet potato plants exhibited higher salt tolerance. Wang et al. (2013a,b) cloned the *IbNFU1* gene from sweet potato and the *IbNFU1*-overexpressing sweet potato plants exhibited higher salt tolerance (Liu et al., 2014c). Fan et al. (2015a,b) developed transgenic sweet potato plants with better biomass production and root yield under stressful conditions (salt and cold stresses) by expressing the *AtNHX1* gene. Herbicide-resistant sweet potato plants were produced through the herbicide-resistant gene bar. When sprayed with Basta, the transgenic sweet potato plants were tolerant to the herbicide (Yi et al., 2007). The R2R3-type protein IbMYB1 is a key regulator of anthocyanin biosynthesis in the storage roots of sweet potato. Park et al. (2015) generated a dual-pigmented transgenic with enhanced antioxidant capacity using the *IbMYB1* gene. Examples of GM sweet potatoes resistant to abiotic stress developed by transgenic approaches are listed in Table 11.6.

4.3 TRANSGENIC RESEARCH INTO INCREASED QUALITY AND YIELD IMPROVEMENT

Starch is a storage carbohydrate composed of a linear polymer of sugars and ramified chains, amylose and amylopectin, respectively. The range of amylose content in sweet potato starch is about 10%–25%. By the downregulation of granule-bound starch synthase I (GBSSI) and branching enzyme expression, transgenic sweet potato plants with altered amylose content have been developed (Kimura et al., 2001; Noda et al., 2002; Shimada et al., 2006; Otani et al., 2007). The intrinsic relationship between amylose content and starch physicochemical properties was studied using six representative starch samples (amylose content 0%–65%) produced from transgenic sweet potato. The waxy lines (downregulated GBSSI expression by RNAi) and high-amylose lines (downregulated starch branching enzyme expression by RNAi) showed larger than average granule sizes, fewer short chains, and more medium and long chains compared to wild-type sweet potatoes (Zhou et al., 2015). Wakita et al. (2001) introduced the tobacco microsomal ω-3 fatty acid desaturase (*NtFAD3*) gene driven by the El2Ω promoter into sweet potato to modify the fatty acid composition of the lipids for both functional and nutritional improvement of sweet potato quality, and an increased content of (18:2 and 18:3) linolenic acid in the transgenic sweet potato plants.

The storage roots of sweet potato provide high levels of digestible nutrients and fibers. Noh et al. (2013) demonstrated that storage root development of sweet potato was accelerated in *IbEXP1* antisense plants and suggested that *IbEXP1* plays a negative role in the formation of storage root by suppressing the proliferation of metaxylem and cambium cells to inhibit the initial thickening growth of storage roots.

Table 11.6 Genes Overexpressed in Transgenic Sweet Potato Plants for Abiotic Stress

Gene	Gene Product	Abiotic Stress	References
Cu/Zn SOD and *APX*	Cu/Zn superoxide dismutase and ascorbate peroxidase	Drought tolerance	Li et al. (2006)
swpa4	Plant peroxidases	Increased lignin and phenolic	Kim et al. (2008)
SPCP2	Papain-like cysteine protease	Enhanced drought and salinity tolerance	Chen et al. (2010)
CAD	Cinnamyl alcohol dehydrogenase	Cold response	Kim et al. (2010)
SCOF-1	Cold-inducible zinc-finger protein	Enhanced tolerance to low-temperature stress	Kim et al. (2011)
IbLEA14	Late embryogenesis abundant 14	Accumulation of lignin and enhanced salt tolerance	Park et al. (2011)
BADH	Encoding betaine aldehyde dehydrogenase	Enhanced tolerance to salt, oxidative stress, and low temperature	Fan et al. (2012)
LCY-ε	Lycopene ε-cyclase	Defended against salt-mediated oxidative stress	Kim et al. (2013c)
DFR	Dihydroflavonol-4-reductase	Enhanced tolerance to oxidative stress	Wang et al. (2013a,b)
ERD15	Dehydration 15	Responded to drought stress	Shao et al. (2014)
IbMas	α/β-Hydrolase	Improved salt tolerance	Liu et al. (2014a)
MTs	Metallothioneins	Enhanced tolerance to heavy metals, methyl viologen, and NaCl	Kim et al. (2014)
AtNHX1	Vacuolar Na(+)/H(+) antiporter	Improved salt and cold stress tolerance	Fan et al. (2015a,b)
CuZnSOD and *APX*	Cu/Zn superoxide dismutase and ascorbate peroxidase	Sulfur dioxide (SO_2) tolerance	Kim et al. (2015)
IbZFP1	Cys2/His2 zinc-finger protein	Salt and drought tolerance	Wang et al. (2016)

5. GENETIC ENGINEERING OF TARO [*COLOCASIA ESCULENTA* (L.) SCHOTT]

Taro [*Colocasia esculenta* (L.) Schott] is a tropical root crop grown primarily for its starchy corm or underground stem. It is one of the most important staple food crops in the Pacific Islands and is widely grown throughout the South Pacific, Asia, and Africa (Kreike et al., 2004). Traditionally, taro is propagated vegetatively through suckers or stem cuttings. Fukino et al. (2000) transformed the β-glucuronidase (*gus*) gene into taro using particle bombardment; however, the efficiency of transformation was very low.

Sclerotium or southern blight, caused by *Sclerotium rolfsii* Sacc., is a major fungal disease of dryland-grown (nonflooded) taro. He et al. (2008) inserted a rice chitinase gene into taro through *Agrobacterium*-mediated transformation. In a laboratory bioassay, the transgenic taro plants exhibited increased tolerance to the fungal pathogen *S. rolfsii*, with a 42% to 63% reduction in lesion expansion. He et al. (2010) also introduced the rice chitinase gene into taro calluses through particle bombardment. Growth and morphology of the transgenic plants appeared normal and similar to nontransformed

controls. In pathogenicity tests, the transgenic line exhibited improved resistance to the fungal pathogen *S. rolfsii*. He et al. (2013) generated transgenic taro plants with improved resistance to pathogen *Phytophthora colocasiae* by transformation of the wheat oxalate oxidase gene through *Agrobacterium tumefaciens*.

6. GENETIC ENGINEERING OF GINGER AND TURMERIC

Ginger (*Z. officinale* Rosc.) is a widely used spice, flavoring agent, and herbal medicine and is also employed in the perfume industry. Ginger originated in India or Southeast Asia, and is nowadays is cultivated in many tropical and subtropical areas. The main producers are India, China, Indonesia, and Nigeria. Suma et al. (2008) established a genetic transformation system of ginger mediated by *A. tumefaciens*. Transformants were recovered on selection media containing 100 mg/L kanamycin and a combination of 1.0 mg/L 2,4-D and 0.5 mg/L BA, and regenerated in half-strength MS media of 3.0 mg/L BA and 0.5 mg/L 2,4-D.

Turmeric (*C. longa* L.) is a tropical perennial herb mainly cultivated in India, Pakistan, Sri Lanka, Bangladesh, and China. Turmeric is used to add flavor and color to food. Its bright yellow color is caused by the presence of curcumin pigment, which is a strong antioxidant. Turmeric is a rarely flowering, sterile triploid plant. The plant is propagated vegetatively through its underground rhizomes. It is difficult to improve the crop by conventional breeding (Shirgurkar et al., 2001). Shirgurkar et al. (2006) developed an efficient method for the stable transformation of turmeric with plasmid pAHC25 containing the *bar* and *gusA* genes using particle bombardment. Transformed plantlets were resistant to the herbicide glufosinate. He and Gang (2014) developed a leaf-based transient expression system and callus-based stable transformation system mediated by *A. tumefaciens* for turmeric, which offered opportunities for assaying gene function in turmeric and for improving turmeric properties.

7. BIOSAFETY ISSUES INVOLVED IN TRANSGENIC TUBER AND ROOT CROPS

Despite the growth and use of transgenic crops in many areas of the world, some governments, organizations, and individuals still hesitate to acknowledge that transgenic crops provide economic and environmental benefits that are unobtainable in a timely manner via nontransgenic advances in plant breeding. Conner et al. (2003) and Nap et al. (2003) provided an overview of the approaches used for regulating GM crop release into the environment and presented a detailed description of risk assessments and how they are performed. Craig et al. (2008) summarized general features of risk assessments of GM crops.

GM crops have been facing increased disapproval and lack of consumer acceptance because of the associated risks to the environment and food safety (Kamthan et al., 2016). One of the main public concerns that prevent the widespread use of crops developed using the process of transgenesis is the introduction of foreign DNA in the plant genome without utilizing the plant's native genetic repertoire to achieve the desirable traits. The presence of selectable marker genes in crops has also provoked public concern worldwide. The use of antibiotic or herbicide markers to select GM organisms can lead to unpredictable changes in the transgenic plants (Mehrotra and Goyal, 2012). The development of

marker-free transgenic plants could solve the issues of biosafety in genetically engineered crops. Tuteja et al. (2012) discussed the regulation and biosafety concerns of GM crops and described the current technologies to eliminate the selectable marker genes to develop marker-free transgenic plants.

Kyndt et al. (2015) found that the genome of cultivated sweet potato contains *Agrobacterium* T-DNAs with expressed genes. Their finding is that sweet potato is naturally transgenic while being a widely and traditionally consumed food crop, which could affect the current consumer distrust of the safety of transgenic food crops.

One of the major concerns of the general public regarding transgenic crops relates to the mixing of genetic materials between species that cannot hybridize by natural means. Considering public concerns about safety issues regarding transgenic crops, cisgenesis and intragenesis have been developed as new tools in crop modification and plant breeding (Espinoza et al., 2013). Based on the use of native genes in comparison with the use of hybrid genes, cisgenesis can be considered much closer to traditional breeding than intragenesis. Cisgenic and intragenic plants should also be free from other nonplant sequences, such as vector backbone and selection markers.

These intragenic approaches have been performed through the silencing of unwanted genes. The first intragenic potato was developed to produce high amylopectin content (de Vetten et al., 2003). This approach was based on the silencing of the granule-bound starch synthase gene GBSS, which is responsible for the synthesis of amylase in potato. Another improved trait was reduction in enzymatic browning of potato tubers (Rommens, 2004). Cisgenic plants are presumably considered safer than those produced through conventionally bred plants because of the lack of linkage drag. In cisgenesis, only the desired genes are introduced (Telem et al., 2013). Cisgenic plants should not be assessed as transgenics for environmental impacts (Hou et al., 2014). Cisgenesis will show the way toward sustainable crop improvement programs.

8. CONCLUSION AND FUTURE PROSPECTS

Agrobacterium-mediated transformation has been successfully used for genetic modification of tuber and root crops for improving biotic and abiotic stresses, nutritional content, and increasing productivity. However, traditional methods of crop transformation such as *Agrobacterium*-mediated transformation and particle bombardment are dependent on the random insertion of multiple copies of transgenes into the plant genome leading to gene silencing and unpredictable expression patterns (Kamthan et al., 2016). Site-specific recombination technology can lead to precise insertion of transgenes into known target sites in the plant genome, remove unwanted DNA, and resolve complex transgene insertion into single copies (Halpin, 2005).

Public perception has proven to be essential for the approval of GM crops. Several surveys and focus group interviews in the United States and Europe clearly show that both intragenic and cisgenic crops are more acceptable to a greater number of people than transgenic crops (Holme et al., 2013). It is accordingly possible that the intragenic/cisgenic route will be of major significance for future plant breeding.

To meet the food demand of the booming world population, introduction of a single gene for development of a single trait is not sufficient. There is an increasing need to develop crops with complex traits, such as stress tolerance and nutrient-use efficiency as well as combinations of multiple traits (Marra et al., 2010). The breakthrough technology of genome editing can overcome

some limitations of conventional breeding. Genome editing technology involving engineered nucleases such as zinc-finger nucleases, transcription activator-like effector nucleases, and clustered regularly interspersed short palindromic repeats/CRISPR associated nuclease can be used to generate improved crops and allow the precise insertion of specific genes for modification or replacement of genes at their specific genomic location without involving any other source of DNA (Samanta et al., 2016). This would open a new door for the development of crops with superior phenotypes and permit their commercialization even in countries where GM crops are poorly accepted.

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CHAPTER

GENETIC ENGINEERING IN MEDICINAL AND AROMATIC PLANTS

12

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1. INTRODUCTION

Plant secondary metabolites (PSMs) are natural products derived from primary metabolites with diverse physiological activities. These PSMs are involved in plants' interactions with the environment for their survival and fitness, which makes them essential as primary metabolites (Kliebenstein et al., 2012). Plants used primarily for their medicinal or aromatic properties in pharmacy or perfumery are defined as medicinal and aromatic plants (MAPs) (Planta Europa, 2010). MAPs are cultivated for their secondary metabolites, which have diverse applications and are used as essential oils, pharmaceuticals, herbal medicines, dyes, colorants, cosmetics, personal care products, and plant protection products. An estimated 50,000–70,000 plant species are used in traditional and modern medicine throughout the world. Around 70%–80% of people worldwide predominantly depend on traditional, largely herbal, medicines to meet their primary healthcare needs. The demand for herbal medicine globally is not only large, but increasing. More than 25% of the pharmaceutical drugs used in the world today are derived from plant natural products. The commonly used analgesic aspirin is derived from *Salix* and *Spiraea* species and some of the most valuable anticancer agents such as paclitaxel and vinblastine are derived solely from plant species *Taxus* and *Catharanthus*, respectively. A total of about 252 drugs are listed in the World Health Organization's essential medicine list of which 11% is exclusively of plant origin. MAPs are not only a major resource base for the traditional medicine and herbal industry, they also provide livelihood and health security to a large segment of the world population. MAPs are cultivated in smaller areas and can be classified as low-volume and high-value crops. Although plants are renewable resources, in many plants, obtaining sufficient amounts to meet increasing demands is a major limitation. Destruction of natural habitats and difficulties in cultivation also are reasons for reduced plant availability. Synthesis of alternatives in place of natural chemicals is possible but the higher cost involved and ecological impacts of chemical synthesis are major drawbacks (Farnsworth, 1979; Schmidt et al., 2008a,b; Lubbe and Verpoorte, 2011).

2. PLANT SECONDARY METABOLITES AND THEIR ROLE

About 100,000 compounds are now known from plants, with about 4000 new ones being discovered every year (Verpoorte et al., 1999). PSMs are classified into three major groups, namely, terpenes (or isoprenoids), phenolic compounds (phenylpropanoids and flavonoids), and nitrogen-containing

Genetic Engineering of Horticultural Crops. http://dx.doi.org/10.1016/B978-0-12-810439-2.00012-X

compounds (alkaloids, glucosinolates, and cyanogenic glycosides) (Fang et al., 2011). Terpenes are synthesized in plants through two different pathways, the mevalonate and methyl erythritol 4-phosphate pathways; one occurs in cytoplasm and the other in plastids. Based on five carbon units (isoprene units), these terpenes are classified into different groups such as monoterpenes, sesquiterpenes, diterpenes, triterpenes, tetraterpenes, and polyterpenoids. Terpenes play a vital role in plant growth (gibberellins, sterols, carotenoids, and abscisic acid) and plant defense mechanisms. Some plants have volatile terpenes known as essential oils and terpene glycosides called saponins. The second major group of PSMs belongs to aromatic phenolic compounds, which have a phenyl ring bearing one or more acidic hydroxyl groups. The phenolic compounds are formed by malonic and shikimic acid pathways and the shikimic acid pathway is very common in plants. Phenolics play many physiological roles in plants including reproduction, growth, and defense against different biotic or abiotic stresses and have antioxidant and allelopathic activity. The third important category of secondary metabolites includes nitrogen-containing compounds such as alkaloids, glucosinolates, and cyanogenic glycosides. The alkaloids are synthesized from a few amino acids such as lysine, tyrosine, tryptophan, etc. Alkaloids can be further classified into different groups such as terpenoid indole alkaloids (TIAs), benzylisoquinoline alkaloids (BIAs), purine alkaloids, tropane alkaloids (TPAs), nicotine, etc. TIAs include more than 3000 compounds such as antineoplastic agents (vinblastine and camptothecin), antimalarial drugs (quinine), and strychnine (a rat poison), which are primarily synthesized from tryptophan. Medicinally important TIAs such as vinblastine for cancer and ajmaline for heart disorders are produced by *Catharanthus roseus* and *Rauvolfia serpentina*. BIAs contain approximately 2500 compounds with pharmacological properties such as morphine (analgesic), codeine (cough suppressant), papaverine (muscle relaxant), sanguinarine, berberine (antimicrobial agent), etc. TPAs include hyoscyamine and scopolamine, which are derived from *Hyoscyamus*, *Atropa*, and *Datura* plant species (Taiz and Zeiger, 2006). Examples of secondary metabolites and source plant species and their uses are shown in Table 12.1.

Table 12.1 Plants and Their Secondary Metabolites and Uses

Secondary Metabolite	Plant Species	Uses
Atropine, scopolamine	*Atropa belladona*	Antispasmodic, sedative
Codeine, morphine	*Papaver somniferum*	Analgesic
Quinine	*Cinchona officinalis*	Antimalarial
Artimisinin	*Artemisia annua*	Antimalarial
Quinine	*Cinchona* sp.	Antimalarial
Taxol	*Taxus brevifolia*	Anticancer
Vincristine, vinblastin	*Catharanthus roseus*	Anticancer
Reserpine	*Rauvolfia serpentina*	Hypotensive
Atropine	*Hyoscyamus niger*	Anticholinergic
Stevioside	*Stevia rebaudiana*	Sweetener
Vanillin	*Vanilla planifolia*	Flavouring agent
Naphthoquinone	*Lawsonia inermis*	Dye
Indigo	*Indigofera*	Dye

Molecular breeding through genetic engineering is a preferred approach for the production of PSMs in overcoming agronomic and environmental problems that have not been solved by conventional plant breeding programs. Genetic transformation has been used effectively for the production of plants with desired traits in many crops. Through genetic engineering it is possible to produce plants with increased levels of fine chemicals, new compounds for screening for biological activity, new colors, fragrances, and improved resistance against pest and diseases. Genetic engineering can also improve nutritional quality with reduced unwanted compounds in food and fodder. Progress in tissue culture, combined with development in genetic engineering and omics techniques, has opened new vistas for enhanced production of pharmaceuticals, nutraceuticals, and other secondary metabolites.

3. MOLECULAR ELUCIDATION OF PLANT SECONDARY METABOLITES

PSMs in MAPs are an important group of phytochemicals that exhibit immense chemical diversity and pharmacological activities. To understand and use the secondary metabolism in MAPs a vast array of biotechnological tools is available. Pathway elucidation and metabolite engineering have been useful to obtain increased yield of the metabolites of interest or for producing novel metabolites. Thus molecular elucidation consists of finding out the precise chemical routes of metabolite biosynthesis, enzymes catalyzing the biosynthetic reactions, genes encoding the biosynthetic enzymes, and regulatory factors that control secondary metabolite production. The availability of molecular information with regard to production and regulation of PSMs enables biotechnologists to rationally tinker with biosynthetic machinery. Molecular elucidation involves several approaches and several steps starting with the identification of genes or regulatory mechanisms such as transcription factors that control the secondary metabolite pathways up to the cloning of the genes involved. The techniques include precursor labeling, gene overexpression and inhibition, mutant selection, or differential gene expression studies using elicitation to create varied phenotypic states. The recent technique of "omics," which take advantage of readily accessible sequencing technologies, is yet another method of molecular elucidation. Once a reference genome has been established, high-throughput sequencing technologies can be used to identify the genes responsible for specific phenotypes through quantitative trait loci and genome-wide association studies.

3.1 GENOMIC cDNA SEQUENCES AND REGULATION OF GENETIC CONTROL

Apart from the genes, studies have shown that transcription factors, i.e., the sequence-specific DNA binding proteins that interact with the promoter regions of target genes and modulate the rate of initiation of messenger RNA (mRNA) synthesis, are also specifically involved in the regulation of secondary metabolism. Understanding the role of transcription factors in a secondary metabolism pathway will aid in metabolic engineering for increased yield of secondary metabolites and also the development of new production techniques for valuable metabolites. There are many examples where transcription factors are used to improve the production of pharmaceutically important PSMs such as terpenoids, flavonoids, and alkaloids.

3.1.1 Metabolic Engineering of Terpene Biosynthesis With Transcription Factors

In spearmint (*Mentha spicata*), the sites of secondary metabolite production are tiny specialized structures called peltate glandular trichomes (PGTs). In a study, Wang et al. (2016) isolated and functionally

characterized a novel *MsYABBY5* gene that is preferentially expressed in the PGTs of spearmint. Transgenic plants were developed in which *MsYABBY5* was either overexpressed or silenced using RNA interference (RNAi) to confirm whether the expression of the gene is related to terpene production in spearmint, and the results showed that the reduced expression of *MsYABBY5* led to increased levels of terpenes and that overexpression decreased terpene levels. Additionally, ectopic expression of *MsYABBY5* in *Ocimum basilicum* and *Nicotiana sylvestris* also decreased secondary metabolite production in them, suggesting that the encoded transcription factor is probably a repressor of secondary metabolism.

3.1.2 Metabolic Engineering of Flavonoid Biosynthesis With Transcription Factors

Flavonoids are formed from the amino acid phenylalanine and they belong to the phenylpropanoid group of compounds. Several molecular families, such as anthocyanin pigments, condensed tannins, antimicrobial phlobaphenes, etc. are derived from the flavonoid biosynthesis pathway. Among these, the anthocyanin pigmentation biosynthesis pathway has been extensively studied since anthocyanin pigments determine the colors of flowers and seed kernels and thus provide a convenient, visible marker of mutant phenotypes. In maize, anthocyanin biosynthesis is regulated by a combination of two transcription factor species that are encoded by two families of regulatory genes, *R/B* and *C1/Pl*. *R* and *C1* interact to regulate anthocyanin biosynthesis in the maize kernel. Homologous regulatory genes regulate anthocyanin synthesis in other parts of maize and other plant species. When *R* and *C1* are expressed ectopically in unpigmented maize cells cultured in vitro, they induce metabolic differentiation that leads to the biosynthesis and accumulation of anthocyanins. Thus it was shown that ectopic expression of specific transcription factors can redirect the metabolic differentiation of plant cells by acting simultaneously and coordinately on different events, including the regulation of the expression of genes that encode biosynthetic enzymes and proteins necessary for metabolite storage and differentiation of appropriate subcellular compartments.

3.1.3 Engineering Alkaloid Biosynthesis With Transcription Factors

Alkaloids are the largest group of plant pharmaceutical compounds. Alkaloid biosynthesis pathways are often more complex than the flavonoid pathway and only a few structural genes from the alkaloid pathways have been isolated such as tropane and benzylisoquinoline pathways. In *C. roseus*, molecular studies were carried out to identify tools to improve alkaloid production by regulating the TIA biosynthetic pathway. Focus was given on the promoter sequences that regulate TIA biosynthetic genes to find transcription factors that control this pathway. Promoter analysis of the genes isolated a short *STR* promoter sequence called the JERE (jasmonate and elicitor-responsive element), which is responsible for elicitor-responsive and jasmonate-responsive gene expression. Using the JERE as bait in yeast one-hybrid screening, a cDNA that encodes *ORCA2* [octadecanoid responsive *Catharanthus* (AP2-domain protein 2)] was isolated. *ORCA2* is a transcription factor induced by jasmonate, and *ORCA2* activates *STR* expression by interacting with the JERE. These data indicated that ORCA2 controls the jasmonate-responsive expression of *STR* and, possibly, other TIA biosynthesis genes (Table 12.2).

3.2 CLONING OF SECONDARY METABOLITE PATHWAY GENES

Precursor labeling and retrosynthetic studies provide the framework to trace the precise chemical route of biosynthesis and hence they are a prerequisite to identifying the genes or enzymes involved in the

Table 12.2 Examples of Transcription Factors That Control Secondary Metabolism in Plants

Plant Transcription Factor	Metabolite Class	DNA Binding Domain
C1	Anthocyanins	MYB[c]
P	Phlobaphenes	
TT2	Condensed tannins	
PAP1	Anthocyanins	
AtMYB4	Sinapate esters	(*Arabidopsis thaliana*)
CrBPF1	Alkaloids	
R	Anthocyanins	bHLH
TT8	Condensed tannins	
CrMYC2	Alkaloids	
ORCA2	Alkaloids	AP2/ERF(*Catharanthus roseus*)
ORCA3	Alkaloids	
CrGBF1	Alkaloids	bZIP (*C. roseus*)
CrGBF2	Alkaloids	

From Gantet, P., Memelink, J., 2002. Transcription factors: tools to engineer the production of pharmacologically active plant metabolites. Trends Pharmacol. Sci. 23 (12), 563–569.

secondary metabolite pathway. As an example, terpenoids that contribute one-third of all known secondary metabolites were shown to be produced by condensation of C5 units—isopentenyl pyrophosphate (IPP) and dimethylallyl pyrophosphate (DMAP) (Poulter et al., 1981). Earlier it was thought that only the cytosolic mevalonate pathway produces IPP, the universal precursor of all terpenoids. However, with the use of 13C-labeled intermediates, it was shown that certain terpenoids are produced not only from mevalonate pathways, but also from another pathway also producing IPP/DMAPP in microorganisms and plants (Rohmer, 1999). The approaches for molecular elucidation can be broadly divided into the following.

3.3 BIOCHEMICAL APPROACH

The biochemical approach for molecular elucidation of biosynthetic pathways has been very useful in the pregenomic era. In this, foremost is knowledge of the chemical route of the particular metabolite synthesis; once it is known, a hypothetical reaction is set. Enzyme activity is detected and purification of the enzyme is done using various chromatographic techniques. This is followed by sequencing of the purified protein or enzyme and, using degenerate primers, partial cDNA is amplified. The sequence of partial cDNA is used to design rapid amplification of cDNA ends primers and full-length cDNA is cloned. Heterologously expressed protein is checked for enzyme activity against purified substrates. For example, the enzyme phenylalanine aminomutase (PAM) that catalyzes the first step in the C-13 side chain of Taxol biosynthesis was cloned from *Taxus chinensis* cell cultures using this approach. Peptide sequences derived from the purified protein/enzyme were used to design and synthesize degenerate primers enabling polymerase chain reaction (PCR) synthesis of the PAM cDNA. The PAM cDNA was cloned and expressed in *Escherichia coli*, and PAM activity was demonstrated (Steele et al., 2005).

3.4 POSITIONAL CLONING, TAGGING, AND EXPRESSION LIBRARIES

Positional cloning, also referred to as map-based cloning, is an approach that discovers the gene of interest based on the creation of mutants that are defective in secondary metabolite synthesis. This approach can be used mainly for those metabolites whose deficiency results in scorable phenotypes, such as color, aroma, and flavor. The mutants created will be hybridized and a large segregating population will be screened for normal phenotype. By cloning the homologous sequences of the two mutants and the restored line, one can arrive at the gene of interest. If the restored line contains the same sequence as the predicted gene, and the two mutants have unique changes in the gene sequence not found in the normal gene, it shows that the putative sequence is the gene of interest. The open reading frames in the cloned DNA are expressed in the heterologous system and assayed for enzyme activity.

Also a cDNA expression library can be used for screening and identifying the requisite enzyme activity against purified substrates. Once the expected enzyme activity is detected, the clone is sequenced. This approach has been used for the cloning of several cytochrome P450 enzymes that catalyze various steps in many secondary metabolite pathways.

3.5 HOMOLOGY-BASED CLONING

This approach is based on the principle that certain enzymes share considerable sequence homology among themselves at both the DNA and protein level. This provides an advantage to design degenerate primers and clone related genes in closely related plant species. This approach reduces the time required for cloning of secondary metabolite pathway genes, and has been successfully employed for several important PSMs.

3.6 DIFFERENTIAL EXPRESSION ANALYSIS, EXPRESSED SEQUENCE TAG LIBRARIES, NEXT-GENERATION SEQUENCING

With the development of DNA sequencing technologies, availability of large-scale proteomics platforms, and development of better bioinformatic tools, many recent approaches have emerged for cloning secondary metabolite genes. A differential expression-based transcriptomics study is one of such approaches. By this, one can identify genes whose patterns of expression differ according to phenotype or experimental condition. In medicinal plants, trichomes are the plant parts producing secondary metabolites, so a differential gene expression study such as suppression subtractive hybridization can be used for identifying the trichome-expressed genes that may be involved in metabolite biosynthesis. This is followed by sequencing the expressed sequence tag library. Recent technologies such as next-generation sequencing have considerably reduced the time required for sequencing of differentially expressed transcriptomes. Wherever genomic resources preexist, a microarray-based differential expression study may be conducted. Differentially expressed RNAs (or proteins, in the case of comparison of 2D PAGE profiles) are analyzed by bioinformatics tools. Furthermore, their expression pattern helps to predict with some degree of certainty whether they could be involved in secondary metabolite biosynthesis. Once the genes are predicted, one generally fishes out the full-length cDNA and heterologous expression followed by in vitro enzyme activity determination. To further prove the role of the gene of interest in secondary metabolite biosynthesis, knockout or knockdown lines may be

created using transgenesis and then accumulation of preceding intermediates may be tested, as per the proposed biosynthetic pathway. These methods have been employed for characterizing several secondary metabolite pathway genes such as those involved in the production of anticancer compounds—vincristine and vinblastine in *C. roseus* (Miettinen et al., 2014).

4. GENETIC TRANSFORMATION SYSTEMS

4.1 *AGROBACTERIUM*-MEDIATED TRANSFORMATION FOR INDUCTION OF HAIRY ROOTS

Roots serve as the storage organs for most secondary metabolites. Extraction of metabolites results in the destruction of whole plants. Hence culturing roots in vitro through induction of hairy root cultures seems to be the most viable option for enhancing the production of secondary metabolites. Hairy roots are induced upon transformation of the plant material with the natural vector system *Agrobacterium rhizogenes*. The production of hairy roots has facilitated rapid multiplication of roots with extensive branching without loss of potential for production of original metabolites synthesized in the mother plant (Nader et al., 2006). The high growth rate, maintenance of genotypic and phenotypic stability over long culture periods, and ability to multiply indefinitely without the need of plant growth regulators, with its potential to produce high levels of secondary metabolites (Srivastava and Srivastava, 2007), are some of the advantages offered by hairy roots.

Hairy roots can also be used in metabolite engineering through the introduction of additional genes along with the Ri T-DNA for production of either useful or novel metabolites (Giri and Narasu, 2001). The technique of production of hairy roots is fairly simple as well as rapid. Unlike *Agrobacterium tumefaciens*-based transformation, which takes about 6 months to regenerate plants, hairy roots are produced within a few weeks of inoculation. The random insertion of genes from the T-DNA of Ri plasmid in the plant genome during *A. rhizogenes*-mediated transformation is indicative of the variable expression of the inserted genes. This variability in gene expression has been correlated to the amount of secondary metabolite production and can help in the selection of lines with enhanced metabolite production (Gandhi et al., 2015). Unraveling of the roles of genes in T-DNA of Ri plasmid revealed that rol gene loci (rolA, rolB, and rolC) played a major role in increasing phytochemical production in transformed plant cells (Bulgakov, 2008; Shkryl et al., 2008), while the other genes in the T-DNA were shown to influence morphology, growth, biosynthetic gene expression, and metabolite accumulation in *C. roseus* hairy roots (Taneja et al., 2010).

Because of the ability of hairy root cultures to accumulate secondary metabolite stability in liquid systems, it offers great potential for scaling up production through bioreactors. A wide variety of bioreactor designs have been tested and used for hairy root cultures such as stirred tank, wave, and rotating drum reactors, which are based on mechanically driven reactors; bubble column and airlift reactors (pneumatically driven reactors); and trickle bed and mist reactors (bed reactors) (Ramakrishnan and Curtis, 2004; Georgiev et al., 2007; Eibl et al., 2009; Liu, 2009; Georgiev et al., 2010; Sivakumar et al., 2010). Exploitation of hairy root cultures for economically sustainable production of phytochemicals depends on the type and suitability of the bioreactor system used. The lack of homogeneity in hairy root growth is one of the major challenges for designing an appropriate bioreactor. Measurement of root growth, shear sensitivity, and uneven distribution of nutrients and gases are some of the other consequences of the interlocked hairy root matrix. Computer models taking into account the rheological

characteristics of hairy roots should be developed for designing the most suitable bioreactor. Successful commercial application of ginsenoside production in scaled-up bioreactors of 10,000 L^{-1} capacity from adventitious roots of *P. ginseng* can provide a number of insights into the scaling-up of hairy root cultures (Georgiev et al., 2010). Hairy root cultures have now become a commercially successful technology for the production of pharmaceuticals as evidenced by the success of several private companies. Green2chem is one such Belgian private company (http://www.green2chem.com) that is involved in the production of nutraceutical, cosmetic, and pharmaceutical metabolites. Research and motivation in academic laboratories are driven by the success of such companies (Guillon et al., 2006).

4.2 STRATEGIES FOR IMPROVEMENT OF SECONDARY METABOLITE PRODUCTION FROM HAIRY ROOT CULTURES

Commercial viability of hairy root culture technology depends on the satisfactory yield of the secondary metabolite. Various strategies have been developed for enhancing the production and productivity of hairy root biomass for the synthesis of secondary compounds, such as strain improvement, optimization of medium, and culture environments, elicitation, precursor feeding, metabolic engineering, permeabilization, immobilization, biotransformation methods, and bioreactor cultures.

4.2.1 Optimization of Transformation

Efficiency of hairy root induction determines the success of *A. rhizogenes*-mediated transformation. This is the first parameter in the plethora of factors that can influence the production of secondary metabolites. Efficiency of the transformation process is determined by the strain of *Agrobacterium* used, the concentration of the bacterial culture (Park and Facchini, 2000), and age and type of plant tissue (Sevon and Oksman-Caldentey, 2002) used.

4.2.2 Optimization of Hairy Root Culture and Secondary Metabolite Production

Maximum growth of *P. ginseng* hairy root cultures was obtained with inoculation of 0.7% (w/v) and growth was significantly reduced with a reduction in inoculum used. A subculture cycle of 10 days was optimal (for *P. ginseng*), while it was 14 days for hairy roots of *Beta vulgaris* (Pavlov et al., 2003; Jeong et al., 2004). Nutrient content of the culture medium has a significant influence on the growth of hairy root cultures. Because there are innumerable components and their combinations in the culture medium can influence its growth, researchers are now attempting to develop computational models to predict the optimal growth conditions for high biomass and phytochemical production.

Important abiotic stresses such as drought and salinity are known to affect plant growth and development processes, alter a wide array of physiological and metabolic processes, and act as abiotic elicitors for enhancing the production of secondary metabolites. Other abiotic elicitors include light, metallic ions, and hormones. Similar elicitation is also brought about by biotic factors (Karuppusamy, 2010).

4.2.2.1 Abiotic Elicitors

Production of catharanthine and flavonoid in cell suspension cultures of *C. roseus* and *Passiflora quadrangularis* L. was enhanced by UV radiation (Antognoni et al., 2007; Ramani and Chelliah, 2007). The light is a physical factor that can affect metabolite production. Temperature and light quality were found to influence the production of ginsenoside in hairy root culture of *P. ginseng* (Yu et al., 2005).

Successful enhancement (100×) in diterpenoid tanshinone production in *Salvia miltiorrhiza* hairy root cultures through bioreactor technology was brought about by hyperosmotic stress created by high sorbitol concentrations in conjunction with a yeast elicitor (Wu and Shi, 2008). Electric current given at sublethal doses to *Pisum sativum* hairy root culture increased (+)-pisatin accumulation and similar levels were accumulated upon treatment with $CuCl_2$, another abiotic elicitor (Kaimoyo et al., 2008). In the same lines, use of silver nitrate or cadmium chloride in hairy root cultures of *Brugmansia candida* was found to elicit the overproduction of two tropane alkaloids, scopolamine and hyoscyamine (Angelova et al., 2006). Electric current is thought to bring about its effect by altering cell membrane integrity and activating the metabolic pathway for synthesis of secondary metabolites (Cuell Yue, 2008). Successful application of electro-elicitation is being used in bioreactor technology because it serves to penetrate the heterogeneous interconnected matrix of hairy roots. The extraction or secretion of secondary metabolites such as betalaine from red beet hairy roots (Thimmaraju et al., 2003a,b) and serpentine from *C. roseus* hairy roots (Moreno-Valenzuela et al., 2003) into the culture medium has been accomplished through facilitation from the use of various elicitors and permeabilisant agents (e.g., detergents, solvents, calcium chelators, pH, sonication, temperature, oxygen stresses, etc.). Use of Tween 20 in *Datura* hairy roots resulted in a three- to eightfold increase in alkaloid production (Boitel-Conti et al., 1996).

Jasmonates and salicylic acid (SA) are important signaling molecules, which are produced in response to pathogen attack and other stresses (Pauwels et al., 2009). There are several examples of their use in eliciting secondary metabolites and thus constitute an important class of elicitors. Jasmonic acid (JA) and/or its more active derivative methyl jasmonate (MeJA) along with SA have been implicated in the production of plumbagin in hairy roots of *Plumbago indica* (Gangopadhyay et al., 2011), stilbene biosynthesis in *Vitis rotundifolia* hairy root cultures (Nopo–Olazabal et al., 2014), withanolide A, withanone, and withaferin A (Sivanandhan et al., 2013) in hairy root culture of *Withania somnifera*, and vincristine and vinblastine production in periwinkle (Idrees et al., 2010). Furthermore, elicitation can be used as a tool that helps in the identification of genes through its real-time expression. Using this approach, genes that are limiting in the ginsenoside biosynthetic pathway were identified upon elicitation of ginseng hairy roots with MeJA (Choi et al., 2005).

4.2.2.2 Biotic Elicitors

Biotic elicitors are usually compounds derived from fungal or bacterial origin. Biotic elicitors have been successfully used in enhancing the production of secondary metabolites from plants. One of the most successful examples of fungal elicitation was demonstrated in *T. chinensis* cell culture through the enhancement of Taxol production (Wang et al., 2001). In many cases, fungal elicitation is brought about using autoclaved cell wall filtrates from the fungus as seen in *Ambrosia artemisiifolia* hairy root cultures, which enhanced threefold by the use of autoclaved cell wall filtrates from the fungus *Protomyces gravidus* (Bhagwath and Hjortsø, 2000). The production of artemisinin from hairy root cultures of *Artemesia annua* was increased by treatment with filtered and autoclaved mycelial extract of *Verticillium dahlia* (Wang et al., 2000a,b). Enhanced production of azadirachtin by hairy root cultures of *Azadirachta indica* was achieved by incorporation of biotic elicitors from *Claviceps purpurea.* Bacterial elicitors enhanced production of scopolamine in adventitious hairy root cultures of *Scopolia parviflora* (Jung et al., 2003). Elicitation in cell cultures is one of the important strategies used for improvement of secondary metabolite production from hairy root cultures. Better understanding of the elicitation mechanisms will help in successful exploitation of hairy root culture technology for targeted phytochemical production and extraction.

5. FUNCTIONAL GENOMICS APPROACHES FOR ENGINEERING OF SECONDARY METABOLIC PATHWAYS

Root cultures can be engineered with heterologous genes to alter secondary metabolite production using *A. tumefaciens* or *A. rhizogenes* in the host plant or hairy roots, respectively. The natural genetic engineers *A. tumefaciens/rhizogenes* are capable of transferring the genes of interest that are placed in the T-DNA region of the Ti/Ri plasmid, respectively. Alternately, hairy roots can be induced in stably transformed host plants by additional transformation with *A. rhizogenes* (Georgiev et al., 2007; Ono and Tian, 2011). Hairy root cultures also provide the potential for metabolic engineering in cultures by introducing or altering gene expression (Ludwig-Müller et al., 2014).

5.1 UPREGULATION OR OVEREXPRESSION OF THE GENE(S)/ENZYME(S)

5.1.1 Single Gene(s) Engineering

A plant cell is a chemical factory producing a multitude of compounds as by-products of several intertwined biosynthetic pathways. These pathways can operate either singly or through interactions at various steps in several other pathways in operation. Considering the complexity of the biosynthetic and regulatory process associated with the production of secondary metabolites, complete knowledge of the biosynthetic pathway including the various intermediates and enzymes that are involved is critical for the success of metabolic engineering. Furthermore, knowledge from computational biology and omics science will complement our endeavor for enhancing the production of secondary metabolites while reducing toxic chemicals and producing novel chemicals (Yang et al., 2014). Because the production of secondary metabolites is often dependent on the networking of several biosynthetic pathways, it is not surprising that a particular intermediate becomes rate limiting because the intermediate may be utilized by other competing pathways. Targeting this rate limiting through overexpression of the enzyme associated with conversion of this intermediate toward the desired pathway for metabolite synthesis has been used as a strategy in many secondary metabolite synthesis pathways. For example, overexpression of chalcone isomerase 1 (CHI1), a key gene in the flavonoid pathway in tomato, and overexpression of strictosidine synthase, an early enzyme in the alkaloid biosynthetic pathway, in *C. roseus* could enhance flavonoid levels in transgenic tomato (Muir et al., 2001) and alkaloid levels in *C. roseus* cells (Whitmer et al., 1998), respectively. Likewise, two- to threefold increases in artemisinin production were achieved through overexpression of farnesyl diphosphate synthase in *A. annua* (Chen et al., 2000).

Secondary metabolite production has also been improved through transgenic hairy roots overexpressing genes of interest. Engineering the T-DNA with genes of interest in Ri plasmid of *A. rhizogenes* and using such strains for transformation facilitates the overexpression of secondary metabolites in hairy root cultures. There are several examples in the literature wherein enhancement of PSM production in transgenic hairy root lines was achieved as a consequence of overexpressing genes of interest. In *C. roseus*, transgenic hairy root lines were developed for production of hörhammericine, ajmalicine, and serpentine through overexpression of both deacetylvindoline-4-*O*-acetyltransferase (Magnotta et al., 2007) and the peroxidase gene (CrPrx) (Jaggi et al., 2011), respectively. Other examples include more than a 200% increase in paclitaxel production in hairy roots of *Taxus* media obtained through upregulation of taxadiene synthase (Exposito et al., 2010), increased methylputrescine through overexpression of putrescine *N*-methyltransferase in *Hyoscyamus niger* hairy root line (Zhang et al., 2007), increased flavonoids through overexpression of chalcone isomerase gene (Chi) in hairy roots of

Glycyrrhiza uralensis Fisch (Zhang et al., 2009a), enhanced L-ascorbic acid production in transgenic hairy root line of tomato upon overexpression of GalUR (D-galacturonic acid reductase) gene (Oller et al., 2009), and increase of squalene contents in *Centella asiatica* hairy roots brought about by overexpression of *P. ginseng* farnesyl diphosphate synthase (PgFPS) (Kim et al., 2010).

5.1.2 Multiple Gene(s) Engineering (Simultaneously or Sequentially)

Use of single genes in metabolic engineering has not always been successful. For example, in *C. roseus* hairy root cultures, overexpression of a single gene (1-deoxy-D-xylulose synthase, DXS) within the TIA biosynthetic pathway resulted in mixed results; however, co-overexpression of two genes (DXS with geraniol-10-hydroxylase or anthranilate synthase α subunit) within the pathway led to a significant increase in the accumulation of multiple TIA metabolites (Peebles et al., 2011). Similarly, increased yields (9×) of scopolamine over control plants were obtained in transgenic *H. niger* L. hairy root line through overexpression of hyocyamine 6β-hydroxylase and putrescine *N*-methyltransferase (Zhang et al., 2004). As a result, more complex engineering strategies that manipulate expression of multiple pathway genes and/or regulators are necessary to balance flux toward the product of interest (Morandini, 2013).

Transcription factors are known to bring about a cascading effect by turning on several genes downstream in a metabolic pathway. However, in *C. roseus* transgenic hairy root line, catharanthine content could not be increased by overexpression of transcription factor ORCA 3 (octadecanoid-responsive *Catharanthus* apetala2/ethylene response factor domain) alone (Zhou et al., 2010), while its use in conjunction with G10H (cytochrome P450 monooxygenase) gene brought about enhancement in catharanthine content (Wang et al., 2010). The flux of the metabolites in the pathway could be manipulated through the use of single or multiple genes or a combination of genes used in *C. roseus* hairy root cultures. Peebles et al. (2011) advocated this strategy for increasing the content of vinblastine and vincristine. Transcription factors are known to bring about their effect by binding to the hormone or elicitor response domain in the promoter region suggesting that a combination of multiple genes/transcription factors and use of elicitors can greatly enhance the efficiency of secondary metabolite production.

5.2 ENGINEERING OF REGULATORY GENES

Regulatory genes through their influence on several downstream genes are known to increase the production of secondary metabolites. Transcription factors are one such regulatory gene known to bind to the promoter region and upregulate genes in a metabolic pathway. For example, ORCA3, a transcription factor, enhances the production of alkaloids in *C. roseus* by upregulating the genes of the TIA pathway and has been shown to respond to jasmonate elicitor treatment. Transcription factors C1 and R are known to bring about increased anthocyanins in maize suspension cells (Grotewold et al., 1998). Similarly, transgenic apples overexpressing maize regulatory gene leaf color (Lc) resulted in increased flavonoid content (Li et al., 2007). Alternatively, increased apocarotenoid and flavonoid content in tomato fruits could be brought about by silencing the DET1 regulatory gene (Davuluri et al., 2005).

5.3 DOWNREGULATION OR FUNCTIONAL KNOCKOUT OF THE GENE(S)/ENZYME(S)

With the advancement of molecular biology and enzymology, many new approaches have emerged to increase the secondary metabolite content in medicinal plants. One of the approaches is downregulation or functional knockout of the genes to decrease the production of a certain unwanted group of

compounds and increase the concentration of a desired secondary metabolite. The basic principle behind these approaches is knocking out an enzymatic step in the particular pathway by reducing the level of corresponding mRNA or protein.

5.3.1. Antisense RNA Technology

Antisense RNA technology is one of the approaches that are used for the inhibition of gene expression or downregulation of a gene. This technology works on the principle that an antisense nucleic acid sequence base pairs with its complementary sense RNA strand and prevents it from being translated into protein. The complementary nucleic acid sequence can be either a synthetic oligonucleotide, often oligodeoxyribonucleotides of less than 30 nucleotides, or longer antisense RNA sequences. The complementary sense and antisense RNA molecules in the same cell can lead to the formation of a stable duplex, which may interfere with gene expression at the level of transcription, RNA processing, or possibly translation (Table 12.3).

5.3.2 Co-suppression

Co-suppression refers to gene silencing mediated by a sense transgene. In this, the expression of a homologous gene is suppressed by a sense transgene. Co-suppression involves either transcriptional gene silencing or posttranscriptional gene silencing. The suppression of the expression of an endogenous gene may not associate with the alteration in the developmental timing of mRNA expression but will reduce the level of mRNA produced by this gene thereby suppressing its expression.

5.3.3. RNA Interference

Increasing metabolic flux is one of the strategies for enhancing secondary metabolite production, which can be achieved by inhibiting or blocking competitive pathways. The RNAi approach was used to enhance the accumulation of the preceding intermediate by downregulating the gene expression of the succeeding gene in the metabolic pathway. RNAi technology provides an alternative whenever the use of antisense RNA and co-suppression approaches have failed to block the activity of an enzyme that is coded by multigenes. RNAi is a process of double-strand RNA (dsRNA)-mediated gene silencing in which only the mRNA associated with dsRNA is specifically degraded. This type of RNAi-mediated gene silencing is also referred to as co-suppression or posttranscriptional gene silencing in plants (Price and Gatehouse, 2008; Nakayashiki and Nguyen, 2008). RNAi is a powerful tool for gene silencing applications in medicinal plant research but has not been fully exploited in engineering of secondary metabolites (Borgio, 2009). RNAi technology has been used to engineer secondary metabolites and identify genes responsible for the synthesis of secondary metabolites in MAPs. This dsRNA-mediated gene silencing system has been successfully employed to modify the production of pharmaceutically important secondary metabolites in plants such as *Papaver somniferum*, *P. ginseng*, *A. annua*, and *W. somnifera* (Allen et al., 2004, 2008; Han et al., 2006; Zhang et al., 2009a,b; Smrati et al., 2016), essential oils in peppermint and *S. miltiorrhiza*. Opium poppy (*P. somniferum*) is a key source of the narcotic analgesic alkaloids codeine and morphine. Accumulation of the precursor alkaloid (*S*)-reticuline was observed in transgenic plants developed through silencing of codeinone reductase by RNAi using a chimeric hairpin RNA construct (Allen et al., 2004). A feedback mechanism may be the cause for the accumulation of (*S*)-reticuline precursor alkaloid at the expense of morphine, codeine, oripavine, and thebaine. Analysis revealed the loss of *Cor* gene transcript and reduction of enzyme activity. In another study, DNA-encoded hairpin RNA-mediated suppression of gene encoding the morphinan pathway

Table 12.3 Metabolic Engineering of Plant Secondary Metabolism

Name of Compound	Source Plant	Approach Used	Gene(s) Involved	Results	References
Alkaloids	*Catharanthus roseus* (L.) G Don	Overexpression of pathway gene	Strictosidine synthase	Increased content	Whitmer et al. (1998)
Scopolamine	*Hyoscyamus niger* L.	Overexpression of pathway gene	Hyocyamine 6β-hydroxylase and putrescine *N*-methyltransferase	Increased content	Zhang et al. (2004)
Flavonoids	*Solanum lycopersicum* L.	Overexpression of pathway gene	Chalcone isomerase	Increased content	Muir et al. (2001)
Artemisinin	*Artemisia annua* L.	Overexpression of pathway gene	Farnesyl diphosphate synthase	Increased content	Chen et al. (2000)
Menthol	*Mentha* × *piperita* L.	Inhibiting competitive pathway (through gene silencing/RNA)	Menthofuran synthase	Increased content	Mahmoud and Croteau (2001)
Pinoresinol	*Forsythia koreana* (Rehder) Nakai	Inhibiting competitive pathway (through gene silencing/rNA)	Pinoresinol lariciresinol reductase (PLR)	Increased content	Kim et al. (2009)
Apocarotenoids and flavonoids	*S. lycopersicum* L.	Engineering regulatory mechanism (through gene silencing/RNA)	Deetiolated (DETI)	Increased content	Davuluri et al. (2005)
Flavonoids	*Zea mays* L.	Engineering regulatory mechanism (through gene silencing/RNA)	Leaf color (Lc)	Increased content	Li et al. (2007)
Anthocyanins	*Z. mays* L.	Engineering regulatory mechanism (through gene silencing/RNA)	CI and R (transcription factors)	Increased content	Grotewold et al. (1998)
Nornicotine	*Nicotiana tabacum* L.	Gene silencing (RNA interference)	CYP82E4	Decreased content	Gavilano et al. (2006)
Caffeine	*Coffea arabica* L.	Gene silencing (RNA interference)	Theobromine synthase (CaXMTI, CoMXMTI) and caffeine synthase (CaDXMTI)	Decreased caffeine content	Ogita et al. (2003)
p-Hydroxybenzy glucosinolates	*Arabidopsis thaliana* (L.) Heynh.	Heterologous expression	CYP79A1	Production of novel compound	Bak et al. (1999)

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enzyme salutaridinol 7-*O*-acetyltransferase (SalAT) resulted in the novel accumulation of the alkaloid salutaridine at up to 23% of total alkaloid (Allen et al., 2008). Alkaloid production was also effected in opium poppy by suppression of the gene encoding the morphinan pathway enzyme SalAT. Transgenic *P. somniferum* plants in which the salAT transcript has been reduced using RNAi technology showed accumulation of the intermediate compounds salutarydine and salutaridinol in a ratio ranging from 2:1 to 56:1 (Kempe et al., 2009). Han et al. (2006) demonstrated the posttranscriptional gene silencing of dammarenediol synthase through RNAi technology in *P. ginseng*. Dammarenediol synthase mRNA expression was strongly suppressed because of gene silencing, manifesting in reduced ginsenoside accumulation (85.4%) in transgenic roots of *P. ginseng*. Runguphan et al. (2009) reported that the RNA-mediated suppression of tryptamine biosynthesis in *C. roseus* during hairy root culture eliminates all production of monoterpene indole alkaloids. They introduced an unnatural tryptamine analog to the media and revealed that the silenced *C. roseus* culture could produce a variety of novel products derived from this unnatural starting substrate. Artemisinin isolated from *A. annua* L. has very potent antimalarial activity. The artemisinin content of *A. annua* was increased by suppressing the expression of squalene synthase, a key enzyme of the sterol pathway, by means of a hairpin-RNA-mediated RNAi technique. Some transgenic plants recorded significantly enhanced artemisinin content by 3.14-fold as compared to untransformed control plants (Zhang et al., 2009a,b; Jagtap et al., 2011). Kumar et al. (2016) identified, cloned, characterized, and silenced the *AaC4H* gene in *A. annua* with the assumption that the elevated internal cinnamic acid caused by knockdown may increase the artemisinin yield. *Cinnamate-4-hydroxylase* (C4H) converts *trans*-cinnamic acid to *p*-coumaric acid in the phenylpropanoid/lignin biosynthesis pathway. *AaC4H* knockdown resulted in the accumulation of *trans*-cinnamic acid with the reduction of *p*-coumaric acid, total phenolics, anthocyanin, C4H, and phenylalanine ammonia lyase activities in the plant but an increase in SA and artemisinin. SA was reported earlier to be inducing the artemisinin yield. This report demonstrates the link between the phenylpropanoid/lignin pathway and the artemisinin pathway through SA, triggered by accumulation of *trans*-cinnamic acid because of the blockage at C4H. In *W. somnifera*, dunal cycloartenol synthase (*CAS*) is an important enzyme in the withanolide biosynthetic pathway, catalyzing cyclization of 2,3-oxidosqualene into cycloartenol. Smrati et al. (2016) cloned a full-length *CAS* from *W. somnifera* and designed three RNAi gene silencing constructs and a full-length overexpression construct, which were employed in the transformation of *W. somnifera*. The analysis showed that the expression of *WsCAS* gene was considerably downregulated in stable transgenic-silenced *Withania* lines compared with the nontransformed control, and withanolide content was greatly reduced in silenced lines. The 5′ inverted repeat (IR) construct (WsRNAi 1) gave a higher silencing efficiency in the range from 93.3% to 98.9% than the middle (WsRNAi 2) and 3′ IR (WsRNAi 3) construct showing the downregulation mediated by RNAi of *WsCAS*. Transgenic plants overexpressing *CAS* gene displayed an enhanced level of *CAS* transcript and withanolide content compared to nontransformed controls. Mahmoud and Croteau (2001) achieved transgenic peppermint (*Mentha*×*piperita* L.) with a homologous sense version of the 1-deoxy-D-xylulose-5-phosphate reductoisomerase cDNA and with a homologous antisense version of the menthofuran synthase cDNA. Regenerated transgenic plants with normal appearance and development expressed the reductoisomerase transgene strongly and constitutively, and accumulated substantially more essential oil (about 50% yield increase). Reductoisomerase mRNA or enzyme activity was not detected in transgenic plants deficient in chlorophyll production, which recorded slow growth and less essential oil indicating co-suppression of the reductoisomerase gene. Transformed plants with the antisense version

of the menthofuran synthase cDNA were normal in appearance but produced less than half of undesirable monoterpene oil component than did wild-type mint. The study showed that the alteration of the mevalonate-independent pathway for the supply of terpenoid precursors improved flux through the pathway leading to increased monoterpene production, and antisense manipulation of a selected downstream monoterpene biosynthetic step led to improved oil composition.

In the case of *Eschscholzia californica*, RNAi-mediated suppression of berberine bridge-forming enzyme resulted in accumulation of reticuline which is the precursor of isoquinoline alkaloids like morphine, codeine, and beberine. Sanguinarine, an end-product of this pathway, was considerably reduced. However, laudanine, a methylated derivative of berberine, accumulated in the transgenic plants (Fujii et al., 2007). In medicinal *S. miltiorrhiza* phenylalanine ammonia-lyase (PAL) catalyzes the first step in the phenylpropanoid pathway and is critical in the production of rosmarinic acid and its derivatives. Genetically engineered PAL-suppressed salvia plants with RNAi construct exhibited several unusual phenotypes such as stunted growth, delayed root formation, altered leaves, and reduced lignin deposition. PAL-suppressed lines expressed decreased production of total phenolics by 20%–70% and reduced rosmarinic acid and salvianolic acid B, which are major water-soluble pharmaceutical ingredients. Downregulation of PAL also affected the expression of C4H, 4CL2, and TAT, which are related genes in the rosmarinic acid pathway (Song and Wang, 2011). Examples for RNAi-mediated gene silencing for bioactive products in medicinal plants are presented in Table 12.4.

Through rapid progress made in molecular biology, RNAi has become the preferred technology and has wider applications in the areas of developing plants with abiotic and biotic stress resistance that are rich in nutrition and devoid of toxins. This technology has proved very useful in the study of gene

Table 12.4 RNAi-Mediated Gene Silencing in Medicinal Plants

SI No.	Plant	Product	Enzyme	References
1	*Papaver somniferum*	Codeine and morphine	Codeinone reductase (COR)	Allen et al. (2004)
		Morphinan alkaloids	Salutaridinol 7-*O*-acetyltransferase	Allen et al. (2008) Kempe et al. (2009)
		Morphine, codeine, sanguinarine	Berberine bridge enzyme (BBE) and *N*-methyl coclaurine 3′-hydroxylase (CYP80B1)	Frick et al. (2004)
2	*Mentha×piperita*	Menthofuran	Cytochrome P450 (+) menthofuran synthase	Mahmoud and Croteau (2001)
		Limonene	Limonene-3-hydroxylase gene	Mahmoud et al. (2004)
3	*Catharanthus roseus*	Tryptamine	Tryptophan decarboxylase	Runguphan et al. (2009)
4	*Salvia miltiorrhiza*	Rosmarinic acid	Phenylalanine ammonia-lyase	Song and Wang (2011)
5	*Panax ginseng*	Ginsenoside	Dammarenediol synthase gene	Han et al. (2006)
6	*Withania somnifera*	Withanalides	Cycloartenol synthase	Smrati et al. (2016)
7	*Artemisia annua*	Artemisinin	Squalene synthase	Zhang et al. (2009a,b)
		Salicylic acid and artemisinin	*Cinnamate-4-hydroxylase*	Kumar et al. (2016)

functions and manipulating plants to create novel characteristics. RNAi might prove useful for studies of the production of important biomedical products by medicinal plants, which in turn can provide novel and rapid applications with potential benefits such as development of gene-specific therapeutics or a complete understanding of genomics. Compounds such as ginsenoside, morphinan alkaloid, and (*S*)-reticuline may be produced from RNAi-incorporated medicinal plants in the near future. Drawbacks such as off-target effects, nontarget effects, and the impact of genetic mutations and polymorphisms need to be addressed. To facilitate gene silencing expression, time-specific and inducible promoters active in the target tissues, which could when required minimize "off-target" effects, are needed (Auer and Frederick, 2009; Borgio, 2009; Jagtap et al., 2011).

Agrobacterium-mediated transformation with either a rice chitinase gene or a thaumatin-like protein gene to enhance tolerance to fungal diseases in American ginseng (*Panax quinquefolius* L.) has been demonstrated (Chen and Punja, 2002; Punja and Chen, 2003). Transgenic plantlets were developed from somatic embryos of confirmed transgenic lines and the expression of the chitinase and TLP genes was demonstrated by Western analysis. In *Mentha* spp. (mints), biosynthetic pathways have been engineered to modify essential oil production in the trichomes and to enhance the resistance of the plants to fungal infection and abiotic stresses (Veronese et al., 2001).

6. GENETIC ENGINEERING FOR ABIOTIC STRESS RESISTANCE

Successful application of transformation for conferring herbicide resistance in *Atropa belladonna* was reported. Transgenic plants were regenerated from transformed hairy roots with an Ri plasmid binary vector containing the *bar* gene encoding phosphinothricin acetyltransferase. The transgenic plants showed resistance toward bialaphos and phosphinothricin with normal production of tropane alkaloids. The transgenic plants had short internodes, wrinkly, narrow, and small leaves, and multiple branches (Saito et al., 1992). Transgenic *P. ginseng* plants resistant to the herbicide Basta were produced through *Agrobacterium*-mediated transformation with the *phosphinothricin acetyl transferase* (PAT) gene. Integration of the transgene into plants was confirmed by PCR and Southern analyses. Transgenic ginseng plantlets developed from somatic embryos were transferred to soil and they exhibited resistance to the herbicide Basta (Choi et al., 2003). An *A. tumefaciens* binary vector with isopentenyl transferase gene (*ipt*) was used to transform *A. annua* L. The *ipt* gene integration was confirmed through reverse transcrition PCR and Northern blotting analyses. Transgenic plants expressed elevated cytokinins (iPA and iP) up to two- to threefold, chlorophyll content by 20%–60%, and artemisinin by 30%–70% compared with the control plants, respectively. A direct correlation was reported between the contents of cytokinins, chlorophyll, and artemisinin (Geng et al., 2001). Liu (2015) produced herbicide-resistant transformants of *S. miltiorrhiza* through *Agrobacterium*-mediated genetic transformation. Leaf discs of *S. miltiorrhiza* were infected with *A. tumefaciens* EHA105 harboring pCAMBIA 3301containing an intron-containing *gus* reporter and a bar selection marker. By employing two-step selection with herbicide resistance and *gus* expression an efficient screening system for the transformed plant containing bar gene was developed. It was also identified that 0.6 mg/L phosphinothricin is suitable for selecting putatively transformed callus because non-transformed callus growth was effectively inhibited under this concentration. The transgenic *S. miltiorrhiza* plants were tolerant to the herbicide Basta demonstrating successful transformation of the bar gene conferring herbicide resistance.

Salinity and drought are important abiotic stresses limiting plant growth and development of MAPs. Han et al. (2007) reported transgenic *S. miltiorrhiza* for salt and drought resistance. *S. miltiorrhiza* was transformed with *TaLEA1*, a gene encoding a late-embryogenesis-abundant protein, which was cloned from wheat. Seven transgenic lines were obtained after kanamycin (50 mg/L) screening. Six positive lines were obtained by PCR amplification, and after four additional generations, one stable line was obtained by Southern hybridization. Transgenic plants had better growth states than control plants on medium containing 1% NaCl and 8% PEG-6000, which demonstrated that *TaLEA1* played an important role in increasing the salt and drought tolerance of *S. miltiorrhiza.* Late embryogenesis abundant (LEA) proteins are a group of proteins associated with tolerance to water-related stress. Wu et al. (2014) reported that overexpression of *SmLEA* enhances salt and drought tolerance in *E. coli* and *S. miltiorrhiza*. *SmLEA* belongs to group LEA14, which is involved in dehydration response, and cloned *SmLEA* was overexpressed in *S. miltiorrhiza* showing faster root elongation and a lower malondialdehyde concentration than the empty vector control plants cultured on MS media supplemented with 60 mM NaCl or 150 mM mannitol. An *SmLEA*-overexpressing transgenic experienced a less rapid rate of water loss, had greater superoxide dismutase activity, and higher glutathione concentration under either salinity or drought. In *A. annua*, transgenic plants were developed with NAC transcription factor gene *AaNAC1*. When *AaNAC1* was overexpressed, artemisinin and dihydroartemisinic acid were increased to 79% and 150%, respectively, with an enhanced expression of artemisinin biosynthetic pathway genes. The transgenic plants also exhibited increased tolerance to drought and resistance to *Botrytis cinerea* indicating that *AaNAC1* can be used in transgenic breeding in *A. annua* (Zongyou et al., 2016).

7. FUTURE PROSPECTS

PSMs with their great chemical diversity, varied biological functions, and pharmacological activities constitute interesting and important research. Metabolic engineering and biotechnological approaches can provide an alternative production system for PSM compounds for commercial exploitation. These approaches hold great promise and have not been fully exploited in MAPs as in the case in other crops. Genetic transformation and metabolic engineering techniques require an understanding of the regulation of the secondary metabolite pathways involved and identification of enzymes and genes, knowledge of which is very limited in MAPs. RNAi might prove to be useful in the development of gene-specific therapeutics or a complete understanding of genomics. Increased focus on time-specific and inducible promoters active in the target tissues and generation of additional RNAi protocols for genome-wide screening might aid in successful PSM production. Obstacles such as gene silencing, unpredictable results because of the complex network of genes, production of stable and desirable concentrations of secondary metabolites, and ethical and biosafety issues concerning transgenics need to be addressed. Continued efforts to identify more functional genes and enzymes that control secondary metabolite production and a multidisciplinary approach with integration of information from genomics, proteomics, metabolomics, and synthetic biology are essential for rapid progress and successful and economically viable biotechnological production of secondary metabolites. Superior materials for the breeder can be developed modifying complex secondary metabolite pathways through advanced genomic approaches, efficient methods for gene isolation, and genetic transformation.

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CHAPTER

BIOSAFETY AND BIOREGULATORY MECHANISMS FOR TRANSGENIC CROPS

13

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1. INTRODUCTION

Anxiety over the probable ill effects that genetically modified (GM) foods and organisms can impart on the human system as well as on the ecosystem has invited burgeoning discussions ever since the birth of the genetic engineering concept itself. The unending questions mainly revolve around the effects of such crops on the environment, the human body, microbes, weeds, flora, and related species. Perhaps the introduction of more profit-motivated, monopoly-intended, technically rigorous strategies such as traitor and terminator technologies have forced the world to ascertain the need for regulating the extent of genetic modifications that have to be imposed on living beings. Thus biosafety regulations have been initiated across the world to systematically assess the technology involved, the extent of genomic modifications, and the possible impacts on the environment and other living forms. These goals were initially achieved through the imposition of Acts and rules, which were the building blocks of safety measures and subsequent rigorous protocols to ensure safety for all spheres. This chapter details the Acts and regulations and the subsequent biosafety and bioregulatory procedures in India, the United States, the European Union, and Canada.

2. DEFINING BIOTECHNOLOGY, BIOSAFETY, AND RISKS

Biotechnology is defined as the application of scientific and engineering principles to the processing of materials by biological agents to provide goods and services (OECD, 1982). Biosafety is the protected status of human and animal health and the environment from the possible adverse effects of the products of modern biological techniques and the consortium of precautionary approaches for its assessment and assurance.

Environmental risk assessment is defined by Directive 2001/18/EC of the European Union as the evaluation of risks to human health and the environment, whether direct or indirect, immediate or delayed, which experimental deliberate release or deliberate release by placing genetically modified organisms (GMOs) on the market may pose. Directive 2001/18/EC details the scientific elements and major strategies to be used in the risk assessment of GMOs (European Parliament and Council, 2001).

Genetic Engineering of Horticultural Crops. http://dx.doi.org/10.1016/B978-0-12-810439-2.00013-1

Risk analysis, the path for biosafety, consists of risk assessment, risk management, and risk communication. Risk assessment is carried out by scientists and navigated in physical space, whereas risk management is entrusted to policymakers in decision space and risk communication is with the public in perpetual space. Steps in environmental risk assessment include identification of characteristics that may cause adverse effects, evaluation of the potential consequences of each adverse effect if it occurs, evaluation of the likelihood of the occurrence of each identified potential adverse effect, estimation of risk posed by each identified characteristic of the GMO, application of management strategies for risks from the deliberate release or marketing of the GMO, and determination of the overall risk of the GMO (König et al., 2004). Through paragraph 10 of the Revised Guidelines for Safety in Biotechnology (1994), the Department of Biotechnology (DBT), India, suggests 19 factors that should be set as criteria for risk assessment (discussed under Section 4.1.5).

3. RISKS ASSOCIATED WITH GM CROPS AND ITS ANALYSIS

Risks associated with GM crops are the potential to transfer the newly introduced genetic material to other crops or weeds via cross-pollination or to other organisms through horizontal gene transfer (depending on the transferred trait such gene transfer might not present a hazard), potential to confer an ecological fitness advantage to the GM crop causing persistence and invasiveness (superweeds), potential of reversing downregulation of a naturally occurring hazardous trait, potential for production of substances that are toxic or allergenic to human beings or other species, potential to negatively influence decomposition processes in the soil and thus cause changes in nitrogen and carbon recycling, and potential that genetic modification leads to unintended effects, e.g., influencing other genes of the organisms, which might lead to unexpected hazards (Bock, 2005).

3.1 RISK ASSESSMENT

3.1.1 Assessing Environmental Risks

3.1.1.1 Gene Escape

Gene escape could happen through vertical gene transfer or horizontal gene transfer. Vertical gene transfer refers to the transfer of genes from parents to offspring, through the pollen fertilizing the female plant belonging to the same or related genera. Horizontal gene transfer represents nonsexual gene transfer, more frequently between the microorganisms or rarely between plants and microbes. Since microbes have the capability to integrate these genes from plants into their genome, though the event may be extremely rare, this has to be viewed seriously in the context of the antibiotic resistance genes used in the transformation protocol. As of now there are many strategies that avoid the use of these marker genes through recombinase enzymes and specialized vectors (Yoder and Goldsbrough, 1994; Ow, 2001; Krens et al., 2004; Afolabi, 2007; Tuteja et al., 2012). Furthermore, most breeders adopt large-scale crossing of the transgenic with the parent non-GM plants to select the recombinant plants with transgenes devoid of antibiotic resistance genes. However, the presence of the marker has to be confirmed through polymerase chain reaction (PCR)-based techniques using the flanking primers for these genes. Gene escape by vertical transfer could be easily assessed by random sampling of seeds from the vicinity of the lineage-specific transcripts (LSTs) and subjecting the seeds to PCR analysis using the gene-specific primers.

The initial data generated should be submitted to the regulatory authorities, with respect to gene escape, and should include details on: economic, agronomic, and other benefits and rationale behind developing such a transgenic; source and sequence of transgene; promoter sequence; promoter characteristics (e.g., tissue specific); enhancers and other elements in the construct; copy number; event position; tracking bracket molecular markers; marker genes used; regulatory mechanism used in the expression cassette; diagram of the expression cassette to describe fully the marker genes used; cell lines used for shuttling and amplification of the cassette; cloning strategy; characteristic of expression vectors; stability of transgene over generations; level of expression in each tissue; backcrossing methods; seed-setting characteristics; germination rates; phenotypic characteristics; target gene efficacy tests; unanticipated genomic or transcriptomic changes, if any; characteristics of new proteins and secondary products; homology models of new protein/s with identified major allergens/toxins; toxicity and allergenicity, if any, during handling; and several other details of the procedure, and the consequences thereof, used for production of the transgenic plants. Planned field experiments with transgenic plants are permitted only after a stepwise (laboratory to growth chamber and greenhouse) evaluation, either in India or elsewhere, to generate data on all the points submitted by the party.

3.1.1.2 Aggressiveness/Invasiveness and Weediness

The invasiveness of the conventional crop and assessment whether the transgene has helped the GMO to be more invasive in a particular situation has to be performed. It is generally presumed that the transgenes coding for herbicide tolerance and abiotic and biotic stress tolerances shall make the crop more aggressive under adverse conditions. To assess the aggressiveness and weediness of a transgenic, primarily its rate of germination and vigor will be compared with the near-isogenic nontransgenic counterparts. Invasiveness should be assessed in terms of the postharvest survival of the transgenic in the field/vicinity under adverse conditions, in comparison with the non-GE parent.

3.1.1.3 Effect on Nontarget Organisms

Rigorous studies on any kind of toxic effects on nontarget species should be done. For example, in the case of *Bt* cotton, the toxic effects on nontarget species such as sucking pests (aphids, jassids, whitefly, mites) and the beneficial insects (ladybird beetle, honeybees, spiders) were analyzed.

3.1.1.4 Unintended Residues in the Environment

The secretion and spread of unintended chemicals into the soil and air by genetically engineered (GE) plants has to be thoroughly studied. Similarly, outcrossing of *Bt* crops with the related weeds, leading to the development of *Bt* weeds, shall be a great disadvantage. In the case of *Bt* cotton, the possible risk of accumulation of *Bt* toxin in the soil and its influence on soil microflora was assessed. The half-life of Cry1Ac protein in plant tissues was 41 days, comparable to the degradation rates reported for microbial formulations of *Bt*. Thus there was no significant difference in the population of microbes and soil invertebrates among *Bt* and non-*Bt* samples.

3.1.2 Food Safety

The Indian Council of Medical Research (ICMR) has laid out the methods for food safety analysis through "Guidelines for the Safety Assessment of Foods Derived from Genetically Engineered Plants in India (2008; http://envfor.nic.in)." For evaluating food safety of the GE crop, extensive studies on the compositional analysis, allergenicity studies, toxicological studies, studies on the presence of transgene

protein in food and feed, and feeding studies on cows, buffaloes, poultry, and fish have to be completed.

3.1.2.1 Compositional Analysis

Any possible change in the composition of the GE and non-GE produce has to be assessed with respect to the proteins, carbohydrates, oil composition, energy content (calories), and ash content. The "Guidelines for the Safety Assessment of Foods Derived from Genetically Engineered Plants in India (2008)" directs that appropriate analyses must be performed on all parts of the plant that may be used as food in India (discussed under Section 4.1.9).

1. Proximate composition, e.g., ash, moisture content, crude protein, crude fat, crude carbohydrate;
2. Content of true protein, nonprotein nitrogenous material (e.g., nucleic acids and aminoglycosides), amino acid profile [unusual amino acids should be determined if their presence is suspected (e.g., D-amino acids from bacterial proteins)];
3. Quantitative and qualitative composition of total lipids, i.e., saponifiable and nonsaponifiable components, complete fatty acid profile, phospholipids, sterols, cyclic fatty acids, and known toxic fatty acids;
4. Composition of the carbohydrate fraction, e.g., sugars, starches, chitin, tannins, nonstarch polysaccharides, and lignin;
5. Qualitative and quantitative composition of micronutrients, i.e., significant vitamin and mineral analysis;
6. Presence of naturally occurring or adventitious antinutritional factors, e.g., phytates, trypsin inhibitors, etc.;
7. Predictable secondary metabolites, physiologically active (bioactive) substances, and other detected substances.

Characterization of the product by techniques such as high-performance liquid chromatography, gas chromatography mass spectrometry, and conventional analytical methods is considered appropriate. The statistical significance of any observed differences will be assessed in the context of the range of natural variations for that parameter to determine its biological significance. If the composition of the GM food is judged not to be nutritionally equivalent to that of its parent and commercial varieties, i.e., significant differences (statistical and biological) exist in the nutrient data, additional nutritional data may be required on a case-by-case basis.

3.1.2.2 Allergenicity Studies on Proteins

Allergenicity studies have to be completed on any model organism feeding on GM produce. The primary consideration in allergenicity assessment of a newly expressed novel protein in a food derived from GE plant is the prevention of unexpected exposure of sensitized individuals to food allergens. This includes the assessment of the potential for foods containing such novel proteins to cross-react with known food allergens or to lead to the development of de novo hypersensitivity. In the case of *Bt* cotton, feeding of brown Norway rats with the seeds has shown no significant difference in feed consumption, weight gain, and general health. Furthermore, there was no significant change in endogenous allergens for *Bt* seed compared to non-*Bt* seed. The "Guidelines for the Safety Assessment of Foods Derived from Genetically Engineered Plants in India (2008)" directs the following.

3.1.2.2.1 Assessment Strategy for Allergenicity-Related Risks Genes derived from known allergenic sources should be assumed to encode an allergen unless scientific evidence demonstrates otherwise.

The initial steps in assessing possible allergenicity of any newly expressed proteins are the determination of:

1. The source of the introduced protein.
2. Any significant similarity between the amino acid sequence of the protein and that of known allergens. As there is no single test that can predict the likely human IgE response to oral exposure, the first step to characterize newly expressed proteins should be the comparison of the amino acid sequence and certain physicochemical characteristics of the newly expressed protein with those of established allergens in a weight of evidence approach.
3. Its structural properties, including but not limited to, its susceptibility to enzymatic degradation, heat stability, and/or acid and enzymatic treatment.
4. Isolation of any newly expressed proteins from the GE plant, or the synthesis or production of the substance from an alternative source, in which case the material should be shown to be structurally, functionally, and biochemically equivalent to that produced in the GE plant.
5. The choice of the expression host, since posttranslational modifications allowed by different hosts (i.e., eukaryotic vs. prokaryotic systems) may have an impact on the allergenic potential of the protein.

3.1.2.2.2 Source of the Protein Allergenic sources of genes are defined as those organisms for which reasonable evidence of IgE-mediated oral, respiratory, or contact allergy is available. Knowledge of the source of the introduced protein allows the identification of tools and relevant data to be considered in the allergenicity assessment. These include:

1. The availability of sera for screening purposes;
2. Documented type, severity, and frequency of allergic reactions;
3. Structural characteristics and amino acid sequence; and
4. Physicochemical and immunological properties (when available) of known allergenic proteins from that source.

3.1.2.2.3 Amino Acid Sequence Homology Amino acid sequence homology comparisons need to be used to assess the extent to which a newly expressed protein is similar in structure to known allergens to determine whether that protein has allergenic or cross-reactivity potential. Sequence homology searches comparing the structure of all newly expressed proteins with all known allergens are required to be undertaken. Searches should be conducted using various algorithms such as FASTA or BLASTP to predict overall structural similarities.

3.1.2.2.4 Pepsin Resistance Resistance to pepsin digestion has been observed in several food allergens; thus a correlation exists between resistance to digestion by pepsin and allergenic potential. The resistance of a protein to degradation in the presence of pepsin under appropriate conditions indicates that further analysis has to be conducted to determine the likelihood of the newly expressed protein being allergenic. A consistent and well-validated pepsin degradation protocol may enhance the utility of this method and is strongly recommended. However, it is recognized that other enzyme susceptibility protocols also exist and these may be used with adequate justification.

3.1.2.2.5 Specific Serum Screening For those proteins that originate from a source known to be allergenic, or have sequence homology with a known allergen, testing in immunological assays needs to be performed where sera are available. Sera from individuals with a clinically validated allergy to

the source of the protein can be used to test the specific binding to IgE class antibodies of the protein in vitro assays. A critical issue for testing will be the availability of human sera from sufficient numbers of individuals. The quality of the sera and the assay procedure need to be standardized to produce a valid test result.

3.1.2.3 Toxicological Studies

Toxicological testing is required for substances of unknown safety that are introduced to the food supply. The in vitro nucleic acid techniques enable the introduction of DNA that can result in the synthesis of new substances in plants. These include the protein expression product and other substances, which may be generated as a result of enzymatic activity of the protein expression product. The new substances can be conventional components of plant foods such as proteins, fats, carbohydrates, and vitamins, which are novel in the context of that GE plant.

ICMR "Guidelines for the Safety Assessment of Foods Derived from Genetically Engineered Plants in India (2008)" directs the following analyses:

1. The chemical nature and function of the newly expressed substance;
2. The concentration of the substance in the edible parts of the GE plant, including variations and mean values;
3. Current dietary exposure and possible effects on population subgroups, if applicable;
4. Information, if any, that genes coding for known toxins or antinutrients present in the donor organisms are not transferred to GE plants that do not normally express those toxins or antinutrient characteristics.

This assurance is particularly important in cases where the GE plant is processed differently from a donor plant, since conventional food-processing techniques associated with the donor organisms may deactivate, degrade, or eliminate antinutrients or toxicants. Toxicology studies are not considered necessary where the substance or a closely related substance has been consumed safely in food at equivalent intakes or where the new substance is not present in the food. Otherwise, the use of conventional toxicology studies on the new substance will be necessary. This may require the isolation of the new substance from the GE plant, or the production of the substance from an alternative source, in which case the material has to be shown to be biochemically and functionally equivalent to that produced in the GE plant.

In the case of proteins, the assessment of potential toxicity needs to focus on amino acid sequence similarity between the protein and known protein toxins and antinutrients (e.g., protease inhibitors, lectins) as well as stability to heat or processing and to degradation in appropriate representative gastric and intestinal model systems.

Proteins that are enzymes have never been shown to be direct-acting carcinogens, mutagens, teratogens, or reproductive toxicants (Pariza and Foster, 1983). Hence it is generally not necessary to test proteins for these toxicological endpoints when exposure occurs by the oral route. Protein toxins act through acute mechanisms after the administration of doses in the nanogram-to-milligram per kilogram body weight range. Therefore acute oral toxicity studies using gram per kilogram body weight doses of the novel protein are appropriate for assessing their potential toxicity. A negative result using doses in the gram per kilogram body weight range together with evidence that the protein is digested to small peptides and amino acids would provide assurance that the protein is not a toxin and is digested to amino acids, similar to other dietary proteins.

Potential toxicity of nonprotein substances that have not been safely consumed in food should be assessed on a case-by-case basis depending on the identity and biological function in the plant of the substance and dietary exposure. Studies on metabolism, toxicokinetics, subchronic toxicity, chronic toxicity/carcinogenicity, and reproduction and development toxicity may be performed according to the traditional toxicological approach.

3.1.2.4 Unintended Residues/Nutritional Modifications

The level of presence of unintended residues, especially the protein product of the transgene in the edible part of the GE crop, has to be assessed. For example, in *Bt* cotton the presence of Cry1Ac protein in the cotton seed oil was assessed and found absent. When the modification results in a food product with a composition that is significantly different from its conventional counterpart (e.g., vegetable oil), it may be appropriate to use additional conventional foods or food components whose nutritional composition is closer to that of the food derived from the GE plant.

Nutritional changes to a specific food may have a greater impact in some geographical areas or in some cultural populations than in others because of variations in food consumption patterns. The nutrient and the populations affected need to be identified. Some foods require additional testing. For example, animal feeding studies may be warranted for foods derived from GE plants if changes in the bioavailability of nutrients are expected or if the composition is not comparable to conventional foods. Also foods designed for health benefits may require specific nutritional, toxicological, or other appropriate studies. If the characterization of the food indicates that the available data are insufficient for a thorough safety assessment, properly designed animal studies could be requested on the whole food.

3.1.2.5 Feeding Studies

Feeding studies with GE produce have to be completed on cows, buffaloes, poultry, and fish.

3.1.3 Risk of Acquired Resistance in Pests to the Transgene Proteins

Acquired resistance is said to occur when a particular pest, over a period of time, obtains the ability to resist a particular protein product to which it was previously susceptible. This can result from the mutation of genes involved in normal physiological processes and cellular structures, from the acquisition of foreign resistance genes, or through the evolution of resistant strains through natural selection or from a combination of all these mechanisms. The probability for systemic acquired resistance in the pest population, which is the resistance response that occurs following localized exposure to a toxin, also cannot be ruled out. Thus it may be anticipated that the cultivation of a pest-resistant transgenic cultivar, over many years, leads to the development of strains that withstand the toxins of transgenes.

To prevent the buildup of a resistant pest population, it is recommended to plant sufficient nontransgenic plants (~20% of the transgenic population) to serve as a refuge for susceptible pests. The refuge strategy is designed to ensure that the susceptible pests will be available to mate with resistant ones. Available genetic data indicate that susceptibility is dominant over resistance. Therefore the offspring of these matings would most likely be susceptible, thus mitigating the spread of resistance in the population.

Furthermore, it must be assured that when the cultivar is released for commercial cultivation, the seeds are supplied by mixing with 20% nontransgenic near-isogenic seeds.

3.1.4 Other Safeguards

Another important safeguard to be followed is the assurance that the GE cultivar under trial does not harbor the terminator/traitor genes.

3.2 RISK MANAGEMENT

Complete biosafety or no risk is unachievable through risk assessment and management strategies. Bock (2005) suggests few risk management strategies regarding GM crops, which primarily include confinement strategies (permitting GM crops to be grown in protected greenhouses only), restricted use (restricting GM crops to certain geographical areas), monitoring following experimental/commercial release or monitoring GM food to identify predicted or unforeseen effects, adherence to guidelines and technical support (e.g., introduction of refuge areas to minimize resistance development of pests or advice for good agricultural practices such as crop rotation and weed control to avoid weediness of GM crops and GM volunteer plants), and record keeping [as foreseen in Regulation (EC) 1830/2003 on traceability of GM crops and food] (European Parliament and Council, 2003a). Male sterility, if introduced in GM cultivars, shall be an added advantage.

3.3 RISK COMMUNICATION

Unbiased communication of risk to all the parties involved is mandatory. Key rules, identified by the Scientific Steering Committee of the European Commission (EC), include completeness of information, public access to documentation, transparency of discussions and motivations, frank acknowledgment of the various positions and contrasting views including speculations, clarity in wording and accuracy in use of specific expressions, recognition of different interests and stakeholders, and recognition of social, cultural, and ethical issues (Bock, 2005).

4. BIOREGULATORY MECHANISMS FOR GM CROPS IN INDIA

4.1 ACTS AND GUIDELINES

4.1.1 Cartagena Protocol on Biosafety (2003)

Although many countries have enacted national biosafety legislations to ensure the safe use of GMOs and products thereof, biotechnology being a global industry and GMOs traded across borders, international rules are needed as well. The Cartagena Protocol on Biosafety (CPB) is an attempt to produce a globally harmonized regime for biosafety under the Convention of Biological Diversity (CBD). The protocol, named after the Colombian city where the final round of talks was launched, sets out a comprehensive regulatory system for ensuring the safe transfer, handling, and use of living modified organisms (LMOs) subject to transboundary movement. In everyday usage, LMOs are considered to be the same as GMOs although definitions and interpretations vary widely. The protocol deals primarily with LMOs that are to be intentionally introduced into the environment (such as seeds, trees, or fish) and with GM farm commodities (such as corn and grain used for food, animal feed, or processing). It does not cover pharmaceuticals for humans addressed by other international agreements and organizations or products derived from LMOs, such as cooking oil from GM corn.

The CPB, drafted on January 29, 2000 and ratified by India on January 23, 2001, entered into force on September 11, 2003 (UNEP, 2003). The international legally binding instrument is conceived on the provisions of Article 19.3 of the CBD and its objective is to contribute to ensuring an adequate level of protection in the field of the safe transfer, handling, and use of LMOs resulting from modern biotechnology, which may have adverse effects on the conservation and sustainable use of biodiversity, taking also into account risks to human health, and specifically focusing on transboundary movements. Scope of the protocol applies to the transboundary movement, transit, handling, and use of all LMOs that may have adverse effects on the conservation and sustainable use of biological diversity, taking also into account risks to human health.

The governing body of the protocol is the Conference of the Parties to the Convention serving as the meeting of the parties to the protocol. The main function of this body is to review the implementation of the protocol and make decisions necessary to promote its effective operation.

Parties are required to designate national institutions to perform functions relating to the protocol. Each party needs to designate one national focal point to be responsible on its behalf for liaison with the secretariat. Each party also needs to designate one or more competent national authorities, which are responsible for performing the administrative functions required by the protocol and which shall be authorized to act on its behalf with respect to those functions. In accordance with the foregoing requirement, the Ministry of Environment, Forest and Climate Change (MoEFCC) has been designated as the competent national authority. The National Focal Point for the CPB is the Joint Secretary, MoEFCC, and the biosafety clearing house (BCH) Focal Point is the Director, MoEFCC.

Major elements of the protocol include the Advance Informed Agreement (AIA, Article 7) procedure, risk assessments and risk management (Articles 15 and 16), handling, transport, and packaging identification (Article 18), information sharing and BCH (Article 20), capacity building (Article 22), socioeconomic considerations (Article 26), liability and redress (Article 27), monitoring and reporting (Article 33), compliance (Article 34), and public awareness and participation (Article 23). Other elements include export documentation and finance (Biosafety GMO Portal, 2016).

The AIA applies prior to the first international transboundary movement of an LMO for intentional introduction into the environment. Major steps involved are notification, risk assessment, and decision making. Pharmaceuticals, LMOs in transit, LMOs for contained use, and LMOs intended for direct use as food or feed or for processing, are exempted from the AIA.

Annex III of the protocol specifies risk assessment principles and scientific methodology for scientific, case-by-case, and transparent assessment. It also directs that the lack of scientific knowledge or scientific consensus should not necessarily be interpreted as indicating a particular level of risk, an absence of risk, or an acceptable risk.

Article 18 (paragraph 1) of the protocol imposes a general obligation on each party to set standards for safe handling, packaging, and transport of LMOs subjected to intentional transboundary movement, in consultation with other international bodies.

The BCH (http://bch.cbd.int/) facilitates exchange of information including laws, scientific data, risk assessments, decisions, etc. and assists parties to implement the protocol. Under the Intergovernmental Committee for the Cartagena Protocol on Biosafety (ICCP, established to undertake the preparations necessary for the first meeting of the parties to the protocol), a pilot phase of the BCH has been developed and is operational. In accordance with Article 20 of the CPB, the India BCH was launched in 2006.

Article 22 mandates parties to cooperate in capacity building relevant to the protocol, including development and strengthening of human resources and institutional capacities. Activities under the ICCP process included training in use of the BCH and development of a Roster of Experts. The MoEFCC successfully completed the Phase-I Capacity Building Project on Biosafety through Global Environment Facility (GEF)-World Bank assistance in June 2007. The MoEFCC has initiated the Phase-II Capacity Building Project on Biosafety through GEF-United Nations Environment Programme assistance. The Project Identification Form was approved by the GEF council in its meeting held in October 2008. The most pressing needs in general are the capacity to use and provide all required information to the BCH and the capacity to make informed decisions based on risk assessment and other factors.

4.1.2 Environment (Protection) Act (1986)

The Environment (Protection) Act (EPA) (1986) provides for the protection and improvement of the environment and for matters connected therewith (EPA, 1986). Environment includes water, air, and land and the interrelationship that exists among and between water, air, land, human beings, other living creatures, plants, microorganisms, and property. The EPA furnishes definitions for environment, environmental pollutant, environmental pollution, "handling" in relation to any substance, hazardous substance, and "occupier" in relation to a premise, and there are rules governing each in relation to environmental protection.

4.1.3 Ministry of Environment and Forests Rules (1989)

Ministry of Environment and Forests (MoEF) Rules 1989 include a set of rules governing the manufacture, use, import, export, and storage of hazardous microbes or GMOs or cells under the EPA (1986). It covers rules for all activities involving:

- Research and development of products containing GMOs/microorganisms and cells and correspondingly to any substance and products and food stuffs, etc., of which such cells, organisms, or tissues form part;
- Field and clinical trials, deliberate and unintended release, exchange or manufacture of organisms or cells carrying genomic modifications;
- Manufacture, import, and storage of gene technological products, new gene technologies in addition to cell hybridization and genetic engineering.

Approval and prohibitions under the Rules 1989 (Rules 7–11):

1. No person shall import, export, transport, manufacture, process, use or sell any hazardous microorganisms or GE organisms/substances or cells except with the approval of the Genetic Engineering Approval Committee (GEAC). This is applicable for:
   a. All activities involving research and development of products containing GMOs/HMOs including transgenic crops, pharma products, industrial products, and food and foodstuffs;
   b. Field trials on GMOs;
   c. Deliberate/unintentional release of GMOs.
2. The GEAC shall give directions to the occupier to determine or take measures concerning the discharge of microorganisms/GE organisms or cells mentioned in the schedule. Further details on MoEF Rules (1989) are available at http://envfor.nic.in/sites/default/files/geac/rules1989.pdf.

4.1.4 Recombinant DNA Safety Guidelines and Regulations (1990)

The DBT has issued these guidelines and set up the Recombinant DNA Advisory Committee for implementing these guidelines. Guidelines cover areas of research involving GE organisms, genetic transformation of green plants, animals, rDNA technology in vaccine development and large-scale production, and deliberate/accidental release of organisms, plants, animals, and products derived by rDNA technology (http://www.envfor.nic.in/divisions/csurv/geac/annex-5.pdf).

The guidelines define the physical containments (good laboratory practices, safety equipment, and laboratory design and facilities) and biological containments (which limit the infectivity of vector-to-specific hosts and control host–vector survival in the environment) required in rDNA experiments, identify the Institutional Biosafety Committee (IBSC) as the nodal point for interaction within the institution for implementation of guidelines, and define the duties of the IBSC, Review Committee on Genetic Manipulation (RCGM), and GEAC. Furthermore, these guidelines carry the directions to be followed during large-scale rDNA experiments and manufacture, the handling of biologicals produced by rDNA technology, and release of products to the environment and field experiments. These guidelines stipulate that prerelease tests of GE organisms in agricultural applications should include elucidation of genetic markers, host range, requirements for vegetative growth, persistence and stability in small plots, and experimental field trials for 2 years. Soil samples in experiments under controlled containment conditions should be tested for the absence of viable cells before disposal into the environment.

Additionally, these guidelines include good laboratory practices for rDNA experiments, strategies for aerosol minimization, and details on glasshouse containments for plant experiments. Glasshouse containment A is used when no plant pathogens are used, and where nonpathogenic vector systems and regeneration from single cells are used. Glasshouse containment B is recommended for experiments involving (1) genetically manipulated plant pathogens, including plant viruses, and (2) the growth of plants regenerated from cells transformed by genetically manipulated pathogen vector systems, which still contain the pathogen. Inspection of the glasshouse containment B facility by the IBSC will be required before approval. Application for recognition of a research facility to carry out genetic manipulation should be made to the Department of the Environment before the commencement of work.

4.1.5 Revised Guidelines for Safety in Biotechnology (1994)

These revised guidelines issued by the DBT cover the same areas of research as the Recombinant DNA Safety Guidelines and Regulations (1990), and, in addition, the guidelines for import and shipment of GMOs for laboratory research and large-scale use (http://jipmer.edu.in/wp-content/uploads/2013/12/guidelines_94.pdf). These guidelines classify the pathogenic microorganisms into four groups based on pathogenicity, modes of transmission and host range of the agent, availability of preventive treatments, capability to cause diseases to human, animal, and plants, and capability to cause epidemics. The functions of the IBSC, RCGM, GEAC, State Biotechnology Coordination Committee (SBCC), and District Level Committee (DLC) are defined and guidelines are detailed for: large-scale industrial processes and operations (operations requiring the handling of 20 L or more capacities are defined as large-scale operations); field trials of transgenic plants and their release to the environment; and postharvest handling of transgenic plants.

These guidelines also include the factors on which the risk assessment should be done. These factors are: geographical location, size, and nature of the site of release, physical and biological proximity to humans, and proximity to plants that might be cross-pollinated; target ecosystem and the predicted

effects on this ecosystem; method and amount of release and rate frequency and duration of application; monitoring capabilities and intentions, how many novel organisms can be traced; on-site worker safety procedures; contingency plans in the event of unanticipated effects of a novel organism; nature of the organism to be released; procedure used to introduce genetic modification; nature of any altered nucleic acid and extent to which this change is characterized; verification of the genetic structure of the novel organism; genetic stability of the novel organism; behavioral changes that the manipulation brings on the organism in its habitat; ability of the organism to form long-term survival forms; details of any target biota; growth and survival characteristics of the host organism and the changes that the manipulation brings; susceptibility to environmental and ecological stresses; effect of any modification to the organism designed to affect its ability to survive and transfer genetic material; and potential for transfer of inserted DNA to other organisms including methods for monitoring survival and transfer and methods to control or eliminate any superfluous organism or nucleic acid surviving in the environment or possibly in a product.

Apart from the guidelines for import and shipment of GMOs, quality control of biological produce by DNA technology is also included. Detailed appendices are provided on good laboratory practices, recommended biosafety levels for infectious agents, glasshouse containment conditions for plant experiments, and good industrial large-scale practices.

4.1.6 Revised Guidelines for Research in Transgenic Plants and Guidelines for Toxicity and Allergenicity Evaluation of Transgenic Seeds, Plants and Plant Parts (1998)

These revised guidelines are issued by the DBT (http://envfor.nic.in/sites/default/files/geac/annex-6.htm). These guidelines cover areas of recombinant DNA research on plants including the development of transgenic plants, their growth in soil for molecular and field evaluation, and import and shipment of GM plants for research use only. These guidelines detail the directions for statutory bodies such as the IBSC, RCGM, and GEAC and especially for the RCGM. These guidelines identify three categories of genetic engineering experiments on plants.

Category I—Routine rDNA experiments: Routine cloning of defined genes, defined noncoding stretches of DNA, and open reading frames in defined genes in *Escherichia coli* or other bacterial and fungal hosts that are generally considered as safe (GRAS) to humans, animals, and plants. A list of such microorganisms will be prepared by the RCGM and shall be made available to the principal investigator (PI) on request. This category involves experiments in the lab in a contained environment and includes the following: (1) routine cloning of defined DNA fragments of microbial, animal, and plant origin in GRAS organisms; (2) transfer of defined cloned genes into *Agrobacterium*; (3) use of defined reporter genes to study transient expression in plant cells to study genetic transformation conditions; and (4) molecular analysis of transgenic plants grown in vitro. Category I experiments need only intimation to the IBSC and should be performed using routine good laboratory practices.

Category II: Lab and greenhouse/net house experiments in a contained environment where defined DNA fragments nonpathogenic to humans and animals are used for genetic transformation of plants, both model species and crop species, and the plants are grown in the greenhouse/net house for molecular and phenotypic evaluation.

This category includes the following experiments: (1) transgenics with constitutive, tissue-specific, and chimeric promoters used for experimenting expression of defined DNA fragments; (2) marker genes extensively used in genetic transformation of plants in lab and greenhouse/net house experiments; (3) lab and greenhouse/net house experiments with plants with herbicide resistance

conferring genes; (4) lab and greenhouse/net house experiments with plants using heterologous genes that confer resistance to biotic and abiotic stresses; (5) lab and greenhouse/net house experiments with genes from plants, animals, and microbial sources that would confer resistance to plant pathogens; (6) lab and greenhouse/net house experiments with transgenics with genes for the production of antibodies; and (7) greenhouse/net house experiments on transgenics with transposable elements for gene tagging.

Category II and the foregoing experiments should be carried out in a greenhouse/net house, the specification of which is significantly stringent and is described in Appendix II of the guidelines to ensure arrest of transgenes within the contained facility, near complete isolation from the environment, and to prevent entry of insects. Permission for performing Category II experiments will be provided by the IBSC. The decision of the IBSC would be intimated to the RCGM before execution of the experiments and the RCGM would put this information on record.

Category III and above: High-risk experiments where the escape of transgenic traits into the open environment could cause significant alterations to the biosphere, the ecosystem, and plants and animals by dispersing new genetic traits, the effects of which cannot be judged precisely. All experiments conducted in greenhouse and open field conditions not belonging to the above Category II types would fall under Category III risks. Before conducting research under this category, the PI must obtain clearance from the RCGM notified by the DBT. The RCGM authorizes the PI to conduct limited field trails in multilocations in the country. The design of the trial experiments is either provided by the RCGM or it may approve the protocol designed by the PI. The protocol will seek answers related to animal and human health, and data should also be generated on economic advantage of the transgenics over the existing varieties. The RCGM can, if required, direct the applicants to generate toxicity, allergenicity, and any other relevant data on GE cultivars in appropriate systems. The RCGM may design or approve a protocol for conducting experiments to seek answers to the foregoing.

These guidelines discuss in detail the biosafety aspects of transgenic plants.

Necessary information on the donor organisms providing the target nucleic acids are: name of the donor organism with its identification characteristics with relevant reference to published information, if any; pathogenicity and toxicity characteristics to plants and animals; allergenicity characteristics to humans along with allergenic substances, wherever possible; and geographical origin of the organisms, their distribution pattern and survival mechanisms, and method of transfer of their genetic materials to other organisms.

Necessary information on the vector are: origin, identity, and habitat of the vectors used; sequence, frequency of mobilization, specificity, and marker genes, if any, present in the vectors; and abilities of the vectors to become established in other hosts (the hosts are also to be specified).

Necessary information on the transgenic inserts are: specific functions coded by the inserted nucleic stretches including the marker gene inserts; expression of the nucleic acid products and their activities/properties; toxicity of the expression products on the host plant, if any; and toxicity and allergenicity of the nucleic acid products to humans and animals.

Necessary information on the transgenic plants are: methods of detection of the transgenic plant in the environment; methods of detection and characterization of the escaped transgenic traits in the environment; toxicity and pathogenicity of the transgenic plants and their fruits to other plants in the ecosystem and the environment; possibility and extent of transgenic pollen escape and pollen transfer to wild near relatives; and the consequences to the environment and pathogenicity, toxicity, and allergenicity of the transgenic plants and their fruits to humans and animals.

Additionally, these guidelines discuss the steps for minimizing the risk arising from the limited release of transgenic plants, import and shipment of transgenic germplasm for research purposes, good laboratory practices (Appendix I), and a model plan (with figures) for the construction of a greenhouse/net house for experiments using transgenic plants (Appendix II).

4.1.7 Guidelines for Generating Preclinical and Clinical Data for rDNA Vaccines, Diagnostics and Other Biologicals (1999)

Though these guidelines are not directly influential in plant genetic engineering, they are worth mentioning in this discussion on the basis that transgenic plants are currently being attempted as a source for edible vaccines. These guidelines give a description on method of rDNA products such as host cells, gene construct, vector construction including source and diagram of the plasmid(s) used, all intermediate cloning procedures and transfection methods, description of the method of sequence verification (such as restriction enzyme mapping, PCR, etc.), description on physical, chemical, immunological, and biological identity, wherever applicable, potency (for recombinant vaccines and biologicals), general safety tests to be performed in mice and guinea pigs on each lot of rDNA vaccines/biologicals to detect extraneous toxic contaminants potentially introduced during production, data on sterility tests as per Indian Pharmacopeia guidelines, data on purity of recombinant products, description of constituent materials such as preservatives, etc., and data on stability of finished formulation, as per Indian Pharmacopeia guidelines (http://igmoris.nic.in/Files/CD_IBSC/Files/Biologicals.PDF). Detailed guidelines on preclinical trials, in vitro diagnostics for recombinant reagents or monoclonal antibodies and clinical trials (Phase I—human/clinical pharmacology, Phase II—exploratory clinical trials, and Phase III—confirmatory trials) are available at http://dbtbiosafety.nic.in/Files/CD_IBSC/Files/Biologicals.PDF.

4.1.8 Guidelines for the Conduct of Confined Field Trials of Regulated, Genetically Engineered Plants in India and Standard Operating Procedures (2008)

These include the guidelines for conduct (http://igmoris.nic.in), monitoring (http://igmoris.nic.in/), and standard operating procedures (SOPs) (http://igmoris.nic.in/) for field trials of GE plants.

4.1.9 Guidelines for the Safety Assessment of Foods Derived From Genetically Engineered Plants in India (2008)

These guidelines are issued by the ICMR (http://igmoris.nic.in/files%5CCoverpage.pdf). These guidelines describe the concept and framework of safety assessment, and safety assessment protocols for assessment of possible toxicity, assessment of possible allergenicity (proteins), assessment strategy, source of the protein, amino acid sequence homology, pepsin resistance, specific serum screening, other considerations, compositional analyses of key components, intended nutritional modifications, and unintended effects (discussed under Section 3.1.2 in this chapter). Additionally, these guidelines include Dossier Preparation Checklists (Appendix I) and a section on Codex Alimentarius Principles for the Risk Analysis of Foods Derived from Modern Biotechnology (Appendix II).

4.1.10 Protocols for Food and Feed Safety Assessment of GE Crops (2008)

The protocols for acute oral safety limit study in rats or mice, subchronic feeding study in rodents, protein thermal stability, pepsin digestibility assay, and livestock feeding study are developed by the DBT. Details are available at http://igmoris.nic.in/files/Coverpage1.pdf.

4.2 IMPLEMENTATION—AN INDIAN MODEL

Under the "Rules for Manufacture, Use, Import, Export and Storage of Hazardous Microorganisms/ Genetically Engineered Organisms or Cells, 1989 under the EPA (1986)," GMOs and products thereof are regulated articles in India, in view of potential risks to human health and the environment by their indiscriminate use.

4.2.1 Agencies Involved in India

4.2.1.1 Department of Biotechnology

The DBT (http://www.dbtindia.nic.in/), under the Ministry of Science and Technology, is the nodal department for biotechnology in the government of India. In more than a decade of its existence, the department has promoted and accelerated the pace of development of biotechnology in the country. Through several research and development projects, demonstrations, and creation of infrastructural facilities, a clear visible impact of this field has been seen. The department has made significant achievements in the growth and application of biotechnology in the broad areas of agriculture, health care, animal sciences, the environment, and industry. Along with the MoEFCC, the DBT is the implementing agency for biosafety regulations in the country.

4.2.1.2 Ministry of Environment, Forests and Climate Change

The MoEFCC (http://envfor.nic.in/) is the nodal agency in the administrative structure of the central government for the planning, promotion, coordination, and overseeing of the implementation of environmental and forestry programs. The ministry is also the nodal agency for the implementation of the CBD and CPB. The decisions of the GEAC are also hosted on this website.

4.2.1.3 Indian GMO Research Information System

The Indian GMO Research Information System (http://igmoris.nic.in/) is a database on activities involving the use of GMOs and products thereof in India. The primary purpose of this website is to make available objective and realistic scientific information relating to GMOs and products thereof under research and commercial use to all stakeholders including scientists, regulators, industries, and the public in general. It is also expected to promote collaborations and avoid duplication of work.

4.2.1.4 ICAR—National Research Centre on Plant Biotechnology

The National Research Centre on Plant Biotechnology has established an integrated database on postrelease risk assessment and monitoring transgenics to share the information from India and other countries. The database is divided into two main modules, i.e., Transgenics and GM Publications. The Transgenics module contains information on GM crops. It has been divided into several subdatabases, which have information on genes and promoters that are being used for the production of transgenic crops, unique features of transgenic crops, effect of GM crops on soil microflora, possible environmental hazards, and gene flow. The second module, GM Publications, contains published literature on biosafety and all issues related to GMOs.

4.2.1.5 Biotech Consortium India Limited

The Biotech Consortium India Limited (http://www.bcil.nic.in) provides linkages among research institutions, industry, government, and funding institutions to facilitate accelerated commercialization of biotechnology. The company is engaged in technology transfer, project consultancy, fund syndication, information dissemination, and workforce training and placement related to biotechnology.

4.2.2 Statutory Bodies Involved

4.2.2.1 Committee With Advisory Functions

4.2.2.1.1 Recombinant DNA Advisory Committee This committee shall review developments in biotechnology at national and international levels and shall recommend suitable and appropriate safety regulations for India in recombinant research, use, and applications from time to time. The committee shall function in the DBT (Ministry of Science and Technology, government of India). A panel of members nominated from the DBT forms the committee.

4.2.2.2 Committees With Approval/Regulatory Functions

4.2.2.2.1 Institutional Biosafety Committee This committee shall be constituted by an occupier or any person including research institutions handling microorganism/GE organisms and its activities are regulated by the IBSC guidelines by the DBT (DBT, 2011). The committee shall comprise the head of the institution, scientists engaged in DNA work, a medical expert, and a nominee of the DBT.

This statutory committee shall operate from a research organization in compliance with Rules (1989) and will be the nodal point for interaction of the organization with the government. It is the competent authority to which experiments that are likely to have biohazard potential and genetic manipulation of plants under containment have to be accountable. Accordingly, the IBSC shall conduct onsite evaluation, assessment, and monitoring of adherence to the biosafety guidelines with overall oversight of the regulatory process, and the decision taken by the RCGM depends on the approval of the IBSC. It also authorizes interstate exchange of etiologic agents, diagnostic specimens, etc. The IBSC will meet at least twice a year and provide half-yearly reports on ongoing projects to the RCGM. Additionally, the IBSC shall prepare an up-to-date onsite emergency plan as per the guidelines of the RCGM and make available copies to the DLC/SBCC and the GEAC. Revised Guidelines for Safety in Biotechnology (1994) assigns the IBSC with the additional functions of training personnel on biosafety, and instituting health monitoring programs (medical checkup including pathological tests) for laboratory personnel.

4.2.2.2.2 Review Committee on Genetic Manipulation This committee shall function in the DBT to monitor the safety-related aspects in respect of ongoing research projects and activities involving GE organisms/hazardous microorganisms. The RCGM shall include representatives of the (1) DBT, (2) ICMR, (3) Indian Council of Agricultural Research (ICAR), (4) Council of Scientific and Industrial Research, and (5) other experts in their individual capacities. The RCGM may also appoint subgroups. For example, "Revised Guidelines for Research in Transgenic Plants and Guidelines for Toxicity and Allergenicity Evaluation of Transgenic Seeds, Plants and Plant Parts (1998)" directs the RCGM to monitor, over a period of time, the impact of transgenic plants on the environment, greenhouse/net house experiments, and limited field trials in the open environment, through setting up a Special Monitoring cum Evaluation Committee (SMEC). Secretary, DBT and Secretary, DARE (Department of Agricultural Research and Education) shall jointly discuss and elect a chairman of the committee. Other members of the SMEC include three to four eminent plant biotechnologists nominated by the RCGM, two to three seed technologists nominated by the ICAR, up to two plant breeders nominated by the ICAR, up to two plant ecologists or environmentalists nominated by the RCGM, a National Bureau of Plant Genetic Resources (NBPGR) representative nominated by the ICAR, a representative of the MoEFCC nominated by the Chairman of the GEAC, and one member secretary from the RCGM.

Major duties assigned to the RCGM through rDNA Safety Guidelines (1990) include the following:

- The RCGM should meet at least twice a year and review the reports on all approved ongoing research projects involving high-risk category and controlled field experiments.
- To visit sites of experimental facilities periodically where projects with a biohazard potential are being pursued and also at a time prior to the commencement of the activity to ensure that adequate safety measures are taken as per the guidelines.
- To issue authorization for import and receipt of etiologic agents and vectors, germplasms, organelles, etc. needed for experimental work/training and research.
- Manuals of guidelines shall be published specifying procedures for regulatory processes with respect to activities involving GE organisms in research, use, and applications including industry with a view to ensuring environmental safety.
- All ongoing projects involving high-risk category and controlled field experiments shall be reviewed to ensure that adequate precautions and containment conditions are followed as per the guidelines.
- Procedures shall be laid down restricting or prohibiting the production, sale, importation, and use of such GE organisms of cells.

Revised Guidelines for Safety in Biotechnology (1994) direct that the funding agencies offering grant-in-aid for rDNA experiments must submit the list of approved projects to the RCGM annually and obtain clearance.

4.2.2.2.3 Genetic Engineering Approval Committee The GEAC is an interministerial committee functioning in the MoEFCC, government of India. The committee shall also be responsible for the approval of proposals relating to the release of GE organisms and products into the environment including experimental field trials. Additional to the Secretary to the Department of the Environment and Forests will be the chairman of the committee and a representative of the DBT will be the co-chairman. Representatives of concerned agencies and departments, namely, the Ministry of Industrial Development, the DBT, and the Department of Atomic Energy, will be members. Expert members of the GEAC include the Director-General of the ICAR, the Director-General of the ICMR, the Director-General of the Council of Scientific and Industrial Research, the Director of the General Health Services, Plant Protection Adviser, Directorate of Plant Protection, Quarantine and Storage, the Chairman of the Central Pollution Control Board, and three outside experts in individual capacities. Additionally, an official of the Department of the Environment and Forests will be the member secretary.

Recombinant DNA Safety Guidelines and Regulations (1990) assign the following duties to the GEAC:

- Field use permits for testing of genetically altered organisms, transgenic animals, plant material tested against pathogens, and products in the environment shall be issued by the GEAC, following regulatory guidelines.
- Issue licenses for large-scale planned release of organisms into the environment both for environmental and agricultural applications. Applications will be examined on a case-by-case basis and the validity of approval is for 4 years.
- Maintain regulatory control over exports and imports for large-scale use, and manufacture, process, sale, and use of any GE substances or cells including food stuffs and additives that contain rDNA products.

Revised Guidelines for Safety in Biotechnology (1994) assign the additional duty of issuing clearance for scale-up or pilot operations for facilities using GMOs on a case-by-case basis. For approval of large-scale industrial processes and operations, fundamental design of a strategy of the process, together with operational details as well as methods for proper neutralization/disposal of harmful by-products, should be submitted. The GEAC will implement regular monitoring, both in-house and by outer agencies, and a stringent code of conduct for containment, process control and execution, and final dissemination will be laid down and implemented.

The GEAC shall supervise the implementation of the terms and conditions laid down during the approval accorded through the SBCC/DLC/State Pollution Control Boards.

4.2.2.3 Committees With Monitoring Functions

4.2.2.3.1 State Biotechnology Coordination Committee There shall be an SBCC in the states wherever necessary. It shall have powers to inspect, investigate, and take punitive action in case of violations of statutory provisions through the Nodal Department and the State Pollution Control Board/Directorate of Health/Medical Services. The committee shall review periodically the safety and control measures in the various industries/institutions handling GE organisms/hazardous microorganisms. Chief secretary to the government of the respective state will be the chairman of the team and Secretary to the Department of Environment will be member secretary. Members include secretaries of the departments of Health, Agriculture, Industries and Commerce, and Forests; chief engineers/secretaries of the departments of Public Works and Public Health Engineering; state microbiologists and pathologists; and Chairman of the State Pollution Control Board. The committee may coopt other members if necessary.

4.2.2.3.2 District Level Committee There shall be a District Level Biotechnology Committee (DLC) in districts wherever necessary under District Collectors to monitor the safety regulations in installations engaged in the use of GM organisms/hazardous microorganisms and their applications in the environment. The DLC or any other person authorized in this behalf shall visit the installation engaged in activity involving GE organisms and hazardous microorganisms, and formulate an information chart, discover hazards and risks associated with each of these installations, and coordinate activities with a view to meeting any emergency. The DLC shall regularly submit its report to the SBCC/GEAC.

The District Collector will be the chairman of the DLC. A factory inspector, a representative of the Pollution Control Board, District Health Officer, District Agricultural Officer, a representative from the Public Health Engineering Department, District Microbiologist/Pathologist, and Commissioner of Municipal Corporation will be included as members. This committee can also opt other experts if necessary.

4.3 IMPLEMENTATION OF BIOREGULATORY MECHANISMS RELATED TO GM CROPS IN INDIA

4.3.1 General Outline

The IBSC is the starting point for assessing an application for confined field trials. The application from the principal investigator for permission for further evaluation of a selection event, based on information generated in a lab/greenhouse and on preliminary phenotypic evaluation of selection for one to a few events, will be received by the IBSC.

The details that should be submitted at this phase include: (1) *rationale for development*—benefits to farming communities, nutritional benefits, or environmental benefits; (2) *details on vector construct*—transgene source and characteristics of donor, construct layout, transgene sequence, cloning and transformation procedures followed, details on selection markers, antibiotic resistance marker genes used, transgene function in donor and expected function in recipient, details on promoter and expression vector, event location(s), copy number, amino acid sequence anticipated transgene protein, marker and primer details to track the transgene, sequence of flanking regions; and (3) *preliminary contained GMO/transgenic screening results*—biological features of organisms/GE plants, mammalian allergenicity or toxicity reported during the lab or greenhouse trials, transgene expression, and laboratory and greenhouse screening procedure and results.

Based on the scientific discussions, the committee will review the need for genetic modification, biosafety precautions taken by the research team, and progress and decide if the continuation should be permitted. If permitted, the application will be forwarded to the RCGM.

Once the program is approved by the IBSC, the RCGM assesses the biosafety of the event along with necessary requirements. A minimum of three seasons of Biosafety Research Level (BRL) trials are required for generating sufficient biosafety data for an event. Before initiating the BRL I trials, the RCGM generates information on basic facts such as description of the GE plant, description of the biology of the nontransgenic host plant, description of the genetic modification(s), assessment of possible toxicity and allergenicity, and conformation of inheritance of the new trait(s) over multiple generations. For screening a particular event, two options are available. In the first option, BRL I screening for the first two seasons followed by one season of BRL II screening, and in the second option, BRL I level screening in the first season followed by BRL II in subsequent seasons. The choice of system depends on several factors such as crop-raising conditions, transgene features, etc., and for screening of GE mustard (*Brassica juncea*), hybrid DMH-11 by the Centre for Genetic Manipulation of Crop Plants, University of Delhi (South Campus), the second system was followed. The RCGM is the regulatory authority for BRL I trials, which should be limited to one acre per trial site location and a maximum cumulative total of 20 acres (8.1 ha) for all locations for each plant species per construct combination, per applicant, per crop season. The GEAC is the regulatory authority for BRL II trials and these trials are limited in size to no more than 2.5 acres per trial site location and to no more than eight locations within India for each plant species per construct combination, per applicant, per crop season. The RCGM and GEAC perform the evaluation of field trials by the Monitoring-cum-Evaluation Committee (MEC) through State Agricultural Universities (SAUs).

4.3.2 Composition and Responsibilities of Prerelease MEC

A prerelease monitoring team will be constituted for monitoring the multilocation field trials (MLTs) and large-scale field trials (LSTs) taken up by SAUs or other public sector scientific bodies.

The Director of Research of the SAU will be the nodal person and team leader. The plant breeder for the concerned crop in the SAU, heads of the departments of Entomology, Agronomy, Plant Pathology, and Plant Biotechnology in the SAU, the Joint Director/Deputy Director from the Department of Agriculture of the state government, the Agricultural Officer of the concerned region/district of the state government, and nominees of the RCGM and GEAC will be included as members.

A prerelease monitoring team will be responsible for monitoring GE cultivar field trials conducted in the jurisdiction of the SAU by constituting monitoring teams as per the composition given. In addition, this team will be responsible for maintenance of grants received from the government of India/fees

collected from the applicants for this purpose. The team will visit the fields for a minimum of two times during the crop season, matching with the specified transgene expression critical phases and other important stages of the crop. All the replicated field trials by the applicants in its SAU's jurisdiction and at least 25% of large-scale field trials in its jurisdiction as per the conditions given in the experimental trial permits issued by the DBT/MoEFCC will be monitored. The team will observe and advise on collection of data by the applicants on the objectives of large-scale and replicated field trials on transgenic crops and advise minor modifications in the collection of data based on the nature of gene expression in transgenes and prevailing situations at the site of experimentation. The team will collect the data during its visit and hand over a copy of the data sheet to the applicant for their records, along with suggestions, if any, for improvement on the conduct of the trial, and submit the monitoring team report on LSTs and replicated MLTs to the MEC/RCGM within 15 days from conclusion of the last visit. The Director of Research shall maintain the records of monitoring, which may be called for by the government of India if required. The monitoring team(s) shall maintain all the information provided by the applicant and/or collected by the team as confidential.

The GEAC is the regulatory authority for BRL II trials; the size and number of trials depend on the nature of cases (Table 13.1). The field studies under BRL I and BRL II should complete the trials for at least confirmation of expression levels of new proteins, reproductive and survival biology in comparison with nontransformed counterparts, impact on nontarget organisms, compositional analysis of key components, and livestock feeding performance studies. Other biosafety experiments to be run parallel with the field trials in GE crop plants are presented in Table 13.2.

4.3.3 Guidelines for Confined Field Trials of Regulated GE Plants

A confined field trial is a field experiment for growing a regulated GE plant in the environment under specified terms and conditions that are intended to mitigate the establishment and spread of the plant. The trial is conducted under conditions known to mitigate the pollen- or seed-mediated dissemination of the experimental plant, persistence of the GE plant or its progeny in the environment, and introduction of the GE plant or plant products into the human food or livestock feed pathways. Guidelines for Research in Transgenic Crops (1998), issued by the DBT, government of India, briefly describe the considerations for limited field experiments.

Members of the MEC, SBCCs, DLCs, and monitoring teams of SAUs have the authority to inspect confined field trial sites at the time of planting, during the growing and harvesting season, and in the period of postharvest land use restriction for compliance with the terms and conditions of

Table 13.1 Details on the Biosafety Research Level (BRL) II Trials Regulated by the Genetic Engineering Approval Committee (GEAC) and Monitored by the Prerelease Monitoring-cum-Evaluation Committee (MEC) Through State Agricultural Universities (SAUs)

	Field Trials			
Stage of Trial	**LSTs**	**ICAR—MLTs**	**Lab Trials**	**Seed Production**
BRL II year 1	Yes	Yes	Yes	10 ha
BRL II year 2	Yes	Yes	Yes	100 ha

ICAR, *Indian Council of Agricultural Research;* LSTs, *lineage-specific transcripts;* MLTs, *multilocation field trials.*

Table 13.2 Experiments to Be Completed in Biosafety Research Level (BRL) I and BRL II for the Safety Assessment of Genetically Engineered Plants

Sl. No.	Name of Experiment	Food and Feed Safety Assessment		Environmental Risk Assessment	
		Field Experiments	Laboratory Experiments	Field Experiments	Laboratory Experiments
1	Acute oral safety limit		√		
2	Pepsin digestibility assay		√		
3	Protein thermal stability		√		
4	Subchronic feeding study in rodents (if required)		√		
5	Livestock feeding study (if required)	√			
6	Molecular characterization		√		√
7	Inheritance of introduced trait	√		√	
8	Stability of introduced trait	√		√	
9	Expression of introduced protein(s)	√		√	
10	Compositional analysis	√			
11	Reproductive and survival biology			√	
12	Impact on nontarget organisms: tier I testing				√
13	Impact on nontarget organisms: tier II testing			√	

authorization. Records of all confined field trials, including pre- and postharvest site monitoring, activities related to trial site compliance (including subcontracts), cleaning of equipment, transportation, disposition and storage of all surplus, and harvested seed and plant material shall be maintained by the Permitted Party and shall be made available to the RCGM/GEAC or the designated monitoring agencies upon request. Mandatory recording formats on all these are available at http://igmoris.nic.in (IGMORIS, 2016). The Permitted Party shall submit a field trial report to the RCGM/GEAC within 3 months after termination/harvest of a confined field trial. The field trial report must summarize the completed trial, including methods, observations, data, and analysis of any effects of the trial plants on other plants, nontarget organisms, or the environment.

4.3.3.1 Information to Be Submitted to the RCGM/GEAC by the Firm

4.3.3.1.1 Planting Intimation The RCGM/GEAC shall be informed in writing within 7 working days of planting at a trial site. A Record of Planting shall be submitted and must reference the confined trial permit number and document the amount of material planted, the planting date, the transportation of plant material to the trial site, the cleaning of any equipment used during planting, and the disposition of any surplus plant material remaining after planting. If it was not provided with the application, this notification must also include a detailed map of the trial site.

4.3.3.1.2 Harvest Intimation A Record of Harvest/Termination shall be prepared for each confined field trial site and shall document the date and method of harvest, the amount of harvested material, the disposition of any harvested materials, the cleaning of any equipment used during harvest, and the method of destruction of any residual plant material on the trial site.

4.3.3.1.3 Accidental Release Intimation The Permitted Party shall notify the RCGM/GEAC immediately upon discovery, by telephone/email within 24 h, of any incident involving an accidental or unauthorized escape such as spillage, theft, encroachment by unauthorized persons, vandalization, etc. of regulated GE plant material during transportation, and storage within a contained facility or during any other activity associated with the conduct of a confined field trial.

4.3.3.2 Standard Operating Procedures for Confined Field Trials of Regulated GE Plants

SOPs have been prepared to provide guidance on the transport of regulated GE plant material, storage of regulated GE plant material, labeling of storage area, inspection of storage area, inspection by regulatory officials, management of confined field trials, management of harvest or termination of confined field trials, postharvest management of confined field trials, occurrence of noncompliance, and corrective action in the event of accidental release of regulated GE crops.

The general requirements on SOPs for the transport of all regulated GE seed or propagule insist that they must be stored in secure containers for transportation, kept separate (secured in a primary container) from other plant material during transport, and clearly labeled, and the Permitted Party will ensure that appropriate containers/packaging materials are supplied to all agents. Additionally, SOPs for transport include the specific requirements for transport, details on accompanying documentation for transport, receipt on transported goods, and corrective action in the event of accidental release.

SOPs for the management of confined field trials of GE plants include details of the requirements for planting confined field trials, performance requirements for confined field trials, monitoring of the field trial by the Trial In-Charge, inspection by regulatory officials, corrective action in the event of an accidental release, and record keeping.

Recommendations in the requirements for planting are: all equipment and tools used to seed or plant confined field trials or used in the maintenance of the trial site must be cleaned on the trial site prior to their removal to eliminate unintended transport of regulated plant material from the trial site; a map of the trial site must be prepared by the Trial In-Charge and appended to the Record of Planting; a Record of Planting must be completed for each field trial site and a copy of the Record of Planting with the appended map must be submitted to the RCGM/GEAC within 7 days following the completion of planting; the Trial In-Charge must mount a notice board at the trial site indicating the purpose and duration of the confined field trials conducted at the trial site and the authorization under which the confined field trials were approved; and the Trial In-Charge must ensure that only personnel authorized by the Permitted Party are permitted on the trial site.

Recommendations on the performance requirements are: all corners of each trial site will be clearly marked with reference to physical markers, any plant material removed during maintenance of the trial must be rendered nonviable by burning or burial on the trial site, trial sites must be reproductively isolated from plants of the same or any other sexually compatible species (isolation distance will be indicated by the RCGM/GEAC in the letter of approval for the confined field trial), isolation areas must be

continuous and completely surround the confined trial site, trial sites and surrounding isolation areas are kept free of all prohibited plants by implementing a program of regular monitoring and removal of any prohibited plants, and any prohibited plants within the isolation area must be removed before they flower and rendered nonviable by burning or burial on the trial site.

An SOP for the harvest or termination of confined field trials details the requirements for harvest of confined field trials, destruction of regulated transgenic plant material, transport of harvested materials from the trial site, inspection by regulatory officials, occurrence of noncompliance, corrective action in the event of an accidental release, and record keeping. An SOP for the postharvest management includes details on general requirements, requirements for postharvest management of trial sites, case-by-case, as specified by regulatory authorities, monitoring of the postharvest trial site, corrective action in the event of an accidental release, and record keeping.

These SOPs will be reviewed by the RCGM/GEAC at least annually and after review any revised SOPs will be posted to the DBT website (www.dbtbiosafety.nic.in). Copies will be provided to all MECs, SAUs, IBSCs, SBCCs, and DLCs, and will be referred to in confined field trial permits.

4.3.3.2.1 Reproductive Isolation of Confined Field Trials The Permitted Party will ensure that the conditions for reproductive isolation of all trial plants are met by providing the isolation distance applicable to foundation seed of the crop all round the transgenic crop field during both the current growing season and the postharvest period. A few rows of the same crop as the transgenic one should be planted beyond the isolation distance to act as pollen trap. Additionally, nontransgenic plants should be grown within the isolation distance at 1 or 5 m intervals to determine the distance of pollen escape.

No harvested material or by-product from a confined field trial may be used as human food or livestock feed. Progeny from any confined field trial cannot be retained for future planting without prior written authorization from the RCGM/GEAC. Seed or other plant material harvested from confined trials (including border rows) that has not been previously authorized by the RCGM/GEAC is to be retained for future research work and must be disposed of by dry heat, steam heat, incineration, deep burial, chemical treatment, or crushing or burying on the trial site.

4.3.3.2.2 Postharvest Land Use Restrictions and Postharvest Monitoring The area under restriction must be monitored during the postharvest period to ensure that any prohibited plants (volunteers or sexually compatible species) are destroyed prior to flowering. All the vegetative plants and leftover seeds must be destroyed by burning after the experiments are concluded. After the experiment, the land may be left fallow and all plants that emerge must be destroyed. No plants of the same or a sexually compatible species may be planted in the restricted area during the postharvest period. Land use of the restricted area must be compatible with requirements for monitoring and removal of prohibited plants. No plants that could interfere with monitoring for prohibited plants can be planted.

4.3.3.2.3 Conditions for Large-Scale Trials As already detailed, new GE cultivars should undergo a minimum of 2 years of LSTs and 2 years of MTLs by the ICAR prior to their consideration for commercial release. LSTs and MLTs may also be conducted simultaneously. In the case of *Bt* cotton, LSTs have been conducted at 40 locations as per the protocol by the GEAC, and SAUs were involved in the monitoring of these trials.

The firm shall provide to the GEAC/MEC/State Department of Agriculture/District Collector and other field functionaries under the Seed Act the state/district-wise details of locations (area, village,

name of the farmer) where it intends to undertake large-scale field trials within 30 days of issue of this clearance letter. The location of LSTs should be carefully chosen so as to represent adequately the various agro-climatic zones and agricultural practices in the region. Detailed justification for the selection of LST locations shall also be furnished.

The firm shall make available socioeconomic data such as cost of GE seed, projected demand of GE seeds, cost of GE crop production versus non-GE crop production under various agro-climatic conditions and agricultural practices, cost–benefit analysis, etc.

The MEC set up by the RCGM would evaluate the performance of GE cultivars during the LSTs on a random and representative sampling basis and submit its 2-year combined report to the GEAC. The MEC may seek additional information/stipulate additional conditions if so necessitated based on the observations made during the monitoring of LSTs.

The firm shall be completely liable to pay compensation for damages to the environment caused by them while conducting the field trials. The permission letter for first and second year LSTs is valid only for the period of normal cultivation, from the date of issue, and would lapse automatically after the season.

4.3.3.2.4 Conditions for Seed Production The GE cultivar may undertake seed production in an area of 10 ha during first year LST and 100 ha during second year LST subject to the following conditions.

The firm shall provide to the GEAC/MEC/State Department of Agriculture/District Collector and other field functionaries under the Seed Act the state-wise details of location (area, village, name of the farmer) where it intends to undertake seed production within 30 days of issue of the clearance letter.

The seeds produced by the firm shall be regulated as per the provisions of the Seeds Act (1966) and subsequent rules/amendments. The firm shall maintain records of seed production and shall make them available for inspection if it so desires by the GEAC/MEC/State Department of Agriculture/District Collector and other field functionaries under the Seed Act. Seed generated shall not be sold or diverted for commercial purpose without the approval of the GEAC.

Plant residue after harvesting should be destroyed by burning and records to this effect need to be maintained and submitted to the GEAC/State Department of Agriculture/District Collector.

4.3.3.2.5 Conditions for Commercial Release In connection with the granting of approval with terms and conditions shall be stipulated, including terms and conditions as to the control to be exercised by the applicant, supervision, restriction on use, layout of the enterprise, and as to the submission of information to the SBCC or to the DLC. All approvals of the GEAC shall be for a specified period not exceeding 4 years at the first instance, renewable for 2 years at a time, entrusting the following additional recommendations:

- The GEAC shall have powers to revoke such approval in case of nonstipulation by the agency, with any of the pertinent rules.
- The sale must be effected by meeting the 20% refuge requirement. Each packet of seeds of the approved varieties should also contain a separate packet of the seeds of the same non-*Bt* cotton variety.
- A minimum of 100 g seed of each of approved hybrids and parental lines will be deposited at the NBPGR. Additionally, DNA fingerprints of the approved varieties and testing procedures for identifying transgenic traits in the approved varieties by DNA and protein methods shall also be deposited.

- Information and agreements with their dealers/agents to provide details about the sale of seeds, acreage cultivated, and state/regions of cultivation should be provide to government at regular intervals.
- Annual reports on dealers, acreage, and region of cultivation will be submitted to the government agencies.
- A thorough monitoring mechanism on the performance or breakdown of the transgene should operate. For example, in case of *Bt* cotton, the susceptibility of bollworms to the *Bt* gene vis-à-vis baseline susceptibility data should be monitored annually and the data relating to resistance development, if any, should be submitted to the GEAC. Monitoring of susceptibility of bollworms to the *Bt* gene also will be assessed by India's premier institute for cotton research, the Central Institute for Cotton Research, Nagpur, at the applicant's cost.
- Labels indicating the contents and the description of the *Bt* hybrid including the name of the transgenes, the GEAC approval reference, physical and genetic purity of the seeds along with agricultural practice with detailed directions for use including sowing pattern, pest management, suitability of the hybrids specifically for irrigated conditions, etc. in vernacular language should be clearly presented to the buyers.
- The government agencies must be entrusted with the duty to develop GE cultivar-based packages for cultivation, including the integrated pest management (IPM) program.
- Education and awareness programs must be undertaken to convey the benefits and risks regarding the specific GE cultivar.
- Additional conditions, if so necessitated on the basis of feedback received from the MEC/State Department of Agriculture/District Collector/other field functionaries under the Seed Act and other sources, shall also be obliged.

4.3.3.2.6 Postrelease Monitoring The GEAC may supervise the implementation of the terms and conditions laid down in connection with the approvals accorded by it. The GEAC may carry out this supervision through the SBCC/State Pollution Control Boards/DLC or through any person authorized in this behalf. Statutory provisions for implementation of pre- and postrelease monitoring are given by the GEAC by empowering the seed inspectors, seed analysts, and seed testing laboratories notified under the Seed Act (1966) and Seed Control Order (1983) under EPA (1986), through Gazette Notifications issued on September 1, 2006 (Extra Ordinary Gazette, 2006).

Composition and responsibilities of postrelease MEC: Similar to the prerelease monitoring team, a postrelease monitoring team will be constituted to monitor the commercial cultivation and seed production practices of GE cultivars. The Director of Extension in the SAU will be the nodal person and team leader. The plant breeder for the concerned crop in the SAU, heads of the departments of Entomology, Agronomy, Plant Pathology, and Plant Biotechnology in the SAU, and a biostatistician will be included as members.

This team will be responsible for monitoring of the GE crop through a scientifically designed survey and maintenance of grants received from the government of India/fees collected from the applicants for this purpose. The monitoring team shall visit the fields for a minimum of two times during the crop season, matching with the critical phases of transgene outcome and other important stages. The team will record information such as date of sowing, seed rate, method of planting, spacing, fertilizer application, micronutrient application, irrigation schedule, measures taken to manage pests/diseases, IPM practices followed, method of harvesting, performance of the hybrid, transgene expression and economic benefits, views of public acceptability and other comments, compliance of GEAC conditions, and any other parameter of relevance.

4.3.3.3 Guidelines for the Monitoring of Confined Field Trials of Regulated GE Plants

These guidelines issue the following terms of reference to all members of monitoring teams:

1. *Ethical conduct*: Trust, integrity, confidentiality, and discretion are essential to monitoring activities and all members of monitoring teams shall conduct themselves in a professional and ethical manner.
2. *Fair presentation*: The findings, conclusions, and reports of monitoring teams will truthfully and accurately reflect the monitoring activities.
3. *Due professional care*: Monitoring teams will exercise care in accordance with the importance of the task they perform and the confidence placed in them by the Regulatory Authority.
4. *Independence*: Members of the monitoring teams should be independent of the activity being inspected and free from bias and conflict of interest.
5. *Evidence-based approach*: Reports of monitoring teams, upon which conclusions and regulatory actions may be based, must be verifiable. Such evidence may include photographs of trial site conditions, measurements of trial site dimensions and isolation distances, samples of documents and/or records, and first-hand interviews with technical personnel.

4.3.3.3.1 Procedures for the Monitoring Teams The monitoring process will be intensive and the first step is preparation for the site visit. The second step is the documentation inspection, which should be performed in accordance with DBT's Standard Operating Procedures for Confined Field Trials of Genetically Engineered Plants. The compliance documentation that should be available for review may include a *Letter of Permit* authorizing conduct of the confined field trial, a *Record of Transport*—transport documentation for shipments of regulated plant material to, and between, field trial sites and contained facilities, a *Record of Storage*—storage facility documentation, a *Record of Storage Inspection*—current season documentation, a *Record of Planting*, *Record of Spatial Isolation*, and/or records for other methods of reproductive isolation, a *Record of Harvest/Termination and Disposition*—trial harvest and/or termination documentation, a *Record of Postharvest Inspection*—postharvest management documentation, and a *Record of Corrective Action*—any records related to compliance or corrective actions.

The third step is storage facility monitoring to verify that storage facilities meet the minimum physical requirements stipulated in any applicable regulations, guidelines, or SOPs, and that material management and monitoring processes are in place and being followed. Additionally, it will confirm that: regulated plant material is appropriately labeled and stored separately from any conventional seed or plant material in a fully enclosed, lockable space, access to storage areas is limited to authorized personnel, and there must be evidence of some active access control system; areas or units designated for storage of regulated plant material must be cleaned prior to, and immediately following, the period of storage, and there should be records documenting these activities; the storage area is clearly marked as containing regulated plant material and used exclusively for that purpose; all regulated plant material in storage is recorded on an inventory record, which also records all additions to, or removals from, storage; and storage facilities are checked regularly to ensure they are secure, free of any waste or debris, and that material packaging or labeling has not been compromised, and this activity should be documented on records of storage inspection completed at least once every 4 weeks.

The next step in field trial monitoring includes site location monitoring and monitoring for reproductive isolation. Monitoring of the trial site may occur at any time but the most useful times from a risk management perspective may include prior to authorization, during planting, during

the period of crop flowering and prior to seed set, during harvest or trial termination, and during the postharvest period.

Other steps in monitoring are monitoring of transport, storage and labeling, termination, harvest, and disposition, and postharvest site monitoring.

4.3.3.4 Preparation of Offsite Emergency Plan by the DLC

The DLC shall prepare an offsite emergency plan detailing how emergencies relating to a possible major accident at a site will be dealt with, in consultation with the occupier and others. For the purpose of enabling the DLC to prepare the emergency plan, the occupier shall provide the DLC with necessary information relating to the handling of hazardous microorganisms/GMOs under his control as the DLC may require, including the nature, extent, and likely offsite effects of a possible major accident, and the DLC shall provide the occupier with any information from the offside emergency plan to help him to carry out the necessary responsibility of notifying the DLC regarding the interruptions or accidents in field experiments.

4.3.3.5 Penalties

If an order is not complied with, the SBCC/DLC may take measures at the expense of the person who is responsible. In cases where immediate intervention is required to prevent any damage to the environment, nature, or health, the SBCC/DLC may take the necessary steps without issuing any orders or notices. The expenses incurred for this purpose will be repayable by the person responsible for such damage. The SBCC/DLC may take samples for a more detailed examination of organisms and cells and shall be competent to ask for assistance from any other government authority to carry out its instructions.

4.3.3.6 Appeal and Exemption Mechanism

The agency/person penalized by the GEAC/SBCC/DLC may appeal to the National Environment Appellate Authority within 30 days. The MoEFCC, wherever necessary, may exempt an occupier handling particular GMOs from the provisions of the 1989 rules (7–11) (discussed under Section 4.1.3).

5. BIOREGULATORY MECHANISMS FOR GM CROPS IN THE EUROPEAN UNION

Regulation (EC) No. 1829/2003 of the European Parliament and of the Council of September 22, 2003 on Genetically Modified Food and Feed (European Parliament and Council, 2003b) directs the bioregulatory mechanism of GE plants in the European Union. The objective of the regulation is to provide the basis for ensuring a high level of protection of human life and health, animal health and welfare, and environmental and consumer interests in relation to GM food and feed, while ensuring the effective functioning of the internal market; laying down Community procedures for the authorization and supervision of genetically modified food and feed; and laying down provisions for the labeling of genetically modified food and feed. The scope of the EU regulations on GMOs is restricted to food and feed for all intents and practical purposes, in deference to the original definition, which encompasses animals.

Process summary on GMO authorization: The EU uses the "precautionary principle" demanding a premarket authorization for any GMO to enter the market and postmarket environmental monitoring. Both the European Food Safety Authority (EFSA) and the member states author a risk assessment. This assessment must show that the food or feed is safe for human and animal health and the environment "under its intended conditions of use." The EU treats all GM crops, along with irradiated food, as "new food" and they are subject to extensive, case-by-case, science-based food evaluation by the EFSA. The EFSA uses independent scientific research to prepare risk assessment reports to the EC on how to regulate a particular GM food to protect consumers and the environment through molecular characterization, potential toxicity, and potential environmental impact. Based on ESFA reports, the EC drafts proposals for granting or refusing authorization. Each proposal is submitted to the Section on GM Food and Feed of the Standing Committee on the Food Chain and Animal Health. If accepted, it is either adopted by the EC or passed on to the Council of Agricultural Ministers. The Council has 3 months to reach a qualified majority for or against the proposal. If no majority is reached, the proposal is passed back to the EC, which then adopts the proposal.

5.1 AUTHORIZATION PROCESS IN DETAIL

5.1.1 Submission of Application for Authorization

For authorization of a GMO [referred to in Article 4(2) and available at http://eur-lex.europa.eu/legal-content/en/ALL/?uri=CELEX%3A32003R1829], a party shall submit the application to the national competent authority of a member state. The application should include: the name and address of the applicant; designation of the food, and its specification, including the transformation event(s) used; the information to be provided for the purpose of complying with Annex II to the Cartagena Protocol; a detailed description of the method of production and manufacturing; a copy of the studies, including, where available, independent peer-reviewed studies that have been carried out and any other material that is available to demonstrate that the food complies with the regulation [criteria as in Article 4(1)]; either an analysis, supported by appropriate information and data, showing that the characteristics of the food are not different from those of its conventional counterpart, having regard to the accepted limits of natural variations for such characteristics and to the criteria specified in Article 13(2) (a), or a proposal for labeling the food in accordance with Article 13(2) (a) and (3); either a reasoned statement that the food does not give rise to ethical or religious concerns, or a proposal for labeling it in accordance with Article 13(2) (b); the conditions for placing on the market the food or foods produced from it, including specific conditions for use and handling; methods for detection, sampling (including references to existing official or standardized sampling methods), and identification of the transformation event and, where applicable, for the detection and identification of the transformation event in the food and/or in foods produced from it; samples of the food and their control samples, and information as to the place where the reference material can be accessed; a proposal for postmarket monitoring regarding use of the food for human consumption; and a summary of the dossier in a standardized form.

The national competent authority shall acknowledge receipt of the application in writing to the applicant within 14 days of its receipt, along with the date of receipt of the application. With no delay, the application will be communicated to the EFSA along with the supplementary information. The EFSA will inform other member states and the EC about the application, and the application and any supplementary information will be made available to them. The EFSA will make the summary of the dossier available to the public.

5.1.2 Role of the EFSA

The EFSA will prepare its "opinion" within 6 months by verifying the particulars and documents. It may also ask the appropriate body of a member state to carry out a food safety assessment and a competent authority to carry out an environmental risk assessment, and a Community reference laboratory shall test and validate the method of detection and identification proposed by the applicant. An opinion in favor of authorizing the food shall include: the designation of the food and its specification; the information required under Annex II to the Cartagena Protocol; a proposal for the labeling of the food produced from it; any conditions or restrictions that should be imposed on the placing on the market and/or specific conditions or restrictions for use and handling, including postmarket monitoring requirements based on the outcome of the risk assessment; the method, validated by the Community reference laboratory, for detection, including sampling, identification of the transformation event, and the monitoring plan.

The EFSA will forward its opinion to the EC, the member states, and the applicant, along with an assessment report, reasons for its opinion, and the information on which this opinion is based, including the opinions of the competent authorities when consulted. Finally, the EFSA will make its opinion public, and the public may make comments to the EC within 30 days from such publication.

5.1.3 Authorization

Within 3 months of receiving the opinion, the EC submits a draft decision proposal to the Standing Committee on the Food Chain and Animal Health. The final decision shall be drawn with the help of the committee, quickly intimated to the applicant, and details of the decision published in the *Official Journal of the European Union*.

The authorized food shall be entered in the EC's Community Register along with the date of authorization and other particulars. Member states may invoke a safeguard clause to temporarily restrict or prohibit use and/or sale of a GMO crop within their territory if they have justifiable reasons to consider that an approved GMO crop may be a risk to human health or the environment. The EC is obliged to investigate and either overturn the original registrations or ask the country to withdraw its temporary restriction.

5.1.4 Postmarket Monitoring

The authorization-holder makes sure that products not covered by the authorization are not placed on the market as food or feed, and monitoring ensures that conditions or restrictions imposed in the authorization are followed. Monitoring for environmental effects and the use of food for human consumption will be made. Monitoring reports will be submitted to the EC and made accessible to the public.

In addition, applicants who wish to cultivate or process GMOs must provide a detailed surveillance plan after authorization. This ensures that the EFSA will know if risk to consumers or the environment heightens and that they can then act to lower the risk or deauthorize the GMO.

5.1.5 Modification, Suspension, and Revocation of Authorization

On request from a member state or from the EC or on its own, the EFSA issues an opinion on whether an authorization still meets the conditions set by this Regulation and transmits this to the EC, authorization-holder, and the member states. The EFSA makes its opinion public and the public can submit feedback to the EC within 30 days. The EC examines the opinion and authorization shall be aptly modified, suspended, or revoked.

Table 13.3 Labeling Requirements on Genetically Modified Foods as Necessitated by EU Regulations

Sl. No.	Condition	Label	Remarks
1	Food consists of more than one ingredient	*Genetically modified* or *Produced from genetically modified* (name of the ingredient)	Appears in the list of ingredients, in parentheses, immediately following the ingredient concerned, in a font sufficiently large for easy identification and reading
2	Ingredient is designated by the name of a category	*Contains genetically modified* (name of organism), *Contains* (name of ingredient), or *Produced from genetically modified* (name of organism)	Appears in the list of ingredients
3	There is no list of ingredients	*Genetically modified* or *Produced from genetically modified* (name of organism)	Appears clearly on the labeling
4	Food is offered for sale to the final consumer as nonprepackaged food, or as prepackaged food in small containers of which the largest surface has an area of less than 10 cm^2	As suited from above	Displayed either on the food display or immediately next to it, or on the packaging material

5.1.6 Renewal of Authorization

Each GMO must be reassessed every 10 years and the application for the same, along with copies of authorization, results of monitoring, and new information on food safety and risks to consumers and environment, should be submitted at the latest 1 year before the expiry date of the authorization.

5.2 LABELING

Foods produced from GMOs in a proportion less than 0.9% of the food ingredients considered individually, or food consisting of a single ingredient, provided that this presence is adventitious or technically unavoidable, need not be labeled. The labeling requirements are summarized in Table 13.3. Labeling shall also mention any characteristic or property, where a food is different from its conventional counterpart regarding its composition, nutritional value or nutritional effects, intended use of the food, implications for the health of certain sections of the population, or where it may give rise to ethical or religious concerns.

6. BIOREGULATORY MECHANISMS FOR GM CROPS IN UNITED STATES OF AMERICA

The history of US bioregulatory mechanisms starts with the formation of an rDNA Advisory Committee by the National Institute of Health (NIH) and establishment of a set of rules to regulate rDNA research (NIH, 1978). This was followed by the allocation of compulsory mandates to the United States

Department of Agriculture (USDA), Environmental Protection Agency, and Food and Drug Administration (FDA) to function as a "Federal Coordinated Framework for the Regulation of Biotechnology." In 1982, the Organization for Economic Cooperation and Development (OECD) first developed a report on probable risks by transgenics, and subsequently the National Academy of Sciences (NAS) issued a paper on risk assessment strategies to be followed in the United States (NAS, 1983). In the same year, the NIH authorized the first environmental release of a GMO (ice-minus bacterium) and with no delay the first transgenic tobacco plants resistant to methotrexate and kanamycin were reported.

The White House established a committee at the Office of Science and Technology Policy (OSTP) and this committee observed that rDNA is not inherently risky and regulations should focus on the risk of products and not the processes. The OECD *Blue Book on Recombinant DNA Safety Considerations* set out principles and concepts for handling GMOs safely outside of contained laboratory conditions (OECD, 1986). A white paper from the NAS (1987) validated the OSTP regulatory approach. Virtually, it was accepted that every method of genetic modification including traditional breeding can result in hazardous products, and biotech is no more likely to produce hazardous products than traditional methods. The regulatory trigger for risk assessment should be based on product rather than process (NRC, 2000). The OSTP has further assigned regulatory functions to the USDA, FDA, and Environmental Protection Agency. The USDA was directed to evaluate GE plants as *potential pests of agriculture*, the FDA was to evaluate them as *potential threats to food and feed supply*, and the Environmental Protection Agency was to evaluate them with pesticidal properties (McHughen and Smyth, 2008).

A short description of the regulatory functions of the three authorities in the Federal Coordinated Framework for the Regulation of Biotechnology is furnished hereunder.

6.1 ROLE OF THE USDA

Legislative authority for the USDA comes from the Federal Plant Protection Act (FPPA) of 2000. The USDA regulates all GE plants prior to environmental release including import, interstate transport, field trials, and commercial cultivation. Regulated procedures for GE field trials were first published in 1987. A plant and its progeny arising from a specific event are considered as "Regulated Articles" and each event will be separately regulated. Currently, the regulations on GE crops are implemented through the Biotechnology Regulatory Services (BRS) office within the Animal and Plant Health Inspection Service (APHIS). APHIS coordinates the responsibilities along with other federal agencies as part of the Federal Coordinated Framework for the Regulation of Biotechnology.

For screening a regulated product, APHIS follows two systems: Notifications and Permits. The simple GE issues and their field trials are completed through the Notification path. The party should generate an eAuthentication account with the USDA and submit the Notification (https://www.eauth.usda.gov). Application should detail how the GE plant meets the set standards. The Notification is used for field trial approval, import, and transport within the United States.

Permits, a more complicated path for deregularization, generally apply to GE cases not meeting the Notification requirements (http://www.aphis.usda.gov/). A genuine GE plant, which may be a result of the production of an unexpected allergen, is disqualified to enter the Notification path. The USDA needs molecular, biochemical, and cellular characterization of GE plants along with data or life cycle, reproductive characteristics, and any unexpected changes from the nontransgenic counterpart.

There will be five site inspections during field trials, followed by two inspections in the coming season (Table 13.4). The isolation distance has to be maintained from the trial site and no food crop could be grown on the site in the following season (Stewart and Knight, 2005).

Regulations for lifting the regulated status of GE plants, which are tested and proven genuine, were proposed by the USDA in 1992 and approved in 1993. The parties should apply to the USDA to bring

Table 13.4 Permit Systems for Deregulation Genetically Engineered (GE) Plants

Permit Type of GE Plant	Attributes of GE Plant	Procedural Requirements for Permits	Permit Conditions
Type 1	**1.** Plants having a low potential as plant pests and noxious weeds based on meeting criteria including a food safety evaluation of newly expressed substances, unless acreage is very low **2.** Plants reclassified from type 2, based on high familiarity	**1.** Information requirements: Simplified formats **2.** Review by BRS: Applications reviewed for compliance with criteria **3.** Scope of notifications: Required only for field tests, single year	*Confinement*: APHIS presets standard confinement requirements as part of the permit conditions. Confinement will be similar to that typically used for field tests for notifications under current system *Inspections*: A percentage is chosen for inspection
Type 2	**1.** Plants having higher plant pest or noxious weed potential than type 1 **2.** Food crop plants reclassified from type 3, based on increased familiarity	**1.** Information requirements: Standard formats (APHIS may ask for additional information) **2.** Review by BRS: Case by case **3.** Scope of permits: Single or multiyear permits with SOPs	*Confinement*: Variable depending on plant. For most, applicants will propose confinements for APHIS review. Plants reclassified from type 3 will follow APHIS set standards consistent with those currently used for pharmaceutical and industrial plants *Inspections*: At least one per permit
Type 3	**1.** Plants having higher plant pest or noxious weed potential than type 2 **2.** Food crops engineered with traits with which APHIS is unfamiliar or expressing substances having food safety profiles with which APHIS is unfamiliar	**1.** Information requirements: Standard formats (APHIS may ask for additional information) **2.** Review by BRS: Case by case **3.** Scope of permits: Single or multiyear permits with SOPs	*Confinement*: Very stringent standards likely to exceed those currently used for pharmaceutical and industrial plants *Inspections*: Up to five annual inspections
Type 4	**1.** Plants with highest plant pest or noxious weed profile (likely to pose a hazard to human health and the environment)	**1.** Information requirements: Standard formats (APHIS may ask for additional information) **2.** Review by BRS: Case by case **3.** Scope of permits: Single year permits	*Confinement*: Highly stringent, case by case. Security/surveillance and restrictive access will be required *Inspections*: At least five annual inspections

APHIS, *Animal and Plant Health Inspection Service;* BRS, *Biotechnology Regulatory Services;* SOPs, *standard operating procedures.*

Adapted from USDA, 2007. Introduction of Genetically Engineered Organisms – Draft Programmatic Environmental Impact Statement 2007, United States Department of Agriculture, pp. 310. Available at: https://www.aphis.usda.gov/brs/pdf/complete_eis.pdf.

the material to "deregulated status." Based on trial results, the USDA prepares two reports: an "Environmental Assessment" (EA) to satisfy the environmental safety issues under the FPPA and a "Determination of Nonregulated Status" to satisfy the National Environment Policy Act 1970. An EA critically analyzes the environmental consequences and if the EA is satisfactory with insignificant risks, the USDA prepares the report FONSI (finding of no significant impact), providing rationale for declaring the GE plant environmentally safe.

If the results of laboratory and field trials are not convincing, the USDA publishes a notice of intent in the Federal Register for an elaborate environmental impact statement (EIS). Accordingly, in-depth analyses will be completed and when the draft EIS is ready, a notice of availability (NoA) is published in the Federal Register and a draft will be open for public opinion for at least 45 days. At this phase, the USDA also arranges activities such as meetings to attract more public involvement. Subsequently, the NoA for a final EIS will be notified in the Federal Register, with a 30-day time limit for publishing a Record of Decision publically, but not necessarily in the Federal Register.

6.2 ROLE OF THE FDA

The FDA was established to regulate food and feed, irrespective of method of breeding, under the Federal Food, Drug and Cosmetic Act (FFDCA, 21USC 301) through a policy statement (FDA, 1992). The FDA does not compel GE foods to undergo evaluation just because they are GM. Food and feed will be analyzed, mainly at the Center for Food Safety and Nutrition and the Center for Veterinary Medicine, respectively, and those with unexpected substances such as allergens or toxicants or abnormal levels of normal constituents are considered "adultered." Voluntary screening by the FDA is called "consultation," for which the party submits data regarding the identical non-GE plant/product. The submitted data should include the genetic stability of the plant, composition and nutrition facts, allergenicity and toxicology of substances ordinarily present, and assessment of introduced gene products. While assessing composition, the caseworker may also consider dietary exposure, change in exposure by vulnerable groups such as those who are pregnant, enhanced exposure by any particular ethnic groups, possible reduced availability of any normally present nutrient caused by the overproduction of any particular component, etc. However, other than checking the composition and issuing a memo, the FDA will not formally declare the safety of a GE product.

6.3 ROLE OF THE ENVIRONMENTAL PROTECTION AGENCY

The Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA) (1947) assigned the USDA the responsibility for regulating pesticides. Congress enacted major revisions to the FIFRA in 1972 with the Federal Environmental Pesticide Control Act (FEPCA) and this transferred pesticide regulation responsibility to the Environmental Protection Agency and shifted emphasis to protection of the environment and public health. As of now the FEPCA along with the FFDCA authorizes the Environmental Protection Agency to regulate pesticide properties in GM crops. The Environmental Protection Agency will be concerned about the pesticide-producing plants (for example, *Bt* toxin) and those requiring pesticides for screening (for example, herbicide-tolerant plants). In 1994, the Environmental Protection Agency published regulations in this regard and started evaluating the submissions case by case. The data on description of plant and modification, product, toxicity, effect on nontarget organisms and the environment, source of gene, other elements in the construct, antinutritional compounds produced by the receiving plant, stability of inserted DNA, and number and position of events should be submitted to the agency. Additionally, full details on

Table 13.5 Role of Different Federal Regulatory Agencies in Regulating Genetically Engineered Plants Based on Their Transgene Trait

SI No.	Trait Coded by Transgene	Regulatory Oversight by	Regulatory Authority
1	Insect resistance in food crops	APHIS	Safety for agriculture and environment
		Environmental Protection Agency	Safety for the environment and food and feed safety of pesticidal compound
		FDA	Safety for food and feed use
2	Modified oil content in a food crop	APHIS	Safety for agriculture and the environment
		FDA	Safety and labeling for food and feed use
3	Herbicide tolerance in a food crop	APHIS	Safety for agriculture and the environment
		Environmental Protection Agency	Safe use of companion herbicide
		FDA	Safety for food and feed use
4	Modified flower color in an ornamental crop	APHIS	Safety for agriculture and the environment

APHIS, *Animal and Plant Health Inspection Service;* FDA, *Food and Drug Administration.*
Adapted from USDA, 2007. Introduction of Genetically Engineered Organisms – Draft Programmatic Environmental Impact Statement 2007, United States Department of Agriculture, pp. 310. Available at: https://www.aphis.usda.gov/brs/pdf/complete_eis.pdf.

pesticidal protein with complete amino acid sequence, expression in various tissues, intentional and unintentional modifications in the protein, digestibility assay results, and homology models with known possible allergens should also be submitted. The agency also examines any possible ill effects of gene flow, and in places where the cross-compatible species are abundant, the GE product may not be permitted. It also examines any possible potential toxicity to nontarget organisms such as honeybees and examines its effects on environmental elements including soil, living forms in the rhizosphere, etc. The agency also keeps its own strategies for monitoring every kind of GE crop. For example, in the case of toxin-producing GE products with insect resistance, an insect resistance management program, which directs the strategies to be followed to avoid the development of insect populations with resistance to the specific toxin, will be conducted following a memorandum of understanding with the agencies. For evaluating herbicide-tolerant plants, the strategy followed by the Environmental Protection Agency is to evaluate the use of herbicide on GE products and this will be done with herbicide on plants and not by evaluating plants alone. The different regulatory agencies take up the authority based on the nature of the crop as well as the function of the transgene (Table 13.5).

7. BIOREGULATORY MECHANISMS FOR GM CROPS IN CANADA

Under the Food and Drugs Act 1985 (http://laws-lois.justice.gc.ca/eng/acts/f-27/) and its regulations, Health Canada is responsible for provisions related to public health, food safety, and nutrition (http://www.hc-sc.gc.ca/sr-sr/alt_formats/hpfb-dgpsa/pdf/pubs/reg_gen_mod-eng.pdf). In Canada, foods derived from biotechnology are considered to be one class of novel foods. Health Canada regulates the sale of novel foods in Canada through a premarket notification requirement, which is specified under the Food and Drug Regulations (Division 28 Novel Foods Part B) (http://laws-lois.justice.gc.ca/eng/regulations/C.R.C.,_c._870/page-85.html#h-144).

As per Division 28 of the Food and Drug Regulations, genetic modification is the change in the heritable traits of a plant, animal, or microorganism by means of intentional manipulation.

Novel food is defined as: a substance, including a microorganism, that does not have a history of safe use as a food; a food that has been manufactured, prepared, preserved, or packaged by a process that has not been previously applied to that food and causes the food to undergo a major change; and a food that is derived from a plant, animal, or microorganism that has been genetically modified such that (1) the plant, animal, or microorganism exhibits characteristics that were not previously observed in that plant, animal, or microorganism, (2) the plant, animal, or microorganism no longer exhibits characteristics that were previously observed in that plant, animal, or microorganism, or (3) one or more characteristics of the plant, animal, or microorganism that no longer fall within the anticipated range for that plant, animal, or microorganism.

The steps followed by Health Canada in the regulatory process are as follows.

7.1 PRESUBMISSION CONSULTATION

Prior to the notification of GE plants to Health Canada for safety assessment, the party may consult the Novel Foods Section of the Food Directorate.

7.2 PREMARKET NOTIFICATION

A novel food should be sold or advertised for sale only if the party has notified the Director, Health Canada, in writing of their intention to sell or advertise for sale the novel food and has received a written notice from the Director. When the party has generated sufficient data on the safety of a GM food to address Health Canada's criteria, the notification may be submitted to the Novel Foods Section.

The notification should include the common/trade name and a description of the novel food, including information respecting its development, details of the method by which it is manufactured, prepared, preserved, packaged, and stored, details of the major change, if any, information respecting its intended use and directions for its preparation, information respecting its history of use as a food in a country other than Canada, and information relied on to establish that the novel food is safe for consumption. Additionally, details of the name and address of the principal place of business of the manufacturer/importer, information respecting the estimated levels of consumption by consumers of the novel food, text of all labels to be used in connection with the novel food, and name and title of the person who signed the notification and the date of signing should also be furnished.

If the information provided in the notification for a novel food is not considered adequate to determine the novel food's safety, an additional Safety Assessment Data Package, supporting the safety of the food, will be required. This has to be prepared under the headlines history of use, dietary exposure, detail of novel process, history of organism, characterization of derived line/strain, genetic modification considerations, nutritional considerations, toxicology considerations, allergenicity considerations, chemical considerations, and microbiological considerations.

7.3 SCIENTIFIC ASSESSMENT

Within 45 days after receiving a notification, the Director shall review the information included in the notification. Accordingly, a complete safety assessment of the product, involving rigorous evaluation

by scientific evaluators, will be done following Health Canada's Guidelines for the Safety Assessment of Novel Foods (Health Canada, 2006).

The initial chapters of the Guidelines for the Safety Assessment of Novel Foods explain (1) the notification procedure that includes submission of novel food notification, submission of safety assessment data package, and SoPs and (2) regulatory considerations that include environmental impact, coordination of regulatory decisions, postmarket monitoring, etc. Subsequent chapters include the guidelines for assessing novel foods from plants and microbes, including the procedure for the generation of information on substances with no history of safe use, novel process, and information on genetic modification, in both cases. However, only the part of the guidelines dealing with the generation of information requirements for safety assessment of novel foods derived from plants through genetic modification (Section 4.1.3) requires explanation in this chapter.

7.3.1 Characterization of Derived Line

The safety of novel products is assessed relative to a conventional counterpart having a history of safe use, taking into account both intended and unintended effects. Any significant differences between the novel and the conventional variety are then assessed for potential adverse health effects. Whether the modification has inadvertently increased the toxicity or allergenicity potential of a new variety or reduced the nutritional quality will be assessed.

7.3.2 Genetic Modification Considerations

Traditionally developed plants require a multidisciplinary assessment since details of the modifications may be largely unknown. Conventional breeding may result in a food crop that requires a premarket notification if selected characteristics fall well outside the agronomic, nutritional, and compositional range for that species. The extent of backcrossing should be fully described as the process can remove a large percentage of the donor parents' genetic material from the progeny selected for food use.

For cultivars developed through modern techniques, the following areas should also be addressed:

1. *Description of the genetic modification(s)*: Methods and manipulations involved in the modification of an organism must be provided to identify genetic material potentially inserted, deleted, mutated, or rearranged in the host genome. This includes method of modification (*Agrobacterium* mediated or direct), description of genetic material delivered including marker genes, regulatory, and other elements affecting the function of the DNA, its size and identity, location and orientation of the sequence in the final vector/construct, and function in the organism and other manipulations that may change the amino acid sequences.
2. *Characterization of the genetic modification(s)*: Information on the DNA insertions including characterization and description of all inserted genetic materials, number of insertion sites, data to demonstrate if complete or partial copies have been inserted into the genome, data to demonstrate whether the arrangement of the genetic material used for insertion has been conserved or whether significant rearrangements have occurred upon integration, the organization of the inserted genetic material at each insertion site including copy number and any potential chimeric open reading frames created by the insertion(s) with contiguous plant genomic DNA if the inserted genetic material is truncated.

Information on the expressed substances including gene product (protein or untranslated RNA), gene product's function, phenotypic description of the new trait(s), level and site of expression of the

gene product(s), and the levels of its metabolites should also be presented. It should be shown that deliberate modifications made to the amino acid sequence of the expressed protein result in changes in its posttranslational modification or affect sites critical for its structure or function. When genetic manipulations alter regulation of endogenous genes, the characteristics and level of gene expression should be compared with that of the unmodified host. Evidence for alteration of one or several endogenous genes in the host plant, expression pattern of any new fusion proteins, intended effect that the modification has been achieved, and that traits are expressed and inherited with laws of inheritance should be given.

7.3.3 History of Organism

The history of both donor and host organisms provide important information such as toxin production, which will be important in food safety assessment.

7.3.4 Dietary Exposure

Dietary exposure assessment estimates how much of the food is likely to be consumed at what frequency and what role it is likely to play in the diet, if different from the role of the unmodified food; the potential impact of that food on the dietary intake of nutrients; modifications in the level or nature of bioactive substances, antinutrients, or toxins; and the potential exposure to gene expression products.

A differential impact in subgroups of the population (e.g., children, infants, elderly, ethnic groups, susceptible populations) should be evaluated as well as the impact on the population as a whole.

7.3.5 Nutritional Considerations

Nutritional considerations are broadly dealt under the topics Unintended Nutritional Effects and Intended Nutritional Effects

7.3.5.1 Guidelines for Producing Data for Nutritional Evaluation

1. *Function of the data to be submitted*: Data should show that the assessment of any significant unintended genetic modification affecting the nutritional quality of the food has occurred as a result of the introduction of the novel trait. Data should be provided for the raw food; in other words, the edible part of the plant in its unprocessed state. Data may also be required for the food prepared for human consumption by conventional means to examine the effects of processing and storage. Data on the novel food should be compared, at a minimum, to data on the near-isogenic, nonmodified parent variety.
2. *Hypothesis and appropriate study design*: All sources of potential variation in nutritional composition should be controlled in the study. Modified plants should be grown under the condition for which they are developed and control plants should be grown in suitable conditions. Multilocation trials representing the growing regions should be done, including the near-isogenic lines. A sampling plan should be developed prior to the start of the experiment and sampling should be done at the correct stage of maturity. Appropriate analyses should be done on all consumed parts of the plant, within a reasonable timeframe. The criteria for selecting the nutrients analyzed and the rationale for the exclusion of any nutrient should be given. All components of all samples are analyzed by a single laboratory using internationally approved and validated analytical methods, following consistent and appropriate sample storage and preparation.

3. *Nutrient composition*: The following is a generic list of the components of novel foods that should be analyzed:
   a. Proximate composition (i.e., ash, moisture, protein, fat, fiber, carbohydrate);
   b. Content of true protein, nonprotein nitrogenous material (e.g., nucleic acids and aminoglycosides), amino acid profile;
   c. Complete fatty acid profile, total nonsaponifiable component, total sterols;
   d. Composition of the carbohydrate fraction (e.g., sugars, starches, chitin, tannins, nonstarch polysaccharides, lignin);
   e. Composition of micronutrients, i.e., significant vitamin and mineral analyses;
   f. Presence of naturally occurring or adventitious antinutritional factors e.g., phytates, trypsin inhibitors, etc.;
   g. Predictable secondary metabolites, physiologically active (bioactive) substances, other detected substances.
4. *Nutritional performance of modified plant*: Response of known antinutrients to processes, which are normally expected to neutralize their activity, should be measured using compositional analysis. Storage stability with regard to nutrient degradation and performance of product in relation to the intended benefit should be studied.
5. *Nutrient bioavailability and presence of new or altered antinutrients*: When GM foods form an important component or regular diet, animal studies may be needed to assess nutritional adequacy to determine if there have been changes in the bioavailability of nutrients or if the composition is not comparable to conventional foods.
6. *Information to be included in the submission*: This includes names of all the cultivars that were represented in the study; complete description of the experimental design, experimental conditions, and how sources of variation for nutrient levels were controlled; complete description of sample collection and sample preparation; citation and description of the analytical and statistical methods used to obtain data for the nutritive and nonnutritive components; nutrient and related data for test, control, and commercial cultivars; results of statistical analyses; raw data for all components analyzed from all locations used to grow the plant; published data if available; intended use of the plant as food in Canada, i.e., ingredient type(s); possible end products; level of use if different from current products that it would replace; and known patterns of use,consumption of the food and its derivatives, and any foreseeable unintended uses.
7. *Decision-making process*: If novel food is not nutritionally equivalent to that of its counterparts, additional nutritional data may be required on a case-by-case basis. Detection of a major change caused by an unintended nutritional effect may require limits on the use of the food in food products or a requirement for labeling that goes beyond basic provisions.

While making the nutritional evaluation of expected or unexpected increased levels of a nutrient or bioactive substance, the data should include level of the targeted nutrient or other substance expected in the food, intended target group, expected level of exposure to the substance through consumption of the food by the target group/vulnerable subgroups/all age and sex groups, how the expected level of exposure to the targeted nutrient differs from the current levels of exposure from all sources, any potential use of the product as a replacement of existing foods, and data in support of the safety of the expected level of exposure.

7.3.6 Toxicology Considerations

Toxicology studies are not considered necessary where the substance or a closely related substance has been consumed safely in food at equivalent intakes. For this, the new substance may be isolated and shown biochemically and functionally equivalent to that produced in the recombinant DNA plant. For proteins, the assessment of potential toxicity should focus on amino acid sequence similarity between the protein and known protein toxins and antinutrients, as well as stability to heat or processing and to degradation in appropriate gastric and intestinal model systems. Acute oral toxicity studies using gram per kilogram body weight doses of the novel protein are appropriate for assessing the potential toxicity of proteins. Studies to assess the toxicity of introduced substances other than proteins include assays of metabolism, toxicokinetics, chronic toxicity/carcinogenicity, impact on reproductive function, and teratogenicity.

7.3.7 Allergenicity Considerations

To assess the possible allergenicity of a newly expressed protein, determination of the following are required: the allergenicity of the source of the introduced protein and amino acid sequence similarity with known allergens and certain physicochemical properties, including its susceptibility to enzymatic degradation. Food proteins that are not allergens and that are altered by mutagenesis techniques need only be assessed for the likelihood that the mutagenized protein is a de novo allergen.

7.3.7.1 Initial Assessment

1. *Source of the protein*: Allergenic sources of genes would be defined as those organisms for which reasonable evidence of IgE-mediated oral, respiratory, or contact allergy is available. Describe any reports of allergenicity associated with the donor organism.
2. *Amino acid sequence homology*: Overall structural similarities that can be predicted using sequence homology searches that compare the structure of newly expressed proteins with all known allergens should be conducted using various algorithms, software, and molecular docking.
3. *Pepsin resistance*: A correlation exists between resistance to digestion by pepsin and allergenic potential.

7.3.7.2 Specific Serum Screening

For those proteins that originate from a source known to be allergenic, or have sequence homology with a known allergen, testing in immunological assays is required. Sera from individuals with a clinically validated allergy to the source of the protein can be used to test IgE binding of the protein in in vitro assays.

7.3.8 Chemical Considerations

Identification and levels of chemical contaminants must be reported. To identify the production of chemical contaminants derived from the process of genetic engineering, comparative data with the near-isogenic line should be given. Food additives present in the final food (e.g., anticaking agents, carrier solvents, solid diluents, colors, preservatives) or processing aids used during the course of manufacture should be identified and indicated.

7.4 REQUESTS FOR ADDITIONAL INFORMATION

If any information on GM food is found insufficient, Health Canada will demand the party to specifically furnish the same. Within 90 days after receiving the additional information, the Director shall assess it and if sufficient to establish the safety of the novel food, the party will be intimated in writing that the information is sufficient (Fig. 13.1).

7.5 SUMMARY REPORT OF FINDINGS

Once evaluators have completed their assessments, they summarize their findings and recommendations in a report.

7.6 PREPARATION OF FOOD RULINGS PROPOSAL

A Health Canada Food Rulings Proposal is reviewed by directors and the Director-General in the Food Directorate, and ensures that all issues have been addressed. Once this has been done, a decision is made whether or not to approve the product.

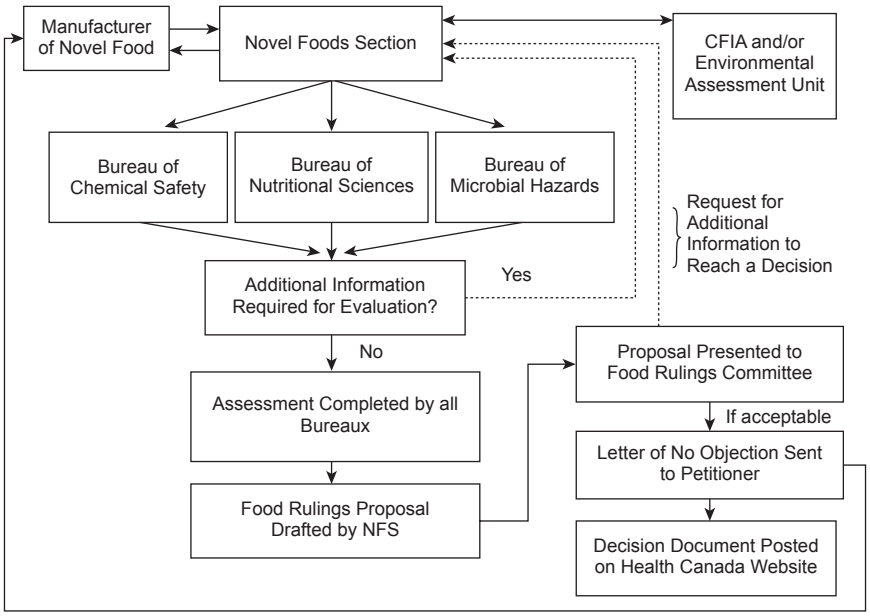

FIGURE 13.1

Processing a novel food notification and requests for additional information in the Food Directorate. *CFIA*, Canadian Food Inspection Agency; *NFS*, Novel Foods Section.

Adapted from Health Canada, 2006. Guidelines for the Safety Assessment of Novel Foods, Food Directorate, Health Products and Food Branch, Health Canada, pp. 111. Available at: http://www.hc-sc.gc.ca/fn-an/legislation/guide-ld/nf-an/guidelines-lignesdirectrices-eng.php.

7.7 LETTER OF NO OBJECTION

Once product evaluation is complete, the Food Rulings Proposal is approved, and other regulatory approvals such as environmental and feed safety are in place, a "Letter of No Objection" is sent to the party.

7.8 DECISION DOCUMENT ON HEALTH CANADA'S WEBPAGE

A decision document, describing the novel food and summarizing the safety information used to determine its safety as a food is posted on the Novel Foods and Ingredients page of the Health Canada website.

8. CONCLUSION

Genetic modifications through transgenesis lead to risks related to food safety, the environment, horizontal gene transfer with special regards to antibiotic resistance genes, and acquired resistance by pests. Transgene introgression in the exonic region of recipient genomes may also lead to the generation of unintended proteins and toxicants or deactivate the vital genes. With the emergence of biotechnological tools for genetic modification, world nations have developed their own robust systems to monitor the research and commercialization of these techniques, mostly following the guidelines in the CPB. As of now, every nation holds their own methodologies to assess and manage every possible risk associated with the release of GMOs into their environments. In this chapter we have discussed biosafety mechanisms in general and bioregulatory mechanisms in particular with respect to the leaders in this field, India, the European Union, the United States, and Canada.

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CHAPTER

14

CRITICAL EVALUATION OF THE BENEFITS AND RISKS OF GENETICALLY MODIFIED HORTICULTURAL CROPS

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1. INTRODUCTION

Natural resources (light, water, air, and soil) are the key driving forces on Earth. Humans started using soil and water for harvesting the light and air through plants to cater for the food, feed, fiber, and fuel (4fs) needs of themselves and others. Although the concept of agriculture has been in vogue from time immemorial, sustainable agriculture came into existence 800–1000 years later. The latter tries to provide the same 4fs to the population with a concern over the environment and economic sustainability by use of decreased agricultural inputs. The world population is projected to increase approximately to 9 billion in the next 30 years, and the increased demand for 4fs will pose a great challenge to the agricultural sector. In addition to increasing population pressure, inadequacy of arable land and water, climate change, and associated biotic and abiotic stresses will affect agricultural productivity and lead to food insecurity. This necessitates the development of varieties that can outperform the existing varieties, conversion of more land into cultivated acres, and more inputs so as to meet societal needs. Furthermore, it is estimated that an additional 1 billion ha of cultivated land will be required by 2050 to achieve the targeted production (Tilman et al., 2002), besides the fact that 1 ha of land is being lost every 7.7 s as estimated by the International Rice Research Institute in the Philippines. Achieving food security against these challenges, without increasing the environmental footprint, will require an integrated and diversified approach. This is possible through improved crop performance in terms of high yield and tolerance to biotic and abiotic stresses, which can be easily achieved by supplementing conventional breeding with modern biotechnological techniques such as "transgenics."

Global adoption of genetically modified (GM) field crops is on the high side as compared to horticultural crops, though horticultural biotechnology has been at the forefront for more than 2 decades, right from the commercialization of the first ever transgenic crop in the form of Flavr-Savr transgenic tomato with enhanced shelf-life. This entered the US market as fresh tomato in 1994 and reached the United Kingdom in the form of tomato paste in 1996. Herbicide tolerance is dominating the GM horticultural crops acreage followed by insect and virus resistance. GM potatoes, tomatoes, papaya, and sugar beet contribute to approximately 80%–90% of globally cultivated GM horticultural crops, and other horticultural crops include apple, sweet pepper, eggplant, squash, melon, etc.

Genetic Engineering of Horticultural Crops. http://dx.doi.org/10.1016/B978-0-12-810439-2.00014-3

2. NEEDS-BASED TRANSGENIC RESEARCH IN HORTICULTURAL CROPS

2.1 FRUITS

The production of new varieties by conventional breeding is a complex and time-consuming process, especially for fruit trees, because of high levels of heterozygosity, pronounced inbreeding depression, long juvenile phase, and complex reproductive biology. Because they are large perennial crops, a relatively small number of seedling progenies will be available for evaluation and it takes many years for breeders to evaluate fruit quality. In addition, the characters that constitute a good cultivar in most instances are polygenic in their inheritance; thus the probability of recombination of gene sets that govern the essential characteristics of a given cultivar is low (Perl and Eshdat, 2007).

The overall objectives of fruit breeding are to get maximum quality production per unit area with low cost, besides tolerance to biotic and abiotic stresses. Generally, the objectives are distinct and vary with fruit crops, location, and requirements of consumers. A wide variety of transformation (*Agrobacterium*-mediated transformation, biolistic bombardment, transient expression, rootstock transformation) and regeneration protocols are used to produce transgenic fruit trees. Most of the basic protocols available are old enough and they need subsequent efforts for optimization of the technique to new genotypes. Unfortunately, the time required to produce transgenic fruit trees is too long, especially for the analysis of fruit characters, and there is a need for faster and stable transformation systems. The production of marker-free transgenic plants is another growing area of interest with concern over end users.

2.2 VEGETABLES

The tools of biotechnology have been applied to vegetable crops to achieve foreign gene insertion and expression of additional and, in some cases, novel traits since 1995. The traits engineered are diverse and they include insect and nematode resistance, disease resistance (to viruses, fungi, and bacteria), tolerance to abiotic stresses (salinity and drought), production of edible vaccines and therapeutic products, nutritional enhancement, and metabolic engineering. Genetically engineered tomato (Flavr Savr) with enhanced fruit quality was the first example of a transgenic vegetable crop, which reached the market rapidly like other transgenic field crops, namely, canola, corn, potato, and soybean. Transgenic squash and zucchini with virus resistance followed tomato in the US market. While an increasing number of vegetable crops are becoming amenable for transformation technologies, it appears that only a few techniques are available to introduce foreign genes into vegetable crop species, with the most popular being *Agrobacterium*-mediated transformation. Optimization of gene transfer methods, transformation efficiencies, and tissue-culture protocols still remains a challenge for some vegetable species. The genetic basis of complex agronomic traits such as flavor and texture, maturity and yield is currently challenging to be defined in potato, because many genes are responsible for these phenotypes. Transgenic vegetable crops that are consumed fresh warrant additional consumer concern compared to field crops. Vitamin-enhanced tomatoes or high-antioxidant carrots could provide a strong example of the potential benefits of transgenic produce. Improvement of some important traits is hampered because of the lack of genetic diversity in vegetable crops. Crops and their corresponding traits are presented in Table 14.1.

Table 14.1 Vegetable Crops With Traits for Transgenic Study

Crop	Traits
Solanaceae	Photoperiod insensitivity, fertilizer use efficiency, better keeping quality
Beans	Suitability for freezing and canning
Watermelon	Attractive round/spherical fruit shape, thick flesh with an attractive color, small seed cavity, sweet juicy taste, musky flavorsome fruits
Musk melon	Total soluble solids not less than 10%, tough netted skin to withstand long-distance transportation
Cucumbers	Early and high total marketable yield, pistillate flowers at lower node numbers
Onions	Longer bulb storage life, bulb quality (size, shape, color, dormancy, amount of soluble solids)
Radish	White long stump and nonpithy roots with thin tap root and nonbranching habit, pungency of roots

Table 14.2 Summary of Approved Transgenic Ornamental Crops as of October 2015

Crop	Traits	Gene(s) Introduced	No. of Events
Carnation	Sulfonylurea herbicide tolerance	*sur*B	19
	Modified flower color	*dfr*, *hfl* (f3′5′h), *bp*40 (f3′5′h), *sfl* (f3′5′h), *dfr-diaca*, *acc* (truncated)	
Rose	Modified flower color	5*AT*, *bp*40 (f3′5′h)	2

Source: ISAAA GMO Approval Database.

2.3 ORNAMENTALS

Ornamental crops can be classified as floriculture and nursery plants, shrubs, trees, and foliage plants for outdoor and indoor use. Ornamental crops are produced with the purpose of beautifying, decorating, or enhancing the environment, and exclude plants intended for commercial food production such as vegetables and fruits. Target quality traits, apart from the usual agronomic traits such as biotic and abiotic stress tolerance, include flower color, size, volatiles, fragrance, flower and leaf longevity, morphology, and plant architecture. A list of approved transgenic ornamental crops is presented in Table 14.2. Many genes involved in plant volatile synthesis are cloned and manipulated in transgenic plants resulting in altered volatile profiles. These studies demonstrate that plant volatile profiles can be genetically engineered and will be useful in improving floral fragrance and plant defense (Clark et al., 2009). Application of transgenic technology to ornamentals to improve their postharvest attributes provides an opportunity to cut down postharvest costs associated with labor, chemicals, and environmental control, contributing to environmentally sustainable practices.

Despite the documented success of the techniques, utility, and enormous benefits rewarded by genetic engineering, there are only a limited number of commercialized transgenic ornamentals in the market. Presently, the only commercialized ornamental plant in the market is carnations engineered for qualitative traits. Reports indicated that consumer perception of genetically modified organisms (GMOs) is mixed, but most surveys are conducted with regard to the presence of GMOs in food and not ornamental plants. A survey of master gardeners revealed that ~73% of survey respondents expressed interest in purchasing a GM product for the garden.

2.4 PLANTATION CROPS

Both direct and indirect DNA delivery systems were employed to transform coffee by various workers. The major objectives for using the genetic engineering technique in coffee are to introduce new traits into elite coffee genotypes, develop new cultivars with desirable traits such as pest and disease resistance, herbicide resistance, drought and frost tolerance, and improve cup quality, which is difficult through classical breeding. Despite significant advances over the last 20 years, coffee transformation is far from a routine procedure in many laboratories. The concerted efforts on coffee genomics led to the identification of many candidate genes, some of which are being cloned and characterized. These include caffeine biosynthesis gene (Satyanarayana et al., 2005), sucrose synthase gene, osmotic stress response genes (Hinniger et al., 2006), genes for seed oil content (Simkin et al., 2006), and several pathogen resistance genes such as *Mex-1* gene (Noir et al., 2003), SH_3 gene (Prakash et al., 2004), and *Ck-1* gene to coffee berry disease (Gichuru et al., 2008) and would be a potential *cisgene* candidate for future coffee improvement.

3. CROP PRODUCTIVITY WITH SPECIAL REFERENCE TO ABIOTIC AND BIOTIC STRESSES

Biotic stresses such as insect pests and weeds are the major concern globally for sustainable agricultural production. Various ways and means to manage these biotic factors have been developed through intensive research and updated as and when the situation demands. However, no environmentally sustainable solutions exist until now for the control of insect pest and weeds. Genetic engineering for abiotic stress resistance is now being focused across the globe. Genetic engineering of abiotic stress resistance in horticultural crops is in its infancy and data on the yield and other benefits are available only at the laboratory level. The current data (1996–2016) on commercialized genetically engineered horticultural crops for biotic and abiotic stress resistance revealed that they have benefited very little from biotechnology when compared to field crops (Table 14.3).

4. CONTRIBUTION OF BIOTECH CROPS TO FOOD SECURITY, SUSTAINABILITY, AND CLIMATE CHANGE

Genetically engineered crops in general increase crop productivity through reduction in cost of cultivation, hence they help farmers to earn a reasonable income, provide more affordable food to poor pupils, and indirectly contribute to alleviation of poverty and hunger. They also act as an agent for biodiversity conservation by reducing the agriculture eco footprint by means of lower pesticide and other inputs use, which leads to decreased CO_2 emissions. Genetically engineered crops also help to mitigate climate change through savings on fossil-based fuel thereby reducing greenhouse gas emissions. Biotech cotton has made a significant contribution to the incomes of 16.5 million poor farmers and their families in India, China, Pakistan, Brazil, Argentina, Burkina Faso, Myanmar, Mexico, Sudan, Paraguay, and South Africa. In addition to economic gains, farmers benefited enormously by at least a 50% reduction in the number of insecticide applications, thereby reducing farmers' exposure to insecticides, and importantly contributing to a more sustainable environment and better quality of life.

Table 14.3 Summary of Approved Transgenic Horticultural Crops as of October 2015

Crop	Traits	Gene(s) Introduced	No. of Events
Apple	Modified product quality (nonbrowning)	*PGAS* (PPO suppression gene)	3
Bean	Viral disease resistance	*Ac*1 (viral replication protein, Rep)	1
Chicory	Herbicide tolerance + pollination control system	*bar*, *barnase*	3
Eggplant	Lepidopteran insect resistance	*cry1Ac*	1
Melon	Delayed ripening/senescence	*sam-k*	2
Papaya	Viral disease resistance	*prsv_cp*, *prsv_rep*	4
Plum	Viral disease resistance	*ppv_cp*	1
Potato	Coleopteran insect resistance	*cry3A*	46
	Viral disease resistance	*pvy_cp*	
	Coleopteran insect resistance, viral disease resistance	*cry3A*, *pvy_cp*	
	Modified starch/carbohydrate	*gbss* (antisense fragment)	
	Reduced acrylamide potential + black spot bruise tolerance	*asn1*, *ppo5*	
	Modified starch/carbohydrate + reduced acrylamide potential + black spot bruise tolerance	*asn1*, *pPhL*, *ppo5*, *pR1*	
	Modified starch/carbohydrate + reduced acrylamide potential + black spot bruise tolerance + late blight resistance	*asn1*, *pPhL*, *ppo5*, *pR1*, *Rpi-vnt1*	
Squash	Stacked viral disease resistance	*cmv_cp*, *zymv_cp*, *wmv_cp*	2
Sugar beet	Glufosinate herbicide tolerance	*Pat*	3
Sweet pepper	Viral disease resistance	*cmv_cp*	1
Tomato	Delayed ripening/senescence	*anti-efe*, *sam k*, *pg* (sense or antisense), *acc* (truncated), *accd*, *pg* (sense or antisense)	11
	Lepidopteran insect resistance	*cry1Ac*	
	Delayed fruit softening	*pg* (sense or antisense)	
	Viral disease resistance	*cmv_cp*	

Source: ISAAA GMO Approval Database.

The metaanalysis on biotech crops in last 20 years concluded that on an average 37% reduction of chemical pesticide, 22% higher crop yield, and 68% increase of farmer profits (Klumper and Qaim, 2014). "Yield gains and pesticide reductions are larger for insect-resistant crops than for herbicide-tolerant crops. Yield and profit gains are higher in developing countries than in developed countries. Continued opposition to technologies that were shown to be beneficial and safe entails unnecessary human suffering and environmental degradation" (Qaim, 2016).

5. BENEFITS OF GENETICALLY ENGINEERED CROPS

5.1 IMPROVED PRODUCTIVITY

The insect-resistant technology used in cotton and corn has consistently delivered yield gains from reduced pest damage. The average yield gains over the 1996–2014 period across all users of this technology have been +13.1% for insect-resistant corn and +17.3% for insect-resistant cotton relative to conventional production systems. Furthermore, 2014 was the second year in which insect-resistant soybeans were grown commercially in South America, where farmers have seen an average of +9.4% yield improvements. The herbicide tolerant technology used has also contributed to increased production; improving weed control, providing higher yields in some countries, and helping farmers in Argentina grow "second crop" soybeans after wheat in the same growing season. The higher level of technology gains realized by farmers in developing countries relative to farmers in developed countries reflect weaker provision of intellectual property rights coupled with higher average levels of benefits in developing countries. Between 1996 and 2014, crop biotechnology was responsible for additional global production of 158.4 million tonnes of soybeans and 321.8 million tonnes of corn. The technology has also contributed to an extra 24.7 million tonnes of cotton lint and 9.2 million tonnes of canola. GM crops are allowing farmers to grow and produce more without using additional land. If crop biotechnology had not been available to the (18 million) farmers in 2014, maintaining global production levels would have required additional plantings of 7.5 million ha of soybeans, 8.9 million ha of corn, 3.7 million ha of cotton, and 0.6 million ha of canola.

5.2 ENVIRONMENTAL IMPROVEMENTS

Crop biotechnology has contributed significantly to reducing the release of greenhouse gas emissions from agricultural practices. This resulted in less fuel use and additional soil carbon from reduced tillage in GM crops. In 2014, this was equivalent to removal of 22.4 billion kg of carbon dioxide from the atmosphere or equal to removing 10 million cars from the roads for 1 year. Crop biotechnology has reduced pesticide spraying (1996–2014) by 581 million kg (−8.2%). Consequently, this has decreased the environmental impact associated with herbicide and insecticide use on the area planted with biotech crops by 18.5%.

5.2.1 Abiotic Stress

Traditional breeding strategies are confounded by the complexity of stress tolerance traits, low genetic variance in yield components under stress conditions making phenotyping of segregating population a cumbersome process, and lack of efficient selection techniques (Bohnert and Cushman, 2000). Furthermore, quantitative trait loci (QTL) that are linked to tolerance at one stage of development can differ from those associated with tolerance at other stages (Foolad and Lin, 1997). Once identified, these useful QTLs not only require extensive breeding to restore the desirable traits but are also time consuming while removing the chromosomal segments that interfere with the recurrent parent genome. Today, with the advancement in science and technology, efforts are being made to generate transgenic plants with heterologous genes. Scientists have developed tools to effect interkingdom gene transfer, which facilitates suitable modifications ranging from gene sequence to protein quantity required by the host that imparts the ability to tolerate stresses. In contrast to traditional breeding, genetic engineering with a small number of abiotic stress-tolerant genes appears to be a more attractive and rapid approach. First-generation transgenic engineering strategies rely on the transfer of one or a couple of genes that

encode either biochemical pathways or endpoints of signaling pathways. These gene products can directly or indirectly protect them from stresses.

Many transgenic studies have shown that stress-specific genes employed in molecular breeding can be grouped into three major categories: (1) genes involved in signal transduction pathways and transcriptional control such as mitogen-activated protein kinase, CBL-interacting protein kinase, SOS kinase, and transcription factors such as AP2/ERF, bZIP, MYB, MYC, NAC, Cys2His2 zinc finger, and WRKY; (2) genes involved in membrane and protein protection functions such as heat shock proteins and late embryogenesis abundant proteins, synthesis of osmoprotectants such as proline, betaine, sugars and sugar alcohol, polyamines, and detoxification, or elimination of reactive oxygen species (ROS) such as various enzymes and nonenzymatic antioxidants; and (3) genes involved in water and ion uptake and transport such as aquaporins and ion transporters (Bhatnagar-Mathur et al., 2008; Jewell et al., 2010).

5.2.2 Genetic Engineering for Moisture Deficit Stress Tolerance

Improving water use efficiency by precise regulation of stomatal opening and closure events; osmotic adjustments by the overproduction of osmolytes/osmoprotectants and ROS detoxification through nonenzymatic antioxidants (ascorbate, α-tocopherol, glutathione, etc.) and enzymatic antioxidants (superoxide dismutase, catalase, and enzymes of ascorbate-glutathione cycle); and insertion of master regulators of all the foregoing among others will be a wise strategy mainly for engineering the tolerance to moisture deficit stress (Moller, 2001; Zhu, 2002). Osmotic adjustment is one of the vital cellular defenses to osmotic stress. This is also associated with an increase in crop yield under drought in many crop plants (Zhang et al., 1999).

The data include a list of fruits and vegetables that have been genetically engineered for enhanced moisture deficit stress tolerance.

S. No.	Crop	Gene	Target Trait	References
1	Potato	TPS1	Drought tolerance	Yeo et al. (2000)
2	Tomato	*CBF*1	Moisture deficit stress tolerance	Tsai-Hung et al. (2002)
3	Lettuce	ABF3	Drought as well as cold stress tolerance	Enkhchimeg et al. (2005)
4	Apple	*Osmyb4*	Drought stress	Pasquali et al. (2008)
5	Tomato	Bacterial mannitol-1-phosphate dehydrogenase (*mtlD*)	Drought and salinity	Khare et al. (2010)
6	Potato	*CBP*80	Drought	Pieczynski et al. (2013)
7	Fruit crops	*RCAR*1, *PYR*1, PYR1-like (*PYL*) genes (*PP2C SnRK2*)	Drought, salt, and cold tolerance	Mickelbart et al. (2015).
8	Tomato	*CBF*1	Oxidative stress as well as water deficit stress tolerance	Hsieh et al. (2002a,b)
9	Banana	*MusaWRKY*71	Oxidative and salt stress tolerance	Shekhawat and Ganapathi (2013)
10	Tomato	*SlAGO*4A	Salt and drought tolerance	Huang et al. (2016)

5.2.3 Genetic Engineering for Salinity Tolerance

A high K^+/Na^+ ratio is essential for cellular metabolism, which is disrupted under salt stress. In plants, Na^+ competes with K^+ for uptake under saline conditions. Hence expression of cation transport systems that specifically transport K^+ into the cell might help in maintaining ionic balance. Overexpression of yeast *HAL*1, a regulator of K^+ transport, in tomato resulted in an increased K^+ accumulation and better salt tolerance under NaCl stress (Gisbert et al., 2000; Rus et al., 2001), which suggests that K^+ accumulation can be genetically manipulated to improve salt tolerance of vegetable crops. The tonoplast Na^+/H^+ antiporters transport Na^+ into the vacuole by using electrochemical gradient of protons generated by the vacuolar H^+-adenosine triphosphatase and H^+ inorganic pyrophosphatase. This vacuolar compartmentation prevents deleterious effects of Na^+ and helps in osmotic adjustment. The table shows the list of fruits and vegetables that are genetically modified for improved salinity tolerance.

S. No.	Crop	Gene	Target Trait	References
1	Tomato	*AtNHX*1	Salinity tolerance	Zhang and Blumwald (2001)
2	Banana	*AhSIPR*10	Salinity and mannitol-induced drought	Rustagi et al. (2015)
		*MusaSAP*1	Stress amelioration pathways of banana	Sreedharan et al. (2012)
3	*Capsicum annum*	Osmotin	Salt tolerance	Subramanyam et al. (2011)
4	Strawberry	Osmotin	Salt tolerance	Husaini and Abdin (2008)
5	Carrot	Betaine aldehyde dehydrogenase, (*BADH*)	Salt tolerance	Kumar et al. (2004)
6	Apple	Cytosolic malate dehydrogenase (*MdcyMDH*)	Salt and cold tolerance in other fruit crops	Wang et al. (2016).
7	Tomato	*SlMBP*11	Salt stress tolerance	Guo et al. (2016)

5.2.4 Genetic Engineering for Cold Tolerance

Genetic engineering, with either one or a small number of genes being introduced into a crop species, has achieved considerable progress in improving tolerance to low-temperature (LT) stresses, including chilling and freezing. Biochemical and physiological changes that occur during cold acclimation are regulated by LTs through modifications in gene expression. Therefore cold regulated (COR) gene expression is critical to plants for imparting tolerance to both chilling (Gong et al., 2002; Hsieh et al., 2002a) and freezing (Thomashow, 1999). Moreover, the expression of specific genes upregulated by LT is highly correlated with the development of freezing tolerance (Thomashow, 1999). Some candidates for cold tolerance include COR genes (Chinnusamy and Zhu, 2002), *Crt/Dre Binding Factors* (*Cbf*s; Stockinger et al., 2001; Liu et al., 1998), *Inducer of CBF Expression* (*ICE*; Chinnusamy et al., 2003), and protein kinases (calmodulin and $Ca2^+$-dependent protein kinases) (Saijo et al., 2000). Goulas et al. (2007) demonstrated that vegetative

storage protein, a homolog of PR-10 protein, exhibited an in vitro cryoprotective role in autumn and winter conditions, which may bestow chilling stress tolerance in plants. The table shows the genes targeted in various fruits and vegetables for improving cold tolerance.

S. No.	Crop	Gene	Target Trait	References
1	Tomato	*CML44*(calmodulin-like)	Multiple abiotic stresses, namely, drought, salinity, and cold tolerance	Munir et al. (2016) and Du et al. (2011)
2	Tomato	*codA*	Chilling tolerance	Park et al. (2004)
		SAMDC	High- and low-temperature tolerance	Cheng et al. (2009)
3	Apple	*MsDREB*6.2	Drought tolerance	Liao et al. (2016)
4	Cucumber	*Dehydrin DHN10 DHN24*	Chilling tolerance	Yin et al. (2004)
5	Grapevine	*DREB*1*b*	Cold tolerance	Jin et al. (2007)

5.2.5 Biotic Stress Resistance

One of the major limitations of transgenic approaches for disease resistance is that some defense responses are only effective against certain pathogens (Punja and Raharjo, 1996). This is because of a huge variation (taxonomic and physiological) in lifestyle among fungal, bacterial, and viral pathogens, which made it impossible to develop effective broad-spectrum disease resistance. Furthermore, other challenges such as risks to the environment and consumer needs need to be addressed during the introduction of disease resistance traits into a plant species (Collinge et al., 2010).

The following four strategies have been applied to develop resistance against fungal and bacterial diseases using genes: (1) encoding pathogenesis-related proteins (PR proteins), antimicrobial peptides, or antimicrobial metabolites, (2) encoding detoxification mechanisms (3) with a role in pathogen recognition and (4) which regulate defense mechanisms (Collinge et al., 2010). Transgenic plants expressing chitinase and glucanase genes showed enhanced resistance to fungal disease in many fruit crops (Nookaraju and Agrawal, 2012; Gambino and Gribaudo, 2012; Litz and Padilla, 2012). Defensins, one of the classical examples of small antimicrobial peptides, interact with fungal-specific membrane components and subsequently permeabilize them to inhibit fungal growth (Coninck et al., 2013).

All viral molecules including genomes and three types of proteins, coat proteins (cp), movement proteins (MP), and proteins involved in genome replication (Rep), represent potential targets for the development of GM resistance to viral diseases (Collinge et al., 2010). This approach is based on the concept of pathogen-derived resistance in which pathogenic virus itself is used as a gene source for developing genetically engineered viral-resistant plants (Dasgupta et al., 2003). Some of the earliest success stories for producing virus-resistant plants by genetic engineering used viral CP as a transgene (Vigne et al., 2004; Litz and Padilla, 2012). Several studies on genetic engineering of fruit crops with CP gene suggest that transgene-conferred viral resistance is mediated by RNA via posttranscriptional gene silencing. Different RNA-mediated strategies such as antisense, small hairpin RNA, intron spliced hairpin RNA, or self-complementary inverted repeats were also employed to develop viral resistance to viruses (Jardak-Jamoussi et al., 2009).

5.2.5.1 Apple

In recent years the most important diseases of apple have been fire blight, caused by the bacterium *Erwinia amylovora*, and scab, caused by the fungus *Venturia inaequalis*. Fire blight caused serious losses all over the world (Norelli et al., 2003).

S. No.	Gene	Target Trait	References
1	Lytic peptide attacin E	Fire blight resistance	Ko et al. (2000)
2	Chitinase *Puroindoline* B, EPS-depolymerase	Scab resistance	Bolar et al. (2000) and Faize et al. (2003)
	Amp1, *AFP2*, *hordothionine*, *endochitinase*		Hanke et al. (2002), Borejsza-Wysocka et al. (2007), Malnoy et al. (2007), and Vinatzer et al. (2001)
	Vf gene orthologs		Xu and Korban (2000)
3	R gene	Partial fire blight resistance	Belfanti et al. (2004)

5.2.5.2 Banana

Genetic transformation and tissue culture regeneration systems are well established in banana. However, until now, there has been no significant progress in transgenic breeding compared to other crop plants. To improve disease resistance, several genes from different organisms have been incorporated in banana and these are listed below.

S. No.	Gene	Target Trait	References
1	*Then*-42, *StSy Cu SOD*, *Zn SOD*	Sigatoka disease resistance	Vishnevetsky et al. (2011)
2	Defensins, *Phdef1*, and *Phdef2*	*Fusarium* wilt resistance	Ghag et al. (2012)
3	*Rep*	Banana bunchy top virus	Tsao (2008)
4	*pflp* and *hrap*	*Xanthomonas* wilt resistance	Tripathi et al. (2009)
5	*Ferredoxin- like protein* (flp)	*Xanthomonas* wilt resistance	Namukwaya et al. (2012)
6	Rice *Xa21*	*Xanthomonas* wilt resistance	Tripathi et al. (2014)
7	Cysteine proteinases	Nematode tolerance	Roderick et al. (2012)
8	Vitamin A, vitamin E, or iron accumulation genes	Vitamin A, vitamin E, or iron	Pillay et al. (2012)

5.2.5.3 Grapes

Transformations were attempted in various cultivars of table and wine grapes with different objectives. Mainly, resistance to *Botrytis cinerea*, the causal agent of gray mold or botrytis bunch rot in grapes, downy mildew, caused by *Plasmopara viticola* (Berk. & M.A.Curtis), powdery mildew caused by *Uncinula necator* (Schwein.) Burrill (anamorph: *Oidium tuckeri* Berk), and Eutypa dieback, caused by the ascomycete fungus *Eutypa lata*, seriously affect grapevines worldwide, particularly *Vitis vinifera* cultivars.

Efforts were made toward the development of grapes resistant to microbial infections by transforming Thompson seedless with lytic peptide gene shiva 1 and the tomato ringspot virus coat protein (CP) gene as early as 1996 (Scorza et al., 1996). Genes encoding hydrolytic enzymes such

as chitinase, which degrade fungal cell wall components, are attractive candidates for improving disease resistance.

S. No.	Gene	Target Trait	References
1	Rice chitinase gene (*RCC*2)	Powdery mildew and anthracnose	Yamamoto et al. (2000)
2	*Vr-ERE*	Eutypa dieback	Roustan et al. (2000)
3	p*PGIP*	*Botrytis cinerea*	Aguero et al. (2005)
4	*Magainin*-2 (*mag*2)	Crown gall and powdery mildew diseases	Vidal et al. (2006)
5	Translatable and nontranslatable coat protein	Viral resistance	Balázs and Tepfer (1997)
6	Grapevine fanleaf virus, grapevine virus A, grapevine virus B, grapevine chrome mosaic virus, and tomato ringspot virus	Coat protein gene	Krastanova et al. (1995), Golles et al. (2000), and Mauro et al. (1995, 2000)
	Resistance to GFLV	GFLV	Gambino et al. (2005), Maghuly et al. (2006), and Vigne et al. (2004)
7	Replicase gene	GFLV	Smith et al. (2000), Wesley et al. (2001), and Vidal et al. (2006)
8	Antifreezing proteins	Low-temperature tolerance	Gutoranov et al. (2001)
9	*Def* H9, *iaa*M	Parthenocarpy	Mezzetti et al. (2002)

Further driving forces for the modification of constructs were safety considerations concerning (1) selection of viral sequences reducing the potential risk of recombination or (2) mutations of the coat protein (cp) gene suppressing particle assembly, heterologous encapsidation, and complementation. The study further indicated that transgenic grapevines did not favor the development of GFLV recombinant isolates to a detectable level.

5.2.5.4 Citrus

Major objectives of citrus transformation are to develop citrus canker, caused by the bacterial pathogen *Xanthomonas axonopodi* pv. *citrii*, coleopteran resistance, citrus tristeza virus resistance using pathogen-derived genes (Ghorbel et al., 2000), and *Phytophthora citrophthora* resistance using antifungal proteins (Fagoaga et al., 2001). Transgenic research in citrus was first reported by Kobayashi and Uchimiya (1989) and the first transgenic citrus was obtained by Vardi et al. (1990). Besides which, several genes have been targeted for enhanced resistance to various pests and diseases in citrus and the same are tabulated below.

S. No.	Gene	Target Trait	References
1	LEAFY (*LFY*) APETALA1 (*AP*1)	Shortened juvenile phase and precocious flowering	Pena et al. (2001)
2	Antibacterial peptide *D*, x*a21*, *Attacin A*	Canker resistance	Chen et al. (1997), Guo and Grosser (2004), Omar and Grosser (2005), and Boscariol et al. (2006)
3	Bovine lysozyme and defensin		Gonzalez et al. (2005)
4	Tomato *PR1*. *PR-5*		Fagoaga et al. (2001)

Continued

S. No.	Gene	Target Trait	References
5	*CP-CMV*	Virus resistance	Iwanami et al. (2004)
6	Modified endotoxin gene of *Bacillus thuringiensis* subsp. *tenebrionis* (*Btt*)	Insect resistance	Rhim et al. (2004)
7	Chitinase gene	*Phoma tracheiphila* and *Botrytis cinerea*	Gentile et al. (2007)
8	Modified plant thionin	Huanglongbing (*Candidatus Liberibacter asiaticus*, Las) and citrus canker (*Xanthomonos citrii*)	Hao et al. (2016)
9	Synthesized *cecropin* B	Huanglongbing (citrus greening)	Zou et al. (2016)
10	*Bacillus thuringiensis Cyt2Ca*	Diaprepes root weevil	Mahmoud et al. (2016)
11	Citrus psorosis virus coat protein-derived intragenic hairpin RNAi	Citrus psorosis virus resistance	Francesco et al. (2016)

5.2.5.5 Others Fruits

The table below contains the list of genes targeted in other fruit and vegetable crops such as pineapple, kiwi fruit, papaya, water melon, squash, tomato, potato, cabbage, etc.

S. No.	Crop	Gene	Target Trait	References
1	Pine apple	*chitinase* (*chi*), *ap24*	Heart and root rot resistance	Espinosa et al. (2002) and Yabor et al. (2006)
		PMWa-2 coat protein (*cp*) gene	Mealy bug wilt resistance	Perez et al. (2006)
		RNAi	Mealy bug wilt resistance	Hu et al. (2005)
		Rice cystatin gene	Reniform nematode tolerance	Sipes et al. (2002)
2	Kiwi fruit	*rol A, B*, and *C*	Better rooting	Rugini et al. (1991)
		Soybean β-1,3-endoglucanase cDNA	Gray mold resistance (*Botrytis cinerea*)	Nakamura et al. (1999)
		Stilbene synthase gene	Piceid (resveratrol-glucoside)	Kobayashi et al. (2000)
3	Papaya	Coat protein gene	Papaya ringspot virus resistance	Fitch et al. (1992)
		Mutated *replicase (RP)* gene	Papaya ringspot virus resistance	Xiangdong et al. (2007)
4	Water melon	Zucchini yellow mosaic virus and papaya ringspot virus-W *CP* genes	ZYMV and PRSV-W immunity	Yu et al. (2011)
5	Squash	Coat protein genes	CMV, ZYMV, and WMV	Fuchs and Gonsalves (2007)

S. No.	Crop	Gene	Target Trait	References
6	Tomato	*Bt*	*Spodoptera litura* and *Heliothis virescens*	Fischhoff et al. (1987)
		Gene *C1* of TYLCV	Tomato yellow leaf curl virus resistance	Fuentes et al. (2006)
		AC4 gene	Tomato leaf curl virus	Praveen et al. (2010)
		Osmotin-like protein (*OLP*) and chitinase (*Chi11*) belong to pathogenesis-related (PR) proteins	Enhanced tolerance to salt, drought, and fungal stresses	Kumar et al. (2016)
7	Cabbage	Synthetic fusion gene of *Bacillus thuringiensis* encoding a translational fusion product of *cry1B* and *cry1Abd*	Resistance to diamondback moth	Paul et al. (2005)
8	Cauliflower	Synthetic *cryIA (b)*	Resistance to diamondback moth	Chakrabarty et al. (2002)
9	Cucumber	*CMV-cp* gene zucchini green mottle mosaic virus coat protein (*ZGMMV-cp*)	Resistance to CMV	Nishibayashi et al. (1996) and Lee et al. (2002)
		Chitinase gene	Resistance to gray mold	Tabei et al. (1999)
10	Lettuce	Coat protein	Mirafiori lettuce virus	Yoichi et al. (2009)
11	Coffee	Synthetic version of *cry1Ac*	CBB, WSB, and leaf miner	Perthuis et al. (2006)

5.2.5.6 Potato

In 1995, Monsanto released the first biotech potato New Leaf for agricultural production containing the CryIIIA gene to provide resistance to Colorado potato beetle (Shelton et al., 2002). Potato varieties engineered for resistance to Colorado potato beetle have been in commercial production for several years and are technically and agronomically successful, allowing significant reductions in insecticide use (Shelton et al., 2002). Biotechnology-based disease resistance in potato was achieved by using pathogen-associated molecular pattern (PAMP) receptor proteins, i.e., pattern recognition receptors (PRR, ELR protein, which recognize the presence of the INF1 elicitin from *Phytophthora infestans*). A list of available cisgenic resistance genes of potato is given below which could be well exploited in the development of transgenics.

***Solanum* spp.**	**Gene Isolated**
Solanum demissum	R1, R2, and R3a
S. bulbocastanum	*Rpiblb1, Rpi-blb2*, and *Rpiblb3*
S. venturii	*Rpivnt1.1*
S. mochiquense	*Rpi-mcq1*
S. tuberosum ssp. *andigena* and *S. acaule*, respectively	*Rx1* and *Rx2* (potato virus X resistance gene)
S. spegazzinii	*Gro1-4* (root cyst nematode resistance)
S. tuberosum ssp. *andigena*	*Gpa2* (pale cyst nematode resistance gene)
S. chacoense, *S. demissum*, and *S. etuberosum*	*eIF4E* (potato virus Y resistance gene)

Biotech intervention on other traits of potato includes (1) tuber quality traits such as lower acrylamide (lower reducing sugar levels and decreased asparagine), black spot bruise resistance (downregulation of polyphenol oxidase), and increased amylopectin in starch (downregulation of granule bound starch synthase), and (2) nutrition-like vitamin C (overexpression of a single potato gene, galactose phosphorylase) and vitamin A (downregulation of beta-carotene hydroxylase), which have also been reported (Halterman et al., 2016).

6. ROLE OF MARKER-FREE TECHNOLOGY IN TRANSGENIC CROPS

6.1 NEED FOR TRANSGENICS DEVELOPMENT

Multiple strategies are being adopted to improve crop performance and yield, including traditional breeding and transgenic approaches. The traditional breeding approach has several limitations. First, it is time consuming since traits have to be selected over several generations, which is a substantial obstacle that could not be overcome by any means. Second, sexual hybridization between some species is not always successful because of incompatibilities. Lastly, only one trait can be introduced at a time leading to incremental improvements. Consequently, the use of improved breeding technologies is imperative to increase crop production (Tester and Langridge, 2010). Genetic engineering, on the other hand, can be a method of choice to overcome many of the limitations posed by traditional breeding. Once candidate genes for crop improvement have been identified, they are functionally characterized, first often in the model plant *Arabidopsis*, then in the respective crop plant, to manipulate those specific genes by gain- or loss-of-function approaches to improve performance, growth, and stress tolerance. Different kinds of genetic transformation methods are represented in Fig. 14.1.

Genetically engineered *Agrobacterium* strains and microprojectile bombardment are the most commonly used methods to drive foreign DNA into plant cells. The technique chosen for transformation has its own prerequisites and characteristics, which need to be optimized/evaluated first. For instance, in the case of *Agrobacterium*-mediated transformation, we need a reproducible tissue culture protocol, a virulent *Agrobacterium* strain, the basic and complementary sets of *Vir* genes, in vitro environmental factors, such as suitable growth medium, pH, temperature, and osmotic conditions, and phenolic stimulators (for *vir* gene expression). In nature, *Agrobacterium* senses and infects its host plants by phenolic exudation from wounds. Hence provision of phenolic stimulators such as acetosyringone makes an essential component of transformation. This beneficial effect of acetosyringone on transformation is described in several agricultural and horticultural crops. Cocultivation duration (duration of explants in contact with the bacteria) and amount of antibiotics for selection are underlined as important conditions (Petri et al., 2004).

Together with the gene of interest, marker genes required for selection of putative transgenic cells/tissues are also transferred. Marker genes allow selection of transformed cells or tissues by imparting ability to the transformed cell or tissue to grow in the presence of an antibiotic or herbicide. The most commonly used selection genes are neomycin phosphotransferase gene (*npt*II), which confers resistance to aminoglycoside antibiotics, and phosphinothricin acetyl transferase gene, which confers resistance to the herbicide phosphinothricin (Miki and Mchugh, 2004). Because of public concern, methods are being developed to avoid selection of transformed cells with antibiotics (Hohn et al., 2001) and subsequent introduction of antibiotic-resistant genes into food chains or herbicide tolerant genes into wild relatives (Zuo et al., 2001). There are several strategies developed to exclude the selection gene for marker-free transgenics, such as cotransformation (De Block and Debrouwer, 1991), site-specific

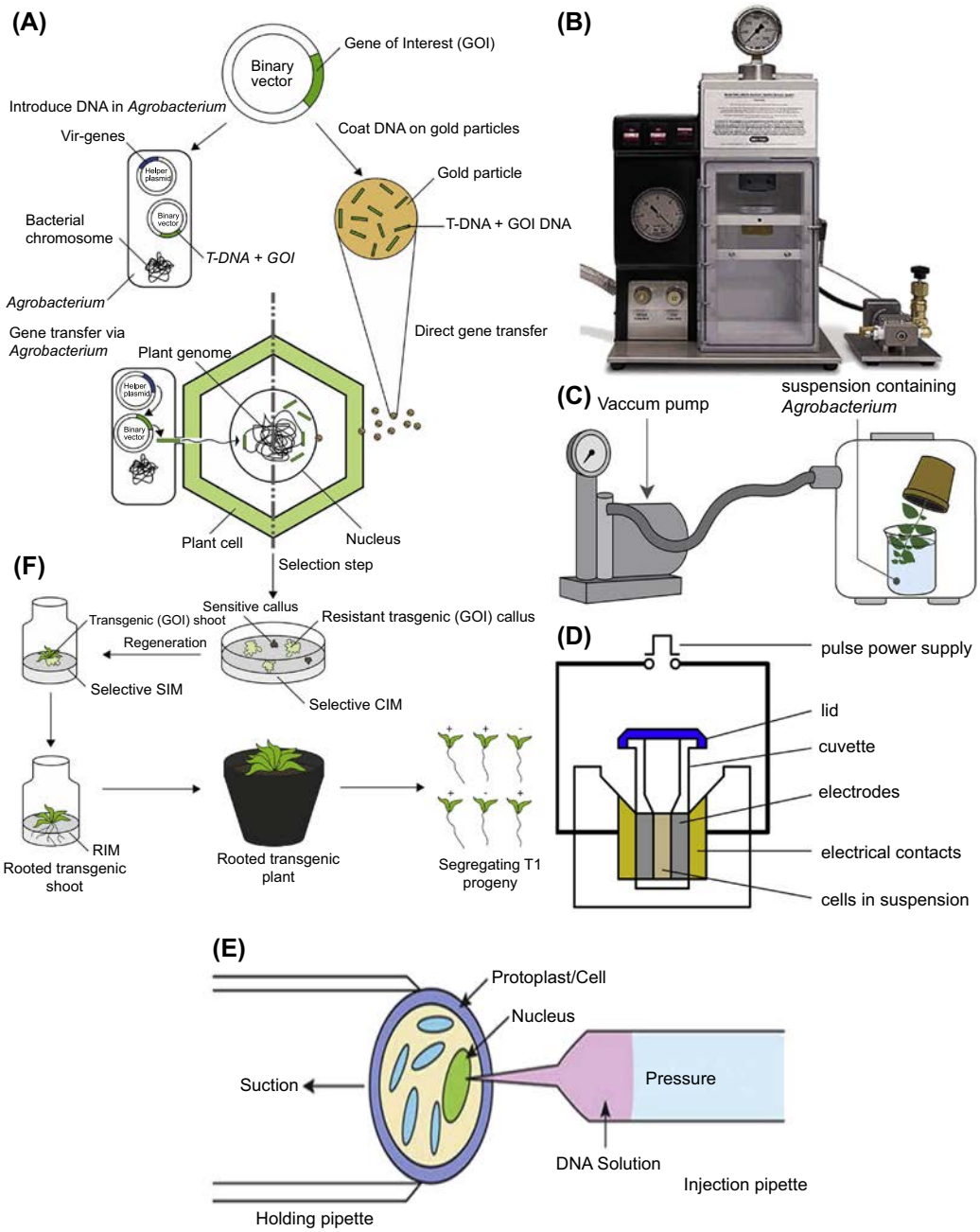

FIGURE 14.1 Different Methods for Genetic Transformation.

(A) General schema of *Agrobacterium*-mediated T-DNA transfer and direct DNA transfer (Anami et al., 2013). (B) Direct DNA transfer through particle bombardment or biolistics. (C) Vacuum infiltration (Rivera et al., 2012). (D) Electroporation. (E) Microinjection. (F) Regeneration and selection of transformed callus and shoots.

recombination (Gleave et al., 1999), Multi-Auto-Transformation (MAT) vector (Ebinuma et al., 1997), transposition system (Goldsbrough et al., 1993), and homologous recombination (Zubko et al., 2000), among which cotransformation is the most widely used method.

6.2 COTRANSFORMATION

Cotransformation is a very simple method to eliminate the marker gene from the nuclear genome. Cotransformation involves transformation with two plasmids. One plasmid carries a selective marker gene and the other carries the gene of interest. The following three methods are used in the cotransformation system: (1) two different vectors carried by different *Agrobacterium* strains (De Neve et al., 1997) and biolistic introduction of two plasmids in the same tissue (Kumar et al., 2010); (2) two different vectors in the same *Agrobacterium* cell (Sripriya et al., 2008); and (3) two T-DNAs borne by a single binary vector (2 T-DNA system) (Miller et al., 2002).

In this method, selectable marker genes can be eliminated from the plant genome at the time of segregation and recombination that occurs during sexual reproduction by selecting only the transgene of interest and not the marker gene in progeny. In spite of all this, there are several inevitable limitations. It is very time consuming and compatible only for fertile plants. The tight linkage between cointegrated DNAs limits the efficiency of cotransformation. Indeed, integration of marker gene and transgene is an indiscriminate event: both the marker gene and transgene may integrate in the same loci, which is not feasible for cotransformation.

6.3 SITE-SPECIFIC RECOMBINATION

6.3.1 Cre/loxP Site-Specific Recombination System

The Cre/loxP system consists of two components: (1) two loxP sites each consisting of 34 bp inverted repeats flanking the selection marker gene and (2) the cre gene encoding a 38 kDa recombinase protein that specifically binds to the loxP sites and excises the intervening sequence along with one of the loxP sites. The Cre/loxP system has been tested in several plants including *Oryza sativa* (Sreekala et al., 2005).

One of the greatest advantages of the Cre/lox system is the specificity of enzyme for its 34 bp recognition sequence. Removal of marker genes from transgenic plants using the Cre/lox recombination system of bacteriophage P1 requires retransformation and outcrossing approaches that are laborious and time consuming (Dale and Ow, 1991). To initiate the Cre/lox recombination for removal of the marker gene, other novel inducible systems are used such as chemical inducers (Zhang et al., 2006) and heat shock (Cuellar et al., 2006). Marker-free transgenic tomato plants expressing cry1Ac were obtained by using a chemically regulated Cre/lox-mediated site-specific recombination system (Zhang et al., 2006). Similarly, Ma et al., (2009) reported a marker-free transgenic tomato using a salicylic acid-inducible Cre/loxP recombination system. Through this system they have developed a 41% marker-free transgenic tomato (*npt* II gene) in F_1 generation. This technique has also been utilized in some transgenic apples (Krens Frans et al., 2015; Würdig et al., 2015).

6.3.2 FLP/FRT Recombination System

In the FLP/FRT site-specific system, 2 μm plasmid of *Saccharomyces cerevisiae* is used. FLP enzyme efficiently catalyzes recombination between two directly repeated FLP recombination target (frt) sites and thereby eliminates the sequence between them. By controlled expression of the FLP recombinase

and specific allocation of the FRT sites within transgenic constructs, the system can be applied to eliminate the marker genes after selection (Cho, 2009).

6.3.3 Transposon-Based Marker Systems

Transposon-mediated repositioning of a transgene of interest is proposed as an alternative for generating a wide range of expression levels in selectable marker gene-free transgenic plants. Two transposon-mediated strategies are available to generate marker-free transgenic plants. The first strategy involves *Agrobacterium*-mediated transformation followed by intragenomic relocation of a transgene of interest, and its subsequent segregation from the selectable marker in the T_1 progeny (Goldsbrough et al., 1993). The second involves excision of the marker gene from the genome (Ebinuma et al., 1997). Both strategies were developed using the maize Ac/Ds transposable element but can be adapted to use similar autonomous transposable elements.

The basic advantage of this strategy is that marker-free transgenic plants can easily be screened at the T_0 generation, avoiding the need for sexually crossed plants and thereby making the strategy applicable to vegetatively propagated crops such as banana, potato, grapes, and so on. In spite of all the advantages, the main limitation of this strategy is that the generation of marker-free transgenic plants is very low. The transgenic plants are genomically instable because of the continuous presence of heterologous transposons (Scutt et al., 2002).

6.3.4 Positive Selection System

Marker genes for positive selection enable the identification and selection of GM cells without injury or death of the nontransformed cell population (negative selection). In this case, the selection marker genes give the transformed cell the capacity to metabolize compounds such as mannose (*manA* gene; Joersbo et al., 1998) and xylulose (xylA and DOG^R1 genes; Haldrup et al., 1998a,b) that are not usually metabolized by them. This will give the transformed cells an advantage over the nontransformed ones. The addition of this new compound in the culture medium, as nutrient source during the regeneration process, allows normal growth and differentiation of transformed cells, while nontransformed cells will not grow and regenerate de novo plants. The DOG^R1 gene encoding 2-deoxyglucose-6-phosphate phosphatase was used to develop a positive selection system for tobacco and potato plants (Kunze et al., 2001). The DOG^R1 gene, which is isolated from yeast, gives resistance to 2-deoxyglucose when overexpressed in transgenic plants.

6.4 MULTI-AUTO-TRANSFORMATION

The MAT vector system is a highly sophisticated approach for the removal of nuclear marker genes. It is a positive selection system that gives the advantage of regeneration to the transgenic cells without killing the nontransgenic cells. It is a unique transformation system that uses morphological changes caused by oncogene [the isopentenyl transferase (*ipt*) gene] or rhizogene (the *rol* gene) of *A. tumefaciens*, which control the endogenous levels of plant hormones and the cell responses to plant growth regulators as the selection marker. Expression of the *ipt* gene causes abnormal shoot morphology called extreme shooty phenotype, which subsequently reverts into normal shoots with objective transgenes because of the excision of *ipt* gene by the function of "hit-and-run" cassette system (Ebinuma and Komamine, 2001).

In this MAT system, a chosen gene of interest is placed adjacent to a multigenic element flanked by recombination sites. A copy of the selectable *ipt* gene from *A. tumefaciens* is inserted between these

recombinase sites, together with the yeast R recombinase gene and this entire assembly is situated within a T-DNA element for the *Agrobacterium*-mediated transformation of plant tissues. In this plant transformation system, neither antibiotic nor herbicide resistance genes are necessary as a selection marker. In addition, this system of transformation allows for repeated transformation of genes of interest in a plant (Sugita et al., 2000).

Generally, selectable marker genes are not required once the transgenic plants are regenerated and the genetic analyses completed. The presence of a particular marker gene in a transgenic plant necessarily precludes the use of the same marker in subsequent transformation and the use of a different marker system is required for each transformation round or event. Thus any technique that can remove or eliminate a selection marker gene in transgenic crops is highly desirable if for no other reason than that the same procedure can be used in subsequent transformations.

7. RISK ASSOCIATED IN TRANSGENIC CROPS: AGRICULTURAL, ECOLOGICAL, AND SOCIAL ISSUES

The following is a concise list of concerns related to the release of transgenic crops into the environment. Possible risks related to the plant transformation process itself, risks related to the particular genes and traits introduced, health risks, namely, possible toxicity or allergenicity of substances produced in the plant, introduction of antibiotic resistance genes into human and animal gut microbiome, environmental risks such as vertical gene transfer, horizontal gene transfer, possible unintended effects on nontarget organisms, possible resistance buildup in pest populations, resistance to herbicides in weeds, possible risk of monocultures, possible risk of new strains, risks that are not inherent to the technology but emanate from its mode of application in certain circumstances, i.e., "technology-transcending risks," for example, high-tech applications such as GM crops may benefit developed countries more than developing countries, large farms more than small farms, and rich consumers more than poor ones. There are also fears that patents on crop technologies may contribute to unfair seed prices, new dependencies, industry concentration, and corporate control of the entire food chain. Metabolic engineering with the production of new substances in the plant for nutrition, health, and industrial purposes and plants that produce pharmaceuticals can be associated with health risks that need to be carefully tested with specific regulation to assess risks and to prevent the entry of such products into the regular food chain.

These specific risks can only be assessed case by case and it is important to note that the same risks would also be present for any conventionally produced crops with the same traits. Problems of antibiotic resistance have increased in human medicine, but these are completely unrelated to GM crops. Beyond possible negative health consequences, GM crops may also be associated with positive health effects such as reduced occupational health hazards for farmers through lower chemical pesticide use or lower pesticide residues in foods that consumers eat. In any case, *Bt* is much less harmful for nontarget organisms than most chemical pesticides. Hence the prevalence of beneficial insects in fields cultivated with *Bt* crops was found to be significantly higher than in fields cultivated with conventional crops (Wolfenbarger et al., 2008). No negative effects of *Bt* were found both in soil and aquatic environments. Resistance development is a common problem in pest control even without GM crops. The issue is somewhat different for resistance buildup in *Bt* target pests. Monoculture has to be avoided. Technology-transcending risks are best dealt with by altering the external conditions for the better, for instance, through improved policies and institutions.

7.1 AGRICULTURAL RISKS

7.1.1 Gene Flow

Gene flow is a natural process that occurs among sexually compatible individuals in which cross-pollination can lead to the production of viable seeds. Gene flow between individuals within and among populations occurs via pollen only when they have concurrent geography, overlapping flowering times, and they share common pollinators. Gene flow in GM crops is not desired because there are possibilities of genes from GM crops moving into their wild relatives in conventional or organic crops. In some instances, large economic losses have occurred because of gene flow, leading to zero tolerances for admixtures, none of which were a food or environmental safety concern. Nonetheless, to avoid market impacts and associated economic losses, a comprehensive understanding and control of gene flow as well as realistic thresholds are required for consistent marketing of agricultural commodities.

Gene flow among crop plants has been reviewed from various angles (Kwit et al., 2011; Ding et al., 2014) and compiled information is available on gene flow to wild relatives in the top 25 crops (Gealy et al., 2007). Sexually compatible wild relatives exist for cassava, cotton, grape, oats, oilseed rape, sorghum, sugarcane, sunflower, wheat, and most of the commonly grown forest trees. Examples of gene flow from transgenics to wild or weedy relatives have been reported in at least 13 species. Although hybridization is possible in these species, introgression was studied only in brassica, wheat, and creeping bent grass. In those cases, none of the weedy relatives indicated signs of invasiveness or selective advantage because of herbicide or insect resistance (Kwit et al., 2011). Gene flow studies in crops have been reviewed by Chandler and Dunwell (2008) and trees by Dick et al. (2008; see case studies for recent studies).

7.1.2 Gene Flow Mitigation Strategies

Gene flow mitigation strategies can be classified into those that act prehybridization or posthybridization. Prehybridization strategies include genic and cytoplasmic male sterility, delayed flowering, parthenocarpy (fruit production without fertilization), transgene excision, chloroplast transformation, and cleistogamy (pollination without flower opening) (Kwit et al., 2011; Ding et al., 2014). Posthybridization strategies include transgene mitigation and selective terminal lines (e.g., V-GURTs, see later). With an enhanced knowledge on genetic control of plant reproduction, many novel systems have been developed and evaluated for control of pollen (Stewart, 2007; Verma and Daniell, 2007), seed (Lee and Natesan, 2006), and even flower production (Liu et al., 2008) to address gene flow mitigation. For example, delayed flowering is suggested as a method to mitigate gene flow by naturally selecting, inducing mutations, or modifying the Flowering Locus C (FLC) or TFL1 gene, a repressor of flowering (Boss et al., 2006; Kim, 2007). The use of such systems would be limited to determinate flowering crops, forage, and biomass crops where seed and fruit are not the harvested commodity.

7.2 SOCIAL AND HEALTH RISKS

As of October 2016, 40 countries granted regulatory approvals to GM crops for use as foods, feeds, or for environmental release, encompassing a total of 28 types of GM crops and 404 events (Fig. 14.2). Major health risks potentially associated with GM foods are (1) the ability of these crops to transfer the resistant genes to wild varieties of microbes and the intestinal flora, thus providing a boost to antibiotic

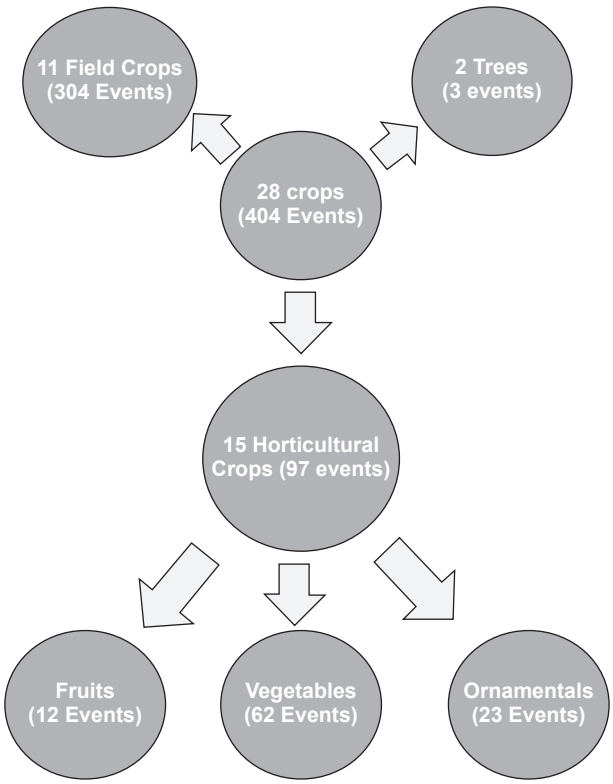

FIGURE 14.2 Commercially Approved Transgenic Crop Plants as of October 2016.

drug resistance (Lack, 2002), (2) potential transfer of the antibiotic, insecticidal genes into pathogenic microbes, which would make them resistant to the host immune system and could lead to pandemic diseases, and (3) toxicity and allergenicity of new proteins to infants, older ones, immune-compromised individuals, etc. (Ivanciuc et al., 2003).

The herbicide glyphosate was introduced in 1974 and its use is accelerating with the advent of herbicide-tolerant genetically engineered crops. Evidence is mounting that glyphosate interferes with many metabolic processes in plants and animals and glyphosate residues have been detected in both. Glyphosate disrupts the endocrine system and the balance of gut bacteria; it damages DNA and is a driver of mutations that lead to cancer. Swanson et al. (2014) surveyed and analyzed US government databases and genetically engineered crop data, glyphosate application data, and disease epidemiological data, respectively. Correlation analyses were then performed on a total of 22 diseases. The Pearson correlation coefficients are highly significant between glyphosate applications ($<10^{-5}$), the percentage of genetically engineered corn and soybean planted in the United States ($<10^{-4}$), and hypertension stroke, diabetes prevalence, diabetes incidence, obesity, lipoprotein metabolism disorder, Alzheimer's, senile dementia, Parkinson's, multiple sclerosis, autism, inflammatory bowel disease, intestinal infections, end-stage renal disease, acute kidney failure, cancers of the thyroid, liver, bladder, pancreas, and kidney, and myeloid leukemia. The significance and strength of the correlations show that the effects of glyphosate and genetically engineered crops on human health should be further investigated.

This does not imply that all these diseases have a single cause because there are many toxic substances and pathogens that can contribute to chronic disease. However, no toxic substance has increased in ubiquity in the last 20 years as much as glyphosate has. The disruption of the detoxification pathways (cytochrome P450 pathways), drug metabolism, oxidation of organic molecules, and endocrine system in the human body by glyphosate can intensify the effect of other toxic chemicals. Particularly for the endocrine system, there is no permitted limit, so the current permitted residue levels in food could be causing multiple health problems that have been documented in the scientific literature to be caused by endocrine disrupting chemicals.

7.3 RISK ASSESSMENT OF FOODS DERIVED FROM BIOTECHNOLOGY

GM crops have been used as a source of oil (GM canola, maize, etc.), sugar (GM sugar beet), paper, biofuel (GM unicellular microalgal strains of *Chlorella*, *Botryococcus*), etc (Flachowsky et al., 2005). Current biotech food crops include white maize in South Africa; sugar beet and sweet corn in the United States and Canada; papaya, squash, potato, and apple in the United States; papaya in China; and *Bt* eggplant in Bangladesh (James, 2015). During the last two decades, at least two important controversies on GM plants, popularly known as the affair Pusztai and the affair Seralini, occupied an important place in the pages of scientific journals. The first one began in 1998, reaching a zenith in 1999. In brief, that "affair" was the result of the stir caused by Pusztai's premature release of information to the mass media even prior to its publication in scientific journals on the adverse effects of GM potatoes fed to rats (Ewen and Pusztai, 1999).

As a result of intense debate generated by that study and doubts generated by some "scientific" groups, which were not even supported by any experimental evidence, Domingo in 2000, 2007, 2011, and 2016 assessed the state-of-the-art regarding both the potential adverse effects and safety assessment of GM plants for human consumption in the PubMed database using key terms. He observed a lot of increase in number of key terms used in the scientific literature in 2001–07 compared to 1980–2000. This resulted in an increased citation number for each key term. Some examples are GM foods increased from 101 to 686, toxicity of transgenic foods increased from 44 to 136, adverse effects of transgenic foods went up from 67 to 199, while health risks of transgenic foods increased from 3 to 23. This makes it clear that references concerning specific risk assessment were much more limited (Domingo, 2007). Most investigations corresponded to short-term studies, mainly nutritional studies, with very limited toxicological information.

For the first time in 2011, equilibrium was reached among the research groups suggesting that a number of varieties of GM products (mainly maize and soybeans) are as safe and nutritious as the respective isogenic lines/conventional non-GM plants. The reported studies during the period from 2010 to 2016 also gave a similar conclusion about the risks of at least four commercialized GM crop plants, namely, soybeans, rice, maize, and wheat. However, long-term studies are still necessary to guarantee that the consumption of GM plants is safe. This does not mean that transgenic crops pose health risks to consumers (Domingo, 2016).

Based on the foregoing discussion, the following conclusions have been arrived at. Neither the risks nor the benefits of GMOs are certain or universal. Both may vary spatially and temporally on a case-by-case basis. Comparisons among transgenic, conventional, and others, such as organic farming, will elucidate the relative risks and benefits of the adoption of GMOs. Our inability to accurately predict ecological consequences, especially long-term, higher-order interactions, increases the uncertainty associated with risk assessment and may require modifications in our risk management strategies. Evaluation of potential environmental benefits is still in its infancy facilitating risk

managers and decision makers to balance these against the extent and irreversibility of any ecological change. The method of benefit documentation is critical. In particular, we should incorporate relative environmental toxicity into analyses of changes in pesticide use and quantify the impacts of herbicide-tolerant crops on soil conservation. Measures that prevent transfer of genes may negatively impact wild populations and can slow down the evolution of resistance in transgenes that can minimize some of the possible ecological risks and prolong the possible benefits associated with genetically engineered plants.

8. BIOSAFETY REGULATION IN TRANSGENIC CROPS

Discussions on appropriate regulatory norms for GM crops and foods in various countries date back to the early 1980s (Cantley et al., 1999). Almost 30 years later, a consensus on norms remains elusive (Herring and Paarlberg, 2016). The safety of GM crops and foods prior to their commercialization are evaluated more or less in a similar way around the world (Kalaitzandonakes et al., 2007). However, countries differ widely on the amount of time they take to complete their regulatory process (Smart et al., 2016; Zilberman et al., 2015) as well as on their treatment of GM foods that have been deemed safe for market introduction. Some countries, including the United States and Canada, consider the deregulated (approved) GM foods substantially equivalent to their conventional counterparts, while others, including the European Union, Japan, China, South Korea, Brazil, and Australia, have introduced various levels of control over where and how GM foods should appear in the market.

8.1 BIOSAFETY REGULATION OF GENETICALLY MODIFIED CROPS IN INDIA

The National Biotechnology Board (1983) was the first Indian government board to be constituted to look after the "biotech research in laboratory and contained use settings." Later, as the time and situation changed, appropriate changes were adopted and several rules and guidelines were formulated and enacted by the government of India (Fig. 14.3). The regulation of GM crops from development and environmental release to commercial approval is covered by three legislative Acts enacted by the Parliament of India and administered by three different ministries. These included the Environment Protection Act 1986 implemented by Ministry of Environment and Forests, the Seed Act 1966 and the Seeds (Control) Order by the Ministry of Agriculture, and the Food Safety and Standards Act 2006 by the Ministry of Health and Family Welfare.

The EPA Rules 1989 (Fig. 14.3) are central to the biosafety regulation of GM crops, whereas others are related to food safety and quality of seeds for sale and other related issues. The next level of legislation (secondary legislation) deals with import of material for R&D, access to biological resources, and intellectual protection of plant varieties. Each Act has been implemented through a set of rules that describes the function, process, power, and composition of different regulating agencies for the implementation of Acts. Apart from those that are presented in Fig. 14.3 there are other guidelines related to the biosafety regulation of certain products that use GMOs/rDNAs: Guidelines for generating preclinical and clinical data for rDNA vaccines, diagnostics and other biologicals (1989) and Guidelines on similar biologics: regulatory requirements for marketing authorization in India (2012).

Considering the ecological consequences and the potential risks associated with the environmental release of GM crops, these guidelines prescribe the biosafety evaluation and risk assessment of the

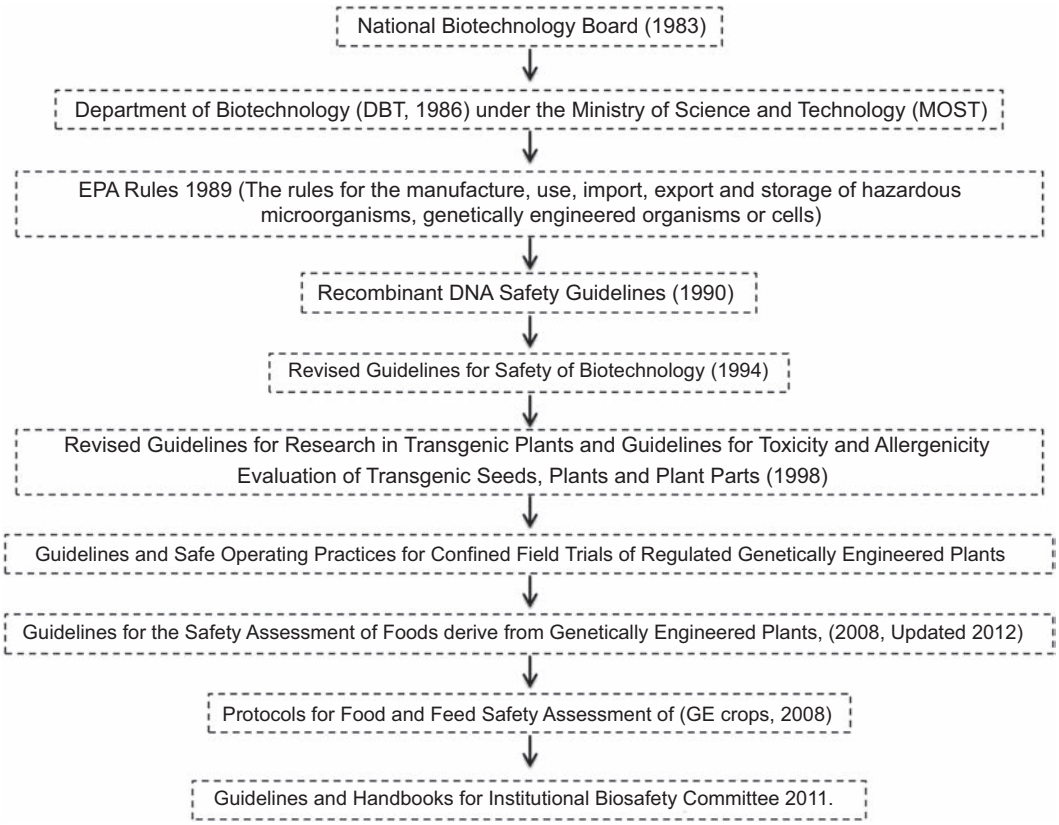

FIGURE 14.3 Timeline of Regulatory Guidelines Development in India.

environmental aspects and agronomic performance on a case-by-case basis taking into consideration specific crops, traits, and agro-ecological systems (Tripathi and Behera, 2008). These guidelines also insist on regulatory measures to ensure safety of imported GM materials in the country (Randhawa and Chhabra, 2009).

The Rules 1989 also define the competent authorities and composition of such authorities for handling of various aspects of the Rules. Presently, there are six committees. Choudhary et al. (2014) presented a schematic of regulatory options in India for GM crops (Fig. 14.4), which is comprehensive.

8.2 DIFFERENCES IN REGULATORY APPROACHES

Risk assessment and risk analysis of GMOs is governed by internationally accepted guidelines, developed by the Codex Alimentarius of the World Health Organization and Food and Agricultural Organization. A leading principle of the Codex Alimentarius is the concept of substantial equivalence, which stipulates that any new GM crop technology should be assessed for its safety by comparing it with an equivalent, conventionally bred variety that has an established history of safe use (Fagerstrom et al., 2012).

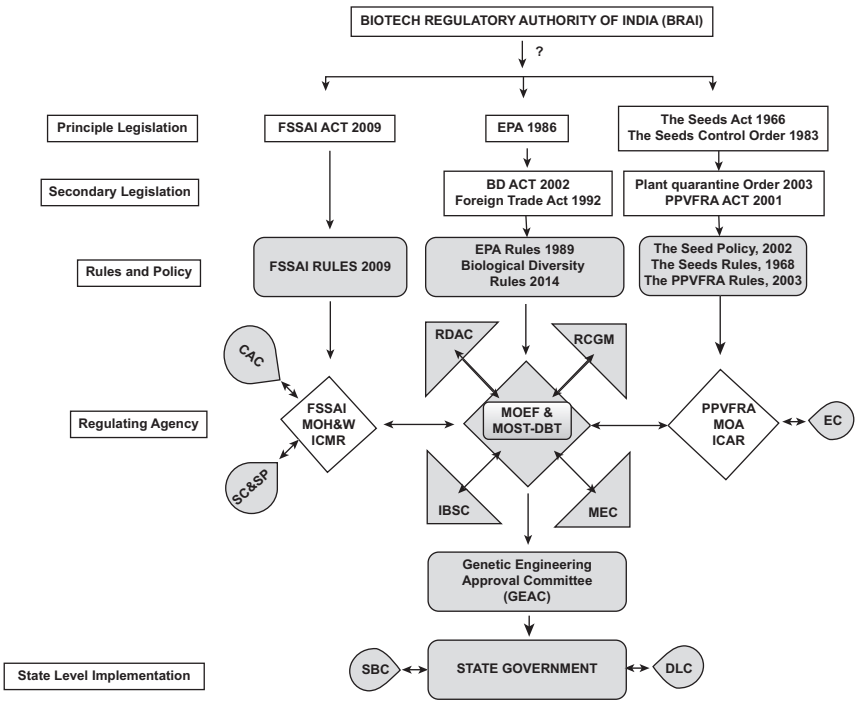

FIGURE 14.4 Schematic Representation of Biosafety Regulation of Transgenic Crops and Derived Products in India.

CAC, Central Advisory Committee; *DLC*, District Level Committee; *EC*, EXIM Committee; *IBSC*, Institute Biosafety Committee; *MEC*, Monitoring and Evaluation Committee; *SBC*, State Biotechnology Committee; *RCGM*, Review Committee on Genetic Modification; *RDAC*, Recombinant DNA Advisory Committee; *SC&SP*, Scientific Committee and Scientific Panel on Genetically Modified Organisms and Foods; *?*, New law has recently been proposed and is yet to be decided (Choudhary et al., 2014).

Despite the fact that the Codex guidelines are globally endorsed, significant differences in the GMO regulatory approaches can be observed between countries. The differences between the European and American approaches are particularly pronounced. While the European Union ratified the Cartagena Protocol with its specific rules for GMOs, the Protocol was not ratified by the United States, Canada, and most other countries.

The regulatory approach in the European Union requires new laws that are specific to GM crops and foods, while in the United States, GMOs are regulated under the same laws that are also used for conventional agricultural technologies. Related to this, the EU approach also requires a separate testing and approval process for GM crops that is overseen by institutions especially established for this purpose. In the United States, existing institutions that also regulate conventional crops, namely, the Food and Drug Administration, the Animal and Plant Health Inspection Service, and the Environmental Protection Agency, screen and approve GMOs. Finally, following the precautionary

principle, even without any evidence of risk, EU regulators can refuse to approve GM crops on grounds of uncertainty alone. In the United States, the precautionary principle is not applied. If the required tests for known risks such as toxicity, allergenicity, environmental invasiveness, and effects on nontarget organisms have been passed successfully, there is no further regulatory hurdle for commercialization of the GM crop in question (Just et al., 2006; Paarlberg, 2014). The regulatory approach for GMOs in Europe is clearly process based. The process of plant genetic engineering is singled out with rules and standards that do not apply to any other breeding method. In contrast, in the United States a product-based approach is followed. Legislation concentrates on the risks of the product, i.e., the crop plant with a specific new trait and not the breeding method because genetic engineering is not considered inherently more risky than conventional methods (Devos et al., 2009).

8.3 FACTORS THAT IMPACT THE REGULATORY APPROVAL PROCESS

The GM crop production and import approval process is hampered by regulatory uncertainty, unpredictable delays, and lengthy timelines that limit the realization of GM crop benefits for farmers and society. This is challenging for the private sector and even more onerous for the public sector that is less experienced and equipped to navigate the different regulatory frameworks and processes around the world. While there can be many reasons for the delay, the following are some prominent examples: (1) extended reviews by risk assessors, administrative delays, and capacity constraints; (2) new and inexperienced regulators, evolving processes, or changing requirements, resulting in added regulatory steps or complexity; (3) new or expanded data requests or limited data transferability between countries despite similar and relevant experimental conditions; and (4) litigation or political influence that interferes with approval processes or decision making by regulatory authorities or introduces uncertainty on the durability of authorizations once granted. While most regulations have general timelines for completion, one way to get past the mandated timelines is to ask multiple rounds of questions, which allows the regulators to " stop the clock " while the developers prepare answers to these questions. For example, European authorities take years to debate modifications in their guidance documents and regulatory requirements. Once finalized, they retroactively apply requirements to products already under review. This results in additional rounds of questions; in some cases the developers must initiate new studies to conform to the data requirements, study designs, or statistical methods, which may have no bearing on the quality of risk assessment.

Uncertainty and delays in the approval process also serve as a barrier to academic institutions, government research laboratories, and small biotechnology start-ups, which could develop meaningful solutions for farmers but are limited because of the high cost and extensive time required to navigate the global regulatory landscape. Unfortunately, the consequences to society caused by delays in regulatory processes are yet to be widely recognized outside the industry and public sector developers, grain traders, and regulatory community. Although the reality today is that regulatory processes are often unpredictable and challenging, there is also hope that continued dialog among regulatory authorities, as well as positive actions taken by progress-oriented countries, will help to bring about improvements in regulatory process and function. Unfortunately, in reality, regulatory processes are neither certain nor predictable, and in some cases not based on science; consequently, the actual situation today is totally different (Sachs, 2016).

9. FUTURE PROSPECTS

The recently developed breakthrough technologies of genome engineering possess the potential to change the pace and course of agricultural research by overcoming some of these limitations of conventional breeding and genetic transformation-based approaches and their associated perceptional risks with respect to regulatory aspects and social issues: for example, targeted genome editing by targeted double-strand DNA breaks at specific loci in the genome or close specific loci using sequence-specific nucleases such as zinc-finger nucleases (Klug, 2010) and transcription activator-like effector nucleases (TALENs) (Joung and Sander, 2013). CRISPR/Cas9 (Belhaj et al., 2013) is a recent form of mutagenesis, which can be used to generate improved crops indistinguishable from naturally occurring mutants and offers a timely and powerful unique set of significant comparative advantages over conventional and GM crops in four domains: precision, speed, cost, and regulation (Kamthan et al., 2015).

Based on the acumen of the CRISPR/Cas system, it can be utilized for introducing desired changes such as targeted single and multiple gene knockouts of detrimental genes in plants (Brouns et al., 2008) and introducing single nucleotide polymorphisms into a gene of interest (Voytas and Gao, 2014) for improvement of economic traits. Gene regulatory elements can also be studied by expressing affinity or fluorescent-tagged proteins at their native loci in the genome (Belhaj et al., 2013; Kim et al., 2014), for molecular stacking of multiple pest resistance genes into plants (D'Halluin et al., 2013), and by providing a breakthrough in generating mutants in previously difficult-to-access genes to mutate multiple loci and to generate large deletions (Mali et al., 2013; Liang et al., 2014). Until now, the CRISPR/Cas system has been successfully validated in many plant species by using multiple single guide RNAs for various functional studies, including *Arabidopsis thaliana*, tobacco (*Nicotiana benthamiana*), sweet orange (*Citrus sinensis*), rice (*O. sativa*), wheat (*Triticum aestivum*), sorghum (*Sorghum bicolor*), maize (*Zea mays*), and liverwort (*Marchantia polymorpha*) (Jinek et al., 2012; Bortesi and Fischer, 2015). Genome editing using CRISPR and TALENs has been applied to generate transgenic wheat, a polyploid crop, by knocking out all six alleles of the mildew resistance locus O gene resulting in resistance to powdery mildew disease affecting wheat (Voytas and Gao, 2014). Furthermore, targeted gene mutagenesis has been successfully applied to edit soybean ALS1 gene to obtain chlorsulfuron herbicide-resistant plants (Li et al., 2015) and readers can consult Xing et al. (2015), Ishii and Araki (2016), and Kim and Kim (2016) for further examples and deliberations on consumer acceptance and regulatory advantages over the usual transgenics.

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CHAPTER

15

TRANSGENIC DEVELOPMENT FOR BIOTIC AND ABIOTIC STRESS MANAGEMENT IN HORTICULTURAL CROPS

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1. INTRODUCTION

Biotechnology has offered tremendous scope and potential to conventional methods of crop improvement, crop protection, crop quality management, and other horticultural traits. Biotechnology extends tremendous opportunities in fruit production by providing new genotypes for breeding purposes, supplying healthy and disease-free planting material, improving fruit quality, and enhancing shelf-life and availability of biopesticides, biofertilizers, etc. Integration of specially desired traits through genetic engineering has been possible in some horticultural crops. Genetic engineering consists of isolation of a gene of interest, ligating that gene with a desirable vector to form the recombinant DNA molecule, and then transferring that gene into the plant genome to create a new function. Transgenic technology has been rated as the fastest-growing technology in agriculture (ISAAA, 2016). It refers to a set of techniques used for transferring desirable gene(s) from any source (plants, microorganisms, or even artificially synthesized genes) across taxonomic boundaries into a certain plant by nonconventional methods. In contrast to conventional breeding, which involves the random mixing of tens of thousands of genes present in both resistant and susceptible plants, recombinant DNA technology allows the transfer of only the desirable genes to the susceptible plants and the preservation of valuable economic traits. Moreover, the genetic sources for resistance are not limited purely to closely related plant species (Lurquin, 2002). Combating various types of biotic and abiotic stresses is the foundation and crux of sustainable agriculture. Although conventional breeding and marker-assisted breeding are being used these days to develop more promising cultivars, in case of biennials or perennial horticultural crops, particularly fruit trees, such techniques are not feasible because of long sexual generation periods. The major advantages of transgenic technology lie in the fact that the genes governing various traits can be sourced from any organism—plants, animals, microorganisms, etc.—and can be employed for plant transformation. Thus novel traits from any background can be incorporated into the target plant with ease. However, for single gene transfer into elite backgrounds, the development and standardization of a high-frequency plant regeneration protocol through direct organogenesis is the utmost prerequisite. A

Genetic Engineering of Horticultural Crops. http://dx.doi.org/10.1016/B978-0-12-810439-2.00015-5

number of studies had been carried out in the past to develop a suitable regeneration protocol in many horticultural species including apple (Rustaee et al., 2007), pomegranate (Parmar et al., 2012, 2013, 2015), chilli (Sharma et al., 2006; Khan et al., 2011a), sweet orange (Singh and Rajam, 2010), etc.

Horticultural biotechnology has been a leading example in many areas for more than two decades, right from the commercialization of the first ever transgenic crop in the form of Flavr Savr transgenic tomato with enhanced shelf-life traits. The first field trials of transgenic horticultural plants were carried out in France and the United States in 1986 (James and Krattiger, 1996). In 1994 transgenic Flavr Savr tomato was the first successful example of a genetically modified (GM) food crop to be approved for commercialization in the United States. Using antisense RNA technology, a gene, namely, *polygalacturonase*, had been silenced in tomato, which led to the softening of the tomato fruit cell wall by dissolving cell wall pectin material, thus the maturity period of tomato was delayed and this trait permitted transgenic tomato fruit to be picked up at a later stage of maturity with enhanced composition of flavor compounds and a better taste (Clark et al., 2004). The main resistant traits introduced into horticultural plants and already commercialized are insect pest resistance (*Bt* toxin gene) and herbicide tolerance. Other studies concern virus resistance, male sterility, etc. Among various GM horticultural crops, GM papaya showing resistance to papaya ringspot virus (PRSV) contributes to approximately 53% of the total share of GM horticultural crops cultivated globally. Herbicide tolerance trait is dominating the GM horticultural crop acreage followed by insect resistance and virus resistance traits.

Also RNA interference (RNAi) technology has found its most powerful expression in plant biology these days. The applications of this technology cover a wide range from producing insect-, viral-, and disease-resistant plants to developing designer flower colors by knocking down the expression of certain endogenous genes. It is being used as a potential tool in tweaking the regulation of various metabolic pathways in plants and assigning functions to the genes involved. The most studied crop so far is tomato, but research activities had already been carried out on many horticultural crops such as fruits, vegetables, and flowers. With the advancement of regeneration and genetic transformation protocols, extensive research efforts have been made to incorporate genes for various biotic and abiotic stress tolerance/resistance traits in a number of horticultural crops, which have been summarized and discussed.

2. TRANSGENIC RESEARCH IN BIOTIC STRESS MANAGEMENT IN HORTICULTURAL CROPS

2.1 INSECT PEST RESISTANCE

At present, insect pest resistance is lacking generally in many crop plants. The use of chemical control measures is proving hazardous to consumers and is also not environmentally sustainable. A large number of insect species attack plants and cause severe damage to yields. From a grower's perspective, any genetic improvement that could reduce the cost of chemical application to combat pests would be of significant benefit. The *Bt* (*Cry*) gene isolated from a soil bacteria *Bacillus thuringiensis* has proven highly effective in controlling various lepidopteran insects in a number of crops. Insect resistance was first reported in tomato using the *Bt* gene in 1987. Transgenic *Bt* tomato plants exhibited resistance against *Spodoptera litura* and *Heliothis virescens* (Fischhoff et al., 1987). Fruit trees such as persimmon transgenic for the *cryI* gene were found resistant to *Plodia interpunctata* and *Monema flavescens* (Tao et al., 1997). Brinjal is among a number of highly consumed vegetables in Asia and specifically in

the Indian subcontinent. However, it is extensively damaged by the lepidopteran insect *Leucinodes orbonalis*. Kumar et al. (1998) transformed a synthetic *cry1Ab* gene coding for an insecticidal crystal protein (*ICP*) to brinjal (*Solanum melongena* cv. Pusa Purple Long) by cocultivating cotyledons with *Agrobacterium tumefaciens*. Gene expression was evaluated by double-antibody sandwich enzyme-linked immunosorbent assay (ELISA) analysis. The transgenic lines displayed significant differences in insect mortality in fruit bioassays. It was suggested to express a very high level of insecticidal crystal protein to confer complete protection against *L. orbonalis* by employing a fruit-specific promoter for better fruit-specific expression. Potato varieties engineered for resistance to Colorado potato beetle were in commercial production for several years and were technically and agronomically successful, allowing significant reductions in insecticide use (Shelton et al., 2002). Chakrabarty et al. (2002) transformed cauliflower var. Pusa Snowball K-1 with a synthetic *cryIA(b)* gene and the transgenic plants indicated the effectiveness of the transgene against infestation by diamondback moth (*Plutella xylostella*) larvae during insect bioassays. Paul et al. (2005) developed transgenic cabbage (*Brassica oleracea* var. *capitata*) line DTC 507 with a synthetic fusion gene of *B. thuringiensis* encoding a translational fusion product of *cry1B* and *cry1Ab* δ-endotoxins to confer resistance against diamondback moth (*P. xylostella*), the most destructive pest of cruciferous plants across the globe. *Bt* cabbage plants expressing the fusion protein in mature leaves caused 100% mortality to all four larval stages of diamondback moth. Complete mortality of the neonate larvae had been observed within 24 h and within a period of 48 h in the case of the other three stages of larvae. The *Bt* gene (*Cry1Ac*) has been successfully transformed and expressed in okra (*Abelmoschus esculentus*) for incorporating resistance against fruit and short borer (*Earias vittella*), which is the most serious insect pest of this crop in Asia (Narendran et al., 2013). Okra is severely affected by *E. vittella* and its larvae bore into pods and shoots of the plant and eat the internal contents leading to withering of the plant and reduction in the market value of the pods. In insect bioassays, fruits from transgenic lines caused 100% larval mortality. Natural as well as synthetic insect resistance genes had been transferred into a number of horticultural crops for imparting resistance against various insect pests. Zhang et al. (2015) transformed kiwifruit plant (*Actinidia chinensis*) with a synthetic chimeric gene *SbtCry1Ac* encoding for protein *btCry1Ac*. When the transgenic plants were screened for insect resistance in insect bioassays, an average of 75.2% *Oraesia excavate* inhibition rate was reported at 10 days postinfection. This technology could be highly useful to protect yield losses of kiwifruit caused by insect attack, which is an economically and nutritionally important fruit crop offering a remarkably high vitamin C content.

Other genes such as protease inhibitors, trypsin inhibitors, lectins, etc. have also been employed for incorporation of insect pest resistance in many crop species. Ding et al. (1998) developed insect-resistant transgenic Taiwan cauliflower *Brassica oleracea* var. *botrytis* cvs. Known You Early no. 2, Snow Lady, and Beauty Lady plants expressing the *trypsin inhibitor* gene, isolated from local sweet potato. The transgenic plants expressed resistance to *S. litura* and *P. xylostella* in in planta bioassays. Transgenic strawberry expressing a cowpea (*Vigna unguiculata*) *protease trypsin inhibitor* (*CpTi*) gene under a constitutive promoter developed resistance against vine weevil (*Otiorhynchus sulcatus*). The *CpTi* transgenic lines reduced the frequency of survival of weevil larvae and pupae during insect bioassays (Graham et al., 2002). Gessler et al. (2006) developed transgenic apple lines with *trypsin inhibitor* encoding a *CpTI* gene from cowpea and a *cry1A(c)* gene of *B. thuringiensis* for incorporation of resistance against codling moth pest. Almost all the genotypes of chrysanthemum are infested by two aphids, namely, *Myzus persicae* and *Aphis gossypii*, lowering the flower quality and also transmitting viruses. Valizadeh et al. (2013) transformed chrysanthemum genotype 1581 by

an *A. tumefaciens*-mediated technique with an *SAE* gene, under the control of chrysanthemum *RbcS* promoter to incorporate aphid resistance. The *protease inhibitor* sea anemone equistatin (*SAE*) has three domains for inhibition of both cysteine and aspartic proteases. In another study, *Chrysanthemum morifolium WRKY48* (*CmWRKY48*) transcription factor overexpressing transgenic chrysanthemum plants was found to inhibit the population growth of aphids (Li et al., 2015).

Root-knot nematode (*Meloidogyne incognita*) causes severe yield losses in many horticultural crops and genetic transformation of various *proteinase inhibitor* genes from plants is considered as the most potential strategy to prevent such yield losses. *Cysteine proteinases* are involved in the digestion process of root-knot nematodes, and binding of various *cystatins* to the active sites of *proteinases* inhibit their activity, thus affecting their proteolytic digestion (Shingles et al., 2007). Roderick et al. (2012) developed transgenic plantain (*Musa* sp.) cv. Gonja manjaya plants expressing a maize *cystatin* gene that inhibits the digestive *cysteine proteinases* and a synthetic peptide that disrupts nematode chemoreception. The best level of resistance exhibited by the transgenic plants against the major pest species *Radopholus similis* was 84% for the *cystatin*, 66% for the peptide, and 70% for the dual defense. In another study, Papolu et al. (2016) developed transgenic brinjal plants expressing a modified rice *cystatin* (*OC-IΔD86*) gene under a root-specific promoter, *TUB-1*, for inducing resistance against root-knot nematode. Transgenic plants were confirmed for gene integration and expression using polymerase chain reaction (PCR), Southern blotting, Western blotting, ELISA, and quantitative PCR assays. When one transgenic line (single copy event) was challenged with root-knot nematode, 78.3% inhibition rate in reproduction of root-knot nematode had been reported. In an earlier study, transgenic banana plants expressing the same *cystatin* gene (*OC-IΔD86*) exhibited 70% resistance to the migratory endoparasite *R. similis* (Atkinson et al., 2004). Lilley et al. (2004) also reported a partial resistance (67%) against *M. incognita* in transgenic potato roots expressing the same gene. Root lesion nematode *Pratylenchus penetrans* is one of the main pests of lily producers, particularly in the United States, where lily (*Lilium longiflorum*) cv. "Nellie white" assumes great economic importance as cut flowers and constitutes one of the most valuable species. Vieira et al. (2015) developed transgenic lilies overexpressing the *OC-IΔD86* gene, which displayed an enhanced resistance to root lesion nematode infection by means of nematode reduction up to 75%. The transgenic lily plants also exhibited an increased biomass and in addition to better growth performance as compared to nontransformed plants.

The Indian Council of Agricultural Research (ICAR), India, had supported crop biotech research at several ICAR institutions (10 institutes, 7 national research centers, and 6 directorates) and state agricultural universities for the development of insect pest-, viral-, and disease-resistant horticultural crops. *Bt* brinjal may become available as the first biotech vegetable food crop in India within next 3–4 years. Efforts are being directed for the development of various vegetable crops such as biotech tomato, broccoli, cabbage, cauliflower, and okra, which require heavy application of insecticides (which can be reduced substantially by a biotech product), and some of them are currently in various stages of field trials and biosafety testing.

2.2 DISEASE RESISTANCE

The next major constraint limiting the production of fruit crops is diseases caused by several fungi and bacteria. Conventional breeding seems to have limited application because of nonavailability of resistant gene(s) in the gene pool of a particular crop. One of the main targets of genetic transformation is to improve tolerance or to incorporate resistance in plants against different pathogens. Genetic

engineering of disease resistance in crops has become popular and valuable in terms of cost and efficacy. For imparting bacterial and fungal resistance, various genes such as *chitinase*, *glucanase*, *osmotin*, *defensin*, etc. are being transferred into various horticultural crops the world over. Various glycolytic enzymes encoded by genes such as *chitinase, glucanase*, *pathogenesis-related* (*PR*) *proteins*, etc. inside the plant cells have cell wall degrading capabilities, which attract their use for developing transgenic plants for incorporation of resistance against fungal pathogens (Ceasar and Ignacimuthu, 2012).

Among the strategies used for genetic engineering for imparting disease resistance, the employment of systemic acquired resistance (*SAR*)-related genes is of paramount importance. SAR is long lasting and often associated with local and systemic accumulation of salicylic acid (SA) and induced expression of many genes including *PR* genes (Ryals et al., 1996). A gene for a PR protein from tomato (*PR-5*) had been expressed in transgenic sweet orange and regenerants showed increased tolerance to *Phytophthora citrophthora* (Fagoaga et al., 2001). Lin et al. (2004) introduced an *Arabidopsis thaliana*-derived *NPR-I* gene into tomato. Transgenic tomato plants developed enhanced heat tolerance and resistance against tomato mosaic virus. The transgenic lines also conferred a significant level of resistance to bacterial wilt and Fusarium wilt along with a moderate degree of enhanced resistance to gray leaf spot and bacterial spot. Malnoy and Aldwinckle (2007) developed transgenic apple lines overexpressing *MpNPR1-1* (ortholog of *AtNPR1*), which exhibited broad-spectrum resistance against *Venturia inaequalis*, *Gymnosporangium juniper-virginianae*, a causative agent of cedar apple rust, and *Erwinia amylovora*, which causes fire blight. The Fuji apple, the most popular and most widely cultivated apple in China, is highly susceptible to powdery mildew disease. Apple powdery mildew, which is caused by *Podosphaera leucotricha*, damages leaves and young fruits, thus leading to huge yield losses (Qu et al., 2009). Chen et al. (2012) introduced a *Malus hupehensis*-derived *NPR1(MhNPR1)* gene into "Fuji" apple for development of resistance against powdery mildew disease. The *NPR1* gene plays a key role in regulating SA-mediated SAR in plants. The *MhNPR1* gene induced the expression of *MdPRs* and *MdMLO* genes, which interact with powdery mildew as revealed by reverse transcription polymerase chain reaction (RT-PCR) and the transgenic apple plants expressed enhanced resistance to powdery mildew disease. Overexpression of an *AtNPR1* gene in tomato and carrot plants exhibited resistance to bacterial and fungal pathogens (Lin et al., 2004; Walley et al., 2009). Commercial sweet orange cultivars are suffering from this deadly disease. In a similar study, overexpression of a *Vitis vinifera NPR1.1* (*Vv NPR1.1*) gene was conferred to enhance resistance to powdery mildew in grapevine (Le et al., 2011). In the United States, Huanglongbing is a very serious disease of citrus, which is associated with a phloem-limited bacterium *Candidatus Liberibacter asiaticus* (Duan et al., 2009). Dutt et al. (2015) overexpressed an *A. thaliana NPR1* gene under a constitutive promoter cauliflower mosaic virus (*CaMV*) *35S* and also under a phloem-specific *Arabidopsis SUC2* (*AtSUC2*) promoter in sweet orange cultivar "Hamlin" and "Valencia." The *NPR1* gene is involved in the induction of expression of several native genes involved in plant defense signaling pathways. The transgenic plants exhibited reduced disease severity and a few lines remained disease free even after 3 years of planting in a high-disease pressure field site.

Another category of genes imparting disease resistance is *chitinases,* which are glycosyl hydrolases that catalyze the degradation of chitin, an insoluble, linear β-1,4-linked polymer of *N*-acetylglucosamine, a cell wall component of various bacteria and fungi, and thus codes for pathogen resistance. The *chitinase* gene has been transferred to a number of crops for harboring fungal resistance. In carrot, the tobacco class I *ChiC* gene has shown resistance against *Botrytis cinerea* (Punja and Raharjo, 1996). The *RCC2* gene, a rice *chitinase*, displayed enhanced resistance to *Sphaerotheca humuli* in transgenic

strawberry plants (Asao et al., 1997). Vellice et al. (2006) expressed a *chitinase* gene from *Phaseolus vulgaris*, a *glucanase* or a *thaumatin-like protein*, both from *Nicotiana tabacum* and a combination of both in strawberry cv. "Pajaro." Two transgenic lines expressing *chitinase* genes showed enhanced tolerance to *B. cinerea*. In another study, Yamamoto et al. (2000) transformed a rice *chitinase* gene (*RCC2*) into the somatic embryos of grapevine cv. Neo Muscat and reported an increased resistance level against powdery mildew fungus *Uncinula necator*. Schestibratov and Dolgov (2005) also developed transgenic strawberry plants expressing the *thaumatin II* gene from *Thaumatococcus daniellii* and reported some level of resistance to *B. cinerea* during in vitro assays. Khan et al. (2008) in an attempt to confer resistance to early blight of potato caused by *Alternaria solani* transformed a *chitinase* gene, *ChiC*, isolated from *Streptomyces griseus* strain HUT 6037, along with a bialaphos resistance (*bar*) gene, into potato. The herbicide-resistant transgenic potato plants demonstrated enhanced resistance against *A. solani* in in vitro bioassays. However, in another study, Moravcıkova et al. (2004) reported that the high level of expression of cucumber class III *ChiC* gene in potato could not enhance resistance against the phytopathogenic fungus *Rhizoctonia solani* (causing black scurf disease in potato) to any considerable level. Das and Rahman (2010) had also expressed the bacterial *chitinase* (*chi B*) gene in litchi cv. Bedana, which, however, showed a low level of *chitinase* activity, and only partial resistance against the *Phomopsis* sp. pathogen had been reported in transgenic plants. Various endochitinase genes such as *CHIT42* and *CHIT33* from *Trichoderma harzianum* had been successfully transformed and expressed in potato to impart increased fungal tolerance (Lorito et al., 1998) to apple (Bolar et al., 2001), broccoli (Mora and Earle, 2001), carrot (Baranski et al., 2008), and lemon (Distefano et al., 2008). Girhepuje and Shinde (2011) developed transgenic tomato plants overexpressing a wheat *chitinase* gene, *chi194*, under the control of maize *ubiquitin 1* promoter. The transgenic tomato lines showing higher expression of *chitinase* activity were found to be highly resistant to Fusarium wilt disease of tomato caused by *Fusarium oxysporum* f. sp. *lycopersici*. In another study, transgenic litchi (*Litchi chinensis*) plants containing a rice *chitinase* gene were developed to increase the antifungal response. The transgenic lines exhibited higher chitinase activity and disease resistance than the nontransformed plants (Das et al., 2012). Guava wilt disease caused by a soilborne fungus *F. oxysporum* f. sp. *psidii* is emanating as a serious threat to guava growers throughout the entire globe. To control this disease, Mishra et al. (2016) transferred a *Trichoderma endochitinase* gene into guava (*Psidium guajava*). In vitro pathogen inhibition assay and spore germination assay revealed that the crude extract of the transformed plants inhibited the germination of fungal conidia and were resistant to wilt disease.

A number of defense mechanisms have evolved in plants over thousands of years to combat pathogen attack/infection and the role of many genes or various pathways has been investigated and identified (Islam, 2006). The disease resistance conferred by the *glucanase* gene may be because it assists in solubilizing elicitors from the fungal cell walls, which induce production of antifungal phytoalexins (Keen and Yoshikawa, 1993). Yoshikawa et al. (1993) also proposed the role of *glucanase* in the induction of the transcription of a plant defense gene, *phenylalanine ammonia lyase*, in response to fungal attack. Furthermore, *glucanase* is a hydrolytic enzyme, which breaks down the cell wall component glucan of many necrotrophic fungal pathogens. An increased level of resistance in transgenic potato plants expressing a soybean *glucanase* gene against *Phytophthora infestans* has been reported because of the increased *glucanase* activity (Borkowoska et al., 1998). Transgenic kiwifruit overexpressing a soybean *β-1,3-glucanase* gene exhibited sixfold increased enzyme activity leading to a decrease in the disease lesion area caused by the gray mold fungus *B. cinerea* (Nakamura et al., 1999). Almost all the cultivated varieties of brinjal are susceptible to wilt disease caused by *Verticillium dahliae* and *F.*

oxysporum, which cause considerable yield losses annually (Najar et al., 2011). To generate wilt disease resistance in brinjal, Singh et al. (2014) transformed alfalfa *glucanase* gene coding for an acidic *glucanase* into brinjal cv. Pusa Purple Long. The selected transgenic lines, confirmed with DNA and protein blotting techniques, showed enhanced level of resistance against these wilt-causing fungi with a delay of 5–7 days in disease development as compared to the nontransgenic plants. Sometimes it has been found that the transgenes were capable of inducing a disease resistance trait, but have altered the plant growth processes because of the use of a constitutive promoter. In a study, Mercado et al. (2015) expressed *β-1,3-glucanase* gene *bgn13.1*, isolated from *T. harzianum* in strawberry under the control of *CaMV 35S* promoter. The transgenic lines showed reduced anthracnose symptoms (from 61% to 16.5%) in leaf and crown that control plants after inoculation with *Colletotrichum acutatum*. However, most of the transgenic lines displayed stunted phenotype and reduced yield because of the reduction in number of fruit per plant and a reduced fruit size.

The use of various antimicrobial protein-coding genes such as defensins had been advocated for combating a large class of fungal and bacterial pathogens (Collinge et al., 2010). Defensins represent a class of antimicrobial peptides that play an important defensive role against fungi, bacteria, and protozoa, but are nontoxic to mammalian cells and plants. Defensin gene-encoded proteins react by creating certain pores in the fungal hyphal membrane and thus disturb the ion influx–outflux and kill the fungal pathogens. Zainal et al. (2009) reported an enhanced level of resistance in transgenic tomato expressing *Capsicum annuum defensin* genes against various fungal pathogens. A bell pepper *J1 defensin* gene was also reported to confer resistance against anthracnose disease of mango, which is caused by *Colletotrichum gloeosporioides* (Rivera-Dominguez et al., 2011). Protein extract from processed embryos of transgenic mango cv. "Ataulfo" inhibited the growth of *C. gloeosporioides*, *Aspergillus niger*, and *Fusarium* sp. Transgenic banana plants overexpressing two defensin genes—*PhDef1* and *PhDef2*—had been found resistant against *F. oxysporum* f. sp. *cubense* (Ghag et al., 2012).

In addition, genetic transformation of many nonplant antimicrobial compounds such as cercopin, attacin, and phytoalexins had been reported to enhance resistance levels in plants expressing them (Mondal et al., 2012; Ahuja et al., 2012). Transgenic apple expressing maize Leaf color (*Lc*) gene exhibited resistance to fire blight and scab diseases (Flachowsky et al., 2010). Gray mold disease, which is caused by the fungus *B. cinerea*, is one of the most destructive diseases of strawberry, causing the majority of yield losses. In another study, transformation of a biotin-binding protein (Markwick et al., 2003) and a *proteinase inhibitor* gene from *Nicotiana alata* exhibited resistance against light brown apple moth disease. Constitutive expression of a fungus-inducible *carboxy esterase* gene (*PepEST*) under *CaMV 35S* promoter was reported to increase the anthracnose disease resistance in transgenic pepper (*C. annuum*) (Ko et al., 2016). *PepEST* gene expression in fruits leads to disease resistance development by generation of hydrogen peroxide (H_2O_2) and expression of *PR* genes, which encode for a number of small proteins having antimicrobial activity. On infection of the anthracnose fungus *C. gloeosporioides* on the transgenic fruits of pepper cv. Nokkang, a higher level of expression of *PR* genes, namely, *PR3*, *PR5*, *PR10*, and *PepThi*, was reported compared to the nontransgenic plants. Furthermore, a lower rate of disease occurrence was reported in the transgenic fruits, approximately 30% less than in the wild-type plants.

Various types of polyamines including putrescine, spermidine, and spermine play key roles in imparting tolerance/resistance to both biotic and abiotic stresses. Hazarika and Rajam (2011) transformed tomato cv. Pusa Ruby with a human *S-adenosyl methionine decarboxylase* (*samdc*) gene, which is involved in the biosynthesis of polyamines, namely, spermidine and spermine. The transgenic tomato

plants synthesized higher levels of polyamines and also expressed enhanced levels of resistance against *F. oxysporum* causing wilt disease and *A. solani*, the early blight-causing fungus. In addition, the transgenic lines also expressed better tolerance to a variety of abiotic stresses including high temperature, drought, salinity, and chilling stress. Banana (*Musa* sp.) is one of the most important fruit crops being cultivated in about 120 countries across the globe. India is the largest global producer of banana. Banana Xanthomonas wilt (BXW), which is caused by *Xanthomonas campestris* pv. *musacearum*, is considered as one of the most destructive diseases of this fruit crop, particularly in East and Central Africa (Tripathi et al., 2009). In a study, Namukwaya et al. (2012) expressed the *plant ferredoxin-like protein* (*Pf1p*) gene under the control of *CaMV 35S* promoter in transgenic banana cv. "Sukali Ndiizi" and "Nakinyika" to develop resistance against BXW disease. In bioassay studies, 67% of the transgenic lines were found resistant to BXW and did not show any disease symptoms, while the wild-type plants expressed severe symptoms of wilting.

In another study, grapevine rootstock of *Vitis berlandieri* × *Vitis rupestris* cv. Richter 110 had been transformed with an *Agrobacterium* oncogene silencing gene to develop crown gall-resistant lines (Galambos et al., 2013). Lindow et al. (2014) reported reduced severity of Pierce's disease and pathogen mobility in transgenic grape cv. Freedom by the overexpression of an *rpfF* gene (from *Xylella fastidiosa*), which codes for the *synthase* for diffusible signal factors. Cheng et al. (2016) transformed *Vitis vinifera* Thompson seedless grape with a *stilbene synthase* gene, *VqSTS6*, isolated from Chinese wild-type *Vitis quinquangulari*s accession Danfeng-2 under a fruit-specific promoter to develop resistance against powdery mildew disease caused by *U. necator*. The transgenic plants synthesized enhanced quantities of trans-resveratrol and other stilbene compounds as compared to the control plants and expressed enhanced resistance to powdery mildew fungus. It has been found that the *VqSTS6* gene is involved in the resveratrol biosynthetic pathway in grapes and thus plays a key role in imparting protection against invading pathogens. Shin et al. (2002) developed transgenic chilli pepper plants (*C. annuum* cv. Nockwang) with a *Tsi1* (tobacco stress-induced 1) gene via an *A. tumefaciens*-mediated gene transfer technique using cotyledon and hypocotyl explants. The protein product of the *Tsi1* gene has a role in regulating stress-responsive genes and *PR* genes. The transgenic chilli plants expressed enhanced resistance to pepper mild mottle virus, cucumber mosaic virus, a bacterial pathogen *X. campestris* pv. *vesicatoria*, and a fungal pathogen *Phytophthora capsici*. Genetic transformation of a *Vf* gene, imparting scab disease resistance caused by *V. inaequalis* under *CaMV 35S* promoter in apple, had been found to impart scab resistance in susceptible cultivars of apple in a number of studies (Malnoy et al., 2008; Szankowski et al., 2009; Joshi et al., 2009). In 2012, Jiwan et al. reported that antisense expression of the peach MLO gene in strawberry (*Fragaria* × *ananassa*) conferred cross-species resistance to *Fragaria*-specific powdery mildew. RNAi technology is being used these days quite successfully in controlling various bacterial and fungal diseases in plants by switching off the expression of certain endogenous genes. Transgenic tomato plants expressing hairpin RNA constructs against *Agrobacterium iaaM* and *ipt* oncogenes were found to be resistant to crown gall disease (Escobar et al., 2001).

2.3 VIRUS RESISTANCE

In fruit crops, the *coat protein* (CP)-mediated approach to engineer virus resistance has been in application to introduce resistance against various viral diseases. Papayas are grown in many tropical countries, but its cultivation is being threatened by PRSV, a disease that is considerably lowering its yield.

Using biotechnological interventions, the *CP* gene of the virus has been transferred to papaya to confer PRSV resistance. Since 1998, GM papayas have been cultivated in Hawaii, USA, which have shown considerable resistance to PRSV. PRSV-resistant transgenic papaya varieties "SunUp" and "Rainbow" have now occupied >80% shelf-space in the US market. Strawberry is susceptible to various devastating fungi, bacteria, and viruses. In a study, Finstad and Martin (1995) developed transgenic strawberry plants expressing a *CP* gene from strawberry mild yellow edge potexvirus (*SMYELV-CP*) and these lines conferred resistance to the virus. In another study, Lee et al. (2009) developed transgenic chilli pepper plants with a *CP* gene (*CMVP0-CP*). Three independent transgenic events, which were earlier highly tolerant to CMVP1 pathogen, were also found to be tolerant to CMVP0 pathogen. The production and productivity of watermelon (*Citrullus lanatus*) has been affected considerably by two viruses, namely, zucchini yellow mosaic virus (ZYMV) and PRSV type W (PRSV W) worldwide. In an attempt to get rid of these two viruses altogether, Yu et al. (2011) transformed three watermelon cultivars, namely, "Feeling," "China rose," and "Quality," with a chimeric construct containing truncated *ZYMV CP* and *PRSV W CP* genes via an *A. tumefaciens*-mediated gene transfer technique. Two completely immune transgenic lines of "Feeling" cultivar had been obtained during greenhouse bioassays where these two lines showed complete resistance to ZYMV and PRSV W. Also no virus accumulation could be detected by Western blotting from these transgenic lines.

Also transgenic papaya plants with the mutated *replicase* (*RP*) gene from PRSV showed high resistance or immunity against PRSV in the field (Xiangdong et al., 2007). Borth et al. (2011) developed transgenic banana (cv. Dwarf Brazilian) plants resistant to banana bunchy top virus (BBTV) by transforming four gene constructs derived from the *replicase associated protein* (*Rep*) gene of the Hawaiian isolate of BBTV. The transgenic plants showed no bunchy top symptoms, while the nontransgenic plants expressed bunchy top symptoms. Azadi et al. (2011) transformed lily cv. "Acapulco" plants with a defective cucumber mosaic virus *replicase* gene and four transgenic lines were found to show enhanced levels of virus resistance.

RNAi technology has been found successful to impart resistance to various viral diseases in plants. The expression of a self-complementary hairpin RNA under the control of *rolC* promoter controlled the systemic disease spread caused by plum pox virus without preventing local infection (Pandolfini et al., 2003). Using a hairpin RNA gene silencing strategy, transgenic poinsettia plants resistant to poinsettia mosaic virus have been developed (Clarke et al., 2008). Praveen et al. (2010) developed transgenic plants of tomato with an *AC4* gene–RNAi construct and the transgenic plants were found to show the suppression of tomato leaf curl virus activity. In other study, transgenic banana plants expressing small interfering RNA targeted against the viral replication initiation (*Rep*) gene were developed by Shekhawat et al. (2012), which displayed a high level of resistance to BBTV infection. Transgenic development work in various horticultural crops for imparting biotic stress resistance is summarized in Table 15.1.

3. TRANSGENIC RESEARCH IN ABIOTIC STRESS MANAGEMENT IN HORTICULTURAL CROPS

Abiotic stresses such as heat, drought, cold, and salinity are the major environmental constraints affecting production and productivity of almost all horticultural crops. Conventional plant breeding has not proved that successful in addressing abiotic stress tolerance traits so far. The reason being that these traits are controlled by a number of genes present at a quantitative trait locus (QTL). To combat the

Table 15.1 Transgenic Horticultural Crops for Biotic Stress Resistance/Management

S. No.	Crop Species/ Cultivar	Gene Used	Mechanism of Action	Target Trait	Trait Improvement (Transgenic vs. Normal Plants)	References
A. Insect Pest Resistance						
1	Brinjal (eggplant) cv. Pusa Purple Long	Synthetic *cry 1Ab* gene	Insect midgut dissolution	Resistance against fruit and shoot borer *Leucinodes orbonalis*	The transgenic lines displayed significant differences in the insect mortality in fruit bioassays	Kumar et al. (1998)
	Brinjal	Modified rice cystatin gene (*OC-IΔD86*)	Binding of cystatin to the active sites of proteinases inhibits their activity, thus affecting their proteolytic digestion	Resistance against root-knot nematode (*Meloidogyne incognita*)	78.3% inhibition rate in reproduction of root-knot nematode in transgenic plants	Papolu et al. (2016)
2	Banana cv. Gonja manjaya	Maize cystatin gene (*CC-II*)	Binding of cystatins to the active sites of proteinases inhibits their activity, thus affecting their proteolytic digestion	Resistance against reniform nematodes *Potylenchulus reniformis* and *Helicotylenchus multicinctus*	Reduced infection of *P. reniformis* and *H. multicinctus* in transgenic plants	Roderick et al. (2012)
	Banana	Cystatin gene (*OC-IΔD86*)	Binding of cystatins to the active sites of proteinases inhibits their activity, thus affecting their proteolytic digestion	Resistance against *Radopholus similis*	Transgenic banana plants exhibited 70% resistance to the migratory endoparasite *R. similis*	Atkinson et al. (2004)
3	Strawberry (*Fragaria×ananassa*)	*CpTi* (cowpea protease trypsin inhibitor) gene	*CpTi* protein inhibits the internal proteases within the plant cell on pest attack and thus protects the integral protein functions	Resistance against vine weevil (*Otiorhynchus sulcatus*)	*CpTi* transgenic lines reduced the frequency of survival of weevil larvae and pupae during insect bioassays	Graham et al. (2002)
4	Cabbage (*Brassica oleracea* var. *capitata*)	*Cry1B-cry1Ab* fusion gene	Insect midgut dissolution	Resistance against diamondback moth (*Plutella xylostella*)	Reduced infestation of *P. xylostella* in transgenic plants	Paul et al. (2005)
5	Okra (*Abelmoschus esculentus*), an inbred line of the Maharashtra Hybrid Seeds Company Ltd., Jalna, India	*Cry1Ac* gene	Insect midgut dissolution	Insect resistance (fruit and shoot borer)	In insect bioassays, fruits from transgenic lines caused 100% larval mortality	Narendran et al. (2013)
6	Kiwifruit (*Actinidia chinensis*)	Synthetic chimeric gene (*sbtCryIAc*)	Insect midgut dissolution	Insect resistance against *Oraesia excavate*	An average of 75.2% *O. excavate* inhibition rate at 10 days of postinfection during in vitro insect bioassay	Zhang et al. (2015)
7	Potato	Cystatin gene (*OC-IΔD86*)	Binding of cystatins to the active sites of proteinases inhibits their activity, thus affecting their proteolytic digestion	Resistance against *M. incognita*	A partial resistance (67%) against *M. incognita* in transgenic potato roots	Lilley et al. (2004)

8	Lily (*Lilium longiflorum*) cv. "Nellie white"	Rice cystatin gene (*OC-IΔD86*)	Binding of cystatins to the active sites of proteinases inhibits their activity, thus affecting their proteolytic digestion	Root lesion nematode (*Patrylenchus penetrans*) resistance	Enhanced resistance to root lesion nematode infection by means of nematode reduction up to 75% and an increased biomass and better growth performance as compared to nontransformed plants	Vieira et al. (2015)
9	*Chrysanthemum morifolium*	*CmWRKY48* gene	*WRKY48TF* works as positive regulator in various biotic stress responses in plants	Aphid resistance	Inhibition in the population growth of aphids in transgenic chrysanthemum plants	Li et al. (2015)
	C. morifolium genotype 1581	Protease inhibitor sea anemone equistatin (*SAE*) gene	*SAE* has three domains for inhibition of both cysteine and aspartic proteases of aphids	Aphid resistance	Enhanced resistance against *Myzus persicae* and *Aphis gossypi*	Valizadeh et al. (2013)
10	Taiwan cauliflower (*Brassica oleracea* var. *botrytis*) cvs. Known You Early no. 2, Snow Lady, and Beauty Lady	Trypsin inhibitor gene	Inhibits trypsin	Insect resistance	Enhanced resistance to *Spodoptera litura* and *P. xylostella* in transgenic plants in in planta bioassays	Ding et al. (1998)
B. Fungal Resistance						
1	Strawberry	A rice chitinase gene	Chitinase degrades chitin, which constitutes 3%–60% of cell wall composition of fungi, by hydrolysis and thus codes for disease resistance	Resistance against *Sphaerotheca humuli*	Enhanced resistance to *S. humuli* in transgenic strawberry plants	Asao et al. (1997)
	Strawberry	Osmotin	Increase the level of proline accumulation	Resistance against gray mold disease caused by *Botrytis cinerea*	The transgenic plants showed higher levels of antifungal activity and significantly increased resistance to wilt and gray mold diseases	Martinelli et al. (1997)
	Strawberry	Thaumatin II gene (from *Thaumatococcus daniellii*)	Some role in fungal resistance	Resistance to *B. cinerea*	Enhanced resistance to *B. cinerea*	Schestibratov and Dolgov (2005)
	Strawberry cv. Pajaro	Chitinase and a glucanase gene	Chitinase and glucanase degrades chitin and glucan, cell wall composition of fungi, by hydrolysis and thus codes for disease resistance	Resistance to *B. cinerea*	Two transgenic lines expressing chitinase gene showed enhanced tolerance to *B. cinerea*	Vellice et al. (2006)
	Strawberry	β-1,3-Glucanase	Dissolves glucan component of cell wall of fungi, thus imparting fungal resistance	Anthracnose crown rot caused by *Colletotrichum acutatum*	Reduced crown rot lesions in transgenic events as compared to the nontransgenic lines	Mercado et al. (2007)
	Strawnberry (*F.* × *ananassa*) cv. Camarosa	β-1,3-Glucanase gene (*bgn13.1* from *Trichoderma harzianum*)	Dissolves glucan component of cell wall of fungi, thus imparting fungal resistance	Resistance to crown gall disease	The transgenic lines showed reduced anthracnose symptoms (from 61% to 16.5%) in leaf and crown compared to control plants	Mercado et al. (2015)

Continued

Table 15.1 Transgenic Horticultural Crops for Biotic Stress Resistance/Management—cont'd

S. No.	Crop Species/ Cultivar	Gene Used	Mechanism of Action	Target Trait	Trait Improvement (Transgenic vs. Normal Plants)	References
2	Carrot (*Daucus carota*)	Tobacco class I *ChiC* gene	Chitinase degrades chitin, which constitutes 3%–60% of cell wall composition of fungi, by hydrolysis and thus codes for disease resistance	Resistance against *B. cinerea*	Enhanced resistance against *B. cinerea* in transgenic carrot plants	Punja and Raharjo (1996)
	Carrot	*AtNPR1* gene (derived from *Arabidopsis thaliana*)	*NPR1* gene plays a key role in regulating salicylic acid (SA)-mediated systemic acquired resistance (SAR) in plants	Resistance against bacterial and fungal pathogen	Enhanced resistance to bacterial and fungal pathogens	Walley et al. (2009)
3	Potato	Soybean glucanase gene	Dissolves glucan component of cell wall of fungi, thus imparting fungal resistance	Resistance against *Phytophthora infestans*	An increased level of resistance in transgenic potato against *P. infestans*	Borkowoska et al. (1998)
	Potato	A cucumber class III *ChiC* gene	Chitinase degrades chitin, which constitutes 3%–60% of cell wall composition of fungi, by hydrolysis and thus codes for disease resistance	Resistance against *Rhizoctonia solani* causing black scurf disease in potato	Enhanced resistance against the phytopathogenic fungus *R. solani*	Moravcıkova et al. (2004)
	Potato cvs. Waseshiro, Benimaru, and May Queen	A chitinase gene *ChiC* from *Streptomyces griseus* and *bar* gene	Chitinases are glycosyl hydrolases that catalyze the degradation of chitin, an insoluble, linear β-1,4-linked polymer of *N*-acetyl glucosamine, a cell wall component of various bacteria and fungi, and thus codes for pathogen resistance	Resistance against early blight of potato caused by *Alternaria solani*	The herbicide-resistant transgenic potato plants demonstrated enhanced resistance against *A. solani* in in vitro bioassays	Khan et al. (2008)
4	Kiwifruit	Soybean β-1,3-glucanase gene	Dissolves glucan component of cell wall of fungi, thus imparting fungal resistance	Resistance against gray mold fungus *B. cinerea*	Transgenic kiwifruit exhibited a sixfold increased enzyme activity leading to a decrease in the disease lesion area caused by the gray mold fungus *B. cinerea*	Nakamura et al. (1999)

5	Grapevine (*Vitis vinifera*)	Rice chitinase gene (*RCC2*)	Chitinase degrades chitin, which constitutes 3%–60% of cell wall composition of fungi, by hydrolysis and thus codes for disease resistance	Powdery mildew resistance	Increased resistance level against powdery mildew fungus *Uncinula necator*	Yamamoto et al. (2000)
	Grapevine	*Vv NPR1.1* gene (derived from *V. vinifera*)	*NPR1* gene plays a key role in regulating SA-mediated SAR in plants	Resistance against powdery mildew	Enhanced resistance to powdery mildew disease	Le et al. (2011)
	Grapevine rootstock of *Vitis berlandieri* × *Vitis rupestris* cv. Richter 110	*Agrobacterium* oncogene-silencing gene	Oncogenes when silenced lead to no crown gall formation	Resistance against crown gall disease	Enhanced resistance to crown gall disease	Galambos et al. (2013)
	Grapevine cv. Freedom	*rpfF* gene (from *Xylella fastidiosa*)	*rpfF* gene codes for the synthase for diffusible signal factors imparting resistance against Pierce's disease	Resistance against Pierce's disease	A reduced severity of Pierce's disease and pathogen mobility in transgenic grape	Lindow et al. (2014)
	Grapevine cv. Thompson seedless grape	A stilbene synthase gene (*VqSTS6*) from wild grapevine, *Vitis quinquangularis*	*VqSTS6* gene is involved in resveratrol biosynthetic pathway and plays a key role in imparting protection against invading pathogens	Resistance against powdery mildew disease caused by *U. necator*	The transgenic plants synthesized enhanced quantity of trans-resveratrol and other stilbene compounds and expressed enhanced resistance to powdery mildew fungus	Cheng et al. (2016)
6	Apple (*Malus domestica*)	*Malus pumila NPR1* (*MpNPR1*) gene	*NPR1* gene plays a key role in regulating SA-mediated SAR in plants	Fungal and bacterial resistance	Resistance to two fungal pathogens *Venturia inaequalis* and *Gymnosporangium juniperi-vergini-anae*, and a bacteria *Erwinia amylovora*	Malnoy et al. (2007)
	Fuji apple (Fuji Naga-fu no. 6)	*Mh-NPR1* gene (derived from *Malus hupehensis*)	*NPR1* gene plays a key role in regulating SA-mediated SAR in plants	Resistance against apple powdery mildew, caused by *Podosphaera leucotricha*	*MhNPR1* gene induced the expression of *MdPRs* and *MdMLO* genes, which interact with powdery mildew, and the transgenic apple plants expressed enhanced resistance to powdery mildew disease	Chen et al. (2012)
7	Litchi (*Litchi chinensis*) cv. Bedana	Bacterial chitinase gene (*ChiB*)	Chitinase degrades chitin, which constitutes 3%–60% of cell wall composition of fungi, by hydrolysis and thus codes for disease resistance	Resistance against *Phomopsis* sp.	Transgenic plants showed low level of chitinase activity and only partial resistance against *Phomopsis* sp. pathogen	Das and Rahman (2010)
	Litchi cv. Bedana	Chitinase gene (rice)	Chitinase degrades chitin, which constitutes 3%–60% of cell wall composition of fungi, by hydrolysis and thus codes for disease resistance	Fungal resistance	The transgenic lines exhibited higher chitinase activity and disease resistance than the nontransformed plants	Das et al. (2012)

Continued

Table 15.1 Transgenic Horticultural Crops for Biotic Stress Resistance/Management—cont'd

S. No.	Crop Species/ Cultivar	Gene Used	Mechanism of Action	Target Trait	Trait Improvement (Transgenic vs. Normal Plants)	References
8	Mango (*Mangifera indica*) cv. Ataulfo	Defensin *J1* gene from bell pepper	Defensin gene acts as proteinase inhibitor, also creates pores in cell membrane of fungal hyphae	Resistance against anthracnose disease caused by *Colletotrichum gloeosporioides*	Protein extract from processed embryos of transgenic mango cv. "Ataulfo" inhibited the growth of *C. gloeosporioides*, *Aspergillus niger*, and *Fusarium* sp.	Rivera-Dominguez et al. (2011)
9	Tomato	Defensin gene from chilli	Defensin gene acts as proteinase inhibitor, also creates pores in cell membrane of fungal hyphae	Various fungal pathogens	Enhanced level of resistance in transgenic tomato plants	Zainal et al. (2009)
	Tomato cv. Pusa Ruby	*Samdc* (a human derived *S*-adenosyl methionine carboxylase gene)	*samdc* is a key gene involved in polyamine biosynthesis, imparting tolerance to a variety of biotic and abiotic stresses	Biotic and abiotic stress resistance/tolerance	The transgenic tomato plants expressed enhanced level of resistance against *F. oxysporum* and *A. solani* and also expressed better tolerance to high temperature, drought, salinity, and chilling stress	Hazarika and Rajam (2011)
	Tomato cv. Pusa Ruby	*chi194*, a wheat endochitinase gene	Chitinase degrades chitin, which constitutes 3%–60% of cell wall composition of fungi, by hydrolysis and thus codes for disease resistance	Resistance against tomato wilt caused by *F. oxysporum* f. sp. *lycopersici*	The transgenic tomato lines were found to be highly resistant to Fusarium wilt	Girhepuje and Shinde (2011)
	Tomato	*At NPR1* gene (derived from *A. thaliana*)	*NPR1* gene plays a key role in regulating SA-mediated SAR in plants	Resistance against bacterial and fungal pathogen	Enhanced resistance to bacterial and fungal pathogens	Lin et al. (2004)
10	Lily cv. "Acapulco"	A defective cucumber mosaic virus (CMV) replicase gene	Defective viral genome-mediated resistance	CMV virus resistance	Four transgenic lines showed enhanced level of virus resistance	Azadi et al. (2011)
11	Brinjal (*Solanum melongena*) cv. Pusa Purple Long	Glucanase gene from alfalfa	Dissolves glucan component of cell wall of fungi, thus imparting fungal resistance	Resistance against *Verticillium dahliae* and *F. oxysporum*	The transgenic lines showed enhanced level of resistance against these wilt-causing fungi with a delay of 5–7 days in disease development as compared to the nontransgenic plants	Singh et al. (2014)
12	Guava (*Psidium guajava*)	*Trichoderma* endochitinase gene	Chitinase degrades chitin, which constitutes 3%–60% of cell wall composition of fungi, by hydrolysis and thus codes for disease resistance	Wilt disease resistance	Crude extract of the transformed plants inhibited the germination of fungal conidia	Mishra et al. (2016)

13	Pepper cv. Nokkang	A pepper esterase gene (*PepEST*)	*PepEST* gene expression leads to generation of H_2O_2 and expression of pathogenesis-related (*PR*) genes, which encode for a number of small proteins having antimicrobial activity	Anthracnose disease resistance (caused by *C. gloeosporioides*)	A lower rate of disease occurrence in the transgenic fruits, approximately 30% less than in the wild-type plants	Ko et al. (2016)
14	Citrus (sweet orange) cv. "Hamlin" and "Valencia"	*AtNPR1* gene (from *A. thaliana*)	*NPR1* gene is involved in the induction of expression of several native genes involved in the plant defense signaling pathways	Resistance against Huanglongbing disease, caused by a bacterium *Candidatus Liberibacter asiaticus* (CLas)	The transgenic plants exhibited reduced disease severity and a few lines remained disease free even after 3 years of planting in a high-disease pressure field site	Duan et al. (2009)
C. Virus Resistance						
1	Potato cv. "Spuncta"	dsRNA of coat protein gene of *PVY*	Antisense gene inhibition	Resistance against potato virus Y (PVY)	Transgenic plants were found highly resistant to three strains of PVY, each belonging to three different subtypes of the virus (PVY^N, PVY^O, and PVY^{NTN})	Missiou et al. (2004)
2	Poinsettia	RNAi	Small interfering (siRNA)-mediated gene silencing	Poinsettia mosaic virus resistance	Enhanced resistance to poinsettia mosaic virus	Clarke et al. (2008)
3	Papaya	Papaya ringspot virus (PRSV) coat protein (*CP*) gene	Coat protein-mediated virus resistance	PRSV	Enhanced resistance against PRSV infection	Suzuki et al. (2008)
4	Watermelon (*Citrullus lanatus*), three cvs., namely, "Feeling," "China rose," and "Quality"	A chimeric gene construct containing truncated *ZYMV-cp* and *PRSV W cp* genes	RNA mediated posttranscriptional gene silencing	Resistance against two viruses, namely, ZYMV and PRSV W	Two completely immune transgenic lines of "Feeling" cultivar showed complete resistance to ZYMV and PRSV W	Yu et al. (2011)
5	Banana cv. Rasthali	*ihp-RNA-Rep* and *ihp-RNA-ProRep*	siRNA, derived from the transgene sequence coded for resistance against banana bunchy top virus (BBTV) by establishing RNA interference mechanism to silence *Rep* gene transcript	Resistance against BBTV	Transgenic plants were found resistant to BBTV	Shekhawat et al. (2012)
6	Strawberry	*CP* gene from strawberry mild yellow edge potexvirus	Coat protein- mediated virus resistance	Resistance against strawberry mild yellow edge potex virus	Transgenic lines were found resistant to strawberry mild yellow edge potex virus	Finstad and Martin (1995)

negative effects of various abiotic stresses, it is a prerequisite to identify potential candidate genes or QTLs (gene networks) associated with broad-spectrum multiple abiotic stress tolerance. Various abiotic stresses including drought, high temperature, salinity, frost, flood, etc. adversely affect overall crop growth and productivity by affecting the vegetative and reproductive stages of growth and development. These stresses generally trigger a series of physiological, biochemical, and molecular changes in the plants, which often result in damage to cellular machinery (Rai et al., 2011). Among these changes include the disruption of cellular osmotic balance leading to dysfunctional homeostasis and ion distribution and oxidative stress, which lead to denaturation of integral proteins of plants. Plants respond to such stresses using a variety of mechanisms that trigger the cell signaling process, transcriptional controls, and production of a number of stress-related tolerant proteins, antioxidants, and osmotic solutes to maintain homeostasis and to protect and repair the damaged integral proteins. Generally, plants that are stress sensitive are unable to synthesize such compounds under stress conditions and thus are rendered liable to various stresses that hamper their overall growth. A number of genes have been identified in a number of plants/organisms, closely or distantly related, which code for the synthesis of these stress-protecting compounds and thus can be targeted for genetic transformation into sensitive genotypes. Such genes have been classified into three categories: (1) genes that code for the synthesis of various osmolytes such as manitol, glycine betaine, proline, heat shock proteins, etc.; (2) genes responsible for ion and water uptake and transport such as aquaporins and ion transporter, etc.; and (3) genes regulating transcriptional controls and signal transduction mechanisms such as *MAPK*, *DREBI*, etc. Nevertheless, abiotic stresses remain the greatest constraint to crop production. Research on genetic modification of various horticultural crops for improved abiotic stress tolerance has been explored.

3.1 DROUGHT TOLERANCE

Various genes controlling for signaling and gene regulatory pathways offer certain key targets for genetic engineering for abiotic stress tolerance. Transcription factors (TFs) that regulate or switch on the expression of a number of genes involved in imparting abiotic stress tolerance in plants have been proposed as the most efficient targets for genetic transformation (Bhatnagar-Mathur et al., 2008). These TFs include the *DREB1* gene family, *Myb* gene family, etc. Tsai-Hung et al. (2002) transformed tomato plants with a DNA cassette containing an *Arabidopsis* C repeat/dehydration-responsive element binding factor 1 (*CBF1*) cDNA and a *nos* terminator, driven by a CaMV *35S* promoter. These transgenic tomato plants were more resistant to deficit water stress than the wild-type plants. In one study, Pasquali et al. (2008) reported improved tolerance to cold and drought stress in transgenic apple by the overexpression of a cold-inducible *Osmyb4* gene from rice, which codes for a TF belonging to the *Myb* family. The overexpression of a *DREB1bTF* gene had also been reported to induce cold tolerance and drought tolerance in transgenic grapevine (Jin et al., 2009). Chrysanthemum is one of the leading ornamental cut flowers across the globe and its production is severely hampered by various environmental conditions (Gao et al., 2012). Drought stress harms this crop to the maximum extent by retarding its growth. *WRKY* TFs work as positive or sometimes negative regulators in various abiotic stress responses in plants. Fan et al. (2016) transformed a *CmWRKY1* TF derived from *C. morifolium* and overexpressed it in chrysanthemum cultivar "Jinba." It was found that *CmWRKY1* regulates an abscisic acid-mediated pathway by suppressing the expression levels of various genes including *PP2C*, *ABI1*, and *ABI2*, and activating the expression levels of genes such as *PYL2*, *SnRK2-2*, *ABF4*, *MYB2*, *RAB18*, and *DREB1A* in a positive regulation. The transgenic plants displayed increased drought tolerance in polyethylene

glycol stress as compared to control plants. Also multiple abiotic stress tolerance in banana had been reported by the overexpression of a *MusaWRKY71* gene, which is a very potential abiotic stress-responsive *WRKY TF* gene cloned from *Musa* sp. cv. Karibale Monthan (Shekhawat and Ganapathi, 2013).

A bacterial *mannitol-1-phosphate dehydrogenase* (*mtlD*) gene driven by the constitutive *CaMV 35S* promoter was transferred into tomato plants in an attempt to improve abiotic stress tolerance in the transformed plants (Khare et al., 2010). Drought (polyethylene glycol in medium) and salinity (sodium chloride in medium) tolerance tests revealed that transgenic lines exhibited a higher tolerance for abiotic stresses than nontransformed plants. To impart tolerance to various abiotic stresses in potato, Gangadhar et al. (2016) transformed a potato-derived gene *StnsLTP1* into potato (*Solanum tuberosum* cv. Desiree) using an *A. tumefaciens*-mediated genetic transformation method. Under stress conditions, transgenic potato lines displayed enhanced cell membrane integrity by reduced membrane lipid peroxidation activity and H_2O_2 content. Also an increased level of antioxidant enzyme activity with enhanced accumulation of ascorbates and upregulation of various stress-related genes including *StAPX*, *StCAT*, *StSOD*, etc. was reported in transgenic potato plants. Glycine betaine plays an important role in drought stress tolerance by scavenging oxidative stress-inducing molecules (free radicals), and it also protects the photosynthetic system in plants. Cheng et al. (2013) transformed *choline oxidase* gene (*Cod A*) isolated from *Arthrobacter globiformis*, which is involved in the biosynthesis of glycine betaine, into potato cv. "Superior" under an oxidative stress-inducible *SWAP2* promoter for inducing a drought stress tolerance trait. Under water-stress conditions, transgenic potato plants showed expression of a *Cod A* gene and an accumulation of glycine betaine with a higher leaf water potential as compared to the nontransformed plants. In the stress-recovery treatment, transgenic potato plants displayed a stronger antioxidant activity, higher chlorophyll content, more efficient photosynthesis, and better recovery.

Plant microRNA regulates several developmental and physiological phenomena inside the plants including drought responses. Zhang et al. (2011) transformed tomato with an *miR169* family member, *Sly-miR169c*, which can effectively downregulate the transcripts of the target genes—three nuclear factor Y subunit genes (*SINF-YA1/2/3*) and one multidrug resistance-associated protein gene (*SIMRP1*), which are downregulated under drought stress. The transgenic tomato plants overexpressing *Sly-miR169c* displayed reduced stomatal opening, reduced leaf water loss, and transcription rate with enhanced drought tolerance traits. In an attempt to improve abiotic stress tolerance in mulberry, a very important plant of the silk industry, an *Hva1* gene encoding for late embryogenesis abundant protein from barley was transformed by the *A. tumefaciens*-mediated method (Checker et al., 2012). Late embryogenesis abundant proteins comprise a group of hydrophillins, which are induced as a response to desiccation in seeds and are also stimulated under various abiotic stress conditions such as dehydration, salinity, chilling, or high-temperature stress in vegetative tissues of plants (Khurana et al., 2008). The transgenic lines displayed an enhanced level of tolerance to drought, salinity, and cold conditions compared to normal plants as quantified by free proline, membrane stability index, and photosystem II activity.

3.2 HEAT TOLERANCE

Under heat stress, many reactive oxygen species (ROS) such as H_2O_2, superoxide, etc. are produced inside the plant cells, leading to various kinds of physiological disorders in plants that affect crop growth and productivity. These ROS denature enzymes and damage various cellular components inside the plant cells. Tolerance to heat stress is straightaway correlated with the increased capacity of plants

to scavenge ROS (Chaitanya et al., 2002). Thus it is very important to scavenge ROS to maintain normal growth and metabolism of plants. Plants have developed a variety of mechanisms to combat ROS by the production of various enzymatic systems such as superoxide dismutase(SOD) to remove superoxide ions, glutathione reductase (GR), peroxidase to scavenge peroxide ions (H_2O_2), etc (Noctor and Foyer, 1998). The nonenzymatic methods involve the production of a variety of chemical compounds including polyamines, carotenoids, ascorbic acid, tocopherols, etc., which directly react with ROS, scavenge them, and thus provide protection to the plants against heat stress. Thus overexpression of ROS scavenging enzymes in plants via genetic transformation offers a potential strategy to combat heat stress. In one study, Wisniewski et al. (2002) reported that the overexpression of a cytosolic ascorbate peroxidase (*cAPX*) gene improved tolerance to heat stress in transgenic apple. Wang et al. (2006) developed transgenic tomato plants that overexpressed a *cAPX* gene with enhanced tolerance to heat (40°C). In field tests, the detached fruits from field grown transgenic tomato plants showed enhanced resistance to exposure to direct sunlight as compared to the fruits from wild-type (nontransgenic) plants. Overexpression of a *Cu/Zn superoxide dismutase (Cu/Zn SOD*) gene (derived from *Manihot esculenta*) under an oxidative stress-inducible promoter *SWPA2* in potato led to enhanced heat stress tolerance (Tang et al., 2006). *Cu/Zn SOD* is an ROS-scavenging enzyme and thus helps in quenching free radicals released under heat stress in plants. Transgenic plants expressed enhanced tolerance to 250 μM methyl viologen and the visible damage caused by heat stress was around 25% in the transgenic plants as compared to the nontransgenics, which were destroyed completely under heat stress.

Polyamines play an important role in imparting thermal stress tolerance in plants. *S-Adenosyl-I-methionine decarboxylase* (*SAMDC*) is one of the key regulatory target enzymes in polyamine biosynthesis. Cheng et al. (2009) overexpressed *SAMDC* cDNA, isolated from *Saccharomyces cerevisae*, in tomato plants for enhanced polyamine production. Transgenic lines produced 1.7–2.4-fold higher levels of spermidine and spermine with enhanced antioxidant enzyme activity and better protection of membrane lipid peroxidation as compared to wild-type plants, leading to enhanced tolerance to high=temperature stress (38°C). Overexpression of heat shock proteins in plants has been proposed as one of the potential strategies to combat heat stress. Heat shock proteins function as molecular chaperons, which are involved in correct protein folding, assembly, translocation, and degradation, and they also provide stability to integral proteins and cell membranes under heat stress (Boston et al., 1996). Song et al. (2014) overexpressed a *CgHSP70* gene conferring for heat tolerance in chrysanthemum. The transgenic lines exhibited an increased peroxidase activity, higher proline content, and reduced malondialdehyde content. Proline is an important osmoprotectant that protects cells from damage under heat stress. The transgenic plants were better able to tolerate heat stress than wild-type plants.

3.3 SALINITY TOLERANCE

Salinity or salt stress is one of the most prevalent abiotic stresses that significantly reduces the overall growth and yield of many horticultural crops. Around 20% of the world's irrigated agricultural land is severely affected by salinity problems (Rengasamy, 2006). Salinity tolerance is a complex mechanism governed by many genes (Bojórquez-Quintal et al., 2014). Plants, when exposed to abiotic stress conditions, produce several PR proteins to compensate the effect of stress conditions. *Osmotin* is one of the important PR proteins, which are produced by the plants to combat various biotic and abiotic stresses. Husaini and Abdin (2008) overexpressed a tobacco *osmotin* gene in strawberry (*F.* × *ananassa* Duch.)

and found that the transgenic strawberry plants exhibited tolerance to salt stress. Chilli plants are not easily amenable to tissue culture and genetic transformation, thus limiting the scope of genetic improvement for various biotic and abiotic stresses (Kothari et al., 2010). Subramanyam et al. (2011) could successfully improve the tolerance of chilli pepper (*C. annuum* L.) cv. Aiswarya 2103 plants by the ectopic expression of a tobacco *osmotin* gene via an *A. tumefaciens*-mediated gene transfer technique. T_2 generation of transgenic pepper plants revealed enhanced levels of chlorophyll, proline, glycine betaine, ascorbate peroxidase (*APX*), *SOD*, *GR*, and relative water content in biochemical analysis and could survive salinity levels up to 300 mM NaCl concentration. In comparison to other horticultural crops, citrus species are the most sensitive to soil salinity, which greatly limits growth and productivity of citrus crops across the globe. Cervera et al. (2000) transformed Carrizo citrange, an excellent rootstock of citrus with a yeast-derived halotolerance gene, *HAL2*, which is involved in the salt tolerance mechanism. The *HAL2* gene is involved in the methionine biosynthetic pathway and confers tolerance to lithium and sodium ions. It encodes for a salt-sensitive bisphosphate nucleotidase, which is required for sulfate accumulation. The transgenic lines expressing *HAL2* protein showed improved tolerance to salinity compared to the wild-type plants. Tomato is considered one of the most important vegetable crops worldwide for the commercial value it offers. Wang et al. (2005) developed transgenic tomato plants expressing tolerance to chilling and salt stress by incorporation of a *cAPX* gene, derived from pea (*Pisum sativum* L.). *APX* plays a key role in quenching H_2O_2 in plant cells, thus providing protection against oxidative injury induced by chilling and salt stress. The transgenic plants showed better seed germination rate (26%–37%) than the wild type (3%) when the seeds were placed at 9°C for 5 weeks. *APX* activity was found 10–25-fold higher in transgenic plants under salinity stress (200–250 mM) conditions, thus ensuring minimum damage to the leaves.

Various abiotic stresses including salinity, chilling, and oxidative stresses are the critical factors limiting the cultivation and productivity of sweet potato (*Ipomoea batatas*), a root crop, used as a vegetable. It has been observed that the increased production of glycine betaine in plant cells improves their tolerance level toward these stresses. Fan et al. (2012) transformed sweet potato cv. Sushu-2 with a chloroplastic *betaine aldehyde dehydrogenase* (*SoBADH*) gene from *Spinacia oleracea*, which is involved in the biosynthesis of glycine betaine. The overexpression of an *SoBADH* gene in transgenic sweet potato improved tolerance toward salinity, oxidative stress, and low temperature by providing protection against cell damage by maintaining cell membrane integrity, stronger photosynthetic activity, reduced ROS production, and activation of the ROS scavenging mechanism. To enhance tolerance of tomato plants to salinity stress, Lim et al. (2016) transformed a strawberry *D-galacturonic acid reductase* (*GalUR*) gene into cherry tomato (*Solanum lycopersicum*) C, H, and F lines to increase the ascorbic acid content. Transgenic tomato plants enriched with high fruit ascorbic acid contents had been found more tolerant to abiotic stress-induced viologen, NaCl, and mannitol as compared to the wild-type plants. The transgenic events could survive salt stress up to 200 mM and also showed higher expression levels of antioxidant genes including *APX* and *catalase*, responsible for imparting additional capabilities to the transgenic plants for salt tolerance.

Under high salinity stress conditions, ion homeostasis within the plant cells is disturbed, altering the overall metabolism. Bulle et al. (2016) developed transgenic chilli pepper (*C. annuum*) plants expressing wheat Na^+/H^+ antiporter gene (*TaNHX2*) to develop tolerance toward salinity stress. Transgene integration and expression was confirmed by PCR, Southern hybridization, and RT-PCR in T_1 generation. In biochemical assays, transgenic lines revealed enhanced levels of proline, chlorophyll, SOD, APX, relative water content, and reduced level of H_2O_2 and malondialdehyde as compared to the

nontransformed plants under salt stress conditions. Overexpression of a *TaNHX2* gene has already been evaluated in tomato to combat salinity stress (Yarra et al., 2012).

Though at present transgenic crops are being cultivated over an area of 179.7 m ha by 70–80 million farmers in 28 countries across the globe, people continue have issues regarding biosafety, health, and environmental risks posed by the consumption and commercialization of these GM crops. This has led to the development of certain new technologies to address these concerns, referred to as marker-free (Clean-gene) transgenic technology and genome editing technology. Transgenic development works in various horticultural crops for imparting abiotic stress resistance and is summarized in Table 15.2.

4. ROLE OF MARKER-FREE TRANSGENIC TECHNOLOGY

Generally, methods of genetic transformation employ selection markers such as antibiotic resistance genes or herbicide tolerance genes for the selection of transformed cells (Bevan et al., 1983; Akama et al., 1995). However, except for the role as selectable marker, these genes do not have any relevant function inside the plant cell and thus they exert an extra burden on the plant genome. Also the constitutive expression of these gene-encoded proteins affects the plant metabolism in a negative way. Furthermore, use of marker genes, particularly those coding for antibiotic resistance, has been facing strong criticism and opposition, particularly in edible crops including fruits and vegetables. Developing marker-free plants or discovering suitable alternatives of antibiotic or herbicide tolerance genes has been proposed with the hope of increasing consumers' acceptance for GM crops. A set of new technologies has been developed that involve the elimination of marker genes after the development of transgenic plants, which come under the umbrella of "marker-free transgenic technology" or "Clean-gene technology." Such technologies would be helpful to minimize biosafety concerns during biosafety research trials and the transgenic products would attract wider consumer acceptance. Several techniques including screening of transformants with PCR only (Vetten et al., 2003; Ballester et al., 2010), cotransformation (Lu et al., 2009), and site-specific recombination (Zhang et al., 2009; Khan et al., 2011b) have been used to develop a selection of marker-free transgenic plants in the past.

For the production of selectable marker-free transgenic plants, the Multi-Auto-Transformation (MAT) vector system (Ebinuma et al., 1997), which combines positive selection using an *isopentenyltransferase* (*ipt*) gene, the first enzyme of cyokinin biosynthesis with a site-specific recombination (Barry et al., 1984) and removal system, offers potential as a really useful tool (Sugita et al., 1999). Marker gene is removed from the transformed cells by the mechanism of homologous recombination. The MAT vector backbone is comprised of a yeast site-specific recombination R/RS system to excise the DNA fragment and the *ipt* gene is cloned between two directly oriented recombination sites (Araki et al., 1987). The MAT vector system has been employed in a number of crops to develop marker-free transgenic plants including *Antirrhinum majus* (Minlong et al., 2000), citrus (Ballester et al., 2007), *Kalanchoe blossfeldiana* (Thirukkumaran et al., 2009), and *Petunia hybrida* (Khan et al., 2010). Khan et al. (2011b) used the MAT vector in which an *ipt* gene was used as a selection marker and a *Wasabi defensin (WD)* gene, isolated from *Wasabia japonica*, as a target gene to transform tomato plants. The marker-free transgenic tomato plants exhibited enhanced resistance against a number of fungi including *A. solani*, *B. cinerea*, *F. oxysporum*, and *Erysiphe lycopersici*. A *phosphomannose isomerase* (*pmi*) gene derived from *Escherichia coli* had been developed as an efficient positive selection marker for apple transformation, which induced the capability to grow on mannose-supplemented medium in the

Table 15.2 Transgenic Horticultural Crops for Abiotic Stress Tolerance/Management

S. No.	Crop Species/ Cultivar	Gene Used	Mechanism of Action	Target Trait	Trait Improvement	References
A. Drought Tolerance						
1	Apple	*Osmyb4*	An *Myb* family transcription factor (TF), leads to accumulation of various solutes compatible with abiotic stress tolerance	Drought and cold tolerance	Enhanced tolerance to drought and low-temperature stress in transgenic plants	Pasquali et al. (2008)
		MdC1PK61	Synthesizes a CBL-interacting protein kinase (*C1PK*)	Salt, drought, and chilling tolerance	Enhanced tolerance to drought, low-temperature, and salt stress in transgenic plants	Wang et al. (2012)
2	Banana	*MusaWRKY71*	Encodes a *WRKY* TF	Multiple abiotic stress tolerance	Enhanced tolerance to drought, salinity and high temperature	Shekhawat et al. (2011)
		MusaSAP1	Encodes for stress-associated proteins	Multiple abiotic stress tolerance	Enhanced tolerance to drought, salinity, and high temperature	Sreedharan et al. (2012)
3	Citrus	*P5CS* gene	Encodes for the biosynthesis of proline	Drought tolerance	Enhanced tolerance to drought and low temperature stress in transgenic plants	De Carvalho et al. (2013)
		P5CSF129A	Provides protection against reactive oxygen species (ROS) by altering enzyme activity	Drought tolerance	Enhanced tolerance to drought and low-temperature stress in transgenic plants	De Campos et al. (2011)
4	Strawberry	Osmotin	Increases the level of proline	Salinity tolerance	Enhanced tolerance to salinity stress in transgenic plants	Husaini and Abdin (2008)
5	Mulberry	Osmotin	Increases the level of proline accumulation	Salinity and drought tolerance	Enhanced tolerance to drought and salinity stress in transgenic plants	Das et al. (2011)
6	Chrysanthemum (*Chrysanthemum morifolium*) cv. "Jinba"	*CmWRKY1* TF (derived from *C. morifolium*)	*CmWRKY1* works as positive regulator in drought stress	Drought stress tolerance	The transgenic plants displayed increased drought tolerance in polyethylene glycol stress as compared to control plants	Fan et al. (2016)
7	Tomato (*Solanum lycopersicum* cv. Aika Craig)	*Sly-miR169c*, an miR169 family member	Downregulates the transcripts of target genes, namely, three nuclear factor Y subunit genes (*S1NF-YA1/2/3*) and one multidrug resistance-associated protein (*slMRP1*) gene	Drought tolerance	Transgenic plants exhibited reduced stomatal opening, decreased transpiration rate, lowered leaf water loss, and enhanced drought tolerance	Zhang et al. (2011)
8	Potato cv. Superior	*Cod A* gene (from *Arthrobacter globiformis*)	*Cod A* gene codes for glycine betaine, which plays an important role in drought stress tolerance by scavenging oxidative stress-inducing molecules (free radicals) and it also protects the photosynthetic system in plants	Drought tolerance	The transgenic potato plants displayed a stronger antioxidant activity, higher chlorophyll content, more efficient photosynthesis, and better recovery	Cheng et al. (2013)

Continued

Table 15.2 Transgenic Horticultural Crops for Abiotic Stress Tolerance/Management—cont'd

S. No.	Crop Species/ Cultivar	Gene Used	Mechanism of Action	Target Trait	Trait Improvement	References
9	China rose (*Rosa chinensis*)	*RcXET* and *MtDREBIC* genes	*XET* and *DREBIC* genes are upregulated in response to various abiotic stresses and impart tolerance to the plant cells	Freezing and drought tolerance	Increased electrolyte conductivity percent, proline content, soluble sugar, photosynthesis rate, negative water potential, and turgor loss point in transgenic plants leading to a better tolerance toward drought and freezing	Chen et al. (2016)
B. Heat Tolerance						
1	Tomato (*S. lycopersicum)*	Yeast *SAMDC* gene	*SAMDC* (*S*-adenosyl-methionine decarboxylase) is one of the key enzymes involved in the biosynthesis of polyamines, which protect the plants against heat stress	Heat stress	Transgenic plants produced 1.7–2.4 times higher level of spermidine and spermine than the wild-type plants and expressed tolerance to heat stress with enhanced antioxidant enzyme activity and protection of membrane lipid peroxidation	Cheng et al. (2009)
	Tomato cv. Zhongshu No. 5	*cAPX* gene	Ascorbate peroxidase (APX) encodes for antioxidant enzyme, which removes H_2O_2, a ROS produced under heat stress	UV-B and heat stress tolerance	Transgenic plants and fruits expressed enhanced tolerance to heat (40°C) and UV-B stress as compared to wild-type plants	Wang et al. (2006)
2	*Chrysanthemum morifolium* cv. "Zhongshanzigui"	*CgHSP70* gene (from *C. morifolium*)	Heat shock proteins function as molecular chaperons, which are involved in correct protein folding, assembly, translocation, degradation, and they also provide stability to integral proteins and cell membranes under heat stress	Heat tolerance	The transgenic lines exhibited an increased peroxidase activity, higher proline content, and reduced malondialdehyde content and were better able to tolerate heat stress than wild-type plants	Song et al. (2014)
3	Potato (*Solanum tuberosum*) cv. Desiree	*StnsLTP1* gene (from potato)	*StnsLTPI* gene imparts tolerance to various abiotic stresses as a result of enhanced activation of antioxidative defense mechanisms via cyclic scavenging of ROS and co-coordinating the expression of stress-related genes	Heat, drought, and salinity tolerance	Transgenic potato lines displayed enhanced cell membrane integrity and an increased level of antioxidant enzyme activity with enhanced accumulation of ascorbates and upregulation of various stress-related genes including *StAPX*, *StCAT*, *StSOD*, etc. under stress conditions	Gangadhar et al. (2016)
	Potato	*Cu/Zn SOD* (from *Manihot esculenta*)	ROS scavenging enzyme and thus helps in quenching of free radicals released under heat stress in plants	Heat stress	Transgenic plant expressed enhanced tolerance to 250 µM methyl viologen and reduced visible damage caused by heat stress	Tang et al. (2006)
C. Salinity Tolerance						
1	Apple	*MdNHX1* gene	Acts as Na^+/H^+ antiporter	Salinity tolerance	Enhanced tolerance to salinity stress in transgenic plants	Li et al. (2010)
2	Banana	*MusaDHN-I* gene	Overexpression of dehydrin gene and a late embryogenesis abundant (LEA) protein	Drought and salinity	Enhanced tolerance to drought and salinity stress	Shekhawat et al. (2011)

3	Kiwi	*AtNHX1* gene	Keeps K^+/Na^+ ratio high during salinity stress conditions	Salinity	Enhanced tolerance to salinity stress in transgenic plants	Tian et al. (2011)
4	Pear	*SAMDC2* gene	Encodes for the biosynthesis of polyamines	Salinity	Enhanced tolerance to salinity stress in transgenic plants	He et al. (2008)
		SPDS gene	Encodes for the biosynthesis of polyamines	Salinity	Enhanced tolerance to salinity stress in transgenic plants	Wen et al. (2010)
5	Tomato (*S. lycopersicum*) cv. Zhongshu No. 5	*cAPX* gene	APX plays a key role in quenching hydrogen peroxide (H_2O_2) in plant cells, thus providing protection against oxidative injury induced by chilling and salt stress	Chilling and salinity	The transgenic plants showed better seed germination rate (26%–37%) than the wild type (3%) when the seeds were placed at 9°C for 5 weeks and higher APX activity under salinity stress (200–250 mM) conditions	Wang et al. (2005)
	Cherry tomato C, H, and F lines	*GalUR* (D-galacturonic acid reductase) gene, derived from strawberry	*GalUR* gene codes for higher level of ascorbic acid biosynthesis, which imparts tolerance to salinity stress	Salinity stress	Transgenic tomato plants were found more tolerant to abiotic stress-induced viologen, NaCl, and mannitol and with higher expression levels of APX and catalase, responsible for imparting additional capabilities to the transgenic plants for salt tolerance	Lim et al. (2016)
	Tomato	Na^+/H^+ antiporter gene (*TaNHX2*) from wheat	*TaNHX2* gene leads to the production of osmolytes to maintain the cell membrane stability	Salinity tolerance	Better salinity tolerance in transgenic plants	Yarra et al. (2012)
6	Citrus (Carrizo citrange root stock)	Yeast *HAL2* gene	*HAL-2* gene is involved in the pathway of methionine biosynthesis and confers tolerance to lithium and sodium	Salinity tolerance	Transgenic plants showed better tolerance to salt stress than nontransgenic plants	Cervera et al. (2000)
7	Sweet potato (*Ipomoea batatas*) cv. Sushu-2	*SoBADH* (*Spinacia oleracea*-derived betaine aldehyde dehydrogenase gene)	Glycine betaine protects the plant cells from abiotic stress by providing protection against cell damage by maintaining cell membrane integrity, stronger photosynthetic activity, reduced ROS production, and activation of ROS scavenging mechanism	Salinity, oxidative stress, and chilling/cold stress	The transgenic plants improved tolerance toward salinity, oxidative stress, and low temperature	Fan et al. (2012)
8	Chilli pepper (*Capsicum annuum* L.) cv. Aiswarya 2103	Osmotin gene	Overexpression of osmotin gene induces biosynthesis of proline and confers tolerance to osmotic stress	Salinity tolerance	Transgenic pepper plants could survive salinity levels up to 300 mM NaCl concentration	Subramanyam et al. (2011)
	Chilli pepper cv. Q4	Na^+/H^+ antiporter gene (*TaNHX2*) from wheat	*TaNHX2* gene leads to the production of osmolytes to maintain the cell membrane stability	Salinity tolerance	Transgenic lines revealed enhanced levels of proline, chlorophyll, superoxide dismutase, APX, relative water content, and reduced levels of H_2O_2 and malondialdehyde as compared to the nontransformed plants under salt stress conditions	Bulle et al. (2016)

Continued

Table 15.2 Transgenic Horticultural Crops for Abiotic Stress Tolerance/Management—cont'd

S. No.	Crop Species/ Cultivar	Gene Used	Mechanism of Action	Target Trait	Trait Improvement	References
9	China rose (*Rosa chinensis*)	*AtDREB2A-CA* gene	Overexpression of *AtDREB2A-CA* gene enhances salinity stress tolerance in Chinese rose by altering the leaf ultrastructure in response to salt stress	Salinity stress	Enhanced salinity tolerance in transgenic plants	Josine et al. (2015)
10	*C. morifolium*	*CmWRKY17* TF	*WRKY* TFs work sometimes as positive and sometimes as negative regulators in a variety of abiotic stress responses in plants	Salinity stress	Overexpression of *CmWRKY17* TF in chrysanthemum increased plant sensitivity to salinity stress	Li et al. (2015)
11	Mulberry (*Morus indica*) cv. K-2	*HvaI* gene (from barley)	LEA proteins (encoded by *HvaI* gene) protect cells against abiotic stresses by sequestering ions, stabilization of macromolecules and membranes, and act as antioxidants	Salinity, drought, and cold tolerance	The transgenic lines displayed an enhanced level of tolerance to drought, salinity, and cold conditions compared to normal plants as quantified by free proline, membrane stability index, and photosystem II activity	Checker et al. (2012)

transformed cells (Degenhardt and Szankowski, 2006; Degenhardt et al., 2007). Furthermore, plastid engineering has also been advocated as one of the most viable techniques to avoid transgene spread to other related crops (nontarget species).

Cisgenic crops represent a step toward a new generation of GM crops. Development of GM crops, which do not possess any selectable marker (e.g., antibiotic resistance or herbicide tolerance) gene in the end product and if the inserted gene is derived from the same organism/plant, would be a welcome step to increase consumers' acceptance for that product and to minimize the environmental risks associated with GM crops. In this direction, Vanblaere et al. (2011) developed cisgenic apple plants by inserting the endogenous scab resistance gene *HcrVf2* under the control of its own regulatory sequences into the scab-susceptible apple cultivar "Gala" using an R/RS vector system to develop marker-free transgenic plants. Dhekney et al. (2011) also used the cisgenic approach to develop disease-resistant apple.

5. GENOME EDITING TECHNOLOGY IN HORTICULTURAL CROP IMPROVEMENT

This technique is based on certain engineered endonucleases that cleave DNA in a sequence-specific manner because of the presence of a sequence-specific DNA-binding domain. These *endonucleases* recognize a specific DNA sequence and thus efficiently and precisely cleave the target genes. The double-strand breaks of DNA result in cellular DNA repair mechanisms, including homology-directed repair and nonhomologous end joining breaks, leading to gene modification at the target sites in the genome of plants. Generally, three types of engineered endonucleases such as zinc-finger nucleases, transcription activator-like effector nucleases,and CRISPR/CaS9 have been used. So far, there has been no report on the use of genome editing technology in horticultural crop improvement.

6. FUTURE PROSPECTS

The applications of recombinant DNA technology or genetic engineering in crop improvement are immense. However, horticultural crops have received less attention in this area so far. In contrast to the increasing global adoption of biotech field crops, biotechnology has had limited commercial success to date in horticultural crops, including fruits, vegetables, flowers, and landscape plants. At this juncture, we cannot ignore the potential of this technology for the genetic enhancement of our horticultural crops to combat various production constraints such as biotic or abiotic stresses and fruit quality improvement. Transgenic technology provides a potential technique for gene analysis in plants. Also there must be a single window regulatory mechanism for commercialization of such GM crops in India so that the real benefits of this technology can be harvested to the maximum. After the advent of next-generation sequencing technologies, many horticultural crops including strawberry, papaya, grapevine, sweet orange, etc. have been sequenced, which has now solved the problem of lack of genomic information and has thus facilitated the target gene/site to be modified using genome editing technology. This has also improved the breeding efficiency because various genes/QTLs coding for various horticulturally important traits have been identified. In addition, transcriptome sequences of a number of horticultural crops are now available in public databases. This vast information will assist in identifying both various genes governing various important traits and the target sites for genome editing and genetic transformation.

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CHAPTER

TRANSGENIC RESEARCH IN SPICES

16

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1. INTRODUCTION

India has been recognized as the "Land of Spices" from prehistoric times and the Western Ghats of India is believed to be the center of origin of many spices, particularly black pepper, cardamom, and other zingiberaceous spices. India grows about 100 spice species among the 112 listed by the International Organization for Standardization, and Indian spices flavor foods in more than 130 countries. Indian spices have obtained geographical indicators such as Malabar pepper, Alleppey Green Cardamom, Coorg Green Cardamom, Naga chilli, etc. because of their intrinsic values in terms of taste, color, and fragrance. Ginger is considered the earliest known oriental spice but the oldest culinary spice is caraway. The most expensive spice in the world is saffron. India possesses many innate advantages over other spice-producing countries—its large genetic base, varied soil, and climatic conditions. The export of Indian spices is remarkable but not spectacular considering its historical importance. Crop loss caused by diseases, lack of resistant varieties, and postharvest losses are the major reasons for low productivity of spices. In perennial spices such as black pepper and cardamom conventional breeding programs are time consuming and cumbersome. No sources of resistance to biotic and abiotic stresses could be located in the cultivated germplasm. In addition, crops such as ginger and turmeric have no or a very rare seed set, making conventional breeding programs ineffective. Under such circumstances, developing high yielding and disease-resistant varieties through biotechnological programs has an important role to play for the improvement of spices and the future of the spice trade. Genetic improvement of plants for resistance to pathogens requires discovering resistance genes and understanding the inherent mechanisms involved. For this purpose, the development of a genetic transformation procedure would help to validate the role of discovered genes.

Transgenic crops represent about 10% of cropland worldwide, and constitute one of the main sources of income for several countries (Alvarez, 2011). Transgenes are introduced into plants to confer novel traits such as improved nutritional qualities, tolerance to pollutants, resistance to pathogens, and for studies of plant metabolism. Now it is possible to transfer genes from plants evolutionary distant from the host plant, as well as from fungi, viruses, bacteria, and even animals. Transgenic plants with special properties have displaced more than half of the varieties generated by standard breeding (Vain, 2007). Moreover, genetic engineering of plants has been utilized in the production of biofuels (Chen et al., 2010). Genetic engineering is not set to replace plant breeding, however; it represents a modern tool for adoption by the plant breeder. Common methods for genetic transformation are usually divided into indirect and direct

Genetic Engineering of Horticultural Crops. http://dx.doi.org/10.1016/B978-0-12-810439-2.00016-7

transformation. The indirect method using *Agrobacterium* is supposed to be advantageous over the direct method because it can introduce larger segments of DNA with minimal rearrangement and with fewer copies of inserted transgenes at higher efficiencies, and at lower cost (He et al., 2010; Hiei et al., 1997; Shewry et al., 2008; Shibata and Liu, 2000).

2. COMPARATIVE GENOMICS AND GENE TAGGING

Comparative genomics is a field in which genomic features such as DNA sequence, genes, gene order, regulatory sequences, and other genomic structural landmarks of different organisms are compared. Identifying the loci of advantageous genes (greater yield, cost-efficiency, quality, and disease resistance) is a key step in breeding. It is a powerful and quick method since it does not require several generations of carefully monitored breeding of parent strains. Johnson et al. (2012) reported the transcriptome of *Piper nigrum* and *Piper colubrinum* in the aspect of host–pathogen interaction in black pepper giving emphasis to Phytophthora foot rot tolerance. The interactive leaf transcriptome of *P. nigrum* and *P. colubrinum* identified a plethora of resistance and defense-related genes that are differentially expressed in response to *Phytophthora capsici* infection. The root transcriptome of black pepper (Gordo et al., 2012) was sequenced by the next-generation sequencing SOLiD platform and an annotated dataset of 10,338 UniGenes will be important for the biotechnological breeding of black pepper. The 4472 predicted proteins showed about 52% homology with the *Arabidopsis* proteome. Two root proteomes identified 615 proteins, which seem to define the plant's root pattern. Hu et al. (2015) described the fruit transcriptome of black pepper along with piperine biosynthetic pathway and identified 40,537 UniGenes genes involved in piperine biosynthesis. The molecular mechanisms underlying foot rot susceptibility were studied by comparing the transcriptome of resistant (*Piper flaviflorum*) and susceptible (*P. nigrum* cv. *Reyin*-1) species. All the genes incorporated in the phenylpropanoid metabolism pathway were upregulated to a relatively high degree in resistant species (Hao et al., 2016).

Transcriptomes of ginger (*Zingiber officinale* Rosc.) and mango ginger (*Curcuma amada* Roxb.) in response to bacterial wilt infection were compared by Prasath et al. (2014). They found 105 genes that were only expressed in *C. amada* (resistant species) in response to infection by *Ralstonia solanacearum*. These genes were directly related to defense against pathogens through salicylic acid (SA)-mediated hypersensitive, systemic acquired, and cell death responses. Among the 54 differentially expressed transcription factors, 32 were upregulated in *C. amada*, which includes WRKY, MYB, leucine zipper protein, zinc finger, and GATA domain transcription factors. Gaur et al. (2016) analyzed the transcriptome of two samples of elite ginger variety Suprabha collected from two different agro-climatic zones of Odisha. Annadurai et al. (2013) reported the presence of novel transcripts related to anticancer and antimalarial terpenoides in the transcriptome of *Curcuma longa*. Comparative transcriptome (rhizome-specific) analysis of *C. longa* and *Curcuma aromatica* contrasting in curcumin content presented an insight into the genetic basis and regulation of biosynthesis of curcumin (Sheeja et al., 2015). Differential expression analysis identified two novel polyketide synthase genes (clpks1 and clpks2) showing similarity to *Musa acuminata* polyketide synthase type 2 (MaPKS2) and *M. acuminata* polyketide synthase type 4 (MaPKS4) that were found to be upregulated in *C. longa*. Sahoo et al. (2016) analyzed transcriptome assembly of the turmeric cultivar Suvarna (CL-Suv-10).

3. CLONING AND ISOLATION OF GENES

Genes responsible for biotic and abiotic stresses and agronomically important characters were identified in major spice crops. Candidate genes responsible for pathogenesis can also be identified from sequence information available in the databases, isolated, then incorporated into promising varieties using transgenic pathways. Wild relatives of the crops within the family or species may be a repertoire of genes for various biotic and abiotic resistance, agronomically important traits, etc. Even though breeding programs involving hybridization to mobilize genes from wild relatives are cumbersome, incorporation of genes through transgenics is an alternative strategy.

3.1 BLACK PEPPER

Foot rot/quick wilt caused by *P. capsici* is a major threat to black pepper cultivation worldwide. One of the *Piper* species, namely, *P. colubrinum*, is found to be highly resistant to all known strains of *P. capsici*. But because of sexual incompatibility, hybridization to mobilize the resistance genes from *P. colubrinum* to cultivated black pepper is difficult. Hence isolation and incorporation of defense/resistance-related genes into the black pepper genome through the transgenic pathway is an alternate way. Differential induction of various pathogenesis-related (PR) proteins such as phenylalanine ammonia lyase and β-1,3-glucanase were identified in black pepper upon inoculation with *P. capsici* (Jebakumar et al., 2001; Nazeem et al., 2008). cDNA fragments encoding the defense-related protein β-1,3-glucanase in black pepper (*P. nigrum* L.) and methylglutaryl CoA reductase were isolated in *P. colubrinum* (Girija et al., 2005a,b).

Differential expression of the chitinase gene was identified in *P. colubrinum* in response to inoculation with *P. capsici* (Sandeep et al., 2009) compared to the uninoculated control plants. An interspecific hybrid between *P. colubrinum* and *P. nigrum* was developed by Vanaja et al. (2008) having partial resistance to the dreaded disease Phytophthora foot rot. Tagging and isolation of genes imparting partial resistance to *P. capsici* from an interspecific hybrid may be a promising alternative in developing foot rot-resistant transgenics in black pepper. Polymerase chain reaction (PCR)-based suppression subtractive hybridization (SSH) was used to identify *P. colubrinum* resistance genes that are differentially expressed in response to the signaling molecule SA (Dicto and Manjusha, 2005). A subtracted library of SA-induced genes were constructed and one of the clones showed sequence homology to osmotin, a PR-5 family protein.

SSH was also used to generate a leaf-specific subtracted cDNA library of *P. nigrum*. A tester population of leaf cDNA was subtracted with a root-derived driver cDNA. Thirty percent of the clones had homology to metallothionein type 2 homologs in the leaf (Alex et al., 2008). The high-level expression of metallothioneins is correlated to exudation of heavy metals in the leaf that were thought to provide first line of defense pathogens.

The two isoforms of *osmotin*, an antifungal *PR-5* gene homolog, were cloned and sequence characterized from a salicylic acid-induced subtracted cDNA library of *P. colubrinum* (Mani and Manjula, 2010). The larger form of the gene is 693 bp long, encoding a 21.5-kDa protein. The smaller form comprises a 543 bp-long coding sequence that codes for a protein of 16.4 kDa. A notable feature of the smaller form was a prominent internal deletion of 150 bp besides certain point mutations. Cloned isoforms of osmotin from resistant species could be candidates for molecular breeding for the improvement of black pepper as well as candidates for the study of a structure-based mechanism of antifungal activity attributed to the PR-5 family.

Isolation and characterization of resistance gene candidates are promising for developing *Phytophthora*-resistant transgenic black pepper. Thirty-three partial resistance gene analogs were identified from *P. nigrum* L. (Suraby et al., 2015) and two resistance gene candidates, which differentially express in *P. nigrum* and *P. colubrinum*, were identified. De Souza et al. (2011) identified differentially expressed genes from black pepper such as transcripts coding for proteins related to oxidative burst and defense response, such as superoxide dismutase, cytochrome p450, and alpha-amylase inhibitors/lipid transfer protein during compatible interactions with *Fusarium solani* f. sp. *piperis*. Bhat et al. (2005) cloned and characterized a cucumber mosaic virus (CMV) coat protein (CP) gene infecting black pepper. The utility of this gene in inducing virus resistance in black pepper is being studied using a transgenic pathway. Gene constructs were prepared in plant transformation vector (pBI 121) and mobilized into *Agrobacterium*: (1) CMV coat protein (CMV-CP) in sense and antisense orientation, and (2) a portion of open reading frame (ORF) III of *piper yellow mottle virus* (PYMoV) in sense and antisense orientation.

High transcript accumulation of genes such as glycoside hydrolase, NPP1, RXLR, and pectate lyase was observed in *P. capsici* during *P. colubrinum–P. capsici* interaction (Vijesh Kumar et al., 2013). Vijesh Kumar et al. (2015a,b) reported the expression analysis of defense-associated transcription factors such as myb, myc, and WRKY during incompatible *P. colubrinum–P. capsici* interactions. The expression of these transcription factors was found to be upregulated during initial phases of *P. capsici* inoculation and this highlights their possible role in signaling events for the activation of defense genes. Johnson et al. (2017) studied the transcriptomic changes during drought stress in tolerant and susceptible cultivars of black pepper and dehydrin, Osmotin and a regulatory protein DREB were found to be differentially expressed. The transcript-level expression of dehydrin was also very high in the drought-induced tolerant variety Acc 4216 (3571-fold) compared to both the drought-susceptible variety Sreekara (108-fold) and the respective control plants.

3.2 GINGER

Isolation and characterization of resistance gene candidates were done using degenerate primers based on conserved motifs from the nucleotide-binding site (NBS) domains of plant resistance (R) genes from cultivated and wild *Zingiber* species (Nair and Thomas, 2006). Priya and Subramanian (2008) reported isolation and molecular analysis of R gene in resistant ginger cultivars against *Fusarium oxysporum* f. sp. *zingiberi*. They observed that the coiled-coil–nucleotide-binding site–leucine-rich repeat (CC–NBS–LRR) class of plant resistance R gene is present only in the resistant cultivars and these cloned R genes provide a new resource for developing *Fusarium* yellows-resistant ginger cultivars. Kavitha and Thomas (2008a,b) employed amplified fragment length polymorphism markers and mRNA differential display to identify genes whose expression was altered in a soft rot-resistant accession of *Zingiber zerumbet* before and after inoculating it with *Pythium aphanidermatum*. A few differentially expressed transcript-derived fragments were isolated, cloned, and sequenced. The clones contain a defense/stress/signaling group that is homologous to genes known to be actively involved in various pathogenesis-related functions in other plant species. They found that *Z. zerumbet* shows adequate variability both at the DNA level and in response to *Pythium*. Nair et al. (2010) identified a member of the PR protein group 5 (PR5) gene family in *Z. zerumbet* that is expressed constitutively but upregulated in response to infection by *P. aphanidermatum*. Isolation of resistance genes from such related species will help to improve ginger via transgenic approaches.

Indian mango ginger, *C. amada* Roxb., exhibited significant resistance to both *R. solanacearum* and *P. aphanidermatum*, proving promising for developing bacterial wilt-resistant ginger (Kumar et al., 2006). Prasath et al. (2011) amplified two putative PR5-like protein genes, CaPR5 and ZoPR5, from *C. amada* and *Z. officinale*, which encode precursor proteins of 227 and 224 amino acid residues. CaPR5 is readily induced by the bacterium in *C. amada*, while ZoPR5 induction was very weak and slow in *Z. officinale*. Promoter analysis indicates the presence of a silencing element binding factor in the ZoPR5 promoter, but not in CaPR5. Prospective promoter elements, such as GT-1 box and TGTCA, implicated as being positive regulatory elements for expression of PR proteins, occur in the 5′-flanking sequences of the CaPR5.

Nair and Thomas (2013) isolated a full-length sequence of the ZzR1 resistance gene from *Z. zerumbet* with potential for imparting resistance to soft rot in ginger. Gene expression studies of ZzR1 transcription in *Z. zerumbet* following pathogen infection demonstrated activation at 3 hpi (hours post inoculation), thus suggesting an involvement of ZzR1 in the *Z. zerumbet* defense mechanism. The ZzR1 gene, showing high homology with other CC–NBS–LRR classes of R genes, represents a valuable genomic resource in designing strategies for engineering resistance in ginger.

Enzymes of the type III polyketide synthase superfamily play an important role in the biosynthesis of gingerols. Radhakrishnan et al. (2009) isolated and characterized a novel form of type III polyketide synthase from *Z. officinale* Rosc. Huang et al. (2007) reported molecular cloning and characterization of violaxanthin deepoxidase in ginger. A full-length cDNA encoding violaxanthin deepoxidase (GVDE) was cloned from ginger and the expression patterns of GVDE in response to light were characterized. GVDE was mainly expressed in leaves.

3.3 TURMERIC

Joshi et al. (2010) reported isolation and characterization of NBS–LRR resistance gene candidates in *C. longa* cv. surama. R-gene conferring resistance to *P. aphanidermatum* was characterized in *Curcuma zedoaria* by Basudeba et al. (2013). Transforming *C. longa* with potential R genes is one of the solutions to obtain disease-resistant cultivars in "golden spice." Annadurai et al. (2013) reports the presence of novel transcripts related to anticancer and antimalarial terpenoides in the transcriptome of *C. longa*.

Katsuyama et al. (2009a) isolated and characterized type III polyketide synthases CURS2 and CURS3 from turmeric. Reshmi and Soniya (2012) isolated and characterized two new type III polyketide synthases, ClPKS9 and ClPKS10, and gene expression studies revealed that they have distinct tissue expression patterns. The studies inferred that polyketide synthase exists as a multigene family in turmeric. Koo et al. (2013), identified genes associated with regulation of tissue growth, development, and transcription. Their studies revealed that both primary and specialized metabolism genes of ginger and turmeric rhizomes are primarily devoted to the utilization of leaf-supplied sucrose for the production and/or storage of specialized metabolites associated with the phenylpropanoid pathway and putative type III polyketide synthase gene products. Mallika et al. (2016) reported the type III polyketide synthase repertoire encoded in the Zingiberaceae family.

3.4 VANILLA

Large gene sequence datasets from vanilla pods at different times of development, and representing different tissue types, including the seeds, hairs, placental, and mesocarp tissues, were generated using

next-generation sequencing technologies for interrogation of pathways of vanillin and C-lignin biosynthesis in the pod and seed, respectively. The combined 454/Illumina RNA-seq platforms provide both deep sequence coverage and high-quality de novo transcriptome assembly for this nonmodel crop species. The annotated sequence data provided a foundation for understanding multiple aspects of the biochemistry and development of the vanilla bean, as exemplified by the identification of candidate genes involved in lignin biosynthesis. Transcriptome data indicated that C-lignin formation in the seed coat involves coordinate expression of monolignol biosynthetic genes with the exception of those encoding the caffeoyl coenzyme A 3-*O*-methyltransferase for conversion of caffeoyl to feruloyl moieties. This database provides a general resource for further studies on this important flavor species (Rao et al., 2014).

Gallage et al. (2014) reported that a single hydratase/lyase-type enzyme designated vanillin synthase (*Vp*VAN) catalyzes direct conversion of ferulic acid and its glucoside into vanillin and its glucoside, respectively. *Vp*VAN localizes to the inner part of the vanilla pod and high transcript levels are found in single cells located a few cell layers from the inner epidermis. Transient expression of *VpVAN* in tobacco and stable expression in barley in combination with the action of endogenous alcohol dehydrogenases and UDP-glycosyltransferases result in vanillyl alcohol glucoside formation from endogenous ferulic acid. A gene encoding an enzyme showing 71% sequence identity to *Vp*VAN was identified in another vanillin-producing plant species *Glechoma hederacea* and was also shown to be a vanillin synthase as demonstrated by transient expression in tobacco.

4. GENETIC TRANSFORMATION

4.1 BLACK PEPPER

Black pepper, the "king of spices," is one of the major export-earning crops. Preliminary attempts of transformation in black pepper were carried out by Sasikumar and Veluthambi (1994, 1996) and primary transformants were obtained for kanamycin resistance in the cotyledons using *Agrobacterium tumefaciens* binary vector strains LBA 4404 and EHA 105. The optimum concentration of kanamycin (50 µg/mL) to completely inhibit callus formation and growth was also standardized but could not attain regeneration of the transformed tissue. Sim et al. (1998) reported genetic transformation of leaf, petiole, and stem explants from axenic seedlings of black pepper with *A. tumefaciens* strain LBA 4404 containing vectors carrying *npt* 11 and *gus* genes. They reported embryo-type protuberances from the basal end of the petiole and multiple shoot induction from stem explants. The transformed status of explants was confirmed through β-glucuronidase (GUS) expression by X-Glu staining but not for regenerated shoots. Reports are available on optimization of an *Agrobacterium*-mediated genetic transformation system in black pepper and related *Piper* species aimed at development of disease-resistant varieties against the most dreaded disease foot rot caused by *P. capsici*. Babu et al. (2005) successfully transformed black pepper leaf tissues with the osmotin gene, a PR protein known for inducing *Phytophthora* resistance. *Agrobacterium*-mediated transformation was attempted using an osmotin gene construct in pGV2260. Among the 70 putative transgenics regenerated, five putative transgenics showed delayed response to infection and decreased spread of foot rot caused by *P. capsici*. Asha and Rajendran (2009) reported *in planta* transformation in black pepper variety Panniyur 2 via pollen tube pathway using the total exogenous DNA of *P. colubrinum*, a wild relative species of *Piper* resistant to *P. capsici*. The resulting putative transformant seeds were germinated in vitro by embryo rescue and the

germinated plantlets were screened in vitro by incorporating the toxic culture filtrate of the pathogen *P. capsici* in the rooting media. The surviving putative transformant plantlets were later screened artificially for disease tolerance under ex vitro conditions: 39.21% of the putative transformants survived the screening and random amplification of polymorphic DNA analysis of these plantlets showed variation in banding pattern compared to the DNA recipient parent *P. nigrum* var. Panniyur-2.

Genetic transformation in black pepper showed retarded regeneration potential from mature tissues because of associated problems such as high phenolic exudation and the presence of endophytic fungi. Embryogenic callus derived from zygotic embryos of black pepper were induced to form somatic embryos on solid and liquid Schenk and Hildebrandt basal medium by Joseph et al. (1996). A very efficient micropropagation strategy through somatic embryogenesis developed by Nair and Gupta (2003) is promising for rapid regeneration of transformed tissues, which can ease genetic manipulations of black pepper. Later in 2006, Nair and Gupta developed a high-frequency plantlet regeneration protocol for black pepper through cyclic secondary somatic embryogenesis. Embryogenic mass derived from primary somatic embryos that were obtained from the micropylar region of mature germinating seeds of black pepper was used by Varghese and Bhat (2011) for efficient *Agrobacterium*-mediated transformation of black pepper plants. Embryogenic mass was cocultivated with *Agrobacterium* strain EHA 105 harboring a binary vector whose transfer DNA (T-DNA) was comprised of the npt 11 gene under the control of the nopaline synthase (NOS) promoter and the GUS reporter gene with an intron–GUS fused to the CaMV35S promoter. Cocultivation was carried out on plant growth regulator-free Schenk and Hildebrandt medium and transformants were selected in medium containing cefotaxime and stepwise increase in kanamycin concentration. Cefotaxime concentration of 100 μg/mL was found to be optimum for *Agrobacterium* control and promotion of somatic embryo proliferation. They also reported successful plantlet regeneration.

The utility of the CMV-CP gene (Bhat et al., 2005) in inducing virus resistance in black pepper was studied using transgenic pathways. Gene constructs were prepared in plant transformation vector (pBI 121) and mobilized into *Agrobacterium*: (1) CMV-CP in sense and antisense orientation, and (2) a portion of ORF III of PYMoV in sense and antisense orientation. Putative transgenics developed in black pepper (IISR Subhakara and Panniyur 1) are under evaluation (IISR, 2011–2012).

Mani and Manjula (2011) attempted the vacuum infiltration method to transform oomycete-resistant wild *Piper* sp. *P. colubrinum* as a rapid transient method for expression of the GUS reporter gene and introduced a hairpin vector for endogenous gene silencing. *Agrobacterium* (EHA 105) harboring a GUS binary vector was vacuum infiltrated into young detached in vitro leaf explants, which showed detectable GUS gene activity within 4 days of infiltration. They also reported the application of transient gene silencing in *P. colubrinum* by the delivery of in vitro a synthesized hairpin vector construct (pHELLSGATE) containing an endogenous serine threonine protein kinase gene homolog into in vitro shoots, which resulted in significant reduction in transcript accumulation of the endogenous gene. Subsequently, Maju and Sonia (2012) established a genetic transformation system for *P. nigrum* L. var. Panniyur-1 plants by infecting seedling-derived explants with *A. tumefaciens* strain EHA105 carrying binary plasmid pCAMBIA 1301, which contains scorable marker β-1,3-glucuronidase and the selectable marker hygromycin phosphotransferase (hpt) gene under the control of a CaMV35S promoter. Sasi et al. (2015) developed loop-mediated isothermal amplification (LAMP) and real-time LAMP-based assays for quick and sensitive detection of transgenic black pepper plants. The sensitivity of LAMP was up to 104 times that of conventional PCR, while real-time LAMP was up to 103 times that of LAMP and 107 times that of PCR. The assays were validated by testing putative transformants of black pepper.

4.2 CARDAMOM

Cardamom (Zingiberaceae), the "queen of spices," is also native to India and the productivity of cardamom is hampered by various diseases of viral etiology. An early attempt on transformation of cardamom was conducted using a biolistics process to study the optimum conditions for gene delivery and the efficiency of the plasmid vector pAHC 25 and promoter Ubi-1 (maize ubiquitin) for transformation and gene expression in cardamom embryogenic callus. Transient expression of the GUS gene was noticed in the bombarded callus tissue (Babu et al., 1998). To develop pathogen-derived resistance in cardamom, Backiyarani et al. (2005) reported cloning of the CP gene of Kursuppara isolate, a highly variable isolate of cardamom mosaic virus infecting cardamom. The PCR-amplified CP and CP + 3′ UTR region were separately cloned in pXcmkn 12 vector and subcloned into a plant expression vector (pAHC17) at the Bam H1 site, under the ubiquitin promoter. Developing virus-resistant lines using CP genes through the transgenic pathway may help in mitigating viral problems.

4.3 GINGER

Ginger (Zingiberaceae) is the third most important spice in South Asia. Since ginger is vegetatively propagated, genetic variability is very much limited, which is a hurdle in crop improvement programs when developing varieties resistant to rhizome rot caused by *P. aphanidermatum* and bacterial wilt caused by *R. solanacearum*. Transient expression of GUS was successfully induced in ginger embryogenic callus bombarded with plasmid vector pAHC 25 and promoter Ubi-1 (maize ubiquitin) callus tissue (Babu, 1997; Babu et al., 1998). Helium bombarded ginger embryogenic calli with microprojectiles (1.6 µm gold particles) using a "BioRad" PDS-1000/He gene gun at 900 and 1100 psi helium pressure with the target distance of either 6 or 9 cm. The pAHC 25 vector used contained GUS and BAR (phosphinothricin acetyltransferase) as reporter and selectable marker genes, respectively. The best GUS score was obtained when the target distance was 9 cm with 900 psi helium pressure. The GUS score of 133 blue spots per cm^2 indicated not only the optimization and efficiency of the biolistics process, but also the ability of the ubiquitin promoter to drive the expression of the reporter gene. Suma et al. (2008) reported the genetic transformation of ginger buds through somatic embryogenesis. They found that young buds had very good embryogenic potential and were superior to other explants. The transformation protocol included 3-day preculture of explants on callus induction medium, bacterial (*A. tumefaciens* strain EHA105/p35SG) dilution of 1:20 (v/v) as the initial inoculum, an infection time of 5 min, 2-day cocultivation with *Agrobacterium*, and postcultivation on callus induction medium with 100 mg/L kanamycin and 300 mg/L cefotaxime under darkness for 2 weeks, followed by a 16/8-h photoperiod regime. Acetosyringone was effective at a concentration of 200 µm for vir induction. With young bud as explant, a transformation frequency of 1.1%–2.2% was noticed. The callus growth was very slow in the presence of antibiotics.

4.4 TURMERIC

Turmeric is conventionally propagated vegetatively through mother or finger rhizomes with one or two buds. It is reported to set seeds rarely in some locations and scarcity of seed set hampers recombination breeding. A good deal of morphological variability is observed in many cultivated types of turmeric, which is mostly because of vegetative mutations or environmental effects. In such circumstances biotechnological tools gain relevance in solving many crop-specific problems and for crop improvement.

An efficient protocol for genetic transformation for turmeric through particle bombardment was reported by Shirgurkar et al. (2006). Callus cultures initiated from shoots were bombarded with gold particles coated with plasmid pAHC25 containing the bar and *gusA* genes each driven by the maize ubiquitin promoter. Transformants were selected on medium containing glufosinate and transgenic lines were established on selection medium from 50% of the bombarded calluses. Transgenic shoots regenerated from these were multiplied and stably transformed plantlets were produced. PCR and histochemical GUS assay confirmed the stable transformation. A protocol for regeneration and genetic transformation was established in *Curcuma alismatifolia* using retarded shoots as explants, and employing *A. tumefaciens* strain AGLO harboring binary vector pBI121 or pBI121-CaACSI. Transformation events were confirmed by PCR, GUS assay, and Southern blotting of regenerated plants (Mahadtanapuk et al., 2006).

Mahadtanapuk et al. (2009) isolated a cDNA fragment encoding 1-aminocyclopropane-1-carboxylate (ACC) synthase from *C. alismatifolia* Gagnep and subcloned into the pGEM-T easy vector to form pCa-ACSI to suppress the expression of the *ACC synthase* gene in the Siam tulip *C. alismatifolia* Gagnep. Northern blot analysis showed that the expression of the gene was in the bracts of curcuma, the highest expression was observed 2 days after cutting the flowers. *Ca-ACS*I was subcloned in pBI121 resulting in pBI121-*Ca-ACS*I and transformed into leaf tissues of *Torenia foumieri* and retarded shoots of *C. alismatifolia* Gagnep via *A. tumefaciens* strain AGLO. Putative transformants, with the gene in an antisense orientation, were investigated by PCR analysis, GUS assay, and Southern blotting. The transgenic plantlets were transferred to pots containing soil for cultivation in a growth chamber.

A stable transformation system with *Agrobacterium* strain EHA105 with plasmid pBISN1 transient expression system was reported by He and Gang (2014). A modified B5 medium was standardized for callus induction from turmeric inflorescence. The stable expression of transgene by transgenic plants and their clonal progeny was confirmed by GUS assay, PCR, and Southern blot analysis. A transient gene expression system involving infiltration of young turmeric leaves with *Agrobacterium* and in vitro regeneration of plantlets was also developed. This system established that an MADS-box green fluorescent protein (GFP) fusion protein was localized to the nucleus of turmeric cells. The first report of a complete in vitro regeneration system from somatic embryos of *C. longa* and *Curcuma manga*, and *Agrobacterium*-mediated transformation of these two species with Diketide CoA synthase (DCS) and curcumin synthase (CURS) genes that are involved in curcuminoid biosynthesis, was published by Pikulthong et al. (2016). The presence of both target and marker genes, *hpt*, in the transformed somatic embryos was confirmed by PCR assay.

4.5 CHILLI

4.5.1 *Capsicum annuum* L.

Capsicum spp. consumed both as vegetable and spice is of high economic importance because of distinctive sensory attributes of aroma, pigments, and taste. Cultivated peppers are affected by biotic and abiotic stresses, the main ones being viral, bacterial, and fungal diseases. Fruit color, pungency, and male sterility are interesting genetic characters of capsicum with high economic importance. The first report on genetic transformation of chilli in vitro seedling explants (hypocotyls, cotyledons, and leaves) cocultured with the wild tumorigenic strains of *A. tumefaciens* (A281 and C58) and with a disarmed strain bearing the plasmid pGV 3850 was published in 1990 (Liu et al., 1990). Only cotyledon and leaf tissues formed callus, leaf-like structures, and occasional shoot buds in the presence

of 200 mg/L kanamycin. No elongation or plan regeneration occurred in the kanamycin-resistant shoot buds obtained. Transformation in leaf-like structures and shoot buds were confirmed by GUS assay. There are various reports available on plant regeneration and transformation studies in *C. annuum* (Liu et al., 1990; Dong et al., 1992; Engler et al., 1993; Christopher and Rajam, 1997; Lee et al., 1993; Ye et al., 1993; Zhu et al., 1996; Kim et al., 1997; Ramírez-Malagón, 1997; Harpster et al., 2002; Kim et al., 2002a,b; Shin et al., 2002; Dabauza and Pena, 2003; Li et al., 2003). Most transformation studies in chilli pepper refer to the use of marker (npt II) or reporter (gus) genes to establish adequate protocols; but some genes have also been employed to generate transgenic plants with tolerance to CMV (Dong et al., 1992; Lee et al., 1993; Zhu et al., 1996; Kim et al., 1997; Chen et al., 2003; Lee et al., 2009) or tolerance to multiple pathogenic organisms (Shin et al., 2002).

Dong et al. (1995) generated two transgenic pepper lines by transforming cotyledonary petioles of pepper cv 89-1 with *Agrobacterium*-containing plasmid with an unspecified CMV satellite RNA gene under the control of CaMV35S promoter. The transgenic lines showed delayed symptoms (from approximately 13 to 24 days) upon virus inoculation. Prior to that, Lee et al. (1993) used a cDNA transgene of CMVI17N satellite RNA under the control of 35S promoter for transforming cotyledonary explants of hot pepper var. Golden Tower, which resulted in four independent transformants. Zhu et al. (1996) reported regeneration of fertile transgenic sweet pepper (*C. annuum* var. *grossum*) plants at a relatively high rate from various explants that were cocultivated with *A. tumefaciens* strain GV3111-SE harboring a plasmid that contains the CMV-CP gene. Use of MADS box genes such as Os MADS1 (MADS box gene isolated from rice) for transformation of chilli pepper by Kim et al. (2001) have shown promise in modifying plant growth habit. Harpster et al. (2002) studied the function of endo-1,4-β-glucanase (AGase) CaCell (ripening-related gene) in fruit softening by suppression of CaCell gene expression in transgenic chilli pepper plants (*C. annuum* L., cv. VS300-1). The protocol described by Engler et al. (1993) was followed. The suppressed lines showed reduced immunodetectable CaCell protein and hydrolase activity on carboxymethylcellulose to at or below the limit detected in ripe mature red fruit.

Kim et al. (2002a,b) introduced a new and refined selection medium for pepper regeneration using mannose. Shin et al. (2002) overexpressed the tobacco stress-induced 1 (Tsi1) gene in transgenic hot pepper plants with a 35S::Tsi1::nos construct and 15 primary transformants were generated. Pathogen challenge of plants of the T_1 generation showed that reduced multiplication of tobacco mosaic virus (TMV) and CMV virus reduced incidence and severity of infection by the pathogen *P. capsici* (late blight) and slightly reduced accumulation of the bacterial pathogen *Xanthomonas compestris* (bacterial spot disease). There is only a single report of transgenic hot pepper plants developed for insect resistance from Korea against the major insect pest oriental tobacco bud worm, which is prevalent there (Kim et al., 2002a,b). Shin et al. (2002) reported the use of *A. tumefaciens* with the binary vector pMPP2 used to transform *C. annuum* cv. Nockwang cotyledon and hypocotyl explants to produce transgenic chilli pepper plants with enhanced resistance to viral (pepper mild mottle virus and CMV), bacterial (*Xanthomonas campestris* pv. *vesicatoria*), and oomycete (*P. capsici*) pathogens by ectopic expression of the Tsi1 gene. The product of the Tsi1 gene seems to be involved in regulating stress responsive genes and PR genes. Cai et al. (2003) also generated transgenic chilli pepper plants with combined CP genes from both TMV and CMV by transforming the hypocotyl explants. Overexpression of transcription factor genes have been used to transform pepper plants as a means for imparting broad-spectrum resistance. Dabauza and Pena (2003) studied the suitability of various *A. tumefaciens* strains, namely, A281, Ach5, C58, 42 CNBP, and 1102, as a means of

selecting appropriate vectors for genetic transformation of sweet pepper genotypes. C58 and 1102 strains showed significantly greater virulence and also induced more tumors per wound than Ach5, 42 CNBP, and A281. A highly efficient genetic transformation system using pepper cotyledons as explants was established by Li et al. (2003).

Mihálka et al. (2003) developed a binary transformation protocol based on the infection of explants with "shooter" mutants of *A. tumefaciens*, which allowed universal gene transfer and marker gene elimination. The procedure also involved a positive selection system utilizing the expression of the isopentenyl transferase (ipt) gene from the T-DNA of *A. tumefaciens* mutants capable of inducing phenotypically normal shoots in leaf explants. Binary vectors were introduced into the shoot-inducing strains and used for genetic transformation of sweet pepper (*C. annuum* L. cv. Fehérözön), tomato, tobacco, and muskmelon. The normal regenerated shoots lacked a stably integrated ipt gene and could be rooted with the same efficiency as nontransformed shoots. Lee et al. (2004) transformed *C. annuum* inbred lines (P915, P409) with two genes, TMV-CP and PPI1 (pepper–PMMV interaction 1 transcription factor), by means of *Agrobacterium* coculture. They also developed a new protocol for the selection and transformation of pepper, callus-mediated shoot formation. Even though the transformation rate was low, transformation via callus-mediated shoot formation proved to be reproducible and was confirmed by Southern and Northern blot analyses. Adventitious bud differentiation, structural changes in hypocotyl explants after coculture with *A. tumefaciens*, and the explants' responses to a selective agent were studied by Delis et al. (2005). Selective medium containing kanamycin and coculture with *A. tumefaciens* strongly affected the organization of the meristematic tissue and brought about necrosis and isolation of the adventitious buds from the vascular bundles. Sobhakumari and Lalithakumari (2005) reported high-frequency shoot regeneration and *Agrobacterium*-mediated DNA transfer in shoot tips of *C. annuum* cv. K1, K2, and PLR 1. Jeon et al. (2007) described an efficient transformation method based on polyethylene glycol treatment for transient expression studies using GUS expression in pepper protoplasts with a long-term goal of developing a gene-tagging system and a method for transposon mutagenesis in pepper.

Kumar et al. (2009) attempted tissue culture-independent *Agrobacterium*-mediated *in planta* transformation in two varieties of *C. annuum*. Transgenic chilli pepper plants highly tolerant to a new CMV pathotype (CMVP1) were obtained by Lee et al. (2009). Capsinoid biosynthesis in transgenic *C. annuum* plants upon *Agrobacterium* transformation using different gene silencing techniques has been reported by Kisaka et al. (2011). Kumar et al. (2012) reported an effective and reproducible auxin-free regeneration method for different red pepper cultivars and efficient *Agrobacterium*-mediated transformation of chilli cultivar Pusa Jwala with βC1 ORF of satellite DNA β molecule associated with chilli leaf curl Joydebpur virus. Transgene integration was confirmed by PCR and Southern hybridization analysis. Verma et al. (2013) standardized an efficient system for *Agrobacterium*-mediated genetic transformation of *C. annuum* L. cv. California Wonder. Cotyledon and hypocotyl explants were cocultured with *A. tumefaciens* LBA 4404 strain harboring a binary vector pBI121, carrying npt-II and gus genes. Transformed status of regenerated plantlets were confirmed by PCR and GUS assay. A virus-induced gene silencing approach has been used to study the functional role of resistance gene (CaRGA2) expression against *P. capsici* in *C. annuum*. The suppression of the CaRGA2 gene renders the pepper plant unable to transduce a signal downstream of the broad-spectrum resistance response, thereby allowing enhanced susceptibility to pepper pathogens (Zhang et al., 2013). Maligeppagol et al. (2016) reported genetic transformation of chilli local cultivar G4 with a transcription factor dreb1A driven by a desiccation-inducible promoter rd29A, known to impart desiccation tolerance, using binary vector

pCAMBIA 2301. The acclimatized transformants showed improved tolerance to drought by lower wilting compared to the control plants. A few reports are available on transformation of chilli pepper by *Agrobacterium rhizogenes*. Jayashankar et al. (1997) employed a binary vector system carrying the wild-type Ri plasmid that confers the hairy root phenotype, and a binary vector harboring the reporter and selectable marker genes. Stably transformed hairy roots were recovered, but could not regenerate transgenic shoots from the transformed roots.

4.5.2 Capsicum frutescens

Transformation protocols have been developed in *C. frutescens* (Wang et al., 1991; Chen et al., 2003; Hasnat et al., 2008; Sharma et al., 2008). A reproducible and efficient transformation protocol for two chilli (*C. frutescens* L.) varieties Nepali and NARC-IV was developed by Hasnat et al. (2008) using various bacterial dilutions to assess its effect on transformation efficiency. The hypocotyl segments were inoculated with *A. tumefaciens* strain EHA 101 harboring the binary vector pTCL5. Plant response, proliferation, and differentiation of calli-forming plantlet and percentage of transformation efficiency were strongly dependent on the bacterial dilutions. Low bacterial density resulted in a drastic increase in plant production from calli along with transformation efficiency, whereas high density brought about the necrosis and death of calli.

Genetic transformation has been reported in other *Capsicum* spp. namely, *Capsicum chinense* (Solís-Ramos et al., 2009) and *Capsicum baccatum* (Subhash and Christopher, 1997). Solís-Ramos et al. (2009) reported genetic transformation of Habanero chilli pepper (*C. chinense*) with the gene WUSCHEL from *Arabidopsis thaliana* as a means of overcoming the somatic embryogenesis formation recalcitrance of this species. After 15 days of induction in the presence of estradiol, the stem segments derived from transformed plants started producing globular structures indicating that heterologous gene WUSCHEL was active and involved in the somatic embryogenesis process. Two different methods of transformation using *Agrobacterium* and a particle gun were adopted by Nianiou et al. (2002) for transgenic plant production in chilli pepper. Since the percentage of transformed plants obtained using *Agrobacterium* was low a biolistics method was employed as an alternative. Pepper hypocotyl explants were bombarded by a hand gene gun. The plasmid employed in the transformation harbored the gus reporter gene under the control of CaMV35S promoter. The particle gun method of gene transfer proved to be more efficient than *Agrobacterium*-mediated transformation.

4.6 GARLIC

Garlic is an important and widely cultivated monocotyledonous crop used both as a condiment and in medicine. Garlic does not produce seed readily because of the absence of fertile flowers and is propagated vegetatively with a low multiplication rate. Though onion is a host for *Agrobacterium*, the *Allium* spp. and the plants of the same family are recalcitrant to transformation (Eady et al., 1996). Though a transient expression upon particle bombardment was reported earlier by Barandiaran et al. (1998), the first report on transgenic garlic was presented by A1 Kondo et al. (2000). They developed a stable *Agrobacterium*-mediated transformation system that employed *A. tumefaciens* strain EHA101 harboring the plasmid pIG121Hm, carrying a kanamycin-resistant gene under the control of *nos* promoter, a hygromycin-resistant gene, and an uid A gene with an intron of a catalase gene from the castor bean both under the CaMV promoter. Integration of the uid A gene was confirmed by Southern blot analysis. Sawahel (2002) reported stable genetic

transformation of an immature clove-derived callus of garlic that was previously treated with aurintricarboxylic acid (an endogenous nuclease inhibitor) with plasmid DNA (pBI221.23), carrying the selectable "hpt" gene for hygromycin resistance and the reporter "*gus*" gene. Robledo-Paz et al. (2004) reported stable transformation of embyrogenic calli derived from the garlic cultivar GT96-1 upon microprojectile bombardment with plasmid pWRG1515 containing *hph* and *gus A* genes. Southern blot analysis and GUS assay confirmed the transgenic nature of the regenerated plants. The first report on the introduction of fungal (*Sclerotium cepivorum*) resistance genes in garlic was published by Lagunes-Fortiz et al. (2013). Garlic embryogenic calli were transformed via *A. tumefaciens* strain LBA4404 harboring pC2301CHGLU plasmid carrying *TaCh*, *glu*, *gus*, and *nptII* genes (coding for chitinase, glucanase, GUS and neomycin phosphotransferase, respectively). Transformed plants were not completely resistant but tolerated infection.

4.7 VANILLA

Vanilla is one of the most widely used flavoring agents in sweetened foods worldwide. It finds its application in the perfumery and cosmetic industries as well. Genetic transformation in *Vanilla planifolia* using thin shoot-tip sections under the influence of external incorporation of putrescine was reported by Malabadi and Nataraja (2007) using an expression vector containing nptII and GUS genes driven by CaMV35S promoter. An efficient transformation protocol using protocorm-like bodies (PLBs) derived from shoot tips was developed for *V. planifolia* by Retheesh and Bhat (2011). PLBs were cocultured with *A. tumefaciens* strain EHA105 harboring the binary vector pBI121 containing the GUS (*gusA*) and neomycin phosphotransferase II (*npt II*) genes. Two new caffeoyl CoA *O*-methyltransferase-like genes were identified by screening a cDNA library from specialized hair cells of the pods of the orchid *V. planifolia* (Widiez et al., 2011). Quantitative analysis of gene expression indicated a dramatic tissue-specific expression pattern for *Vp-OMT4*, which was highly expressed in the hair cells of the developing pod, the likely location of vanillin biosynthesis. Although Vp-OMT4 had a lower activity with the proposed vanillin precursor 3,4-dihydroxybenzaldehyde than with tricetin, the tissue specificity of expression suggests it may be a candidate for an enzyme involved in vanillin biosynthesis. In contrast, the *Vp-OMT5* gene was mainly expressed in leaf tissue and only marginally expressed in pod hair cells. Transient expression of a GFP fusion in tobacco demonstrated that Vp-OMT5 was localized in the plastids.

4.8 SEED AND HERBAL SPICES

4.8.1 Mustard

Genetic transformation has been applied to the improvement of *Brassica juncea* in the area of phytoremediation, herbicide resistance, salt tolerance, hybrid seed production, oil quality, and aphid resistance. Fertile transgenic plants of *Brassica* spp. carrying genes of interest have been produced by different methods such as *Agrobacterium*, electroporation of protoplast, and biolistic transformation. This biotechnological approach has already been applied to the improvement of *B. juncea* in the area of phytoremediation (Zhu et al., 1999), herbicide resistance (Mehra et al., 2000), hybrid seed production (Jagannath et al., 2002), oil quality (Das et al., 2006), and aphid resistance (Kanrar et al., 2002; Dutta et al., 2005). Hypocotyl segments from axenically grown seedlings were transformed with the γ-ECS gene construct, which contains the *Escherichia coli gshI* gene fused to a pea chloroplast transit sequence

and driven by the CaMV35S promoter with a double-enhancer sequence (P70). The construct also contains the *nptII* gene, which confers kanamycin resistance. The γ-ECS transgenic seedlings showed increased tolerance to Cd and had higher concentrations of phytochelatins, γ-GluCys, glutathione, and total nonprotein thiols compared with wild-type seedlings (Zhu et al., 1999). Dutta et al. (2008) reported transformation of leaf piece explants of five mustard cultivars with *A. tumefaciens* strain EHA105 harboring the plasmid pCAMBIA1301, carrying the GUS (uidA) and hygromycin phosphotransferase (hpt) genes driven by CaMV35S promoter. The efficiency of stable transformation was found to be 19% in the T_0 generation, with the transgenic plants and their progeny showing constitutive GUS expression in different plant organs. Genetic transformation of precultured cotyledonary petioles of mustard with a lectin gene from chickpea was achieved by cocultivating with *A. tumefaciens* (gv3101). Confirmation of integration of transgenes in regenerated plants was done by PCR (Singh et al., 2009).

4.8.2 Cumin

Cumin (*Cuminum cyminum* L.) is an annual, herbaceous, spice glycophyte that has various applications as a food and flavoring additive and therapeutic agent. India contributes 70% of the total world production of cumin and abiotic stress such as salinity is the major constraint in cumin production. Low genetic diversity attributes limited scope to improve traits in cumin via conventional breeding. Microprojectile bombardment-mediated genetic transformation has been established using precultured cumin embryos by Singh et al. (2010) and 91% of the embryos showed transient GUS expression after 24 h. Shoot tips and roots of T_0 plantlets also showed GUS expression after 3 months of bombardment exhibiting the possibility of stable transformation in cumin. An efficient method for *Agrobacterium*-mediated genetic transformation and plant regeneration using embryos as explants without callus induction was reported by Pandey et al. (2013) with a transformation efficiency of 1.5% at the hardening stage. Southern blot analysis of hardened plants confirmed single copy gene integration. An efficient *Agrobacterium*-mediated, tissue culture-independent *in planta* genetic transformation method was established in cumin seeds by Pandey et al. (2016). Transgenic cumin plants were obtained by transformation with the *SbNHX1* gene, which encodes a vacuolar Na^+/H^+ antiporter and is involved in the compartmentalization of excess Na^+ ions into the vacuole and maintenance of ion homeostasis, cloned from *Salicornia brachiate*, which is an extreme halophyte. Transgenic lines that overexpressed the *SbNHX1* gene showed higher photosynthetic pigments and lower electrolytic leakage, lipid peroxidation, and proline content as compared to wild-type plants under salinity stress.

4.8.3 Coriander

The herbaceous plant coriander (*Coriandrum sativum* L.) serves culinary and medicinal purposes. Its leaves (cilantro) as well as fruits (coriander) serve as flavoring agents in various dishes. The seeds are also valued for their fatty acid content, in particular, petroselinic acid. Wang and Kumar (2004) developed transgenic coriander plants in an attempt to investigate the role of mutated ethylene receptor ERS1 from *A. thaliana* in tissue senescence of heterologous plants. Transgenic coriander was regenerated by cocultivating hypocotyl segments with *A. tumefaciens* harboring binary vector pCGN1547 that carried the ERS1 gene. The *Arabidopsis* ERS1 mutant effectively conferred ethylene-insensitive phenotype to coriander plants with a transformation efficiency of 6.6%.

4.8.4 Fenugreek

The fenugreek plant is cultivated throughout the world for use in medicines, food, condiments, dyes, and forage. Hairy root cultures of *Trigonella foenum* have been widely used in the production of secondary metabolites. Diosgenin production was established in *T. foenum* by hairy root induction by *A. rhizogenes* strain A4 (Merkli et al., 1997). The highest diosgenin production was observed in half-strength woody plant medium (0.040% dry weight), which represents almost twice the amount detected in nontransformed roots (0.024% dry weight). Stolon and its postulated precursors were detected in hairy root cultures of *T. foenum* (Paraza-Luna et al., 2001). In vitro crown galls were induced in fenugreek by *A. tumefaciens* strain A281 using root, cotyledon, and hypocotyl explants, and the presence of the uidA (gus) gene was confirmed by PCR analysis (Khawar et al., 2004). The production of trigonellin by hairy root cultures of *T. foenum* was described in two Iranian masses—Zanjan and Borazjan (Raheleh et al., 2011). Three different *A. rhizogenes* strains (A4, 9126, and 15834) were tested to investigate the ability for transformation and production of trigonellin by cocultivation injection methods. PCR analysis using the *rolb* gene was used for identification of transformed hairy roots. All the strains of *A. rhizogenes* could produce hairy roots.

5. REGULATORY ISSUES

The basic legal framework governing genetically modified organisms (GMOs) (both GM crops and GM food products) in India is the Environment Protection Act 1986. The Recombinant DNA Advisory Committee (RDAC) and the Review Committee on Genetic Manipulation (RCGM) are the two committees under the Ministry of Science and Technology, government of India. The RDAC is responsible for making recommendations on rules and procedures for ensuring biosafety in research and applications of GMOs. The RCGM is responsible for granting approvals for and monitoring safety aspects of research projects involving GMOs. It can also give approval for controlled field experiments. No food products derived from GM spices are currently available in the market. Under current Indian law, GM crops including spices, before commercialization, require legal approval from the Genetic Engineering Approval Committee (GEAC), the highest body for GM regulation in India, under the Ministry of Environment and Forests. Efforts to regulate biosafety measures are vigorously made in India. As directed by the government of India, the Department of Biotechnology has been entrusted with the responsibility of setting up the National Biotechnology Regulatory Authority (NBRA). This will require the promulgation of new legislation, namely, the "National Biotechnology Regulatory Act" or NBR Act, which is now under the consideration of the Indian Parliament. GM crops must go through a risk assessment procedure where they are evaluated in laboratory tests and field trials and must undergo safety analysis. Depending on the nature and characteristics of the GM crop being evaluated for approval, the RCGM and GEAC often design protocols on an ad hoc basis specifying the parameters for testing. The practical experience of the regulators is that while the guidelines lay down principles for risk assessment, the protocols and tests according to which risk assessment has to be done evolve on a case-by-case approach. The necessary tests include molecular characterization, compositional assessment, and 90-day rat toxicity assays. Agronomic, phenotypic, environmental, and allergenicity testing may also be required.

Various assessment systems have been developed in terms of food safety only in chilli peppers. Chen et al. (2003) proved that the fruits from the CMV-resistant GM sweet pepper *C. frutescens* cv. Zhongjiao plants are comparable to those from nontransgenic plants. The CP gene of CMV derived from a Chinese CMV under the control of CaMV promoter and NOS terminator was used to transform sweet pepper. When assessed in vitro and in vivo, no genotoxicity could be detected or significant differences observed in growth, body weight gain, food consumption, hematology, blood biochemical indices, organ weights, and histopathology between rats or mice of either sex. Assays developed by Shim et al. (2007), Song et al. (2007), and Chaouachi et al. (2008) are useful to detect the presence of transgenic content in chilli peppers. An immunoassay (enzyme-linked immunosorbent assay) for the quantitative detection of phosphinothricin-*N*-acetyltransferase enzyme encoded by the bar gene and qualitative and quantitative PCR analysis based on the detection of the bar gene using capsanthin–capsorubin synthase as the endogenous reference gene in GM chilli pepper tolerant to BASTA herbicide was developed by Shim et al. (2007). Real-time PCR analysis for the differential detection and quantification of GM chilli peppers using a β-fructosidase gene as the endogenous reference along with other solanaceous species (tomato, potato, eggplant) will be useful in terms of food safety (Chaouachi et al., 2008). Kim et al. (2009) studied the gene flow from a GM chilli pepper (*C. annuum* L.) containing the CMVP0-CP (cucumber mosaic virus pathotype 0-coat protein) gene to a nontransformed control variety "P915" and two commercial F hybrids ("Manidda" and "Taesan") over two growing seasons in the field. A gene flow frequency of 17.89% between GM and "Taesan" chilli pepper were observed at the closest distance (0.5 m) from the central GM plot.

6. FUTURE PERSPECTIVES

Preliminary work on the isolation of genes responsible for biotic and abiotic stresses and agronomically important characters are available in the major spice crops. Candidate genes responsible for pathogenesis can also be identified from sequence information available in the database, which can be isolated and incorporated into promising varieties using transgenic pathways. Wild relatives of the crops within the family or species may be a repertoire of genes for various biotic and abiotic resistance, agronomically important traits, etc. Even though breeding programs involving hybridization to mobilize genes from wild relatives are cumbersome, incorporation of genes through transgenics is an alternative strategy. Because of the restricted taxonomic functionality of R genes, no R genes have been successfully expressed in a different family. So, developing resistant genotypes within the family through transgenic pathways may be an alternative way. Even though genetic transformation experiments are now restricted to the "greenhouse" level in the case of spices, it will be a powerful concept to produce pesticide-free spices and high-yielding, drought-, and disease-tolerant spice varieties, especially with the changing climates of today. However, the major concern will be what effect GM material could have on human health. As of now, research groups dealing with various spice crops have used antibiotic-resistant markers to select transformants. The impact of such antibiotic-resistant marker genes in altering nutritional values (Phillips, 1994) and allergic reactions (Nordlee et al., 1996) is either unknown or untested. Plants engineered to contain virus particles as part of a strategy to enhance resistance could facilitate the creation of new viruses in the environment. The possibility of cross-pollination of GM crops having herbicide and insect resistance with wild species (Hileman, 1999), evolution of superweeds, and impact

on genetic diversity especially in the native land of spices should be subjected to critical studies before introducing GM spice crops to address fear among the public. Though great progress has been made in the area of genetic engineering, there still remain many challenges that need to be addressed, including (1) use of *Agrobacterium* for site-directed recombination to avoid random T-DNA integration, (2) stable integration of the transgene and consistent inheritance in further generations without loss or alteration of expression, and (3) introduction of multiple "stacked" transgenes. The detailed analysis of plant proteins involved in the facilitation of T-DNA delivery into the host genome, the extension of the range of transformable genotypes and explants with a better understanding of host–pathogen interactions, and the development of methods for minimizing necrosis in transformed tissue will improve *Agrobacterium*-mediated transformation and provide further applicability and general efficiency. Alternatives such as cisgenics, intragenics, and markerless transgenics (Afolabi et al., 2005) could also be attempted in spice crops. Cisgenesis brings new possibilities for resistance strategy because the stacking of R genes is easier to handle, avoids linkage drag, and, moreover, cisgenic resistance breeding using wild species is safer. There is a repertoire of potential candidate genes identified in wild relatives of various spice crops (Dicto and Manjusha, 2005; Sandeep et al., 2009; Mani et al., 2012; Nair and Thomas, 2007), and research groups studying improvements to spices can focus on developing cisgenics. Even though fewer regulatory measures are expected regarding intragenesis and cisgenesis because it shares the same gene pool as that of conventional breeding, intragenic/cisgenic crops are currently regulated as only transgenic plants.

To conclude, *Agrobacterium*-mediated genetic transformation and biolistics are dominating the various transformation strategies employed in the context of spice transgenics. Plant transformation technologies have been used to study the functional genomics in wild relatives of spices via gene silencing with a promise to extend genetic modification in spice crops. Hairy root transformation of various seed spices is promising for large-scale secondary metabolite production. The adoption rate of genetic transformation in spices is at a slow pace because of the high regulatory burden of genetically engineered technology, market barriers, and fear among the general public. Development of food safety assessment protocols for detection of transgenic content in spices and studies on the possibility of gene flow from GM to conventional plants by the concerned research groups can alleviate the problems. Perfection of current transformation methods and application of new plant breeding technologies such as site-specific mutagenesis, cisgenics, and intragenics, breeding with transgenic inducible lines, grafting techniques on GM rootstock, and agro-infiltration methods will be of enormous value as tools in the genetic improvement of spices against various diseases caused by phytopathogenic fungi, bacteria, and viruses, which together can make a commercial impact on the spice industry.

Transgenics is unlikely to become more popular, however, if the business ethic is seen to prevail over human welfare and the environmental ethic. Food production will, however, have to be increased in the future, and increased use of agrochemicals and mechanized agriculture will contribute further to environmental degradation and loss of biodiversity. If transgenic crops in any way reduce these adverse effects, without themselves causing additional problems, they represent a technical and ethical advance. In retrospect, the "Transgenic Revolution" might not appear to have been particularly special, but until the fears of the public are assuaged it will continue to be ethically contentious. Only by continuing fundamental research on risks and benefits of transgenics will there be a possibility that the public will come to recognize the probable usefulness of genetic engineering in agriculture.

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CHAPTER

GENOMICS OF CUCURBITS 17

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1. INTRODUCTION

The Cucurbitaceae family, also called cucurbits, is composed of economically valuable plants such as *Cucumis sativus* L. (cucumber), *Cucumis melo* L. (melon), *Citrullus lanatus* (watermelon), *Lagenaria siceraria* (calabash), and *Cucurbita* spp. (squash and pumpkin). A total of 98 genera and about 975 species are found in this family. This family species is mainly utilized for food and medicinal purposes. Global cucurbits (including fruits, vegetables, and seeds) production was approximately 233 million tons, which were cultivated in 10 million ha of land in 2014 (http://faostat.fao.org). Because they show a high diversity of sex expression and long-distance signaling events, Cucurbitaceae family members are considered as model organisms and have been selected for sex determination (Tanurdzic and Banks, 2004) and plant vascular biology studies (Lough and Lucas, 2006). The cucumber genome was the first sequenced genome among cucurbits family members (Huang et al., 2009) and its genome became the seventh completed plant genome project among model plants including *Arabidopsis thaliana*, poplar, grapevine, papaya, rice, and sorghum (Baloglu et al., 2014). The second and third completed genome sequencing projects belong to melon (Garcia-Mas et al., 2012) and watermelon (Guo et al., 2013), respectively. In 2013, 115 cucumber lines and wild cucumber genomes were again sequenced for comparison. In that study, cucumber evolution and domestication have been highlighted (Qi et al., 2013). These studies are a milestone in the genomics of the Cucurbitaceae family. Furthermore, there are also some studies related to single nucleotide polymorphism (SNP) genotyping and quantitative trait locus (QTL) mapping. These are examples of such studies. Pumpkin, also known as winter squash, is another cucurbits family member whose high-density genetic map has been produced using genome sequences (Zhang et al., 2015b). Partial genome sequencing of calabash (bottle gourd) was completed in 2011 (Xu et al., 2011). A SNP-based genetic map has been constructed for summer squash (*Cucurbita pepo*), which is a member of the cucurbits family. Using an Illumina GoldenGate platform, QTL analysis has also been performed (Esteras et al., 2012).

The first completed genome project of the Cucurbitaceae family belongs to the cucumber plant. Seven chromosomes of cucumber have been sequenced using a combination of two techniques including conventional Sanger sequencing and next-generation Illumina sequencing in cucumber cultivar *C. sativus* var. *sativus* L., known as Chinese long inbred line 9930 (Huang et al., 2009). Although high genome coverage (about 72.2-fold) has been obtained, only a small quantity of genes has been identified because of there was limited information about whole genome and tandem duplications at that time. Approximately, 26,682 genes were predicted in the assembled genome of cucumber, which was

Genetic Engineering of Horticultural Crops. http://dx.doi.org/10.1016/B978-0-12-810439-2.00017-9

243.5 Mb in length. According to flow cytometry analysis of isolated nuclei, the actual cucumber genome size has been calculated as 367 Mb in length (Arumuganathan and Earle, 1991). Therefore the assembled genome of cucumber is almost 30% smaller than its actual genome size. For gene prediction, different methods were used including cDNA-EST, homology based and ab initio. About 82% of the genes have been functionally classified or their homologs have been found in related databases such as TrEMBL and InterPro. Furthermore, RNA molecules such as ribosomal RNA, transfer RNA, small nucleolar RNA, small nuclear RNA, and microRNA (miRNA) genes have been identified. About 15,669 gene families have been predicted. A total of 4362 and 3784 families belong to cucumber unique families and single-gene families, respectively. The highest rate of synteny was observed between cucumber and papaya with 9842 syntenic blocks. In addition, *Arabidopsis*, poplar, grapevine, and rice showed synteny with cucumber. These results also correlate with phylogenetic distances of these plants to cucumber. Cucumber and melon are found in the same genus. Although cucumber, melon, and watermelon belong to the same family, a total of 7, 12, and 11 chromosomes are found in cucumber, melon, and watermelon, respectively. A total of 348 melon and 136 watermelon markers were arranged on the cucumber chromosomes. Based on chromosomal evolution studies, it was concluded that some intrachromosome rearrangements have taken place and reorganization has probably occurred before deviation of cucumber and melon.

Melon is the second cucurbits whose genome has been sequenced (Garcia-Mas et al., 2012). As a melon cultivar, the homozygous DHL92 double-haploid line was selected for 454 pyrosequencing. A whole genome shotgun strategy was applied to the melon sequencing project. Assembled genome size was about 375 Mb, which represents 83.3% of the melon genome. A total of 27,427 protein coding regions have been predicted. Exhaustive gene annotation has been performed using an automatic pipeline that enables accurate identification of protein signatures, orthology groups, and metabolic pathways. In the melon genome, 411R-genes, also called disease resistance genes, were predicted. They were classified in their functions and domains. Some of them contained the nucleotide-binding site and leucine-rich repeat (NBS-LRR) and Toll interleukin receptor domains, which provide canonical disease resistance for cytoplasmic proteins. The remainder was classified as transmembrane receptors, including receptor-like kinases (RLK), kinases, and receptor-like proteins. In addition to R-genes, some genes related with fruit quality, taste, flavor, and aroma were identified. These genes were mainly associated with sugar and carotenoid accumulation, which directly affect the characteristic sweet taste and flesh color of melons, respectively. Syntenic relationships between melon and cucumber were examined and ancestral five melon chromosome matches with cucumber chromosomes with several inter- and intrachromosome rearrangements were found (Huang et al., 2009; Li et al., 2011a). In the melon genome sequencing study, syntenic relationships between melon and cucumber were also examined. For this purpose, both genomes were aligned. In this study, it was first observed that a great level of synteny at higher resolution between melon and cucumber genomes was obtained, which provides easy detection of small regions in chromosomes. However, it requires identification and purification of the physical maps and sequencing of other cucurbits members to obtain detailed information about genome evolution of the Cucurbitaceae family.

Watermelon is the last cucurbits whose draft genome sequencing project was completed in 2013 (Guo et al., 2013). Chinese elite watermelon cultivar 97103 ($2n=2\times=22$) and Illumina technology were used for genome sequencing. According to earlier flow cytometry analysis, watermelon genome size is about 425 Mb (Arumuganathan and Earle, 1991). It reached a 108.6-fold coverage in final assembly, which equals 353.5 Mb, and represents 83.2% of the watermelon genome. Because the same pattern of unassembled reads with transposable elements was shown, 16.8% of the watermelon genome was not covered.

Table17.1 Comparison of Cucurbitaceae Family Members' Genomes and Their Assemblies

Species	Chromosome Number	Protein-Coding Genes	Genome Assembly Size (Mb)	Estimated Genome Size (Mb)	Genome Covered by Assembly (%)	Sequencing Technologies
Cucumber	7	26,682	243.5	367	66.3	Illumina
Melon	12	27,427	375	450	83.3	Sanger + Roche 454
Watermelon	11	23,440	353.3	425	83.2	Sanger + Illumina

In total, 23,440 protein-coding genes were detected in the watermelon genome, which is similar to gene numbers of cucumber and melon (Table 17.1). Major classes of R-genes, including NBS-LRR, RLK, and lipoxygenase (LOX), were identified in the watermelon genome. Furthermore, genes associated with fruit development, quality, and sugar accumulation were identified and their expressions were examined at different stages of fruit development using RNA-seq analysis. Apart from watermelon genome sequencing analysis, resequencing of 20 watermelon accessions (10 from *C. lanatus* subsp. *vulgaris*, six from semiwild *C. lanatus* subsp. *mucosospermus*, and four from wild *C. lanatus* subsp. *lanatus*) was also performed in the watermelon genome project. Genetic diversity and population structure of *C. lanatus* germplasms were evaluated by examining their SNPs and indels (insertions/deletions) regions. To understand the cucurbits genome structure, syntenic relationship analysis between watermelon, cucumber, melon, and grape was performed. The watermelon genome had about a 60% orthologous relationship with the grape genome because of the close relationship between them. A detailed investigation of each chromosome of watermelon, cucumber, and melon was also performed. This analysis indicated that Cucurbitaceae family members have a high degree of orthologous relationships at the genomic level.

2. GENOME-WIDE CHARACTERIZATION STUDIES IN CUCURBITS

Although genome sequences of Cucurbitaceae family members have been published (Garcia-Mas et al., 2012; Guo et al., 2013; Huang et al., 2009), there are many studies that investigate identification of gene families at the genome scale (Table 17.2). Therefore genome-wide identification and expression analysis in cucurbits have been valuable in recent years. Because of early release of the cucumber genome sequence, genome-wide identification of gene family studies was focused first on cucumber. Some gene families such as transcription factors and structural and functional genes have been identified at a genome scale in cucumber, and their expression analysis has been investigated under abiotic stress conditions (salt, drought, high- and low-temperature, abscisic acid) and different tissue types (roots, male flower buds, female flower buds, leaves, stems, and fruits in five stages of fruit development). This type of study has also been popular among watermelon and melon.

In addition to gene families, some simple sequence repeats have been detected in cucumber, melon, and watermelon genomes (Blanca et al., 2011; Cavagnaro et al., 2010; Zhu et al., 2016). Long intergenic noncoding RNA (lincRNA) (Hao et al., 2015) regions have also been determined in the cucumber genome. Microsatellites are known as short tandemly repeated DNA sequences that are mainly used for comparative mapping and genetic diversity analysis. Intergenic transcripts encode lincRNAs whose length is about 200 nucleotides with no coding potential. They have the ability to

Table 17.2 Genome-Wide Gene Family Identification Studies in Cucurbitaceae

Species	Gene Family	Number of Identified Genes	Gene Expression	References
Cucumber	WRKY gene	55	Cold, drought, salinity	Ling et al. (2011)
Cucumber	AP2/ERF superfamily	103	Tissue specific: leaves, roots, stems, and flowers	Hu and Liu (2011)
Cucumber	Lipoxygenase (LOX)	23	Tissue specific: leaves, roots, stems, flowers, and fruits	Liu et al. (2011)
Cucumber	MADS-box	43	Tissue specific: roots, stems, leaves, and flowers	Hu et al. (2012)
Cucumber	Phenylalanine ammonia-lyase (PAL)	7	Salt Drought Low temperature ABA, SA, and H_2O_2	Shang et al. (2012)
Cucumber	R2R3MYB transcription factors	55	Tissue specific: roots, stems, leaves, male flowers, fruits, and tendrils Salt ABA	Li et al. (2012)
Cucumber	Auxin response factors (ARFs)	15	Tissue specific: leaves, roots, stems, flowers, and fruits	Liu and Hu (2013)
Cucumber	Homeodomain-leucine zipper Class I (HD-Zip I)	13	Salt Low temperature ABA	Liu et al. (2013)
Cucumber	Mildew resistance locus (MLO)	14	NA	Zhou et al. (2013b)
Cucumber	Gibberellin oxidases	16	NA	Pimenta Lange et al. (2013)
Cucumber	Auxin-responsive Aux/IAA gene family	29	IAA treatment	Gan et al. (2013)
Cucumber	Homeodomain-leucine zipper Class IV (HD-Zip IV)	11	Tissue specific: leaves, roots, stems, flowers, tendrils, and fruits	Fu et al. (2013)
Cucumber	Heat shock transcription factor (HSF)	21	NA	Zhou et al. (2013a)
Cucumber	Nucleotide-binding site (NBS)	Cucumber: 57	NA	Wan et al. (2013)
Watermelon		Watermelon: 27		
Melon		Melon: 18		
Bottle gourd		Bottle gourd: 31		
Luffa		Luffa: 2		
Squash		Squash: 7		

Table 17.2 Genome-Wide Gene Family Identification Studies in Cucurbitaceae—cont'd

Species	Gene Family	Number of Identified Genes	Gene Expression	References
Watermelon	Phenylalanine ammonia-lyase (PAL)	12	Tissue specific: roots, stems, cotyledons, mature leaves,male and female flowers, and fruits	Dong and Shang (2013)
Cucumber	bZIP	64	Drought	Baloglu et al. (2014)
Cucumber	WD-repeat (WDR)	191	Salt Low temperature ABA	Li et al. (2014)
Cucumber	D-type cyclin genes (CYCDs)	13	Pollinated and unpollinated ovaries	Cui et al. (2014)
Melon	Lipoxygenase (LOX)	18	Tissue specific: leaves, roots, stems, flowers, and fruits	Zhang et al. (2014)
Melon	SBP-box gene	13	NA	Ma et al. (2014)
Cucumber Watermelon	Polygalacturonase (PG)	Cucumber PG: 53 Watermelon PG: 62	Tissue specific: roots, male flower buds, female flower buds, leaves, stems, and fruits in five stages of fruit development	Yu et al. (2014)
Cucumber Watermelon Melon	Growth regulating factor (GRF)	24	NA	Baloglu (2014)
Cucumber	MAPK, MAPKK, and MAPKKK	MAPKs: 14 MAPKKs: 6 MAPKKKs: 59	Heat, cold, drought, and *Pseudoperonospora cubensis*	Wang et al. (2015b)
Watermelon	MAPK and MAPKK	MAPKs: 15 MAPKKs: 6	Tissue specific: roots, stems, leaves, salt, drought, and high and low temperature ABA	Song et al. (2015)
Watermelon	WUSCHEL-related homeobox (WOX)	11	Tissue specific: roots, stems, leaves, buds, flowers, and fruits	Zhang et al. (2015a)
Melon	AP2/ERF superfamily	136	Tissue specific: fruits, flowers, calli, leaves, roots, cotyledons, and phloem	Ma et al. (2015)
Cucumber Watermelon Melon	The ribosomal protein L24 (RPL24)	7	Drought	Baloglu et al. (2015)

Continued

Table 17.2 Genome-Wide Gene Family Identification Studies in Cucurbitaceae—cont'd

Species	Gene Family	Number of Identified Genes	Gene Expression	References
Cucumber	Lectin domains	146	NA	Dang and Van Damme (2016)
Cucumber	LEA (late embryogenesis abundant)	79	Drought	Celik Altunoglu et al. (2016)
Cucumber	TCP transcription factor	27	Tissue specific: seeds, leaves, internodes, shoot tips, roots, flowers, and fruits	Shi et al. (2016)
Cucumber	Catalase gene	4	Salt, cold, drought, H_2O_2, and ABA	Hu et al. (2016)
Cucumber	Dicer, Argonaute (AGO), and RNA-dependent RNA polymerase (RDR)	Dicer: 5 AGO: 7 RDR: 8	Tissue specific: roots, stems, leaves, male flowers, female flowers, and tendrils	Gan et al. (2016)
Cucumber	Dof transcription factors	36	Tissue specific: leaves, roots, stems, flowers, and tendrils Watermelon mosaic virus and downy mildew	Wen et al. (2016)
Cucumber	Two-component system Histidine kinases (HKs), phosphotransfers (HPs), response regulator proteins (RRs)	Cucumber: HKs: 18 HPs: 7 RRs: 21	Salt	He et al. (2016)
Watermelon		Watermelon: HKs: 19 HPs: 6 RRs: 24	Drought High and low temperature Trans-zeatin ABA treatment	
Melon	Stay-green (SGR) protein	4	Tissue specific: leaves, roots, stems, cotyledons, and fruits in five stages of fruit development	Bade et al. (2016)
Melon	NAC transcription factor	82	Salt	Wei et al. (2016))
Melon	MADS-box	62	Tissue specific: roots, stems, leaves, flowers, and fruits	Hao et al. (2016)
Melon	Flavin monooxygenases (YUC-like FMOs)	12	Tissue specific: roots, stems, leaves, tendrils, flowers, and fruits	Zheng et al. (2016)

Studies are ordered based on publication year of manuscripts.
ABA, *abscisic acid;* IAA, *indole-3-acetic acid;* NA, *not applicable;* SA, *salicylic acid.*

regulate gene expression, which is mainly controlled by targeting miRNAs. These regulatory repeats play important roles together with regulatory and transcription factor genes for gene regulation. Hence identification of microsatellites and small RNAs in Cucurbitaceae family members provides information for determining the functions and characterization of new lincRNAs in other plant species.

2.1 TRANSCRIPTOME ANALYSIS IN CUCURBITS

RNA-seq technology is a widely used technique for gene expression analysis. This technique allows transcripts from transcriptomes of the cells or tissues to be obtained with high coverage and low cost and time. Sequenced transcripts are utilized for prediction of genes. This new technology has measured not only gene expression levels but has also discovered new genes and their structures, and has detected new alternative splicing isoforms (Trapnell et al., 2010). Before the advent of RNA-seq, different microarray platforms were selected for high-throughput gene expression analysis. In the microarray technique, hybridization occurs between probes (representing a known gene portion) and fluorescently labeled target gene transcripts. The intensity of the fluorescence reflects the hybridization level, which actually indicates gene expression changes in samples. Although both technologies provide analysis of thousands of genes simultaneously, RNA-seq has been mostly preferred for transcriptome analysis. Probes are only designed according to known expression sequence tags (ESTs) and genes of organisms in microarray technology. It is not possible to identify new genes or gene structures. However, direct sequencing of whole transcriptome enables measurement of gene expression levels for newly identified genes with known genes. In RNA-seq technology, the only requirement is the availability of the whole genome sequence of organisms.

The first example of a high-throughput gene expression study was performed with a custom-designed oligo-based microarray in melon (Mascarell-Creus et al., 2009). This microarray study examined the gene expression pattern of melon transcriptome after cucumber mosaic virus infection and different fruit-ripening stages. In this study, ESTs from different melon cDNA libraries were used for the construction of oligo-based microarray, and expression of 17,510 melon unigenes was investigated. Two different studies in cucumber fruit (Ando and Grumet, 2010) and cucumber flower (Guo et al., 2010) were performed with the same RNA-seq platform, Roche 454-Pyrosequencing, which was provided for the first RNA-seq analysis of cucumber. The period of fruit developmental stages of cucumber including cell division to expansion, maturation, and ripening has been analyzed, and genes have been identified that played crucial roles in exponential fruit growth in cucumber. In the other study, pistillate and bisexual flowers from cucumber flower buds of two near-isogenic lines were used for transcriptome analysis to improve mechanisms of plant sex determination. About 200 differently expressed genes, simple sequence repeat (SSR) motifs, and some SNPs between two isogenic lines were determined. Both studies provide valuable information for functional genomics analysis, marker development, and cucumber breeding. Furthermore, messenger RNA (mRNA) from 10 cucumber tissues (root, stem, leaf, male flower, female flower, ovary, expanded ovary under fertilization, expanded ovary not fertilized, base part of tendril, and tendril) were sequenced for comparison between protein-coding genes from this study (Li et al., 2011a) and from formerly published protein-coding gene sets (Huang et al., 2009). About 8700 genes showed structural modifications and about 5300 genes were only identified in the reassembled cucumber genome (Li et al., 2011b). It was concluded that RNA-seq analysis has provided increased accuracy for improvement of prediction of protein-coding genes in the reassembled cucumber genome. The transcriptome of watermelon fruit development has also been

explored using the Roche/454 GS-FLX platform (Guo et al., 2011). For gene prediction, two databases, namely, GenBank non-redundant protein database and cucumber protein database, were utilized. Approximately 3000 genes showed different expression patterns in several fruit development stages. Like other fruit studies in cucumber and melon, this study has also provided candidate genes for cucurbits fruit biology and future functional analysis. In addition to mRNA sequencing, miRNAs and their targets can be also sequenced with different next-generation sequencing (NGS) platforms. As a first miRNA sequencing study, cucumber leaves and roots were used for the construction of two small RNA libraries, which were sequenced with the Illumina Solexa system (Mao et al., 2012). Tissue-specific expression of miRNAs, new miRNA families, and their potential targets were identified. Two novel miRNA families, which contained 64 miRNAs, were detected. MiRNAs and their target expression levels were also analyzed and confirmed with quantitative reverse transcription-polymerase chain reaction. This study had a potential to establish miRNA-mediated regulatory networks in cucumber. Another miRNA sequencing analysis was related with interaction between cucumber and downy mildew (Burkhardt and Day, 2016). To construct miRNA libraries, one resistant and one susceptible cucumber line were selected. The novel and existing miRNAs were specified between resistant and susceptible libraries. It was also suggested that there was a complicated interaction and gene regulation between miRNAs and their predicted targets in this plant-pathogen system. Another interesting study examined a grafting mechanism at the transcriptome level in watermelon (Liu et al., 2016a). Three mRNA libraries from watermelon, which was grafted onto bottle gourd and squash rootstocks, and self-grafted watermelon were developed. A total of 787 and 3485 differentially expressed genes were found in libraries of bottle gourd and squash rootstocks, respectively. These genes were associated with metabolism, signaling, and transcription factors. It was suggested that these genes and their networks were responsible for coordination of physiological processes of grafted seedlings. Male sterility has become an important issue for the hybrid seed industry in vegetable crops including watermelon. To illuminate the genetic mechanisms of male sterility, mRNAs from one male sterile and one male fertile isogenic watermelon lines were sequenced with an Illumina HiSeq 2000 system (Rhee et al., 2015). Essential genes related to the development of stamen, pollen, and pollen tube elongation played significant roles for global mechanisms of male sterility in watermelon. Transcriptome analysis in different melon cultivars has been widely studied. One study is related to transcriptomic analysis of sex determination in melon (Gao et al., 2015). RNA sequencing was performed using four melon plant sex types including monoecious (AAGG), gynoecious (AAgg), hermaphrodite (aagg), and andromonoecious (aaGG). Comparison of transcriptome data of paired segregants indicated that genes associated with the serine/threonine protein kinase pathway had a high impact on melon sex determination. In melon, response to salt stress was also examined at the transcriptome level (Wang et al., 2016). Two different melon cultivars, which showed different characteristics under salt stress, were used for sample preparation. RNA-seq analysis demonstrated that many genes gave different responses to salt stress in both cultivars and were mainly functioned in some pathways such as photosynthesis, biosynthesis of secondary metabolites, and signal transduction. In one study, the female and male flowers, leaves, roots, and fruits of two oriental melon cultivars (*Cucumismelo* L. var. *makuwa*) were selected for transcriptome analysis (Kim et al., 2016). According to de novo transcriptome analysis, detection of SNPs and SSRs as molecular markers, examination of tissue-specific gene expression profile, and construction of a genetic linkage map have been conducted. As a conclusion, these RNA-seq and molecular marker data from cucumber, watermelon, and melon provide information for molecular breeding and comparative and functional genomics studies of the Cucurbitaceae family.

3. APPLICATION OF OMICS TECHNOLOGIES FOR MOLECULAR BREEDING OF CUCURBITS

Plant breeding is the process that provides a change for plant traits to construct new phenotypes with desired characteristics. Breeding programs are aimed at improving and increasing traits such as quality, flavor, yield, tolerance of abiotic and biotic stresses, and storage period. Plant breeding programs can be divided into two groups including classical and modern plant breeding. In the first one, plants are selected with desirable characters and elimination of undesirable characters occurs. The modern plant breeding programs have used molecular biology techniques and omics technologies. Association mapping, marker-assisted selection, breeding by design, gene pyramiding, and genomic selection have been the main utilized methodologies in plant breeding programs. Techniques and methodologies used in plant breeding programs are summarized in Fig. 17.1.

Omics technologies have been rapidly developed in areas including genomics, transcriptomics, proteomics, and metabolomics. The advent of NGS has dramatically altered the fields of genomics and transcriptomics. NGS is composed of different sequencing platforms such as Roche, Illumina, Applied Biosystems, and Ion Torrent, which are known as second-generation technologies. Nowadays, third-generation platforms such as PacBio RS and Helicos have been widely chosen for genome and

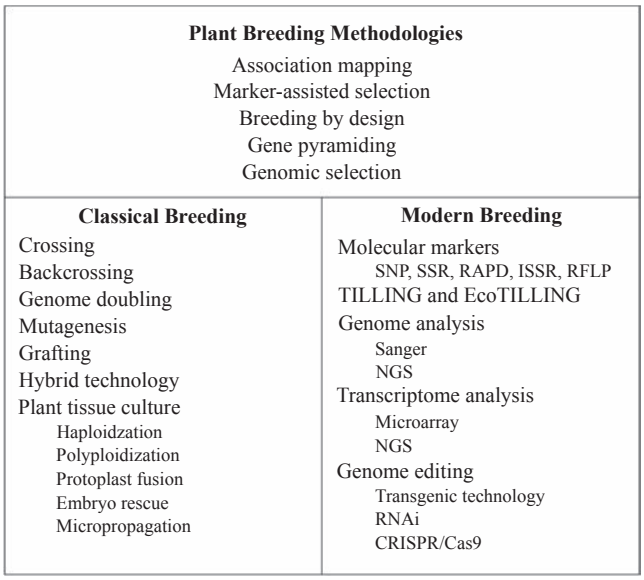

FIGURE 17.1

Classical and modern breeding techniques and methodologies used in plant breeding programs. *CRISPR/Cas9*, clustered regularly interspaced short palindromic repeats/CRISPR-associated protein-9 nuclease; *EcoTILLING*, ecotype Targeting Induced Local Lesions in Genomes; *ISSR*, inter simple sequence repeat; *NGS*, next-generation sequencing; *RAPD*, random amplification of polymorphic DNA; *RFLP*, restriction fragment length polymorphism; *RNAi*, RNA interference; *SNP*, single nucleotide polymorphism; *SSR*, simple sequence repeat; *TILLING*, Targeting Induced Local Lesions in Genomes.

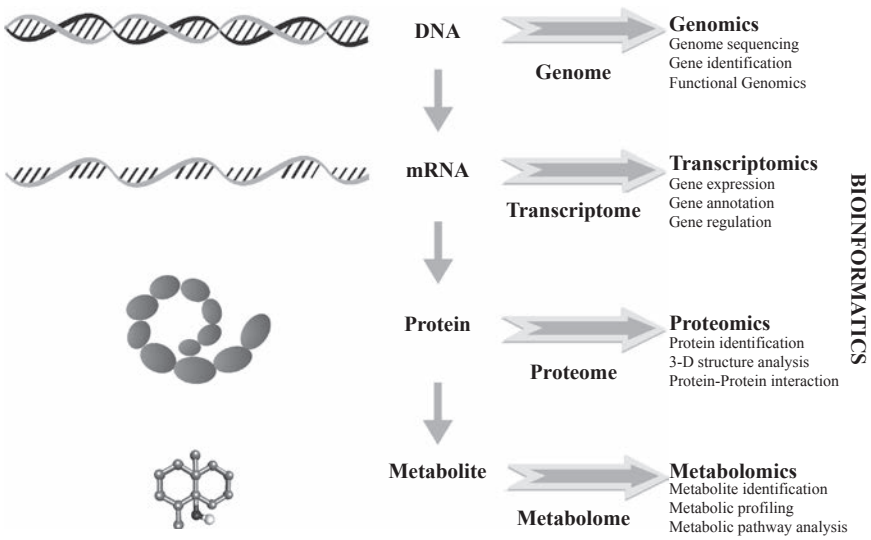

FIGURE 17.2

Details of omics technologies. All omics technologies use bioinformatics as a tool for analysis and interpretation of data.

transcriptome sequencing analysis. Proteomics and metabolomics are the large-scale studies of proteins and metabolites, respectively. Details of omics technologies are shown in Fig. 17.2.

Omics technologies in the Cucurbitaceae family have been mainly focused on many areas including mapping technology, gene expression study, gene and new alleles identification for new traits in plant breeding, and the study of organellar omics (Pawelkowicz et al., 2015). Genome sequencing, mapping, gene identification, and expression studies have already been discussed in former parts of this chapter. Gene and new alleles identification for new traits in cucurbits breeding will now be examined in this part. *Agrobacterium*-mediated, particle bombardment, and infiltration techniques have been commonly used for the introduction of foreign genes into organisms. This technology provides an opportunity for checking gene function and obtaining new traits in crops including cucurbits members. There are many studies associated with regeneration and genetic transformation of Cucurbitaceae family members including cucumber (Plader et al., 2007), watermelon (Pua and Davey, 2007a), and melon (Pua and Davey, 2007b). Many genes (related to tolerance to abiotic stresses, resistance to mosaic virus, pathogens and insects, improvement of quality and yield) have been transferred to gain new traits for cucurbits (Table 17.3). These studies are discussed and reviewed in different type of reports (Pua and Davey, 2007a,b; Wang et al., 2015a). All these studies chiefly investigated the transfer of only one gene or small DNA fragments into cucurbits. However, until now there has been no study associated with the introduction of long fragments of DNA libraries [bacterial artificial chromosome (BAC) or binary bacterial artificial chromosome (BIBAC)] in cucumber and other Cucurbitaceae. BAC and BIBAC libraries have played crucial roles for enhancement of molecular breeding, isolation, characterization, and usage of economically important genes for agricultural production. Only one study has been continued in which an 80 kb-long DNA fragment was inserted into cucumber embryogenic callus (Pawelkowicz et al., 2015).

Table 17.3 Genetic Transformation Studies in Cucurbitaceae Family Members

Gene	Transformation Method	Type of Explant	Agricultural Traits	References
Cucumber				
CMV-C coat-protein	Cucumber mosaic virus	Leaf	CMV resistance	Gonsalves et al. (1992)
CMV-O coat-protein	*Agrobacterium tumefaciens*	Leaf/cotyledon	CMV/ZYMV resistance	Nishibayashi et al. (1996a,b)
gus	*A. tumefaciens*	Hypocotyl	GUS positive	Nishibayashi et al. (1996a,b)
RCC2	*A. tumefaciens*	Leaf	Gray mold resistance	Kishimoto et al. (2002)
Thaumatin II	*A. tumefaciens*	Leaf/fruit	Change in fruit taste	Szwacka et al. (2002)
SOD	*A. tumefaciens*	Leaf/fruit	Increase in SOD activity	Lee et al. (2003)
DHN10	*A. tumefaciens*	Leaf/cotyledon/ hypocotyl	Chilling tolerance	Yin et al. (2004)
pDefH9	*A. tumefaciens*	Leaf/fruit	Parthenocarpic fruits	Yin et al. (2006)
CBF1	*A. tumefaciens*	Leaf	Chilling tolerance	Gupta et al. (2012)
PAC	*A. tumefaciens*	Leaf	Increase in β-carotene	Choi et al. (2016)
NOA1	*A. tumefaciens*	Leaf	Chilling tolerance	Liu et al. (2016c)
Melon				
CMV	*A. tumefaciens*	Cotyledon	CMV resistance	Yoshioka et al. (1993)
ZYMV	*A. tumefaciens*	Cotyledon	ZYMV resistance	Fang and Grumet (1993)
CMV-WL coat protein	*A. tumefaciens*/ biolistics	Cotyledon	CMV resistance	Gonsalves et al. (1994)
CMV/WMV/ZYMV	*A. tumefaciens*	Leaf	ZYMV, WMV, CMV resistance	Clough (1995)
Antisense *ACC oxidase*	*A. tumefaciens*	Cotyledon	Slow ripening	Ayub et al. (1996)
ZYMV, WMV, CMV coat protein	*A. tumefaciens*	Cotyledon	ZYMV, WMV, CMV resistance	Fuchs et al. (1997)
HAL1 yeast gene	*A. tumefaciens*	Leaf/cotyledon	Salt resistance	Bordas et al. (1998) and Serrano et al. (1998)
S-adenosylmethionine hydrolase gene	*A. tumefaciens*	–	Slow ripening	Clendennen et al. (1999)
Bar gene	Potyvirus-vector inoculation	Direct inoculation on whole plant	Herbicide resistance	Shiboleth et al. (2001)
Polyribozyme genes	*A. tumefaciens*	Cotyledon	WMV2, ZYMV resistance	Huttner et al. (2001)

Continued

Table 17.3 Genetic Transformation Studies in Cucurbitaceae Family Members—cont'd

Gene	Transformation Method	Type of Explant	Agricultural Traits	References
Photorespiratory eR genes	*A. tumefaciens*	Cotyledon	Downy mildew resistance	Taler et al. (2004)
Apple ACC oxidase (*ethylene biosynthesis*)	*A. tumefaciens*	Cotyledon	Slow ripening	Silva et al. (2004)
Petunia ACC synthase	*A. tumefaciens*	Cotyledon	Earlier floral development and fruit set	Papadopoulou et al. (2005)
Watermelon				
gus and *npt*II	*A. tumefaciens*/ biolistics	Cotyledon	Antibiotic resistance	Compton et al. (1993)
gus	*A. tumefaciens*	Cotyledon	GUS positive	Choi et al. (1994)
Phosphomannose isomerase	*A. tumefaciens*	Cotyledon	Positive selection	Reed et al. (2001)
WMV-2 coat protein gene	*A. tumefaciens*	Leaf	WMV2 resistance	Wang et al. (2003)
HAL1 yeast gene	*A. tumefaciens*	Cotyledon	Salt resistance	Ellul et al. (2003)
hpt	*A. tumefaciens*	Leaf	Antibiotic resistance	Akashi et al. (2005)
ZYMV, WMV, CMV coat protein	*A. tumefaciens*	Cotyledon	ZYMV, WMV, CMV resistance	Sheng-Niao et al. (2005)
ZYMV, PRSV W coat protein	*A. tumefaciens*	Cotyledon	ZYMV, PRSV W resistance	Yu et al. (2011)
WSMoV coat protein	*A. tumefaciens*	Cotyledon	WSMoV, resistance	Huang et al. (2011)
Pti4	*A. tumefaciens*	Cotyledon	Transcription factor domain	Juan Li et al. (2012)
WSMoV, CGMMV, CMV coat protein	*A. tumefaciens*	Cotyledon	WSMoV, CGMMV, CMV resistance	Lin et al. (2012)
CMV coat protein	*A. tumefaciens*	Cotyledon	Leaf	Liu et al. (2016b)

Studies are ordered based on publication year of manuscripts.
CBF1, *c-repeat binding factor-1;* CGMMV, *cucumber green mottle mosaic virus;* CMV, *cucumber mosaic virus;* DHN10, *gene encoding a* Solanum sogarandium *dehydrin with 10 kDa;* NOA1, *nitric oxide associated 1;* PAC, *phytoene synthase-2a carotene desaturase;* pDefH9, Antirrhinum majus *deficiens homologue 9 promoter;* PRSV W, *papaya ringspot virus type W;* RCC2, *a rice chitinase cDNA;* SOD, *superoxide dismutase;* WMV, *watermelon mosaic virus;* WSMoV, *watermelon silver mottle virus;* ZYMV, *zucchini yellow mosaic virus.*

Transformation and genome editing studies of cucurbits have a great potential to obtain new cucurbits phenotypes with desirable characteristics. A reverse genetic approach called Targeting Induced Local Lesions in Genomes (TILLING) is capable of identifying the allelic version of DNA regions such as multiple alleles of gene(s), missense, nonsense, null alleles, splicing, and *cis*-regulatory elements in artificially mutagenized populations. TILLING generates stable and nontransgenic mutant alleles for the breeding programs of many crops including cucurbits species.

Application of the TILLING approach in different model organisms has contributed to improvement of agronomics traits such as biotic and abiotic stress resistance, an increase in shelf-life, and nutritional value (González et al., 2011; Okabe et al., 2011; Rawat et al., 2012; Vicente-Dólera et al., 2014). Different DNA mutant TILLING libraries have also been constructed in cucurbits (Boualem et al., 2014; Dahmani-Mardas et al., 2010; Fraenkel et al., 2014; González et al., 2011; Vicente-Dólera et al., 2014). TILLING provides detection of new mutant alleles, which cause improvement of agronomically important traits in plant species as well as in Cucurbitaceae family members. This approach has provided a precious source for future functional genomics and plant breeding programs.

4. FUTURE PROSPECT OF CUCURBITS GENOMICS

The advent of NGS has accelerated genomics and transcriptomics studies in Cucurbitaceae family members. Resolution of the whole genome sequence of cucumber is the cornerstone of cucurbits genome studies. Melon and watermelon were the second and third cucurbits whose genomes were sequenced. Evolution of NGS technologies has been rapidly continuing over the past decade. In addition to sequencing technology, the combination of breeding techniques with genomic and bioinformatics tools has also contributed to progress of genomics-based plant breeding. These two important points will directly affect the field of genomics and its application in cucurbits breeding strategies. In the future, new omics analysis tools will emerge and provide further investigation into cucurbits genomes. The extensive genome analysis and editing studies will expand our knowledge and implementation for breeding programs of Cucurbitaceae family members.

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Genetic Engineering of Horticultural Crops

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Genetic Engineering of Horticultural Crops provides key insights into commercialized crops, their improved productivity, disease and pest resistance, and enhanced nutritional or medicinal benefits.

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ISBN 978-0-12-810439-2