CHAPTER 10

RECENT ADVANCES IN THE DEVELOPMENT OF TRANSGENIC CROP PLANTS, BIOSAFETY ASPECTS, AND FUTURE PERSPECTIVES

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ABSTRACT

Genetic improvement of crop plants is not new; we have been modifying plant genomes for thousands of years for our well-being. Development of transgenic crop plants is an outcome of increasing human population and incidence of biotic/abiotic stress determinants. The cost-effective approach of genetic engineering allows for a relatively fast cross-species gene transfer. A number of crop plants have been genetically engineered for resistance to insect pests, fungal and viral pathogens, nematodes etc. using a variety of approaches. Genetically modified crop plants have been developed for tolerance to various abiotic stress conditions such as osmosis, salt, drought, temperature, environmental pollutants and so on. Plants have been engineered for better nutrient utilization as well as enhancement of nutrition quality in food. Crop plants have been engineered for molecular farming in order to generate sufficient antigenic vaccines, antibodies, netraceutical and therapeutic proteins. Recently, plant genomes have also been modified for enhancement in the production of biofuel. It is natural to think about the biosafety aspects of transgenic crop plants especially with respect to health and ecological issues. The cause of concern arises due to the phenomena of various types of gene flow in nature. The selectable markers can be removed from the genetically engineered plants using approaches such as co-transformation, multi-autotransformation, site-specific recombination, Cre/lox recombination system etc. Recently, genome editing technology, which allows plant breeding without introducing a transgene, is expected to generate many new crop varieties with traits that can satisfy various kinds of demands for commercialization genetically improved crop plants.

10.1 INTRODUCTION

The human species emerged on this earth about 300,000 years ago. Since then, we have been working for our well-being and improvement of food quality, quantity, shelter, etc., without thinking of the nature and the mother earth, only to satisfy our needs. Human activities have caused enormous changes in physical, chemical, geological, biological, and atmospheric domains of our planet. Genetic improvement of crop plants is not new; we have been modifying plant genomes for thousands of years. The major crop species were domesticated about 5000–10,000 years ago. This has connection to human civilization of which development is intricately linked to agricultural growth. The biggest challenge even today is to produce sufficient

amount of food for the exploding human population on earth. In 1800, the world population was 1 billion people, whereas in 1900, at the beginning of the 20th century, the population increased to 1.65 billion people, and by 2000, this number increased to over 6 billion (http://www.plantcell.org/site/teachingtools/teaching.xhtml). The world human population is expected to reach 9 billion by the year 2050, and we need to at least double crop production, especially rice, by this time (Sheehy and Mitchell, 2011). Crop plants are under continuous threat by various biotic and abiotic stresses. Therefore, a continuous effort is to be exercised to ensure tomorrow's food security, which is not endorsed by today's food sufficiency. We are still caught up with limited success of cultural practices, environmentally unhealthy use of pesticides, and decreasing arable land area.

To feed the several billion people living on this planet, the production of high-quality food must increase with reduced inputs, but this accomplishment will be particularly challenging in the face of global environmental change. Plant breeders need to focus on traits with the greatest potential to increase yield. Hence, new technologies must be developed to accelerate breeding through improving genotyping and phenotyping methods and by increasing the available genetic diversity in breeding germplasm. Most of the gain will come from delivering these technologies in developing countries, but the technologies will have to be economically viable and readily disseminated. Crop improvement through breeding brings immense value relative to investment and offers an effective approach to improving food security. However, to meet the recent Declaration of the World Summit on Food Security (FAO, 2009) for production of 70% more food by 2050, an average annual increase in production of 44 million metric tons per year is required. Particularly challenging for society will be the changes in weather patterns that will require alterations in farming practices and infrastructure, for example, water storage and transport networks. The likely impacts on global food production are many because one-third of the world's food is produced on irrigated land. Along with agronomic- and management-based approaches to improving food production, improvements in a crop's ability to maintain yields with lower water supply and quality will be critical (Tester and Langridge, 2010). By and large, we need to increase the tolerance of crops to biotic and abiotic stress conditions by several folds.

Modern tools of plant biotechnology can complement conventional plant breeding in an economically useful way to genetically improve crop plants. In genetically modified (GM) crop plants, their genome is engineered using tools of genetic engineering such as recombinant DNA technology, which is complemented by our knowledge of molecular biology. In this approach,

different DNA fragments from various useful sources are put together to create a new molecule that is introduced into the plant genome for desired purposes. Thus, essentially transgenic plants are those plants containing DNA from other organisms. Remarkably, while developing transgenic plants, the genetic engineer enjoys advantage of cross-species gene transfer and considerable reduction in time toward generating an improved transgenic line for a specific crop plant. In the distant and recent past, we have relied on domestication of crop plants, development of hybrid seeds, and experienced "green revolution" through advances in plant breeding technologies. In recent years, we have been witnessing a "gene revolution" that is making remarkable advance in the field of plant biotechnology. Genetic engineering involves cloning of desired genes, development of designer gene constructs, and transfer of transgenes to the organism concerned. Specific changes are introduced in the genome of crop plants using the tools of genetic engineering. Over last three decades, a large number of transgenic plants have been developed across different classes of crops with various improved agronomic characteristics. The main focus has been development of transgenic crop plants for enhanced resistance to bacterial diseases, fungal diseases, virus, nematode, insect pests, etc. and tolerance to drought, salinity, flooding, heavy metals, etc. However, although many GM crop plants have been developed, only a few of them have made their way to the field. On the contrary, the land area under GM crop cultivation has increased steadily over last decade though it has mainly remained restricted to the countries such as the United States, Argentina, Brazil, Canada, India, China, etc. (Table 10.1). Some of the GM crop plants that are being grown in the field are cotton, corn, soybean, canola, sugarbeet, papaya, alfalfa, brinjal, etc.

The improvement of agricultural production and productivity as well as the future versatility of agricultural production are bound to be dependent on the rational utilization of modern plant biotechnology. We stand at the convergence of an unbelievable plethora of new technologies, such as recombinant DNA technology, information technology, and high-throughput genomics, to enhance our understanding of the structure and function of the genomes and to apply this information for improvement of plants and animals. Products arising from modern biotechnology such as GM or transgenic crops are providing new opportunities to achieve sustainable productivity gains in agriculture.

However, ever since GM crop plants were generated, there has been lot of hot debates on application of GM crops over conventional breeding and recently organic farming. Development of GM crop varieties has raised a wide range of ethical, environmental, economic, social, and political

TABLE 10.1 Global Area of Biotech Crops in 2015 and 2016: by Country (Million Hectares**) (reproduced from ISAAA, 2016)

	Country	2015	2016	% increase in 2016 over 2015
1.	USA*	70.9	72.9	3
2.	Brazil*	44.2	49.1	11
3.	Argentina*	24.5	23.8	-3
4.	Canada*	11.0	11.6	5
5.	India*	11.6	10.8	-7
6.	Paraguay	3.6	3.6	0
7.	Pakistan*	2.9	2.9	0
8.	China*	3.7	2.8	-24
9.	South Africa*	2.3	2.7	17
10.	Uruguay*	1.4	1.3	-7
11.	Bolivia*	1.1	1,2	9
12.	Australia*	0.7	0.9	29
13.	Philippines*	0.7	0.8	14
14.	Myanmar*	0.3	0.3	0
15.	Spain*	0.1	0.1	0
16.	Sudan*	0.1	0.1	0
17.	Mexico*	0.1	0.1	0
18.	Colombia*	0.1	0.1	<0.1
19.	Vietnam	<0.1	<0.1	<0.1
20.	Honduras	<0.1	<0.1	<0.1
21.	Chile	<0.1	<0.1	<0.1
22.	Portugal	<0.1	<0.1	<0.1
23.	Bangladesh	<0.1	<0.1	<0.1
24.	Costa Rica	<0.1	<0.1	<0.1
25.	Slovakia	<0.1	<0.1	<0.1
26.	Czech Republic	<0.1	<0.1	<0.1
27.	Burkina Faso	0.5	--	--
28.	Romania	<0.1	--	--
	Total	179.7	185.1	3.0

*Biotech mega-countries growing 50,000 hectares or more
**Rounded-off to the nearest hundred thousand or more

concerns. However, no debates have finally resulted in any unanimously agreed policy. Rather, it has run into a deadlock between various stakeholders such as scientists, farmers, politicians, bureaucrats, and administrators. In the meanwhile, the common man is left in the sidelines only. GM technologies have the potential toward ensuring food security through development of enhanced resistance to biotic stresses, increased tolerance to abiotic stresses, nutritional quality improvement, and use of lesser and lesser pesticides and fertilizers at the same time. On the other hand, organic farming may not ensure increased productivity due to higher incidence of pests and diseases, although it utilizes farmers' knowledge of growing crops while maintaining diversity of crops (Azadi and Ho, 2010). Our choice of going with the GM crops should be determined by the following alarming situations: (1) availability of limited and/or gradually reducing land area for agriculture due to urbanization, (2) exploding human population, (3) constantly changing climatic conditions, (4) limited water availability, (5) increasing incidence of pests and diseases resistant to various pesticides that have been in use so far, (6) biosafety issues, etc.

However, environmental stresses, gradual development of pest resistance to pesticides, alarming increase in population explosion, and food shortage are major concerns of mankind on this globe. Limited natural resources cannot fulfill the food demand of every individual. Thus, we keep experiencing numerous cases of malnutrition especially in the developing and underdeveloped countries. Producing crops with improved quality and quantity is imperative for growing food demand through sustainable agriculture that could be attained using conventional selection and breeding coupled with genetic engineering (Ashraf and Akram, 2009). The application and development of biotechnology have led to newer opportunities and possibilities to enhance qualitative and quantitative enhancement of crop plants (Sun, 2008; Yamaguchi and Blumwald, 2005). Biotechnology for genetic improvement has become a sustainable strategy to combat deficiencies in food by enhancing proteins, carbohydrates, lipids, vitamins, and micronutrient composition (Sun, 2008; Zimmermann and Hurrell, 2002). Major emphasis of agricultural biotechnology has been on traits for improvement in crops related to insect and herbicide resistance, nutritional quality, virus resistance, shelf life, and biofuel production since the 1990s. Thus, to ensure future food security, it is advisable to carefully embrace GM crop plants, especially when genetic engineering offers (1) feasibility of cross-species gene transfer, (2) time saving approach of generating new cultivars, (3) cost-effective strategy toward quality and quantity improvement of crop plants.

In this chapter, we are presenting the work on development of GM crops carried out mostly during the last decade, the major biosafety concerns relevant to the use of transgenic crops, possible precautionary measures for commercial use of GM crops, and future perspectives on generation of "designer biotech crops." However, it must be admitted that the discussion may not still be very exhaustive, and just in case we have missed to acknowledge anybody's work or reference, it is purely unintentional and we sincerely apologize for such lacunae.

10.2 GENETIC ENGINEERING OF RESISTANCE TO BIOTIC STRESS

Some of the limiting factors in crop production are various pests, diseases, and weeds, which are considered as biotic stresses. The limitations associated with chemical methods (mainly, the environmental hazard) and other conventional breeding methods of control necessitated the development of alternative methods for developing new cultivars with higher resistance to biotic stresses. Genetic engineering approach has proven worth to select for the resistance sources from across the species and introduce the agronomical useful genes into the desired plants to provide resistance against different biotic stresses. Numerous strategies have been taken up for last more than three decades for enhancement of resistance against insect pests, nematodes, fungi, bacteria, and virus, which are injurious to crop plants and also cause diseases.

10.3 ENGINEERED RESISTANCE TO INSECT PESTS

A number of transgenic crop plants have been developed for increased resistance to variety of insect pests using variety of strategies (Table 10.2).

10.3.1 USING BT-TOXIN

One of the most skyrocketing achievements in plant biotechnology is development of insect resistant crops expressing crystal proteins from *Bacillus thuringiensis* (*Bt*). *Bt* is a Gram-positive bacterium that produces proteinaceous crystalline (Cry) inclusion bodies during sporulation. It also produces cytotoxins that synergize the activity of Cry toxins (Tohidfar and Khosravi, 2015). It is known that the *Bt* crystal proteins (δ-endotoxin) are toxic to lepidopterans, dipterans, and coleopterans, and at the same time, it is nontoxic to humans and animals (Ahmad et al., 2012).

TABLE 10.2 Recent Developments of Transgenic Crop Plants Resistant to Insect Pests.

Sl. No.	Transgene (Gene Name)	Source of Transgene	Crop and Cultivar	Resistance to	Method of Transformation	Reference
1.	*amiR-24*	*Bacillus thuringiensis*	Tobacco	Cotton bollworm (*Helicoverpa armigera*)	ATMT	Agrawal et al. (2015)
2.	*Bt* (δ-endotoxin gene)	*Bacillus thuringiensis*	Rice	Striped stem borer (*Chilo suppressalis*)	ATMT	Gao et al. (2015)
3.	*HaAK*	*Arabidopsis* sp.	Arabidopsis	Cotton bollworm (*Helicoverpa armigera*)	ATMT	Liu et al. (2015b)
4.	*cry3A*	*Bacillus thuringiensis*	Potato	Colorado potato beetle (*Leptinotarsa decemlineata Say*)	ATMT	Guo et al. (2014)
5.	*cry1Ia8*	*Bacillus thuringiensis* (*Btc008*)	Cabbage	Diamondback moth *Plutella xylostella* (*Linnaeus*)	ATMT	Yi et al. (2013)
6.	*HaHR3*	Cotton bollworm (*Helicoverpa armigera*)	Tobacco	Cotton bollworm (*Helicoverpa armigera*)	ATMT	Xiong et al. (2013)
7.	*EcR*	Cotton bollworm (*Helicoverpaarmigera*)	Tobacco	Cotton bollworm (*Helicoverpa armigera*)	ATMT	Zhu et al. (2012)
8.	*Cry1Ab*	*Bacillus thuringiensis*	Rice	Lepidopteron Leaf folder and stem borer	ATMT	Qi et al. (2012)
9.	*cry1Ab* and *cry1Ac*	*Bacillus thuringiensis*	Chickpea	Cotton bollworm (*Helicoverpa armigera*)	ATMT	Mehrotra et al. (2011)
10.	*Nlsid-1* and *Nlaub*	*Nilaparvata lugens*	Rice	Rice brown plant hopper	ATMT	Zha et al. (2011)
11.	*CYP6AE14* *P450 gene*	Cotton bollworm (*Helicoverpa armigera*)	Cotton	Cotton bollworm (*Helicoverpa armigera*)	ATMT	Mao et al. (2011)
12.	*m-Cry1Ac*	*Bacillus thuringiensis*	Sugarcane	Sugarcane stem borer	Microprojectile bombardment	Weng et al. (2011)

TABLE 10.2 *(Continued)*

Sl. No.	Transgene (Gene Name)	Source of Transgene	Crop and Cultivar	Resistance to	Method of Transformation	Reference
13.	*CpTI* (cowpea trypsin inhibitor) and *Bt/CpTI* (Bt transgene linked to CpTI)	Cowpea and *Bacillus thuringiensis*	Rice	Rice stem borers (*Scirpophaga incertulas*, *Chilo suppressalis*, and *Sesamia inferens*) and rice leaf-folder (*Cnaphalocrocis medinalis*)	ATMT	Yang et al. (2011b)
14.	*Cry1Ab*, *Cry1Ac*, and *Cry2A*, *cry1C*	*Bacillus thuringiensis Berliner* (*Bt*)	Rice	Striped stem borer (*Chilo suppressalis Walker*)	ATMT	Yang et al. (2011a)
15.	*aadA*		Eggplant	Fruit and shoot borer (*Leucinodes orbonalis*)	Microprojectile bombardment	Singh et al. (2010)
16.	*Bt cry1Ab*	*Bacillus thuringiensis*	Tobacco	Lepidopterans	Chloroplast transformation strategy via bombardment	Jabeen et al. (2010)
17.	*cry2Aa*	*Bacillus thuringiensis* (*Btc008*)	Chickpea	Pod borer (*Helicoverpa armigera*)	ATMT	Acharjee et al. (2010)
18.	*NaPI* and *StPin1A*	Potato (*Solanum tuberosum*)	Cotton	*Helicoverpa punctigera* and *Helicoverpa armigera*	ATMT	Dunse et al. (2010)
19.	*SPI2c* (serine protease inhibitors)	*Solanum nigrum*	*Solanum nigrum*	Generalist insect herbivores	ATMT	Hartl et al. (2010)
20.	*Sporamin* (trypsin inhibitor) and *CeCPI* (phytocystatin)	Sweet potato and taro	Tobacco	Cotton bollworm (*Helicoverpa armigera*)	ATMT	Senthilkumar et al. (2010)
21.	*ASAL* (*Allium sativum* leaf agglutinin)	Garlic (*Allium sativum*)	Rice	GLH (*Nephotettix virescens*) and BPH (*Nilaparvata lugens*)	ATMT	Sengupta et al. (2010)

*A*TMT, Agrobacterium tumefaciens-mediated transformation, GLH, green leafhoppers; BPH, brown plant hopper.

A ***cry2Aa*** gene with a sequence-modified open-reading frame encoding an insecticidal crystal protein from *Bt* was introduced into chickpea (*Cicer arietinum* L.) to confer resistance to *Helicoverpa armigera* (Acharjee et al., 2010). Maize has been transformed with either *Bt* ***cry1Ab***, ***cry1Ac***, or ***cry9C*** to protect it against *Ostrinia nubilalis* and *Sesamia nonagriodes*, or with ***cry1F*** to protect it against *Spodoptera frugiperda*, and with ***cry3Bb***, ***cry34Ab***, and ***cry35Ab*** to protect it against the rootworms of the genus *Diabrotica* (James, 2012). *Bt* toxin genes ***cryIA***, ***cryIAc***, and ***cry3A*** have been expressed in soybean (Macrae et al., 2005), chickpea (Sanyal et al., 2005), and alfalfa (Tohidfar et al., 2013), respectively, for insect resistance. Cotton plants were engineerd using *Bt* toxin gene ***cryIAb*** for protection against cotton bollworm (Tohidfar et al., 2008). Transgenic cruciferous vegetables have been developed for use against *Plutella xylostella* (James, 2012). The *Bt* toxins have been introduced in soybean using either one or two cry genes among ***cry1A*****b**, ***cry1Ac***, ***cry1F*** (James, 2013). In field trials, transgenic sugarcane plants expressing high levels of modified ***cry1Ac*** have been shown to provide effective control against stem borers (Weng et al., 2011).

10.3.1.1 MODE OF ACTION OF BT TOXINS

Cry proteins once ingested by the insect are solubilized in the midgut and are then cleaved there by digestive proteases. Some of the resulting polypetides bind to midgut epithelial cell receptors resulting in cell lysis and finally insect death (Gahan et al., 2010).

10.3.2 USING LECTINS

Lectins are carbohydrate-binding peptides or proteins that occur abundantly in seeds and storage tissues of different plants. One of the most important direct defense responses in plants against the attack by phytophagous insects is the production of these peptides or proteins. Lectins have been found to be useful to protect the plants against insect pests, especially the sap-sucking insects (Joshi et al., 2010). The lectins from snowdrop or garlic were found to be injurious to insects but not to mammals (Fitches et al., 2010). The most important protein examined is the lectin from snowdrop (*Galanthus nivalis* agglutinin, GNA). **GNA** has been reported to have the capability to affect the metabolic activity of brown plant hopper (BPH), white-backed plant hopper and green leafhopper pests of rice (Nagadhara et al., 2003). GM rice plant

expressing snowdrop lectin gene [*Galanthus nivalis agglutinin* (GNA)] demonstrated reduced survival and fecundity of insects, impaired insect development, and an inhibitory effect on BPH feeding (Brar et al., 2009; Nagadhara et al., 2004; Tang et al., 2001). Transgenic rice with GNA (snow drop) has shown resistance to BPH (*Nilaparvata lugens*) (Li et al., 2005). Transgenic potato expressing *gna* gene showed reduced damage to leaves (Bell et al., 2001). It has been observed that *Allium sativum* leaf agglutinin, the garlic lectin gene, possesses the insecticidal activity against BPH and green leaf hopper (Saha et al., 2006) as has been observed in rice cv. IR64-induced hopper resistance. *Allium cepa* agglutinin has been reported to show insecticidal property to control sap-sucking insects (Hossain et al., 2006).

10.3.2.1 MODE OF ACTION OF LECTINS

The most likely mechanisms underlying the entomotoxic activity of lectins involve interactions with different glycoproteins or glycan structures in insects, which may interfere with a number of physiological processes in these organisms. Since lectins possess at least one carbohydrate-binding domain and different sugar specificities, and considering the variety of glycan structures in the bodies of insects, possible targets for lectin binding are numerous.

10.3.3 USING INHIBITORS AGAINST PROTEASES

Some cultivars including cotton expressing Cowpea trypsin inhibitor (CpTI) have been commercially released in China in 2000. Oryzacystatin 1 (OC1) isolated from rice seeds has been successfully introduced into various crops like rice (Duan et al., 1996), wheat (Altpeter et al., 1999), oilseed rape (Rahbé et al., 2003), and eggplant (Ribeiro et al., 2006). It protects these plant species against beetle attacks and, in some cases, aphids (Sharma et al., 2004). In a remarkable multigene approach, a *Bt*-corn called *Bt*-Xtra containing three genes including *cry1Ac* from *Bt*, *bar* from *Streptomyces higroscopicus*, and potato proteinase inhibitor (*pinII*) has been produced, where the only inhibitor gene was *pinII.* Potato type I and II serine protease inhibitors (PIs) are produced by solanaceous plants as a defense mechanism against insects and microbes. Co-expression of potato type I and II proteinase inhibitors conferred cotton plants protection against a major insect pest, *Helicoverpa punctigera* (Dunse et al., 2010).

10.3.3.1 MODE OF ACTION OF PROTEASE INHIBITORS

Plant PIs are able to protect plants against insect attacks by interfering with the proteolytic activity of insects' digestive gut. Among the proteinaceous PIs, serine and cysteine PIs are abundant in plant seeds and storage tissues (Reeck et al., 1997) and may contribute to their natural defense system against insect predation. Proteinase inhibitors have been found to affect growth and development of many insects.

10.3.4 USING INHIBITORS AGAINST α-AMYLASES

One potential class of inhibitors is α-amylase inhibitors as they can control seed weevils, which are highly dependent on starch as energy source. The bean (*Phaseolus vulgaris*) amylase inhibitor gene was expressed in seeds of transgenic garden pea (*Pisum sativum*), and other grain legumes and seeds from these transgenics were resistant to stored product pests such as larvae of bruchid beetles and field pests such as larvae of the pea weevil *Bruchus pisorum* (Morton et al., 2000). The α-amylase inhibitor gene from *P. vulgaris* was introduced to chickpea and the transformed plants showed a significant resistance to bruchid weevil (Ignacimuthu and Prakash, 2006). When the same gene was expressed in *Coffea arabica*, the seed extracts from resultant transgenics were had an inhibiting amylolytic enzyme activity up to 88% (Barbosa et al., 2010).

10.3.5 OTHER INSECTICIDAL PROTEINS

Other insecticidal proteins such as antibodies, wasp and spider toxins, microbial insecticides, and insect peptide hormones have also been used to generate various transgenic plants. Some bacterial species like *Bt* has become the source of insecticidal activities during vegetative growth. They produce **Vip3A** protein against lepidopteran insects. Unlike *Bt* toxins, Vips do not need to be solubilized in the insect gut. They bind to receptors in the insect gut different from those targeted by Cry proteins (Lee et al., 2006a). *Vip3Aa20*, the modified form of *vip3Aa1* gene, showed insecticidal effects against a wide host range including the corn earworm, the black cutworm, the fall armyworm, and the Western bean cutworm (Tohidfar and Khosravi, 2015).

10.4 ENGINEERED RESISTANCE TO FUNGAL PATHOGENS

Various transgenic crop plants have been developed for enhanced resistance to number of fungal pathogens using variety of strategies (Table 10.3).

10.4.1 USING GENES FOR CHITINASES AND GLUCANASES

In recent years, several laboratories have transformed plants with genes encoding β-1,3-glucanase and chitinase in order to develop transgenic crops with enhanced resistance to fungal diseases. Chitinase appears to have been used probably most frequently to obtain transgenics in various crops for effective control of fungal pathogens. The genes for **chitinase** from varied sources have been used to generate transgenics in grapevine (Yamamoto et al., 2000), rice (Datta et al., 2001; Kim et al., 2003; Kumar et al., 2003; Mei et al., 2004; Takakura et al., 2000), peanut (Rohini and Rao, 2001), cucumber (Kishimoto et al., 2002), tobacco (Carstens et al., 2003), potato (Chye et al., 2005; Moravčíková et al., 2004, 2007), cotton (Tohidfar et al., 2005), trifoliate orange (Mitani et al., 2006), strawberry (Vellicce et al., 2006), oilseed rape (Melander et al., 2006), taro (He et al., 2008a), pea (Hassan et al., 2009), finger millet (Ignacimuthu and Caesar, 2012), tomato (Girhepuje and Shinde, 2011), etc. Conversely, the gene for **glucanase** has been used to generate transgenics in tobacco (Cheong et al., 2000), flax (Wróbel-Kwiatkowska et al., 2004), rice (Akiyama et al., 2004), Indian mustard (Mondal et al., 2007), etc.

10.4.1.1 MODE OF ACTION OF CHITINASES AND GLUCANASES

Chitin constitutes one of the major components of the cell walls of many fungal pathogens such as *Rhizoctonia solani*, and it can be hydrolyzed by chitinase. β-1,3-Glucanase is known to degrade glucans which are also present in the fungal cell walls.

10.4.2 USING OTHER ANTIFUNGAL GENES

Apart from chitinases and glucanases, many other antifungal proteins and peptides such as thaumatin-like protein (TLP), ribosome-inactivating protein (RIP), *A. cepa* Antimicrobial protein (*Ace*-AMP1b), *Raphanus*

TABLE 10.3 Recent Developments of Transgenic Crop Plants Resistant to Fungal Pathogens.

Sl. No.	Transgene (Gene Name/Notation)	Source of Transgene	Crop and Cultivar	Resistance to	Method of Transformation	Reference
1.	*Chitinase*	*Streptomyces griseus* HUT6037	*Brassica juncea* (RAYA ANMOL)	Wide range of Fungal pathogens	ATMT	Ahmad et al. (2015)
2.	*HaGLP1* (Germin-like proteins)	Sunflower (*Helianthus annuus*)	*Arabidopsis thaliana* plants ecotype Columbia (Col-0, accession CS1092)	*Sclerotinia sclerotiorum* and *Rhizoctonia solani*	ATMT	Beracochea et al. (2015)
3.	*Chi11*	Rice	Finger millet (*Eleusine coracana* (L.) Gaertn. GPU45)	Leaf blast *Pyricularia grisea*	ATMT	Ignacimuthu et al. (2012)
4.	*KP4*	Totivirus (UMV4)	Maize (H99, B73)	*Ustilago maydis*	ATMT	Allen et al. (2011)
5.	*RsAFP2*	Radish (*Raphanus sativus*)	Chinese wheat variety Yangmai 12	*F. graminearum* and *R. cerealis*	Particle bombardment	Li et al. (2011a)
6.	*GbTLP1* (*Gossypium barbadense* thaumatin-like protein gene)	Cotton (*Gossypium barbadense* L.)	Tobacco (*Nicotiana tabacum*)	*Verticillium dahlia*, *Fusarium oxysporum*	ATMT	Munis et al. (2010)
7.	*MsDef* 1 (*Medicago sativa* defensin gene)	Alfalfa (*Medicago sativa*)	Tomato (Castle Rock)	*Fusararium oxysporum*	ATMT	Abdallah et al. (2010)
8.	*ThEn42* (endochitinase); *StSy* (stilbene synthase)	*Trichoderma harzianum*; Grape	Banana *(Musa cavendish*, AAA, cv. Grand Nain)	*Mycosphaerella fijiensis Botrytis cinerea*	Particle bombardment	Vishnevetsky et al. (2010)

TABLE 10.3 *(Continued)*

Sl. No.	Transgene (Gene Name/Notation)	Source of Transgene	Crop and Cultivar	Resistance to	Method of Transformation	Reference
9.	*RCC2* (rice chitinase gene)	Rice	Grape *(Vitis vinifera* L. cv. Neo Muscut)	*Uncinula necator Elisinoe ampelina*	ATMT	Yamamoto et al. (2010)
10.	*NPR1*	*Arabidopsis thaliana*	Cotton	*Verticillium dahliae* isolate TS2 *Fusarium oxysporum* f. sp. *vasinfectum*, *Rhizoctonia solani*, and *Alternaria alternata*	ATMT	Parkhi et al. (2010)
11.	*Pi-d2*	Rice (variety Digu)	Rice variety Lijiangxintuanheu Taipei 309, Nipponbare, and Zhonghua 9	Blast strain ZB15	ATMT	Chen et al. (2010)
12.	*Rs-AFP2* defensin gene	*Raphanus sativus*	(*Oryza sativa* L. cv. Pusa basmati 1)	*Magnaporthe oryzae Rhizoctonia solani*	ATMT	Jha et al. (2010)
14.	*Lc* (leaf color)	Maize (*Zea mays*)	Apple (*Malus* × *domestica* cv. "Holsteiner Cox")	*Venturia inaequalis*	–	Flachowsky et al. (2010)
15.	Wasabi defensin gene	*Wasabia japonica*	Rice (*Oryza sativa* cv. Sasanishiki)	*Magnaporthe grisea*	ATMT	Kanzaki et al. (2002)
16.	*Ech*42 (endochitinase)	*Trichoderma atroviride*	Apple (Marshall McIntosh)	*Venturia inaequalis*	ATMT	Bolar et al. (2001)
17.	p*PGIP* (pear fruit polygalacturonase inhibitor protein)	Pear	Tomato	*Botrytis cinerea*	ATMT	Powell et al. (2000)

ATMT, *Agrobacterium tumefaciens*-mediated transformation.

sativus antifungal protein (*Rs*-AFP2), *Dahlia merckii* antimicrobial protein (*Dm*-AMP1), *Mirabilis jalapa* antimicrobial protein (*Mj*-AMP2), etc. have been very useful in conferring fungal disease resistance in transgenic plants. Wheat plants have been successfully engineered to express ***Ace*-AMP1** to confer resistance against powdery mildew and Karnal bunt diseases (Roy-Barman et al., 2006). Rice plants have also been genetically engineered (GE) using the same gene to have a wide-spectrum increased resistance against both bacterial and fungal pathogens (Patkar and Chattoo, 2006). Transgenic *indica* rice expressing ***Mj*-AMP2** showed enhanced resistance to the rice blast fungus (Prasad et al., 2008). Enhanced resistance to rice blast and sheath blight was achieved in transgenic rice overexpressing ***Rs*-AFP2** (Jha and Chattoo, 2009) and ***Dm*-AMP1** (Jha et al., 2009). Transgenic maize plants expressing the Totivirus antifungal protein, **KP4**, is highly resistant to corn smut fungus *Ustilago maydis* (Allen et al., 2011). Sunflower germin-like protein ***Ha*GLP1** promotes ROS accumulation and enhances protection against fungal pathogens such as *Sclerotinia sclerotiorum* and *R. solani* in transgenic *Arabidopsis thaliana* (Beracochea et al., 2015). Transgenic apple plants overexpressing the ***Lc*** gene of maize showed increased resistance to apple scab caused by *Venturia inaequalis* and fire blight caused by *Erwinia amylovora*) but, also had some altered growth habit (Flachowsky et al., 2010). High resistance to *S. sclerotiorum* in transgenic soybean plants was achieved by expressing ***OXDC*** (oxalate decarboxylase) gene (Cunha et al., 2010). Expression of **defensin** gene from radish in transgenic wheat conferred increased resistance to *Fusarium graminearum* and *Rhizoctonia cerealis* (Li et al., 2011a). Plant defensins are cysteine-rich proteins that play an important role in defense against fungal pathogens. They have a strong potential to be used for engineering disease resistance in crops because of their potent antifungal activity.

10.4.2.1 MODES OF ACTION OF ACE-AMP1, RS-AFP2, DM-AMP1, MJ-AMP2, RIPS, AND TLPS

***Ace*-AMP1** is a lipid-transfer protein with sequence homology and structural analogies to plant nonspecific lipid-transfer proteins (ns-LTPs). In contrast to ns-LTPs isolated from radish and maize, Ace-AMP1 is unable to transfer phospholipids from liposomes to mitochondria due to the presence of aromatic residues in the domain corresponding to a lipid-binding pocket found in true lipid transfer proteins. However, the underlying mechanism of action is not very clear.

***Mj*-AMPs** have been identified in the seeds of *M. jalapa* and their structural and biological properties resemble those of defensins, a class of antimicrobial peptides. The Mj-AMPs exhibit a broad spectrum of antifungal activity since they are active against number of plant pathogens.

***Rs*-AFP2**, a plant defensin from the seeds of *R. sativus*, interacts with glucopyranosylceramide (GlcCer) present in the plasma membrane of fungal hyphae, leads to increased K^+ efflux and Ca^{2+} influx, membrane potential changes, and exerts antifungal activity against a broad spectrum of plant pathogenic filamentous fungi by causing hyperbranching and growth reduction of the hyphal tips.

***Dm*-AMP1**, also a plant defensing, interacts with mannosylated sphingolipids occurring in the outer plasma membrane and displays a broad-spectrum antifungal activity.

RIPs exhibit RNA *N*-glycosidase activity and depurinate the 28S rRNA of the eukaryotic 60S ribosomal subunit. This results in failure of binding of elongation factor-2 and cessation of protein synthesis by the altered ribosome.

TLPs are involved in the acquired systemic resistance and in response to biotic stress, causing the inhibition of hyphal growth and reduction of spore germination, probably by a membrane permeabilization mechanism and/or by interaction with pathogen receptors.

10.4.3 USING GENES FOR RESISTANCE SOURCES

Several *R* genes (resistance) associated with innate immunity of plants have been identified and isolated from various sources (Ballvora et al., 2002; Pel et al., 2009). The ***LpiO*** gene, one of the tested effectors from *Solanum* species, when co-expressed along with ***Rpi-blb1*** (as resistance gene) in *Nicotiana benthamiana*, it led to rapid identification of *Rpi-sto1* and *Rpi-pta1* as resistance genes to late blight (Vleeshouwers et al., 2008). Stacking of three broad-spectrum potato *R* genes (*Rpi*), ***Rpi-sto1*** (*Solanum stoloniferum*), ***Rpi-vnt1.1*** (*Solanum venturii*), and ***Rpi-blb3*** (*Solanum bulbocastanum*) in potato showed HR against pathogenic effects of *Phytophtora* (Zhu et al., 2012). Activating phytoalexins in plants against disease is another strategy for protection against pathogens. Genetic transformation of rice with **stilbene synthase gene** (*STS*) of Vst1, a key enzyme in synthesis of phytoalexin in grape improved resistance to *Piricularia orizae* (Coutos-Thévenot et al., 2001). Similarly, transgenic barley has been developed to resist powdery mildew (Liang et al., 2000).

Ectopic expression of *OsCDR1*, encoding a predicted aspartate protease, in Arabidopsis and rice conferred enhanced resistance against bacterial and fungal pathogens (Prasad et al., 2009). More recently, the role of mitogen-activated protein kinase (MAPK) cascade in the regulation of genes responsible for phytoalexin synthesis in rice in response to UV and blast infestation was reported (Wankhede et al., 2013). MAPK kinase is a key component of MAPK cascade. It was found that expression of phytoalexin in rice increased specifically under UV radiation. Subsequently, generation of transgenic rice lines expressing *OsMKK6* gene was shown to overproduce of phytoalexins. Resistance to several fungal and bacterial diseases has been obtained by overexpressing the nonexpressor of pathogenesis-related genes-1 (NPR1) in various plant species with apparently minimal or no pleiotropic effects. Resistance against various fungal pathogens and reniform nematode in transgenic cotton plants has been achieved by expressing Arabidopsis *NPR1* (Parkhi et al., 2010). Expression of this gene in transgenic cotton plants also enhanced resistance against *Thielaviopsis basicola*. These plants exhibited stronger and faster induction of most of these defense-related genes, particularly *PR1*, *thaumatin*, *glucanase*, *LOX1*, and *chitinase* (Kumar et al., 2013).

10.5 ENGINEERED RESISTANCE TO BACTERIAL PATHOGENS

A variety transgenic crop plants have been developed for improved resistance to number of bacterial pathogens using different strategies (Table 10.4).

10.5.1 USING ERFS

The expression of cotton ethylene responsive transcription factors (ERF) in tobacco showed exhibition of greater level of resistance to *Xanthomonas* (Champion et al., 2009). It is to be noted that bacterial blight is a destructive disease of domesticated rice (*Oryza sativa*) caused by the pathogen *Xanthomonas oryzae* pv. *oryzae*.

10.5.1.1 MODE OF ACTION OF ERF

The ERF have been demonstrated to have a role in controlling the expression of pathogenesis-related (PR) genes (Grennan, 2008).

TABLE 10.4 Recent Developments of Transgenic Crop Plants Resistant to Bacterial Pathogens.

Sl. No.	Transgene (Gene Name/Notation)	Source of Transgene	Crop and Cultivar	Resistance to	Method of transformation	References
1.	*hRPN*	*Erwinia amylovora*	Pears (*Pyrus communis cv.* "*Passe Crassane*")	*Erwinia amylovora*	ATMT	Malnoy et al. (2005)
2.	*FALL39* (precursor for the antimicrobial peptide LL-37)	*Homo sapiens*	Chinese cabbage (*Brassica rapa* cv. Osome)	*Psanthomonas carotovorum*	ATMT	Jung et al. (2012), Gudmundsson et al. (1995)
3.	*Bs2*	Pepper	Tomato (*Solanum lycopersicum)*	*Xanthomonas*	ATMT	Horvath et al. (2012), Tai et al. (1999)
4.	*PR1*	Grape vine (*Vitis* interspecific hybrid)	Tobacco (*N. tabacum* "Samsun")	*Pseudomonas syringae*	ATMT	Li et al. (2011c)
5.	*Pflp*	Sweet pepper (*Capsicum annuum*)	Banana cultivars "SukaliNdiizi" and "Nakinyika"	*Xanthomonas campestris*	ATMT	Namukwaya et al. (2011)
6.	*Xa21*	Rice (conserved)	Tomato (*Lycopersocin esculentum* cultivars Roma, Rio Grande, Pusa Ruby, Pant Bahr, and Avinash) Sweet orange (cvs. Hamlin, Natal, Pera, and Valencia)	*Pseudomonas solanacearum* *Xanthomonas axonopodis*	ATMT	Afroz et al. (2010), Mendes et al. (2010)
7.	*Indolicidin*	Cow (*Bovis* neutrophils)	Tobacco (*Nicotiana tabacum* var. Xanthi)	*Erwinia carotovora*	ATMT	Bhargava et al. (2007), Collinge et al. (2010)
8.	*CB* (Cecropin B)	*Hylophora cecropia*	Tomato (*S. lycopersicum* cv. Microtom	*Ralstonia solanacearum* *Xanthomonas campestris*	ATMT	Jan et al. (2010)

TABLE 10.4 *(Continued)*

Sl. No.	Transgene (Gene Name/Notation)	Source of Transgene	Crop and Cultivar	Resistance to	Method of transformation	References
9.	*EFR*	*Arabidopsis thaliana*	Tobacco (*N. benthamiana*) Tomato (*S. Lycopersicum* var. Moneymaker)	Broad spectrum bacterial resistance	ATMT	Lacombe et al. (2010), Fillatti et al. (1987), Horsch et al. (1985)
10.	*Rxo1*	Maize R gene	Rice	*Xanthomonas oryzae*	ATMT	Wally and Punja (2010), Zhao et al. (2005)
11.	*AtNPR1*	*Arabidopsis thaliana*	Duncan grapefruit Hamlin sweet orange Tomato (*Lycopersicon esculentum*)	*Xanthomonas citri Xanthomonas* sp.	ATMT	Zhang et al. (2010b), Lin et al. (2004)
12.	*βhth*(β-hordothionin gene)	Barley	Tobacco	*Pseudomonas solanaceaurm*	ATMT	Collinge et al. (2010), Charity et al. (2005)
13.	*Lc*	Maize (*Zea mays*)	Apple (*Malus domestica* cv. "Holsteiner Cox")	*Erwinia amylovora*	ATMT	Li et al. (2007a, 2007b)
14.	*SN1*	*S. chacoense*	Potato (*S. tuberosum* subsp. *tuberosum* cv. Kennebec)	*Erwinia arotovora*	ATMT	Almasia et al. (2008)
15.	*Chit33*, *chit42* (chitinase encoding genes)	*Trichoderma harzianum* strain CECT2431	Tobacco (*Nicotiana tabacum* var. Xhanti)	*Pseudomonas syringae*	ATMT	Mercedes et al. (2006)
16.	*GOX* (glucose oxidase)	*Aspergillus niger*	Rice (*Oryza sativa* L. ssp. Japonica cv. Taipei 309)	*Xanthomonas oryzae*	Particle bombardment	Kachroo et al. (2003)
17.	*D4E1*	Synthetic (plasmid ubi7-D4E1)	Poplar hybrid (*Populus tremula* × *P. alba* clone 717 IB 4)	*Agrobacterium tumefaciens* *Xanthomonas populi*	ATMT	Mentag et al. (2003)

TABLE 10.4 *(Continued)*

Sl. No.	Transgene (Gene Name/Notation)	Source of Transgene	Crop and Cultivar	Resistance to	Method of transformation	References
18.	*Asth1* (thionin)	Oat (*Avena sativa* cv. Zenshin	Rice seed (*Oryza sativa* cvs. Nipponbare and Chiyohonami)	*Burkholderia glumae Burkholderia plantarii*	ATMT	Iwai et al. (2002)
19.	*PPO-P1*	Potato (*Solanum tuberosum* cv. Katahdin)	Tomato (*Lycopersicon esculentum* Mill. cv. Money Maker)	*Pseudomonas syringae*	ATMT	Li and Steffens (2002)
20.	*Tsi1*	Tobacco (*Nicotiana tabacum* cv. Samsun NN and cv. Xanthi)	Hot pepper (*C. annuum* cv. Nockwang)	*Xanthomonas campestris*	ATMT	Shin et al. (2002), Park et al. (2001)
21.	*Msi-99* (magainin-2 analog)	Frog (*Xenopus laevis*)	Tobacco (*Nicotiana tabacum* var. Petit Havana)	*Pseudomonas syringae*	Particle bombardment	DeGray et al. (2001)
22.	*myp30* (magainin analog)	Frog (*Xenopus laevis*)	Tobacco (*Nicotiana tabacum* L. cv. Kentucky 14)	*Erwinia arotovora*	ATMT	Li et al. (2001), Zasloff (1987)
23.	*exp1* (*N*-oxoacyle-homoserine biosynthesis)	*E. carotovora*	Tobacco (*Nicotiana tabacum* cv. Samsun)	*Erwinia arotovora*	ATMT	Mäe et al. (2001)
24.	*Msarco* (Sarcotoxin IA gene)	*Sarcophega peregrina*	Tobacco (*Nicotiana tabacum* cv. Sumsun NN)	*Erwinia arotovora Pseudomonas syringae*	ATMT	Mitsuhara et al. (2000)
25.	*cecB* (cecropin B gene)	*Bombyx mori*	Rice (*Oryza sativa* L. Japonica cv. Nipponbare)	*Xanthomonas oryzae*	ATMT	Hiei et al. (1994)

ATMT, *Agrobacterium tumefaciens*-mediated transformation.

10.5.2 USING HAIRPIN GENES

The **harpin** (*hrp*) genes encode type III secretory pathways and are required by many phytopathogenic bacteria for pathogenesis on susceptible hosts and to elicit a hypersensitive response (HR) on nonhost or resistant host plants. Several studies indicated that enhanced **HrpNEa** levels in transgenic plants have effectively increased resistance to bacteria (Malnoy et al., 2005). Harpin NEa (HrpNEa) is encoded by the gene hrpN located on the chromosome of *Erwinia* causing the fire blight disease of apple. HrpNEa is a known inducer of systemic acquired resistance (SAR) in plants. Transgenic plants resistant to bacterial pathogens have been produced making use of this property.

10.5.2.1 MODE OF ACTION OF HAIRPIN PROTEINS

When hrp genes are secreted to the plant cells from bacterial pathogens, localized cell death happens through series of reactions like involving accumulation of reactive oxygen species (ROS).

10.5.3 USING TOXIN DETOXIFYING GENE FROM THE PATHOGEN

Another approach for engineering of plant resistance against bacterial disease is based on the transformation with a gene encoding a toxin-detoxifying enzyme from the pathogen itself. *Pseudomonas syringae* pv. *tabaci* produces the toxin called tabtoxin. In plants, tabtoxin is converted to tabtoxinine-β-lactam, which inhibits glutamine synthase leading to an accumulation of cytotoxic ammonia. The pathogen protects itself against the toxin by expression of the tabtoxin resistance gene (*ttr*), which is able to protect *P. syringae* by acetylating tabtoxin to an inactive form. The transgenic tobacco, expressing *ttr* gene, displayed a reduction in disease symptoms (Batchvarova et al., 1998).

Recently, a plant ferrodoxin-like protein (PFLP) was transferred to *Arabidopsis*. Expression of PFLP enhanced resistance to bacterial disease. PFLP is a photosynthetic type ferredoxin with an N-terminal signal peptide for chloroplast localization. Presence of PFLP in transgenic plants conferred resistance against bacterial disease (Lin et al., 2010). Expression of this gene in transgenic banana also enhanced resistance to wilt disease caused by *Xanthomonas* sp. (Namukwaya et al., 2012). Expression of a synthesized gene encoding cationic peptide Cecropin B in transgenic tomato plants

enhanced resistance against bacterial diseases (Jan et al., 2010). Resistance in the susceptibility to *Xanthomonas axonopodis* pv. *citri* was achieved in transgenic *Citrus sinensis* plants expressing rice ***Xa21*** (Mendes et al., 2010).

10.6 ENGINEERED RESISTANCE TO VIRAL PATHOGENS

A lot of transgenic crop plants have been developed for enhanced resistance to various viral pathogens using different strategies (Table 10.5).

Hundreds plant viruses have been identified till date, which cause various diseases and significant crop losses. Viral diseases are conventionally controlled using certified virus free planting material, eradicating infected plants and spraying chemicals against virus vectors. Additionally, coat protein-mediated resistance to viruses has been one of the successes of plant genetic engineering. Several major crop plants have been engineered using this approach, to resist important viral pathogens. The resistant cultivars that have been commercialized include potato event HLMT15-15, which is tolerant to PYV (Potato Y Virus) or potato event RBMT21-350, which is resistant to PLRV (Potato Leaf Roll Virus) (James, 2013). Transgenic tobacco expressing defective cucumber mosaic virus (CMV) replicase-derived dsRNA was produced to achieve high level of resistance (Ntui et al., 2014). The ability of the sense and antisense RNA for the replication-associated protein encoded by *AC1* (African cassava mosaic virus replication-associated) or *C1* gene of Gemini viruses was also assessed to protect plants against viral infection (Zhang et al., 2005). It was also reported that presence of defective movement proteins in the transgenic plants conferred resistance to viruses, as they are associated with their growth and development in planta (Hallwass et al., 2014; Peiró et al., 2014).

10.7 ENGINEERED RESISTANCE TO NEMATODES

Nematodes, which are not readily controlled by pesticides or other control options, cause an estimated $118b annual loss to world crops (McCarter, 2009). Although natural resistance genes are unavailable for many crops to plant breeders, transgenic plants can provide significant amount of nematode resistance for such crops. Approaches, such as limiting use of dietary protein uptake by nematodes from the crops or by preventing root invasion without a direct lethality or use of RNA interference (RNAi) can take control over wide range of nematodes. A variety of transgenic crop plants have been

TABLE 10.5 Recent Developments of Transgenic Crop Plants Resistant to Viral Pathogens.

Sl. No.	Transgene (Gene Name/Notation)	Source of Transgene	Crop and Cultivar	Resistance to	Method of transformation	Reference
1.	Coat protein gene, V2 gene and replication-associated gene	*Tomato yellow leaf curl virus*-Oman (TYLCV-OM)	*Tomato (Solanum lycopersicum* L.)	TYLCV-OM	ATMT	Ammara et al. (2015)
2.	*pC5*, *pC6*	RGSV	Japonica rice (Nipponbare)	Rice grassy stunt virus (RGSV)	ATMT	Shimizu et al. (2012)
3.	*CP*	Tobacco Streak Virus	Sunflower (*Helianthus annuus* L.)	Tobacco Streak Virus	ATMT	Pradeep et al. (2012)
4.	*Rep* (Replication initiation protein)	*Banana bunchy top virus* (BBTV)	Banana (*Musa* spp.)	*Banana bunchy top virus* (BBTV)	ATMT	Shekhawat et al. (2012)
5.	Capsid protein	AMV BPMV SMV	Soybean (Throne)	*Alfalfa mosaic virus* (AMV), *Bean pod mosaic virus* (BPMV), and *Soybean mosaic virus* (SMV)	ATMT	Zhang et al. (2011b)
6.	FL-CP (coat protein of *Cassava Brown Streak Uganda* virus)	*Cassava Brown Streak Uganda* virus	Cassava (*Manihot esculenta* Crantz)	*Cassava Brown Streak Uganda* virus	ATMT	Yadav et al. (2011)
7.	*CP*	*Soyabean Dwarf virus*	Soybean	Soybean dwarf virus	Particle bombardment	Tougou et al. (2007)
8.	*TOGT* (glucosyl transferase)	Tobacco	Tobacco plants (*N. tabacum* cv. Samsun NN)	Potato virus Y	ATMT	Matros and Mock (2004)
9.	*CP*	*Citrus tristeza virus strain T-305*	Mexican lime (*Citrus aurantifolia* Swing.)	*Citrus tristeza* virus	ATMT	Domínguez et al. (2000)

ATMT, *Agrobacterium tumefaciens*-mediated transformation; CP, coat protein.

developed for improved resistance to various nematode pests using diverse kind of strategies (Table 10.6).

10.8 TRANSGENIC DEFENSE BASED ON PEPTIDES AND PROTEINS

In the GE plants, the feeding of nematodes is targeted and it involves overexpression of cysteine proteinase inhibitors (cystatins) that interfere with intestinal digestion of their dietary protein taken in from the plant. **Cystatins** have a proven wide value against a range of nematodes with differing modes of parasitism (Fuller et al., 2008). A cystatin from the tropical root crop, taro, when expressed in tomato conferred resistance against *Meloidogyne* (Chan et al., 2010). The acetylcholinesterase-inhibiting peptide when expressed in *A. thaliana* suppressed the number of female *Heterodera schachtii* (beet cyst nematode) by more than 80%, while in transgenic potato plants, its expression resulted in almost 95% resistance to *Globodera pallida* (Lilley et al., 2011). When nicotinic acetylcholine receptors (nAChR)-binding peptide was expressed in transgenic potato plants that secreted the peptide from their root tips, it resulted in an effective resistance up to 77% against potato cyst nematode in both containment glasshouse and field trials (Atkinson et al., 2012).

10.8.1 MODE OF ACTION nAChRs-BINDING PEPTIDE

The nAChR-binding peptide is taken up from the environment by certain chemosensory sensilla within the anterior amphidial pouches and it undergoes retrograde transport along some chemoreceptive neurons to their cell bodies and a limited number of interneurons. Chemoreception was only impaired when that transport had been completed.

10.9 TRANSGENIC DEFENSE BASED ON RNAi

In the RNAi process, double-stranded RNA (dsRNA) triggers silencing of specific target genes through mRNA degradation. RNAi in *A. thaliana* plants expressing dsRNA from hairpin and/or inverted repeat constructs reduced transcript abundance of targeted **parasitism genes** in *H. schachtii* (Patel et al., 2008, 2010; Sindhu et al., 2009). This led to a significant reduction in female members (between 23% and 64%) with considerable variation

TABLE 10.6 Recent Developments of Transgenic Crop Plants Resistant to Nematode Pests.

Sl. No.	Transgene (Gene Name/ Notation)	Source of Transgene	Crop and Cultivar	Resistant to	Method of Transformation	References
1.	*16D10*	Conserved root-knot nematode (RKN) gene *16D10*	Wine grape (*V. vinifera* cv. Chardonnay)	Root-knot nematode	ATMT	Yang et al. (2013)
2.	CCII (cystatin)	Maize kernel	Plantain (*Musa AAB* cv. Gonja Manjaya)	*Radopholus similis*, *Helicotylenchus multicinctus*	ATMT	Roderick et al. (2012)
3.	*OC-I* (*Oryza* cystatin I)	Rice Nihonbare (*Oryza sativa* L. *japonica*)	Sweet potato (cv. Xushu 18 and cv. Lizixiang) Alfalfa (*Medicago sativa)* hybrid Regen-SY	*Pratylenchus penetrans*	ATMT	Gao et al. (2011d), Abe et al. (1987)
4.	*NPR1*	Arabidopsis	Tobacco Cotton (*Gossypium hirsutum* cv. Coker 312)	*Meloidogyne incognita*	ATMT	Priya et al. (2011), Parkhi et al. (2010), Sunilkumar and Rathore (2001)
5.	*CeCP1*	Taro (*Colocasia esculenta*) Kaosiang No. 1	Tomato (*Solanum lycopersicum* Mill.) cultivar CLN2468D	*Meloidogyne incognita*	ATMT	Chan et al. (2010)
6.	*Cry5* B	Plant codon-modified from *Bacillus thuringiensis Cry5B*	Tomato (*Lycopersicon esculentum* Mill. var. Rutgers select)	*Meloidogyne incognita*	ATMT	Li et al. (2008)
7.	*CaMi*	Pepper (*Capsium annum* L. (line PR205)	Tomato	Root-knot nematode	ATMT	Chen et al. (2007)

TABLE 10.6 *(Continued)*

Sl. No.	Transgene (Gene Name/ Notation)	Source of Transgene	Crop and Cultivar	Resistant to	Method of Transformation	References
8.	*Cry6 A*	Plant codon-modified from *Bacillus thuringiensis Cry6A*	Tomato	*Meloidogyne incognita*	ATMT	Li et al. (2007a, 2007b)
9.	*Mi-1.2*	Wild-type tomato (*L. peruvianum*)	Eggplant (*S. melongena* cv. HP83) Tomato (*L. esculentum* cv. Moneymaker)	*Meloidogyne javanica*	ATMT	Goggin et al. (2006), Milligan et al. (1998)
10.	*PIN2* (protease inhibitor)	Potato	Wheat (*T. durum* PDW215)	*Heterodera avenae*	ATMT	Vishnudasan et al. (2005)
11.	*Hero A*	Tomato	Tomato line LA1792	Potato cyst nematode resistance		Sobczak et al. (2004), Ernst et al. (2002)
12.	*OC-II* (*Oryza* cystatin II)	Rice Nihonbare (*Oryza sativa* L. *japonica*)	Alfalfa (*Medicago sativa*) hybrid Regen-SY	*Pratylenchus penetrans*	ATMT	Samac and Smigocki (2003), Abe et al. (1987)
13.	*GAD (Glutamate decarboxylase)*	Chimeric or mutant version of tobacco *GAD*	Tomato (*Nicotiana tabacum* L. cvs. Delgold and Samsun NN	*Meloidogyne incognita*	ATMT	McLean et al. (2003)

ATMT, *Agrobacterium tumefaciens*-mediated transformation.

between lines. In chimeric soybean, RNAi for **fibrilin** gene of *H. glycines* resulted in variable and nonsignificant effects (Li et al., 2010). Soybean composite plants derived from hairy root cultures engineered to silence either of **two ribosomal proteins, a spliceosomal protein or synaptobrevin**, of *H. glycines* by RNAi resulted in 81–93% reduction female members in the roots of transgenic plants (Klink et al., 2009). Similarly, by targeting mRNA splicing factor **prp-17** or an uncharacterized gene ***cpn-1***, high reduction in egg production was achieved (Li et al., 2010). A high level of resistance to root-knot nematode was also achieved by targeting a **parasitism gene** expressed in the subventral gland cells of *Meloidogyne incognita*. When dsRNA complementary to the ***16D10*** gene was expressed in transgenic *A. thaliana*, the resulting lines displayed a significant reduction (63–90%) in the number of galls and their size with a corresponding reduction in total egg production. A broad spectrum of resistance against *M. incognita*, *Meloidogyne javanica*, *Meloidogyne arenaria*, and *Meloidogyne hapla* was achieved since there is a high level of homology between the 16D10 sequences of different *Meloidogyne* species. Significant reduction in egg masses were achieved in transgenic Arabidopsis (~60%) and tobacco (~70%) expressing siRNA against the secreted peptide (16D10) of *Meloidogyne chitwoodi* (Dinh et al., 2014). Almost complete resistance to *Meloidogyne* infection was reported in tobacco plants expressing dsRNA corresponding to **splicing factor or integrase** (Yadav et al., 2006) and of four genes targeted in transgenic soybean roots with reduction of gall number by more than 90% (Ibrahim et al., 2011). However, all host-delivered RNAi targeting of *Meloidogyne* genes did not result in a resistance phenotype. Partial silencing of ***MjTis11***, a putative transcription factor of *M. javanica* did not significantly affect either nematode development or fecundity (Fairbairn et al., 2007). Crossing transgenic lines expressing more than a single line of engineered defense provided higher levels of resistance to *M. incognita* than either parent plants (Charlton et al., 2010). Such additive effect may raise the efficacy and durability of RNAi-based defenses. RNAi against nematode effector protein gene (***NULG1a***) from *M. javanica* in Arabidopsis reduced nematode population in the roots by 80% (Lin et al., 2013).

10.10 GENETIC ENGINEERING FOR TOLERANCE TO ABIOTIC STRESS

Plant growth and final yield are often affected due to abiotic stresses such as salt, drought, flooding, extreme temperature, and oxidative stresses. One

of the principal causes of crop failure worldwide is abiotic stress causing a reduction in average yields of most crops by more than 50% (Bray et al., 2000). It is predicted that more than half of the arable land will be affected with salts by 2050. The condition is terrifically alarming and necessitates increasing crop productivity. Conversely, responses of plants under abiotic stresses are complex, involving multiple genes with additive effects (Fig. 10.1) In the field of plant genetic engineering, major emphasis has been given to introduce genes encoding compatible organic osmolytes, heat shock proteins, plant growth regulators, late embryogenesis abundant (LEA) proteins, and transcription factors responsible for activation of a subset of gene expressions. Agricultural productivity could be increased significantly if designer crops can be produced to cope up with environmental stresses. It is also remarkable that with changing environmental conditions in the recent past, most of the efforts have been diverted toward generation of abiotic stress-tolerant transgenic plants.

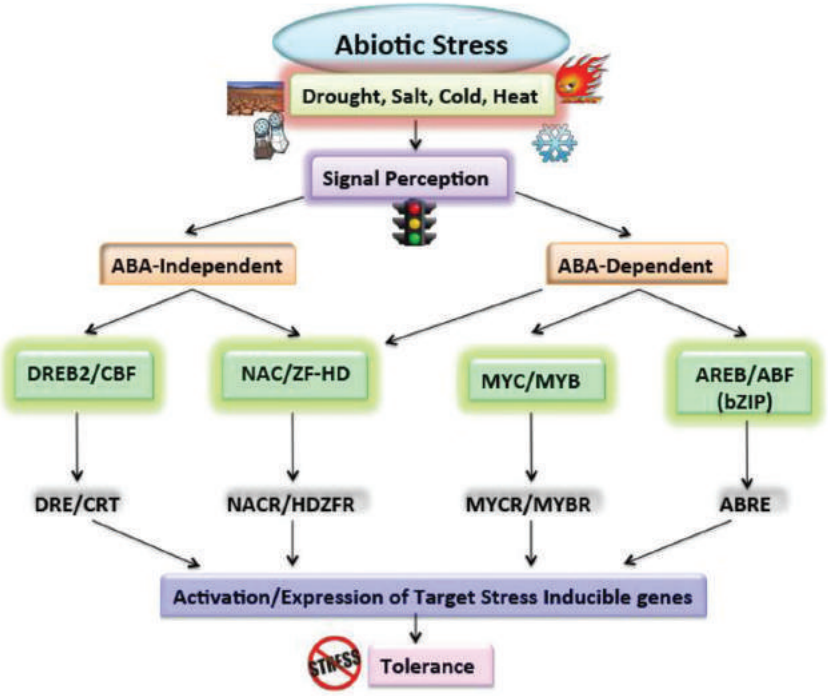

FIGURE 10.1 A schematic explanation of signal transduction pathways and its components involved in gene expression under abiotic stress. Under stress, ABA biosynthesis activates two regulatory ABA-dependent gene expressions: MYC/MYB and bZIP/ABRE. ABA-independent signal transduction pathway involves ERF family of transcription factors.

10.10.1 USING GENES FOR SYNTHESIS OF OSMOTIC PROTECTANTS

It is well known that some organic solutes play an important role in induction of drought tolerance (Ashraf and Foolad, 2007). A number of genes play an important role in the synthesis of osmoprotectants in stress-tolerant plant like proline, glycinebetaine, polyamines, mannitol, trehalose, and galacinol, which are known to accumulate during osmotic adjustment. Some of the genes required for synthesis of such osmotic protectants have been used to engineer crop plants for improved tolerance to various abiotic stress conditions.

10.10.2 USING GENES FOR SYNTHESIS OF GLYCINE BETAINE

Introduction of a gene encoding **choline oxidase** (*codA*) in *Brassica juncea* (Parsad et al., 2000) and rice (Mohanty et al., 2002) resulted in increased tolerance to salt stress due to enhanced levels of glycine betaine. Similarly, increased accumulation of chloroplastic glycine betaine in tomato engineered using the same gene raised the level of stress tolerance (Park et al., 2007). When the *CMO* gene encoding **choline monooxygenase** was expressed in tobacco plants, it resulted in improved tolerance to drought (Shen et al., 2002), and in transgenic rice, it resulted in enhanced tolerance to salt and temperature stress (Shirasawa et al., 2006). Similarly, transgenic cotton (*Gossypium hirsutum*) plants expressing ***AhCMO*** accumulated 26–131% more glycine betaine and showed tolerance to salinity (Zhang et al., 2009). **Choline dehydrogenase** encoding gene *betA* when expressed in maize conferred the plants with higher drought tolerance (Quan et al., 2004), and when expressed in cotton, it resulted in enhanced tolerance to chilling conditions (Zhang et al., 2012). Expression of **choline oxidase** gene *COX* in rice resulted in improved tolerance to saline conditions (Su et al., 2006), whereas in potato, it conferred higher tolerance to oxidative, drought, and salt stress conditions (Ahmad et al., 2008).

10.10.3 USING GENES FOR SYNTHESIS OF PROLINE

Soybean (De Ronde et al., 2004) and petunia (Yamada et al., 2005a) have been GE to produce proline and the transgenics were found to demonstrate enhanced tolerance to heat and drought. Transgenic tobacco plants

expressing *P5CR* encoding **pyrroline-5-carboxylate synthase** showed tolerance to drough.

10.10.4 USING GENES FOR SYNTHESIS OF MANNITOL AND TREHALOSE

Expression of *TPS1* encoding **trehalose-6-phosphate synthase** in tobacco (Karim et al., 2007) and rice (Jang et al., 2003) resulted in increased drought tolerance, whereas in tomato (Cortina and Culiáñez-Macià, 2005), it caused higher tolerance to both, oxidative, drought, and salinity stress. Engineering other trehalose biosynthesis genes such as ***otsA*** and ***otsB*** also improved drought tolerance in transgenic plants (Garg et al., 2002). Transgenic expression of an ***mtID*** involved in the biosynthesis of manitol developed higher tolerant lines against oxidative stress, drought, and salinity stress (Abebe et al., 2003).

10.10.5 USING GENES ENCODING LATE EMBRYOGENESIS ABUNDANT PROTEINS

LEA proteins get accumulated in plants under stress and help them to maintain structure of cellular membranes, ionic balance, water binding, and they also seem to act as molecular chaperons under drought stress conditions. Thus, they are also believed to have vital role in stress tolerance of plants (Babu et al., 2004; Gosal et al., 2009).

When LEA gene ***HVA1*** was transformed into rice and bread wheat, it increased tolerance to drought in both the cases (Sivamani et al., 2000). Expression of LEA gene ***ME-lea n4*** in transgenic *Lactuca sativa* (Park et al., 2005a) and *Brassica camesptris* enhanced drought tolerance in either case (Park et al., 2005b). Transgenic expression of ***PMA1959*** and ***PMA80*** LEA in rice resulted in enhanced dehydration tolerance (Cheng et al., 2002). Overexpression of ***OsLEA 3-1*** in transgenic rice also caused increased tolerance to drought stress under field conditions (Xiao et al., 2007).

10.10.6 USING GENES ENCODING TRANSCRIPTION FACTORS

Transcription factors are DNA-binding proteins required to transcribe and regulate genes. Researchers have been continuously putting their efforts

to identify, characterize, clone, and use the know-how to engineer crop plants to protect them against different stress conditions. Overexpression of ***ZmDREB2A*** in maize (Qin et al., 2007) and groundnut (Bhatnagar-Mathur et al., 2009) promoted stress tolerance. Likewise, through overexpression of ***AtDREB*** in wheat (Pellegrineschi et al., 2004), rice (Kim and Kim, 2009), and groundnut (Bhatnagar-Mathur et al., 2014), enhanced drought tolerance was achieved. Transgenic expression of ***OsDREB*** in rice was found to increase activity of genes involved in the tolerance of drought, high salt, and cold response, whereas overexpression of ***GhDREB1*** in tobacco showed significant chilling tolerance only (Shan et al., 2007). Similarly, when *Arabidopsis* ***DREB1B*** was constitutively expressed in transgenic potato, it enhanced drought and freezing tolerance (Movahedi et al., 2012). Stress-inducible expression of ***GmDREB1*** conferred salt tolerance in transgenic alfalfa (Jin et al., 2010). Drought and salt tolerance was improved in transgenic Arabidopsis expressing ***NAC*** (NAM, ATAF, and CUC) transcriptional factor from *Arachis hypogea* (Liu et al., 2011). Transgenic rice plants expressing ***OsNAC*** in the root system improved drought tolerance under field conditions (Jeong et al., 2010).

10.10.7 USING METAL TOLERANCE

Enhancement of ***TaALMT1*** expression helped increasing Al^{3+} resistance of wheat (Pereira et al., 2010). This was the first report of a major food crop being stably transformed for greater Al^{3+} resistance. Transgenic overexpression of *CcMT1* gene in *A. thaliana* has shown increased plant biomass and chlorophyll content as well as low content of copper and cadmium metals in shoots and roots compared with wild-type plants under copper and cadmium metal stress (Sekhar et al., 2011). Transgenic rice plants expressing cadmium tolerance gene **yeast cadmium factor (*YCF1*)** has been developed (Islam and Khalekuzzaman, 2015). This transgenic rice plants have the ability to uptake cadmium from soil, and it is stored into cell vacuoles and protects rice grain from cadmium. This way soil also will be free from cadmium through the process of phytoremediation. Overexpression of the same gene caused enhancement of heavy metal tolerance in *B. juncea* (Bhuiyan et al., 2011). Overexpression of the *Tamarix hispida* ***ThMT3*** gene not only increased copper tolerance but also the induction of adventitious root in *Salix matsudana* (Yang et al., 2015). The transgenic tobacco plants expressing a *Trichoderma virens* ***GST*** are more tolerant to cadmium, but it did not enhance accumulation of the metal in the plant biomass (Dixit et al., 2011).

Enhanced heavy metal tolerance was achieved and accumulation was also demonstrated in transgenic sugar-beet plants expressing *Streptococcus thermophilus* ***StGCS-GS*** in presence of cadmium, zinc, and copper (Liu et al., 2015a). Some of transgenic crop plants developed for improved tolerance to metal ions are listed in Table 10.7.

10.11 ENGINEERING TOLERANCE TO SALT STRESS

Soil salinity is one of the major constraints in today's agriculture, affecting an estimated 45 million hectares of irrigated land and is expected to increase due to global climate changes and as a consequence of various agricultural practices (Munns and Tester, 2008). The deleterious effects of salt stress include slower growth rates, reduced tillering, and abnormal reproductive development, which, in turn, affect crop yield. Various mechanisms of salinity tolerance of crops such as ion exclusion, osmotic tolerance, and tissue tolerance can be genetically improved.

Osmotic tolerance is regulated by long-distance signals that reduce shoot growth and is triggered before Na^+ accumulation in the shoots. Thus, when ***TmHKT1;5-A*** was introgressed from *Triticum monococcum* into a durum wheat, it resulted in a significant improvement in grain yield under high salt stress by increasing its ion exclusion (James et al., 2012; Munns et al., 2012). Na^+ and Cl^- transport processes in roots reduce the accumulation of toxic concentrations of Na^+ and Cl^- within leaves during ion exclusion. Both, high affinity **potassium transporter (*HKT*)** gene family and the **salt overly sensitive (*SOS*)** pathway have been implicated in having a crucial role in regulating Na^+ transport within a plant system. Genetic engineering of expression of these genes has been frequently reported to alter accumulation of Na^+ in the shoot. However, transgenic approaches to improve salinity tolerance using ***HKT1***s have not been so successful. *HKT2* has been reported to increase salinity tolerance, although not through Na^+ exclusion (Mian et al., 2011). Overexpression of genes in the SOS pathway has been reported to result in increased salt tolerance in transgenic Arabidopsis. Constitutive expression of **CaXTH3, a hot pepper xyloglucan endotransglucosylase/hydrolase** enhanced tolerance to salt in transgenic tomato plants (Choi et al., 2011). Additionally, it also increased drought tolerance in these plants. Overexpression of **osmotin gene** in tomato conferred tolerance to salt and drought. The transgenic plants showed significantly higher relative water content, chlorophyll content, proline content, and leaf expansion than the wild-type plants under stress conditions (Goel et al., 2010). Ectopic

TABLE 10.7 Recent Developments of Transgenic Crop Plants Tolerant to Metal Ions.

Sl. No.	Transgene (Gene Name/Notation)	Source of Transgene	Crop and Cultivar	Tolerance to	Method of transformation	Reference
1.	*YCF1*	*S. cerevisiae*	Rice, BRRI dhan29	Cadmium	ATMT	Islam and Khalekuzzaman (2015)
2.	*ThMT3* (Type III metallothionein gene)	*Tamarix hispida*	*S. matsudana Koidz.* var. *matsudana*	Copper	ATMT	Yang et al. (2015)
3.	*StGCS-GS* (γ-glutamylcysteinesynthetase-glutathione synthetase)	*Streptococcus thermophilus*	*Beta vulgaris* L. US-8916	Copper, zinc, cadmium	ATMT	Liu et al. (2015a)
4.	*YCF1* (yeast cadmium factor 1)	*S. cerevisiae*	*Brassica juncea* cv. *Rai-5*	Cadmium	ATMT	Bhuiyan et al. (2011)
5.	*LMT1* (aluminum metallothionein *TaA*)	Wheat	*Triticum aestivum* Bob White 26	Aluminum	Particle bombardment	Pereira et al. (2010)
6.	*TvGST* (glutathione transferase gene)	*Trichoderma virens*	*Nicotiana tabacum*	Cadmium	ATMT	Dixit et al. (2011)
7.	*MdSPDS1* (spermidine synthase)	*Malus sylvestris*	*Pyrus communis* L. "Ballad"		ATMT	Wen et al. (2008)
8.	*merA18* (bacterial mercury detoxification gene)	*Arabidopsis thaliana*	*Liriodendron tulipifera*	Mercury	Microprojectile bombardment	Rugh et al. (1998)

ATMT, *Agrobacterium tumefaciens*-mediated transformation.

expression of the same gene led to enhanced salt tolerance in transgenic chilli pepper (*Capsicum annum* L.) (Subramanyam et al., 2011). Overexpression of ***TaNHX2*** enhanced salt tolerance of "composite" and whole transgenic soybean (Cao et al., 2011) and tomato (Yarra et al., 2012) plants. Similarly, transgenic sweet potato plants expressing ***LOS5*** gene were developed to tolerate salt stress (Gao et al., 2011a). Stress-inducible transgenic expression of *GmGSTU4* shaped the metabolome of transgenic tobacco plants toward increased salinity tolerance (Kissoudis et al., 2015). Transgenic overexpression of mutagenized version of **Δ^1-pyrroline-5-carboxylate synthetase (*P5CS*)** in transgenic *indica* rice resulted in enhanced proline accumulation and salt stress tolerance (Kumar et al., 2010). ***SUV3*** overexpressing transgenic rice plants are reported not only known to be salt tolerant, but it also conserved physicochemical properties and microbial communities of rhizosphere (Sahoo et al., 2015). Some of transgenic crop plants developed for improved tolerance to salt stress are listed in Table 10.8.

Overexpression of the **ethylene-responsive factor gene *BrERF4*** from *Brassica rapa* increased tolerance to salt and drought in Arabidopsis plants, and it also affected the growth and development significantly (Seo et al., 2010). Similarly, overexpression of ***GsGST*** encoding glutathione-S-transferase, from wild soybean (*Glycine soja*) enhanced drought and salt tolerance in transgenic tobacco (Ji et al., 2010). Expressing a ***BADH*** gene from *Atriplex micrantha* enhanced salinity tolerance in transgenic maize (Di et al., 2015). Conversely, constitutive and stress-inducible overexpression of a native **aquaporin gene (*MusaPIP2;6*)** in transgenic banana plants demonstrated its pivotal role in salt tolerance (Sreedharan et al., 2015).

In case of ***tissue tolerance***, salt is compartmentalized at the cellular and intracellular level under highly saline condition. The mechanisms contributing to tissue tolerance include synthesis of compatible solutes, accumulation of Na^+ in the vacuole, and production of enzymes catalyzing detoxification of ROS. Increasing the abundance of proteins involved in the synthesis of compatible solutes (such as proline and glycinebetaine), vacuolar Na^+/H^+ antiporters (NHX), vacuolar H^+ pyrophosphatases (e.g., AVP1), and enzymes responsible for the detoxification of ROS have had differing levels of success in improving tolerance of crop plants to salinity (Roy et al., 2014). Enhanced salt tolerance in transgenic wheat expressing a **vacuolar Na^+/H^+ antiporter** gene was observed. However, often reports do come about under performance of transgenic plants and low salt stress conditions. Such kind of effects may probably be regulated by use of stress-inducible promoters.

Ca^{2+} mediates many aspects of plant growth and development. Ca^{2+} signaling cascade is activated upon perception of environmental cues on the

TABLE 10.8 Recent Developments of Transgenic Crop Plants Tolerant to Salt Stress.

Sl. No.	Transgene (Gene Name/Notation)	Source of Transgene	Crop and Cultivar	Method of Transformation	Reference
1.	*BADH,* betaine aldehyde dehydrogenase gene	*Atriplex micrantha*	Maize elite inbred lines, Zheng58 and Qi319	ATMT	Di et al. (2015)
2.	*AcPIP2*, plasma membrane Aquaporin gene	*Atriplex canescens*	*Nicotiana benthamiana*, *Arabidopsis thaliana Col-1*	ATMT	Li et al. (2015)
3.	*OCPI2*, chymotrypsin protease inhibitor	*Oryza sativa PB-1*	*Arabidopsis thaliana Columbia*	ATMT	Tiwari et al. (2015)
4.	WT-*PhyA S599A-PhyA*	*Avena sativa*	Zoysia grass (*Zoysia Japonica* Steud.) Creeping bentgrass (*Agrostis stolonifera* L.)	ATMT	Gururani et al. (2015)
5.	*MusaPIP2;6,* aquaporin gene	Banana cv. *Karibale Monthan*	Banana cv. *Karibale Monthan*	ATMT	Sreedharan et al. (2015)
6.	*SOS2*, salt overly sensitive gene	*Populus trichocarpa*	Aspen hybrid clone Shanxin Yang (*Populus davidiana* × *Populus bolleana*)	ATMT	Yang et al. (2015)
7.	*LCY-ε*, lycopene ε-cyclase	*Ipomoea batatas* cv. *Yulmi* wild type	*Ipomoea batatas*	ATMT	Kim et al. (2012)
8.	*AtNHX1*, Na^+/H^+ antiporter gene	*Arabidopsis thaliana*	*Zea mays*	ATMT	Li et al. (2013)
9.	*JcDREB*, stress responsive DNA binding transcription factor	*Jatropha curcas*	*Arabidopsis thaliana*	ATMT	Yu et al. (2013)
10.	(*DREB1B*), dehydration-responsive element-binding factor 1	Arabidopsis	*Solanum tuberosum* cv. Desiree	ATMT	Movahedi et al. (2012)

TABLE 10.8 *(Continued)*

Sl. No.	Transgene (Gene Name/Notation)	Source of Transgene	Crop and Cultivar	Method of Transformation	Reference
11.	*TaWRKY 19 TaWRKY2,* stress-responsive *WRKY* gene	*Triticicum aestivum* L. cultivar Xifeng 20	*Arabidopsis thaliana* Col-0	ATMT	Niu et al. (2012)
12.	Osmotin gene	*Nicotiana tabaccum* cv. Wisconsin 38	*Capsicum annuum*	ATMT	Subramanyam et al. (2011)
13.	*CaXTH3*, bacterial mercury detoxification gene	Hot pepper	*Solanum lycopersicum* cv. Dotaerang	ATMT	Choi et al. (2011)
14.	*AhNAC*, NAC gene	*Arachis hypogea*	*Arabidopsis thaliana* (Col-1)	ATMT	Liu et al. (2010)
15.	*GST*, Glutathione transferase gene	*Suadea salsa*	Arabidopsis	ATMT	Qi et al. (2010)
16.	*ADC1 ADC2*, arginine decarboxylase	*Tritordeum*	*Oryza sativa*	Particle bombardment	Liu et al. (2007)

ATMT, *Agrobacterium tumefaciens*-mediated transformation.

cell membrane, resulting in the regulation of gene expression and protein activities (Batistič and Kudla, 2012). In crop plants, such as rice, apple, barley, tobacco, and tomato, overexpression of genes encoding proteins in Ca^{2+} signaling pathways have been shown to improve the growth of the plants during salt stress.

A gene for **monohydroascorbate reductase** (MDAR) has been isolated from halophytic mangrove *Avicennia marina* and expressed under CaMV 35S promoter in tobacco plants following ATMT (Kavitha et al., 2010). Overexpression of *Am-MDAR* was found to increase salt tolerance in transgenic tobacco compared to untransformed control plants. The protein was localized in the chloroplast of transgenic tobacco as presence of a transit peptide at the N terminus of *Am*-MDAR already suggested. Upregulation of *Am-MDAR* under stress conditions such as salt stress, H_2O_2, high light intensity, and iron load and its localization in the chloroplast point toward a crucial role for this protein in the stress tolerance of *A. marina.*

Various stress factors produce ROS, which can cause damage to plants. Enzymes such as superoxide dismutase, catalase, and peroxidase have the capacity to act as antioxidants and neutralize the effect of ROS (Ahmad et al., 2010). ***OsMT1a*** overexpressing transgenic rice plants, which had enhanced ascorbate peroxidase (APX) activity, showed enhanced tolerance to water limited conditions (Yang et al., 2009). Conversely, chilling tolerance at the booting stage has been increased in rice by transgenic overexpression of the **APX gene, *OsAPXa*** (Sato et al., 2011). Cytosolic APX has been found to help plants acclimatize better under conditions heat and drought stress (Koussevitzky et al., 2008). The increased production of **glutathione reductase (GSH)** can be triggered by the stimulation of pathways involved in the metabolism of sulfur and cysteine. Manipulation of improvement of tolerance to oxidative stress was observed with engineering of GSH biosynthesis pathway (Sirko et al., 2004). Increased salinity tolerance and better growth were reported in transgenic tobacco plants by overexpressing glyoxalate pathway enzymes. In this case, increased GSH content maintained higher reduced to oxidized GSH ratio (GSH:GSSG) and minimized lipid peroxidation (Yadav et al., 2005). Overexpression of ***TaEXPB23***, a wheat expansin gene, improved oxidative stress tolerance in transgenic tobacco plants (Han et al., 2015).

10.12 ENGINEERING DROUGHT TOLERANCE

Drought is one of the prime abiotic stresses in the world. Crop yield losses due to drought stress are considerable. A variety of approaches have been used

to alleviate the problem of drought. Conventional plant breeding or genetic engineering seems to be an efficient and economic means of tailoring crops to enable them to grow successfully in drought-prone environments. It has been observed that in all above approaches discussed in order of achieving tolerance to salt stress and osmotic protection, the resultant transgenics are also found to be tolerant to drought conditions. *AcPIP2* encoding a plasma membrane intrinsic protein from halophyte *Atriplex canescens*, enhanced plant growth rate and abiotic stress tolerance when overexpressed in *A. thaliana* (Li et al., 2015). ***OsSDIR1*** (*O. sativa* SALT-AND DROUGHT-INDUCED RING FINGER 1) overexpression greatly improved drought tolerance in transgenic rice (Gao et al., 2011d). Overexpression of ***TsCBF1*** gene conferred improved drought tolerance in transgenic maize (Zhang et al., 2010a). Both, drought and salinity tolerance were enhanced in transgenic sweet potato (Fan et al., 2012) expressing ***BADH*** from spinach, and in transgenic groundnut expressing ***AtNHX1*** (Asif et al., 2011). When a wheat ***TaMYB30-B*** encoding R2R3-MYB protein was engineered into Arabidopsis, the resulting transgenic plants showed improved drought stress tolerance (Zhang et al., 2012). Remarkably, expression of ***Arabidopsis enhanced drought Tolerance1/HOMEODOMAIN GLABROUS11*** conferred drought tolerance in transgenic rice without compromising the yield factor (Yu et al., 2013). Transgenic tobacco (*Nicotiana tabacum* cv. *Xanthi-nc*) overexpressing Arabidopsis ***LOS5/ABA3*** also resulted in enhanced drought tolerance (Yue et al., 2011). Some of transgenic crop plants developed for improved tolerance to drought stress are listed in Table 10.9.

10.13 ENGINEERING TOLERANCE TO HIGHER OR LOWER TEMPERATURE

The increase in global mean surface temperature is projected to be in the range of 1.5–4°C by the end of the 21st century, which is due to global warming. We have been experiencing a lot of seasonal variations for last several years now due to climate change. The plants in the field are also experiencing increased levels of heat stress. The major world food crops are already underperforming with heat as one of the stress factors (Lobell and Gourdji, 2012; Teixeira et al., 2013). Critical reproductive stages are under threat due to this reason. Extreme temperature regimes in temperate and subtropical agricultural zones cause significant yield loss (Teixeira et al., 2013). Some species and cultivars are more sensitive to heat stress (Lobell and Gourdji, 2012), and in the due course of time, they may somewhat adapt

TABLE 10.9 Recent Developments of Transgenic Crop Plants Tolerant to Drought Stress.

Sl. No.	Transgene (Gene Name/Notation)	Source of Transgene	Crop and Cultivar	Method of Transformation	Reference
1.	*BdWRKY36* (*WRKY* transcription factor)	*Brachypodium distachyon*	*Nicotiana tabaccum*	ATMT	Sun et al. (2015)
2.	*AtEDT1/HDG11* (Homodomain-leucine zipper transcription factor enhanced drought tolerance/HOMEODOMAIN GLABROUS11)	*Arabidopsis thaliana Col-0*	*Oryza sativa japonica*	ATMT	Yu et al. (2013)
3.	*TaMYB30-B* (*MYB* type gene)	Wheat of different ploidy levels	*Arabidopsis thaliana*	ATMT	Zhang et al. (2012)
4.	*OtsA, OtsB* (trehalose-6-P-synthase, trehalose-6-P-phosphatase)	*E. coli*	Rice Pusa Basmati-1 (PB-1)	ATMT	Ahmad et al. (2012)
5.	*TaWRKY19* (WRKY-type transcription factor)	*Triticum aestivum*	*Arabidopsis thaliana*	ATMT	Niu et al. (2012)
6.	*TaWRKY2* (stress-responsive *WRKY* gene)	*Triticicum aestivum* L. cultivar Xifeng 20	*Arabidopsis thaliana* Col-0	Vaccum infiltration method via ATMT	Niu et al. (2012)
7.	*ATHB-7* (homeodomain-leucine zipper (HD-Zip) transcription factor gene)	*Arabidopsis thaliana*	*Lycopersicum lycopersicon DTL 20*	ATMT	Mishra et al. (2012)
8.	*SoBADH* (betain aldehyde dehydrogenas gene)	*Spinacia oleracia*	*Ipomoea batatas* cv. Sushu-2	ATMT	Fan et al. (2012)
9.	*AVP1* (Vacuolar H^+-Pyrophosphatase gene)	*Arabidopsis thaliana*	*Gossypium hirsutum* cv. *Coker 312*	ATMT	Pasapula et al. (2011)
10.	*LOS5* (molybdenum-cofactor sulfurase)	*Arabidopsis thaliana*	*Nicotiana tabacum* cv. *Xanthi*	ATMT	Yue et al. (2011)

TABLE 10.9 *(Continued)*

Sl. No.	Transgene (Gene Name/Notation)	Source of Transgene	Crop and Cultivar	Method of Transformation	Reference
11.	*BADH* (betaine aldehyde dehydrogenase gene)	Spinach	Potato cv. gannongshu	ATMT	Zhang et al. (2011b)
12.	*Osmotin*	Tobacco	*S. lycopersicum* cv. Pusa Ruby	ATMT	Goel et al. (2010)
13.	*TsCBF1* (abiotic stress responsive transcription factor)	*Thellungiella halophila*	Maize	Particle bombardment	Zhang et al. (2010a)
14.	*OsNAC10*	*Oryza sativa*	*Oryza sativa* cv. Nipponbare	ATMT	Jeong et al. (2010)
15.	*TPS* and *TPP* (trehalose synthases)	Yeast	*Arabidopsis thaliana*	ATMT	Miranda et al. (2007)
16.	*SAMDC* (*S*-adenosyl methioninedecarboxy)	Human	*Nicotiana tabacum* var. xanthi	ATMT	Waie and Rajam (2003)
17.	*AtOAT* (Ornithine amino transferase)	*Arabidopsis thaliana*	*Nicotiana tabaccum*	ATMT	Roosens et al. (2002)

ATMT, *Agrobacterium tumefaciens*-mediated transformation.

to heat stress naturally. In some heat tolerant crops, specific thermoprotective genes are constitutively expressed at higher levels (Bita et al., 2011). However, the capacity of plants to evolve naturally against temperature fluctuations, like any other adaptations, will also be a slow process. The conventional plant breeding methods has not been very successful against abiotic stresses, especially higher temperature, because of the complexity of the phenomenon itself. Therefore, genetic engineering of plants for enhanced heat tolerate could be a way to combat the effects of global rise in temperature on crop productivity.

There are at least three approaches that have been used for engineering heat tolerance. A number of proteins associated with diverse cellular metabolic activities have been overexpressed in transgenic experiments with the view of enhancing heat tolerance. These include proteins found to be involved in metabolism of amino acids and their derivatives, protein biosynthesis, photosynthetic activity, redox homeostasis and hormonal regulation, etc. Higher heat tolerance in transgenic plants were achieved through overexpression of **L-aspartate-α-decarboxylase** from *Escherichia coli* in tobacco, gene for **arginine decarboxylase** enzyme (involved in polyamine biosynthesis) from *Avena sativa* in *Solanum melongena*, *Saccharomyces cerevisiae* gene encoding for **S-adenosyl-L-methionine decarboxylase** (SAMDC) enzyme (involved in polyamine biosynthesis) in *Solanum lycopersicum*, **spermine synthase** gene in *A. thaliana*, *Rosa chinesis* gene encoding for **translation initiation factor** in *A. thaliana*, *Zea mays* gene encoding for **elongation factor** in *Triticum aestivum*, ***AtFKBP62*** gene in *A. thaliana*, *Cajanas cajan* gene encoding for **cyclophilin** chaperone in *A. thaliana*, *A. thaliana* gene for **thioredoxin-like protein** (a foldase and holdase chaperone) in *A. thaliana* (Grover et al., 2013), etc. Some of transgenic crop plants developed for improved tolerance to higher temperature stress are listed in Table 10.10.

ROS scavenging pathways also help plants face stress responses. Thus, heat tolerance was improved when *A. thaliana* gene encoding for **nucleotide diphosphate kinase** was overexpressed in *Solanum tuberosum*, *S. lycopersicum* gene for **GDP-mannose pyrophosphorylase** was overexpressed in *N. tabacum*, *O. sativa* gene for **chloroplast protein**-enhancing stress tolerance overexpressed in *A. thaliana* (Grover et al., 2013), and *A. thaliana* gene for **cytokinin oxidase/dehydrogenase** ectopically expressed in *N. tabacum* (Macková et al., 2013).

Sometimes, general stress-related proteins have been ectopically overexpressed. Thus, higher heat tolerance was achieved when *Xerophyta viscosa* gene encoding for **stress-associated protein 1** (SAP1; a cell membrane-binding protein) was overexpressed in *A. thaliana*, *Populus tremula* gene

TABLE 10.10 Recent Developments of Transgenic Crop Plants Tolerant to Heat Stress.

Sl. No.	Transgene (Gene Name and Notation)	Source of Transgene	Crop and Cultivar	Method of Transformation	Reference
1.	*CSD1*, *CSD2* (copper/zinc superoxide dismutase), *CCS*	*Arabidopsis thaliana*	*Arabidopsis thaliana* ecotype Columbia	ATMT	Guan et al. (2013)
2.	*TPS1* (trehalose synthesis)	*Saccharomyces cerevisiae*	*Medicago sativa* L. cv. Regen SY27x	ATMT	Suárez et al. (2009)
3.	*TPS* and *TPP* (trehalose synthesis)	*Saccharomyces cerevisiae* strain W303-1A	*Arabidopsis thaliana* Col-0 ecotype	ATMT	Miranda et al. (2007)

ATMT: *Agrobacterium tumefaciens*-mediated transformation.

encoding for a stable protein overexpressed in *A. thaliana*, and when *A. thaliana* gene for SAP5 was overexpressed in *G. hirsutum* (Grover et al., 2013). *A. thaliana* SAP5 positively regulates salt and osmotic stress tolerance through its E3 ubiquitin ligase activity.

Additionally, a diverse kind of proteins has been employed in development of transgenics for improved heat tolerance, such as overexpression of *Agrobacterium rhizogenes* gene encoding for **β-glucosidase** in *Rubia cordifolia*, *C. cajan* gene for hybrid proline rich in *A. thaliana*, *Malus domestica* gene encoding for vacuolar proton translocating inorganic **pyrophosphatase** in *M. domestica*, *Z. mays* gene encoding for **acetyl cholinesterase** in *N. tabacum*, and *A. thaliana* gene for **CYP710A1** in *A. thaliana* (Grover et al., 2013; Senthil-Kumar et al., 2013), **Annexin protein** from *N. nucifera* in transgenic *A. thaliana* (Chu et al., 2012). On the other hand, overexpression of **SlCZFP1**, a novel TFIIIA-type zinc finger protein from tomato conferred enhanced cold tolerance in transgenic Arabidopsis and rice (Zhang et al., 2011a). Some of transgenic crop plants developed for improved tolerance to lower temperature stress are listed in Table 10.11.

10.14 ENGINEERING FOR REMOVAL OF ENVIRONMENTAL POLLUTANTS

Human activities and industrial development generate large amounts of chemicals that often contaminate soil and water. Prevalent contaminants include petroleum hydrocarbons, polycyclic aromatic hydrocarbons, halogenated hydrocarbons, pesticides, solvents, metals, and salts. Among these, halogenated hydrocarbons, such as polychlorinatedbiphenyls (PCBs) and chlorophenols, are persistent environmental pollutants (Wang et al., 2015). In general, cleaning up environmental pollutants using wild-type plants leads to the accumulation of PCBs in shoots and roots of plants that may be released to the soil or get again into the atmosphere (Akin et al., 2009; Xia et al., 2009). However, it appears like GE plants can handle the situation better. Phytoremediation is now emerging as a promising strategy and attracting much attention due to its advantages of being less expensive, environmentally sustainable, and esthetically acceptable compared to physical and chemical methods (Krämer, 2005). A lot of the studies have shown the removal rate of PCBs or 2,4-DCP using conventional plants is inadequate and slow (Zeeb et al., 2006). The primary reason is that plants lack the necessary enzymatic machinery involved in bacteria or mammals for efficient cleavage of aromatic structure. Alternatively, there is increasing opportunity in using

TABLE 10.11 Recent Developments of Transgenic Crop Plants Tolerant to Cold Stress.

Sl. No.	Transgene (Gene Name and Notation)	Source of Transgene	Crop and Cultivar	Method of Transformation	Reference
1.	*CsTK* (transketolase)	cDNA library	*Cucumis sativa* L. cv. Jinyou 3	ATMT	Bi et al. (2015)
2.	*DREB1B* (dehydration-responsive element-binding factor 1)	*Arabidopsis* sp.	*Solanum tuberosum* L.	ATMT	Movahedi et al. (2012)
3.	*TaWRKY19*	*Triticum aestivum* L. cv. Xifeng 20	*Arabidopsis* ecotype Columbia plants (Col-0)	ATMT	Niu et al. (2012)
4.	*GmbZIP1*	*Glycine max* L.	*Nicotiana tabacum* W38	ATMT	Gao et al. (2011c)
5.	*SlCZFP1* (*Solanum lycopersicum* cold zinc finger protein 1)	*Solanum lycopersicum* var. D. Huang	*Arabidopsis thaliana* (L.) Heynh. (ecotype Wassilewskija, Ws-2), *Oryza sativa* L. cultivar Kita-ake	ATMT	Gao et al. (2011d)
6.	*ZmMKK4* (mitogen-activated protein kinase kinase)	*Z. mays* L. cv. Zhengdan 958	*Arabidopsis thaliana* (ecotype Columbia, Col-0	ATMT	Kong et al. (2011)
7.	*OsAPXa* (ascorbate peroxidase)	*OsAPXa* cDNA library	*L.* cv. Oborozuki	ATMT	Sato et al. (2011)
8.	*At-CBF1* (Arabidopsis C-repeat-binding factor 1)	*Agrobacterium tumefaciens*	*Solanum lycopersicum* var. Shalima	ATMT	Singh et al. (2011)
9.	*JcDREB*	*Jatropha curcas*	*Arabidopsis thaliana* CK	ATMT	Tang et al. (2011)
10.	*mtlD* (mannitol-1-phosphate dehydrogenase)	*Escherichia coli*	*Lycopersicon esculentum* M. cv. Pusa Uphar	ATMT	Khare et al. (2010)
11.	*TERF2/LeERF2* (ethylene responsive factor)	*Solanum lycopersicum*	*Solanum lycopersicum* cv. Lichun and *Nicotiana tabacum* cv. NC89	ATMT	Zhang and Huang (2010c)
12.	*ThCAP* (cold acclimation protein)	*Tamarix hispida*	*Populus davidiana*, *P. bolleana*	ATMT	Guo et al. (2009)
13.	*TPP1* (trehalose synthase)	cDNA library of Nona Bokra	*Oryza sativa* L. ssp. *indica* pv. Nona, *Oryza sativa* L. ssp. *japonica*	ATMT	Ge et al. (2008)

TABLE 10.11 *(Continued)*

Sl. No.	Transgene (Gene Name and Notation)	Source of Transgene	Crop and Cultivar	Method of Transformation	Reference
14.	*Osmyb4* (cold-induced transcription factor)	*Oryza sativa*	*Malus pumila* Mill. Cv. Greensleeves	ATMT	Pasquali et al. (2008)
15.	*WCOR15* (cold-induced gene)	*Triticum aestivum* L. Mironovskaya 808	*Nicotiana tabacum* cv. "Petit Havana"	ATMT	Shimamura et al. (2006)
16.	*CBF3/DREB1A* and *ABF3*	*Arabidopsis thaliana*	*Oryza sativa* cv. Nakdong	ATMT	Oh et al. (2005)
17.	*OsSAP1* (*Oryza sativa* subspecies indica stress-associated protein gene)	*Oryza sativa* subsp. indica var. Pusa Basmati-1	*Nicotiana tabacum* var. Xanthi	ATMT	Mukhopadhyay et al. (2004)
18.	*betA* (Choline dehydrogenase)	*Escherichia coli*	*Nicotiana tabacum* L. cv. samsun	ATMT	Holmström et al. (2000)
19.	*Nt 107* (Glutathione-S-transferase/ glutathione peroxidase)	*Nicotiana tabacum*	*Nicotiana tabacum* L. cv. Xanthi NN	Self-pollination	Roxas et al. (2000)

ATMT, *Agrobacterium tumefaciens*-mediated transformation.

phytoremediation, which will be greatly enhanced by using transgenic plants bearing bacterial genes involved in xenobiotic metabolism, leading to a wider application in the field (Abhilash et al., 2009).

The **2,3-dihydroxybiphenyl-1,2-dioxygenase (BphC.B)**, a key enzyme of aerobic catabolism of a variety of aromatic compounds, was cloned from a soil metagenomic library, then was expressed in alfalfa driven by CaMV 35S promoter using *Agrobacterium*-mediated transformation. The tolerance capability of transgenic line BB11 toward complex contaminants of PCBs/2,4-DCP significantly increased compared with nontransgenic plants (Wang et al., 2015). Strong dissipation of PCBs and high removal efficiency of 2,4-DCP were exhibited in a short time. It was confirmed that expressing BphC.B would be a feasible strategy to help achieving phytoremediation in mixed contaminated soils with PCBs and 2,4-DCP.

10.14.1 MODE OF ACTION OF 2,3-DIHYDROXYBIPHENYL-1,2-DIOXYGENASE

BphC found in a range of Gram-negative and Gram-positive bacteria that aerobically assimilate biphenyl could utilize nonheme ferrous iron to cleave the aromatic nucleus of catechols meta (adjacent) to the yellow substance. BphC is involved in aerobic catabolism of a variety of aromatic compounds including phenol, naphthalene, and polychlorinated biphenyls.

10.15 GENETIC ENGINEERING OF TOLERANCE TO HERBICIDES

The herbicides used in the earlier days have been very destructive for most plants and their use is undesirable for the environment. Among newer herbicides, glyphosate has been widely used for it can be degraded by soil microorganisms. However, with the development of herbicide tolerant crop plants, herbicides can now be applied over the top of crops during the growing season to control weed population more effectively (Ahmad et al., 2012). The glyphosate-tolerant maize, soybean, canola, and cotton are the most abundant lines among those crops (Tohidfar and Khosravi, 2015).

10.15.1 HOW DOES GLYPHOSATE WORK?

Glyphosate, the active component of Roundup®, is used across in the field as nonselective postemergence herbicide. Glyphosate works as an analog of

enolpuruvate by binding to and inhibiting the enzyme **5-enolpyruvylshikimate-3-phosphate synthase (EPSPS)**, which is an active component in the shikimate pathway, leading to the synthesis of chorismate-derived metabolites such as the aromatic amino acids. Thus, inactivation of this enzyme by glyphosate means killing of the plant due to the absence of aromatic amino acids for complete renewal of proteins (Tohidfar and Khosravi, 2015).

10.15.2 STRATEGIES FOR GENERATING HERBICIDE TOLERANT CROPS

Quite a few transgenic plants have been developed for tolerance to various herbicides in crops such as soybean, corn, cotton, and canola more than a decade ago. By and large, there are two approaches that can be used to create herbicide tolerant crops: one-way is to modify the degree of sensitivity of the target enzymes so that the sensitivity of plant to the herbicide is reduced or eliminated. Examples of the first approach include glyphosate and acifluorfen tolerance. Transgenic plants tolerant to the herbicide acifluorfen have been produced through overexpression of the target enzyme involved in chlorophyll biosynthesis (Lermontova and Grimm, 2000). This herbicide inhibits chlorophyll biosynthesis. Herbicide resistant *Amaranthus palmeri* has been developed recently by expressing glyphosate-insensitive herbicide target site gene, ***EPSPS*** involved in the shikimate cycle wherein it catalyzes the reversible addition of the enolpyruvyl moiety of phosphoenolpyruvate to shikimate 3-phosphate (Gaines et al., 2010). A highly glyphosate insensitive EPSPS was created by DNA shuffling in the gene from *Vitisi vinifera* and transgenic introduction of such a gene in rice and Arabidopsis improved tolerance to glyphosate (Tian et al., 2015).

The other approach is to engineer the herbicide detoxification pathway into the plant. Resistance to glufosinate and bromoxynil is based on the second approach. In this approach, introducing a gene in the plant system metabolizes the herbicide concerned. For example, in the case of herbicide Ignite/Basta, the ***bar*** resistance gene from *Streptomyces hygroscopicus* was used to detoxify the herbicide. The expression of *bar* gene responsible for resistance to herbicides was demonstrated in sweet potato (Zang et al., 2009). Previously, various transgenic plants expressing the *bar* gene were developed in sugarbeet, popular plants, aspen, oilseed rape, tomato, potato, alfalfa, and tobacco. Imidazolinone resistance (IR) ***XA17*** gene was incorporated into some maize lines for resistance to imazaquin and nicosulfuron herbicides (Menkir et al., 2010). Transgenic tobacco expressing a tau class GST isoenzyme ***GmGSTU4***

from soybean is active as GSH-dependent peroxidase (GPOX) and shows catalytic activity for diphenyl ether herbicide fluorodifen/alachlor (Benekos et al., 2010). The gene encoding **glyphosate *N*-acetyltransferase** (*Gat*) from *Bacillus licheniformis* into the plant it deactivates glyphosate into a nontoxic *N*-acetylglyphosate (Siehl et al., 2007). The soybean and corn plants expressing *GAT* gene were tolerant to glyphosate (Castle et al., 2004).

10.15.3 MODE OF ACTION OF PHOSPHINOTHRICIN ACETYL TRANSFERASE

The protein phosphinothricin-*N*-acetyl transferase (PAT) is produced in GE plants by genes isolated from *Streptomyces viridochromogenes* (pat gene) or *S. hygroscopicus* (bar gene). PAT is used against selection agents, such as phosphinothricin, bialaphos, and glufosinate ammonium in GM crops. These agents interefere with the functioning of glutamine synthetase/glutamate synthase cycle and the conversion of glutamate and ammonia to glutamine is blocked. The pathway again turns functional only when PAT detoxifies the selection agent by acetylation.

10.15.4 CROP TOLERANCE TO BROADLEAF AND GRASS HERBICIDES

Substrate preferences of bacterial **aryloxyalkanoate dioxygenase enzymes (AADs)** that can effectively degrade 2,4-D were investigated and in addition to their activity on 2,4-D, some members of this class can act on other widely used herbicides. ***AAD-1*** cleaves the aryloxyphenoxy propionate family of grass-active herbicides, and ***AAD-12*** acts on pyridyloxyacetate auxin herbicides such as triclopyr and fluroxypyr. Maize plants transformed with an *AAD-1* gene showed robust crop resistance to aryloxyphenoxy propionate herbicides over four generations and were also not injured by 2,4-D applications at any growth stage. Arabidopsis plants expressing *AAD-12* were resistant to 2,4-D as well as triclopyr and fluroxypyr, and transgenic soybean plants expressing *AAD-12* maintained field resistance to 2,4-D over five generations. These results showed that single *AAD* transgenes can provide simultaneous resistance to a broad group of agronomically important classes of herbicides, including 2,4-D, with utility in both monocot and dicot crops (Wright et al., 2010).

Some of transgenic crop plants developed for improved tolerance to environmental pollutants and herbicides are listed in Table 10.12.

TABLE 10.12 Recent Developments of Transgenic Crop Plants Tolerant to Pollutants and Herbicides.

Sl. No.	Transgene (Gene Name and Notation)	Pollutants	Source of Transgene	Crop and Cultivar	Method of Transformation	Reference
1.	*CuZnSOD* (CuZn superoxide dismutase) and *APX* (ascorbate peroxidase	Sulfur dioxide	oxidative stress-inducible SWPA2 promoter (SSA plants)	*Ipomoea batatas*	Particle bombardment	Kim et al. (2015)
2.	*VvEPSPS* (5-enolpyruvyl shikimate-3-phosphate synthase)	Glyphosate	*Vitis vinifera*	*Oryza sativa* L. ssp. *japonica*, *Arabidopsis* sp.	ATMT, DNA shuffling	Tian et al. (2015)
3.	*BphC.B* (2,3-dihydroxybiphenyl-1,2-dioxygenase)	PCBs and 2,4-DCP	Soil metagenomic library	*Medicago sativa* L. cv. Gongnong No. 1	ATMT	Wang et al. (2014)
4.	*TaEXPB23* (−expansin gene)	Methyl viologen	*Triticum aestivum* L.	*Nicotiana tabacum* L. cv. NC89	ATMT	Han et al. (2015)
5.	*TaALMT1*	Aluminum	*Triticum aestivum*	*Triticum aestivum* Bob White 26 "SH9826" line (BW26)	Particle bombardment	Pereira et al. (2010)
6.	*GmGSTU4*	Diphenyl ether and chloroacetanilide	*Glycine max*	*Nicotiana tabacum* L. cultivar Basmas	ATMT	Kostantinos et al. (2010)
7.	*aad-1*, *aad-12* (aryl oxyalkanoate dioxygenase)	Aryl oxyphenoxypropionate, triclopyr, and fluroxypyr	*Ralstonia eutropha*	*Arabidopsis thaliana*, *Glycine max*, *Zea mays*	ATMT	Wright et al. (2010)

ATMT, *Agrobacterium tumefaciens*-mediated transformation.

10.16 GENETIC ENGINEERING OF PHOSPHORUS UTILIZATION EFFICIENCY IN PLANTS

Phosphorus is one of the three major nutrient requirements of plants. Its low availability, mobility, and high fixation in soils make it a constraint worldwide for crop productivity. However, molecular biology provides great opportunities to improve phosphorus use efficiency in plants. It is also to be noted that phosphorus mainly comes from nonrenewable resource and, therefore, "smart" crop plants have to be developed for better phosphorus use efficiency. Plants have multiple adaptation systems evolved for efficient utilization of phosphorus from soil (Tian et al., 2012). Therefore, it is important to understand, identify, and use the genes involved in various adaptation processes.

Among Pi transporters (PT), Pht1 mainly function in Pi acquisition from soils and translocation from roots to other parts of plants. Biomass and yield of transgenic rice plants were not coincidently improved. However, overexpression of ***NtPht1;1***, ***OsPht1;2***, or ***OsPht1;8*** facilitated Pi acquisition (Jia et al., 2011; Liu et al., 2010; Park et al., 2010). This was due to toxicity of excess phosphorus. Thus, overexpression of Pht1 in crops should be integrated with soil/farm management in order to improve crop phosphorus use efficiency. Similarly, overexpressing a transcription factor, **phosphate starvation response 2** (*OsPHR2*), a major component in phosphorus signaling pathways in rice, resulted in increased phosphorus concentration but inhibited plant growth, which might have been caused due to excessive amounts of P in leaves (Zhou et al., 2008). Similar results were also observed in modifying **SPX** and **miR399**. Suppressing *OsSPX1* in rice and overexpressing ath-miR399d from Arabidopsis in tomato led to excessive phosphorus accumulation in leaves and subsequently inhibited plant growth (Gao et al., 2010; Wang et al., 2009). Conversely, overexpression of a transcription factor, **PTF1** (Pi starvation induced transcription factor 1), enhanced phosphorus use efficiency in both, rice and maize (Li et al., 2011b; Yi et al., 2005). Therefore, improving phosphorus use efficiency through transgenic technology of introducing the critical genes in phosphorus signaling networks requires more insights into the physiological and molecular connections between components.

10.17 GENETIC ENGINEERING OF OILSEED CROPS FOR FISH OIL

Fatty acids (FAs) with 20 carbons or more in length containing three or more cis-double bonds, that is, very long-chain polyunsaturated fatty acids

(VLC-PUFAs) are essential components of human nutrition. These FAs are the major constituents of mammalian retinal, brain and testis membrane phospholipids and play important roles in cellular and tissue metabolism regulating membrane fluidity and thermal adaptation (Sayanova and Napier, 2011). VLC-PUFAs, especially eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) play critical roles in human health and development. Mainly, the fishes, some fungi, marine bacteria, and microalgae are the sources of VLC-PUFAs. Dietary sources of VLC-PUFAs are predominantly met up from marine fish and seafood. However, the mankind has been putting enormous pressure on marine ecosystems due to increasing demand for fish and fish oils and United Nations' Food and Agriculture Organization estimated that more than 70% of world's fish stocks are either exploited or depleted (Sayanova and Napier, 2011). At the same time, commercial cultivation of marine microorganisms and aquaculture are not sustainable and cannot compensate for the shortage in fish supply. Therefore, there is an obvious requirement for an alternative and sustainable source for VLC-PUFAs. Plant oils are relatively inexpensive and are commonly considered to be healthier than animal fats, as they contain relatively high amounts of unsaturated FAs. Plant oils are rich in C18 FA, including the essential FA linoleic acid (LA) and α-linolenic acid (ALA), but are devoid of LC-PUFAs, such as arachidonic acid (ARA), EPA, and DHA, which typically only enter the human diet as oily fish (Ruiz-López et al., 2015). Marine fishes are rich in these beneficial FAs.

These days, it is possible to produce seed oils with a desirable FA composition using latest genetic engineering techniques, which is impossible to achieve by traditional breeding techniques. The health benefits of consumption of oily fish and the ω-3 long-chain polyunsaturated fatty acids (LC-PUFA) such as reducing the risk of cardiovascular disease and related metabolic conditions are now widely recognized. Metabolic engineering demonstrated the feasibility of making EPA and DHA in the seed oils of transgenic *Camelina sativa* plants (Usher et al., 2015). Generation of LC-PUFAs in transgenic plants was demonstrated almost two decades ago in transgenic tobacco and Arabidopsis. The transgenic plants mainly accumulated ω-6 γ-linolenic acid (GLA) and stearidonic acid (SDA) in the leaves. GLA and SDA as high as 70% were achieved using seed-specific promoters (Hong et al., 2002; Qiu et al., 2002; Sato et al., 2004). Similar level of GLA accumulation was achieved in transgenic safflower expressing D6-desaturase from *Saprolegnia diclina* (Nykiforuk et al., 2012). The ALA-specific Δ6-desaturase from *P. vialii* was cloned under a seed-specific promoter and introduced into Arabidopsis and linseed (Ruiz-López et al., 2009). It has

been some years since the successful reconstitution of the ω-3 LC-PUFA biosynthetic pathway in plants using multiple desaturases and elongases was achieved. Genetic transformation of different oilseeds crops with multiple genes is not the technical barrier today. The feasibility of making EPA in a transgenic plant was successfully demonstrated by expressing algal components of the alternative pathway in the leaves of Arabidopsis (Qi et al., 2004). Intriguingly, it generated C20 ω-6 LC-PUFA ARA in addition to moderate amounts of EPA. Seed-specific accumulation of EPA in linseed was achieved where genes of the conventional δ-6-pathway was expressed (Abbadi et al,. 2004). Resultant transgenic seeds contained low levels of EPA, and very high levels of C18 δ6-desaturation products. The authors thus hypothesized that this unwanted build-up of a biosynthetic intermediate was as a consequence of poor acyl exchange between different metabolic pools, and the concept has been defined as "substrate dichotomy" (Napier, 2007). These findings formed the basis for further attempts to increase the levels of target FAs (EPA, DHA) and reduce the levels of undesired biosynthetic intermediates (such as the δ6-desaturation product GLA). Several studies have confirmed the ability to make significant levels of EPA, with minimum levels of GLA (Cheng et al., 2010; Wu et al., 2005). It is now technically possible to accumulate fish oil-like levels of ω-3 LC-PUFAs in the seed oils of transgenic plants similar to that found in fish oils, in which EPA and DHA accumulate up to 20% of total FAs (Napier et al., 2015). Recently, seed oils have been engineered to produce EPA and/or DHA at levels similar to fish oils (Ruiz-López et al., 2014). However, successful conversion of native plant FAs such as LA and ALA to LC-PUFAs such as EPA and DHA in seeds requires a coordinated expression of multiple genes. Recent advances in engineering of oilseed crops has led to the accumulation of ω-3 LC-PUFAs at fish oil levels, demonstrating the efficacy of acyl-CoA desaturases over previously used lipid-linked desaturases, resolving the substrate dichotomy problem (Ruiz-López et al., 2015).

10.18 GENETIC ENGINEERING FOR QUALITY NUTRITION AND HEALTH

Mineral nutrients are found in very poor quantities in staple food crops. Resultant effect, the poorest, especially those surviving on same kind of staple food, is also most vulnerable to mineral deficiency diseases. Malnutrition is more prevalent in the developing world because it is found that the nutritious food is often not reaching the poor and needy. This is also

attributed by poverty, which often occurs due to ill health and an inability to work, the typical consequences of malnutrition. Therefore, poverty, malnutrition, and poor health form a triangle which the poor and needy find difficult to escape (Farre et al., 2011). The mankind relies on food not only to fill the stomach and get enough energy, but also for essential nutrients required to maintain a good state of health and active immune system. Thus, food security is one of the main pillars of health and well-being of the society as a whole. Adequate nutrition is required to ensure lower morbidity and mortality from both infectious and noninfectious diseases. It is particularly important in children and pregnant women where the lack of essential nutrients can lead to irreversible physical and mental damage during development (Hoddinott et al., 2008).

Various strategies have been proposed to deal with micronutrient deficiencies including the provision of mineral supplements, the fortification of processed food, the biofortification of crop plants at source with mineral-rich fertilizers and the implementation of breeding programs and genetic engineering approaches to generate mineral-rich varieties of staple crops (Gómez-Galera et al., 2010). Biofortification focuses on enhancing the qualities of essential mineral nutrients in the edible part of staple crops. Agronomic intervention, plant breeding, or genetic engineering can achieve incorporation of mineral nutrients in crops, whereas plant breeding and genetic engineering can command bioavailability of minerals as well (Gómez-Galera et al., 2010). Some of transgenic crop plants developed for improved nutritional quality, oil production, etc. are listed in Table 10.13.

10.18.1 GENETIC ENGINEERING FOR ENHANCEMENT OF MINERAL MICRONUTRIENTS

Plants take up inorganic nutrients from the environment and metabolically synthesize organic nutrients. We can focus on genetic engineering strategies such as increasing the solubility of these nutrients in the rhizosphere, mobilizing them in the plants, transporting them to storage organs, increasing the storage capacity of the plant, and maximizing bioavailability for enhancement of mineral micronutrients in food crops (Gómez-Galera et al., 2010). Unlike most other minerals, deficiency of nutrients such as iron, zinc, selenium, and calcium have serious implications. Thus, there is a need of designer crops with enhancement of necessary micronutrients (Naqvi et al., 2009).

TABLE 10.13 Recent Developments of Transgenic Crop Plants with Improved Nutritional Quality, Oil Production, etc.

Sl. No.	Transgene (Gene Name and Notation)	Function	Source of Transgene	Crop and Cultivar	Method of Transformation	Reference
1.	*dgat1-1*	Acetyl glyceride oil production	*Arabidopsis thaliana*	*Camelina sativa*	ATMT	Liu et al. (2015c)
2.	*gus* and *nptII*	High seed production	*E. coli*	*Camellia sinensis* L.O. Kuntze	Biolistic mediated	Sandal et al. (2015)
3.	*FAD2*	Oleic acid production	*Linum usitatissimum* L. cDNA library	*Linum usitatissimum* L. cv. Glenelg	ATMT	Chen et al. (2015)
4.	*OtΔ6* (Δ6-desaturase gene), PSE1 *(TcΔ5)* Δ5-desaturase gene, *PsΔ12* (*Δ*12-desaturase gene), Pi-x3 (x3-desaturase)	Omega-3 LC-PUFA production	*Ostrococcus tauri*, *Physcomitrella patens*, *Thraustochytrium* sp. (*Phytophthora sojae*, *Phytophthora infestans*)	*Camelina sativa*	ATMT	Ruiz-López et al. (2014)
5.	*FAD2* (*Δ*12-desaturase)	Oleic acid production	*J. curcas* seed cDNA library	*Jatropha curcas* (Jc-MD)	ATMT	Qu et al. (2012)
6.	*Cg1* (Corngrass1)	Starch content increase	*Zea mays*	*Panicum virgatum*	ATMT	Chuck et al. (2012)
7.	*Δ6-desaturase*, *Δ12-/Δ6-desaturases*	c-Linolenic acid production	*Saprolegnia diclina*, *Mortierella alpina*	*Carthamus tinctorius*	ATMT	Nykiforuk et al. (2012)
8.	*OASS* (*O*-acetylserine sulfhydrylase)	Enhanced levels of cysteine and Bowman–Birk protease inhibitor in seeds	*cDNA library*	*Glycine max* L. cv. Maverick	ATMT	Kim et al. (2012)

TABLE 10.13 *(Continued)*

Sl. No.	Transgene (Gene Name and Notation)	Function	Source of Transgene	Crop and Cultivar	Method of Transformation	Reference
9.	*acyl-CoA D6-desaturase*	Oil production	*Micromonas pusilla*	*S. cerevisiae* strain INVSc1, *Nicotiana benthamiana*, *Arabidopsis thaliana* (Col 0)	ATMT	Petrie et al. (2010a)
10.	*ZmLEC1* (LEAFY COTYLEDON1), *ZmWRI1* (WRINKLED1)	Oil production	*Zea mays*	*Zea mays*	ATMT	Shen et al. (2010)
11.	*LEC2* (LEAFY COTYLEDON2)	DHA biosynthesis	*Arabidopsis thaliana*	*Nicotiana benthamiana*	ATMT	Petrie et al. (2010b)
12.	*OsSPL14*, *SPL16*	Grain size, shape, and quality	*Oryza sativa* Nipponbare	*Oryza sativa* indica Ri22, SNJ	ATMT	Jiao et al. (2010)
13.	*idi*, *crtE*, *crtB*, *crtI*, *crtY*, *crtZ*, and *crtW*	Carotenoid synthesis	*Pantoea ananatis*, *Brevundimonas* sp. strain SD212, and *Paracoccus* sp. strain N81106	*Brassica napus* L. cultivar Westar	ATMT	Fujisawa et al. (2009)
14.	*CrtI* (carotene desaturase), *psy* (phytoene synthase)	Vitamin A synthesis	*Pantoea ananatis*	*Oryza sativa*, GR	Biolistic methods and ATMT	Tang et al. (2009)
15.	*Zmpsy1* (*Zea mays* phytoene synthase 1), *PacrtI* (*Pantoea ananatis* phytoene desaturase), *Gllycb* (*Gentiana lutea* lycopene β-cyclase), *Glbch* (*G. lutea*	Carotenoid synthesis	*Pantoea ananatis*, *Gentiana lutea*, *Paracoccus* sp.	*Zea mays* L. (cv. M37W	Combinatorial nuclear transformation	Zhu et al. (2008)

TABLE 10.13 *(Continued)*

Sl. No.	Transgene (Gene Name and Notation)	Function	Source of Transgene	Crop and Cultivar	Method of Transformation	Reference
	β-carotene hydroxylase), *Paracrt W* (*Paracoccus* β-carotene ketolase)					
16.	*CordapA*, *LKR/SDH* (lysine-ketoglutarate reductase/saccharophine dehydrogenase)	Lysine biosynthesis	Corynebacterium	*Zea mays*	ATMT	Frizzi et al. (2008)
17.	*AVP1* (*Arabidopsis* vacuolar pyrophosphatase)	Enhanced growth under phosphorus limitation	*Arabidopsis thaliana Col-0 ecotype*	*Oryza sativa* var. japonica "Taipei 309," *Lycopersicon esculentum* Mill. cultivar Money Maker	ATMT	Yang et al. (2007)
18.	*Δ6 elongases*	Very long-chain polyunsaturated fatty acids (VLCPUFAs)	*Thraustochytrium* sp.	*Brassica juncea* BJ5	ATMT	Guohai et al. (2005)
19.	*tyrA*, *pds1*, *hpt1*, and *ggh*	Vitamin E synthesis	*Synechocystis* sp. PCC 6803	*Arabidopsis thaliana* var. Columbia *Brassica napus* L. cultivar ebony and *Glycine max* cultivar A3244	ATMT	Karunanandaa et al. (2005)
20.	*Δ6-desaturase gene*	Oil production	*Jatropha curcas*	*Arabidopsis thaliana* D1	ATMT	Reddy and Thomas (1996)

ATMT, *Agrobacterium tumefaciens*-mediated transformation; GR, Golden Rice.

10.18.1.1 IRON

Background: A major challenge with iron is that only the ferrous form (Fe^{2+}) is soluble and available to plants for uptake, whereas the ferric form (Fe^{3+}) is sequestered into insoluble complexes with soil particles (Gómez-Galera et al., 2012). Plants have evolved two counter strategies, first, by secreting reductases into the soil converting ferric iron into the ferrous form, and second, by releasing chelating agents known as phytosiderophores (PS) that can be reabsorbed by the roots as PS-Fe^{3+}.

Strategies for improvement of iron levels in plants include increasing the export of both reductases and PS, overexpression of iron transporter proteins, overexpression of ferritin, which stores large amounts of iron in a bioavailable form and the expression of phytase, which breaks down phytate and makes the stored iron easier to be absorbed in the human digestive system. For example, overexpressing the enzymes nicotianamine synthase (NAS) and/or nicotianamine aminotransferase (NAAT) in transgenic rice significantly increased the iron content (Johnson et al., 2011; Zheng et al., 2010).

10.18.1.2 ZINC

Background: Zinc deficiency affects more than 2 billion people worldwide manifesting as a spectrum of symptoms including hair loss, skin lesions, fluid imbalance (inducing diarrhea), and eventually wasting of body tissues (Hambidge and Krebs, 2007).

Strategies to increase the zinc content of plants have concentrated on transport and accumulation (Palmgren et al., 2008). The expression of NAS/NAAT and transporters such as *Os*ysl15 and *Os*irt1 in rice can increase the levels of both zinc and iron since many PS and transporters can interact with them (Lee et al., 2012).

10.18.1.3 SELENIUM

Background: Selenium is a component of enzymes and other proteins containing the amino acids selenocysteine and selenomethionine, required for the interconversion of thyroid hormones; therefore selenium and iodine deficiency can have similar symptoms (Khalili et al., 2008).

Genetic engineering strategies have focused on storage and accumulation of selenium to increase its levels. Expression of Arabidopsis ATP sulfurylase in mustard increased the selenium content in shoots and roots (Pilon-Smits et al., 1999).

10.18.1.4 CALCIUM

Background: Soluble calcium is an electrolyte and signaling molecule, but most of the calcium in the human body is present in its mineralized form as a component of bones and teeth. The replenishment of serum calcium by bone resorption is slow, so dietary calcium deficiency in the short term can lead to electrolyte imbalance and over the long term can cause osteoporosis.

Genetic engineering strategies to increase the calcium content of plants include the expression of calcium transporters such as *At*CAX1, which increased the calcium content of carrots and potatoes by up to threefold (Connolly, 2008; Park et al., 2005c).

10.18.2 NUTRIENT ENHANCERS AND ANTINUTRIENTS

Background: Mineral bioavailability can be increased by promoting the accumulation of enhancers or eliminating antinutrients that regulate the absorption of plant minerals by the human digestive system (Gibson, 2007). Some key nutrients doubly act as enhancers, like ascorbate and β-carotene, promoting iron uptake by chelating and/or reducing Fe^{3+} and prevent interactions with phytate and polyphenols (García-Casal, 2000). Phytic acid is a key antinutrient abundant in cereals, legumes, and oil seeds where it binds all the principal mineral nutrients and sequesters them into stable complexes that cannot be absorbed (López et al., 2002).

The amount of phytic acid in seeds can be reduced by silencing genes involved in its biosynthesis, such as myo-inositol-1-phosphate synthase (Nunes et al., 2006) or 1D-myo-inositol 3-phosphate synthase (Kuwano et al., 2009). Expression of a thermostable recombinant fungal phytase increased iron bioavailability in wheat (Brinch-Pedersen et al., 2006) and maize (Chen et al., 2008).

10.18.3 GENETIC ENGINEERING FOR ENHANCEMENT OF ORGANIC NUTRIENTS

Human can synthesize almost all the organic compounds needed for normal physiological activity, except the essential nutrients such as some of the amino acids, FAs, and vitamins.

10.18.3.1 ESSENTIAL AMINO ACIDS

Nine amino acids are constitutive essential nutrients because they cannot be synthesized de novo by human, and others are essential under certain specific cases, like child development or metabolic disorders. The most relevant examples are lysine, threonine, tryptophan, methionine, and cysteine. Staple cereals are poor sources of lysine and threonine, and staple legumes are poor sources of tryptophan, methionine, and/or cysteine (Zhu et al., 2007a).

Two strategies to tackle amino acid deficiency are engineering plants to produce proteins containing essential amino acids; and engineering amino acid metabolism to increase the availability of essential amino acids in the product. Lysine was the first target, its content increased up to 4.2% in transgenic rice and wheat (Sindhu et al., 1997; Stöger et al., 2001). In a significant development, 12 and 8 residues of lysine were added to endogenous cereal storage proteins barley hordothionine to produce HT12 and high lysine protein to produce HL8 (Jung and Carl, 2000). Further achievements in improving lysine yield include increment by 55% in maize seeds by the expression of the lysine-rich storage protein (sb401) (Yu et al., 2004), 47% in maize by the expression of lysine-rich animal protein such as porcine α-lactalbumin (Bicar et al., 2008), and 26% in maize seeds by expressing a heterotypical Arabidopsis lysyl tRNA synthetase that inserts lysine residues in place of other amino acids during the synthesis of seed storage proteins (Wu et al., 2007). The lysine content of maize has also been increased by using RNAi silencing one of the zein storage protein genes allowing the protein complement to be filled with lysine-rich storage proteins (Segal et al., 2003).

Amaranthus hypochondriacus seed storage protein is rich in all the essential amino acids and has a composition almost ideal for human consumption. Transgenic maize seeds expressing the AH protein contained up to 32% more protein than wild-type seeds containing higher levels of lysine, tryptophan,

and isoleucine (Rascón-Cruz et al., 2004). Similarly, transgenic potato tubers expressing AH contained 45% more protein than normal (Chakraborty et al., 2000), while transgenic wheat seeds contained nearly 2.5% AH as a proportion of total seed protein, increasing the levels of lysine to 6.4% and tyrosine to 3.8% (Tamás et al., 2009).

Expression of feedback-insensitive dihydrodipicolinate synthase in maize increased lysine levels from to 30%, with concomitant increase in threonine (Frizzi et al., 2008). The key rate-limiting enzyme in tryptophan synthesis—anthranilate synthase—catalyzes the conversion of chorismate to anthranilate. Thus, expressing a feedback-insensitive version, the tryptophan level was increased by 400-fold in rice (Wakasa et al., 2006), 30-fold in potato tubers (Yamada et al., 2005b), and 20-fold in soybean seeds (Ishimoto et al., 2010).

10.18.3.2 ESSENTIAL FATTY ACIDS

The health-promoting ω-3 and ω-6 polyunsaturated fatty acids (PUFAs) need to be obtained from diet (Djoussé et al., 2011). Once acquired, simple ω-3 PUFAs such as ALA can be converted into more complex VLC-PUFAs like ARA, which can be converted back to the simpler species.

The VLC-PUFAs have been biosynthesized by expressing microbial desaturases and elongases in linseed, soybean, and mustard (Abbadi et al., 2004; Kinney et al., 2004; Wu et al., 2005).

10.18.3.2.1 Vitamin A

The reduced form of vitamin A (retinal) is required for the production of rhodopsin, essential for eyesight. Vitamin A deficiency affects more than 4 million children each year, up to 500,000 of who become partially or totally blind (Harrison, 2005).

The overexpression of DXP synthase in tomato produced a carotenoid precursor that increased the pathway flux enhancing the total carotenoid content (Enfissi et al., 2005). Cassava roots expressing the bacterial ***CrtB*** gene accumulated 34 times normal carotenoid level (Welsch et al., 2010). The replacement of the daffodil gene with its maize ortholog in Golden Rice 2 produced significant amounts of β-carotene (Paine et al., 2005). The same genes when expressed in maize yielded kernels with much higher amounts of β-carotene (Naqvi et al., 2010; Zhu et al., 2008). Further, expression of three

Erwinia genes encoding phytoene synthase (CrtB), phytoene desaturase (CrtI), and lycopene beta-cyclase (CrtY) in golden potato, causing diversion of carotenoid synthesis from the α- to the β-branch (Diretto et al., 2007), and expression of the cauliflower ***Or*** gene in tubers increasing the storage capacity for carotenoids have also been observed (López et al., 2008).

10.18.3.2.2 Vitamin C

Ascorbate (vitamin C) is an antioxidant and also cofactor of several enzymes, including those required for the synthesis of collagen, carnitine, cholesterol, and certain amino acid hormones. Vitamin C deficiency causes the ulceration disease scurvy, resulting in the breakdown of connective tissues (Bartholomew, 2002).

Overexpression of L-gulonolactone oxidase (***GLOase***) in lettuce caused a sevenfold improvement in ascorbate fresh weight (Jain and Nessler, 2000). Similarly, a twofold increase by expressing the same gene in potato tubers and a six times increase by expressing the rice dhar gene from the ascorbate recycling pathway in multivitamin maize (Naqvi et al., 2010) were achieved. Co-expression of stylo 9-cis-epoxycarotenoid dioxygenase and yeast D-arabinono-1,4-lactone oxidase improved not only vitamin C level but also the tolerance to drought and chilling in transgenic tobacco and stylo plants (Bao et al., 2016).

10.18.3.2.3 Vitamin B9

Folate (vitamin B9) is the source of tetrahydrofolate essential for DNA synthesis and many other core metabolic reactions. Folate deficiency causes macrocytic anemia and elevated levels of homocysteine, but in pregnant women, it can lead to the neural tube defect—spinal bifida in the fetus (Scholl and Johnson, 2000).

Two transgenic tomato lines, one expressing GCH1 enhancing the cytosolic (pterin) branch and the other ADCS1 enhancing the PABA branch, were crossed (de la Garza et al., 2007). The resultant single line released their individual bottlenecks of only double the enhancement from normal folate level to achieve a 25-fold increase in folate levels. This strategy in rice endosperm resulted in a 100-fold increase in folate levels, indicating its powerful potential in developing-country settings where rice is the staple diet (Storozhenko et al., 2007).

10.18.3.2.4 Vitamin E

Vitamin E comprises eight related molecules known as tocochromanols—powerful antioxidants—protecting FAs, low-density lipoproteins and other components of cell membranes from oxidative stress.

The α/γ tocopherol ratio in transgenic lettuce plants was increased by expressing the Arabidopsis γ-tocopherol methyltransferase (γ-TMT), achieving near complete conversion to α-tocopherol in the best-performing ones (Cho et al., 2005). Similarly, a 10.4-fold increase in α-tocopherol levels and a 14.9-fold increase in β-tocopherol levels in soybean seeds expressing Perilla frutescens γ-TMT was achieved (Tavya et al., 2007). The constitutive expression of two Arabidopsis cDNA clones encoding ρ-hydroxyphenylpyruvate dioxygenase (HPPD) and 2-methyl-6-phytylplastoquinol methyltransferase (MPBQ MT) increased the tocopherol content by threefold in transgenic maize (Naqvi et al., 2011).

10.19 GENETIC ENGINEERING FOR MOLECULAR FARMING

The production of recombinant proteins (including pharmaceuticals and industrial proteins) and other secondary metabolites in plants is known as plant molecular farming. It has long been considered as a promising strategy not only for agriculture and industry but also to produce valuable recombinant proteins for human and veterinary medicine. Some of the products are now commercially available. The process involves the growing, harvesting, transport, storage, and downstream processing of extraction and purification of the protein (De Wilde et al., 2002). It has been proven over the years that plants have the ability to produce even more complex functional mammalian proteins with therapeutic activity, such as human serum proteins and growth regulators, antibodies, vaccines, hormones, cytokines, enzymes, and antibodies (Liénard et al., 2007). Various plant expression platforms such as plant cell suspensions, plant tissues, whole plants; aquatic plants, etc. can be used for production of recombinant proteins (Fig. 10.2). A number plant types and systems have been used for expression of vaccine antigens (Rybicki, 2009). Initially, the systems that were edible by humans and animals, or had "Generally Regarded As Safe" (GRAS) status, were considered with the assumption that the vaccines would be eaten without further processing (Rybicki, 2010). The systems mainly include *Nicotiana* spp., *A. thaliana*, alfalfa, spinach, potatoes, duck-weed, strawberries, carrots, tomatoes, aloe, and single-celled algae. Proteins have also been expressed in seeds of maize,

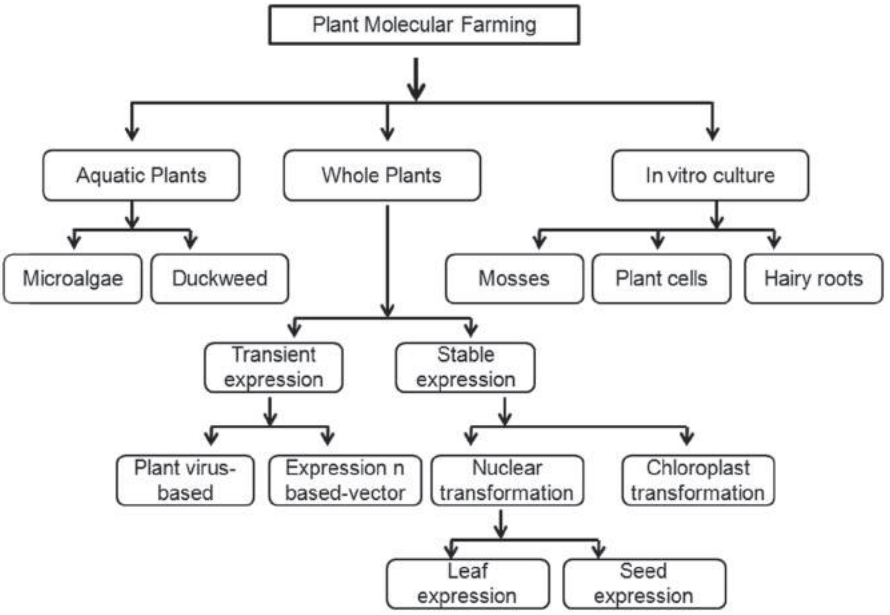

FIGURE 10.2 Plant expression platforms for production of various recombinant proteins. (Adapted from Xu et al., 2012).

rice, beans, and tobacco; in potatoes, tomatoes, and strawberries; in suspension cell cultures of tobacco and maize; in hairy root cultures; and in transformed chloroplasts of a variety of plant species. Human growth hormone, the first recombinant plant-derived pharmaceutical protein and the first recombinant antibody were produced in transgenic plants in 1986 and 1989, respectively (Barta et al., 1986; Hiattet al., 1989). However, avidin, the first recombinant protein was expressed in transgenic maize for commercial purpose in 1997 only (Hood et al., 1997). These trials really demonstrated that plants could be turned into biofactories for the large-scale production of recombinant proteins. This has been possible due to their ability to perform posttranslational modifications that make these recombinant proteins fold properly and maintain their structural and functional integrity. Transgenic plants are gaining more attention as the new generation bioreactors due to increasing demand for biopharmaceuticals, coupled with the high costs and inefficiency of the existing production systems (Knäblein, 2005), which include yeast, microbes, animals cells (Jones et al., 2003), and transgenic animals (Harvey et al., 2002). Thus, transgenic plants are now being seen as suitable alternatives as warehouse of molecular farming. Conventional

hosts have some peculiar limitations. On the contrary, use of plant systems as production units for various vaccines came out with number of potential advantages over the existing systems such as ability for large-scale production, reduction in cost, feasibility of oral delivery, and being useful units for production of glycosylated vaccines (Obeme et al., 2011).

10.19.1 ANTIGENIC VACCINES DEVELOPED IN PLANTS

Largely, vaccine is an antigenic preparation that activates immune system against a given disease. Quite a few such vaccines have been produced in the plant system. These include the hepatitis B surface antigen that has been expressed in transgenic potatoes (Richter et al., 2000), tomato (He et al., 2008b), banana (Kumar et al., 2005), and in tobacco cell suspension culture (Sojikul et al., 2003). Heat labile enterotoxin B subunit of *E. coli* has been expressed in potato (Lauterslager et al., 2001), maize (Chikwamba et al., 2002), tobacco (Rosales-Mendoza et al., 2009), and soybean (Moravec et al., 2007). Similarly, vaccine against gastroenteritis has been raised in maize against corona virus (Tuboly et al., 2000). Several crops, such as tobacco, tomato, and rice, have been used to express the cholera toxin B subunit of *Vibrio cholerae* (Daniell et al., 2001a; Mishra et al., 2006; Nochi et al., 2007). Transgenic tomato expressing RSV (respiratory syncytial virus) fusion (F) protein has been developed to be used as edible vaccine against RSV (Sandhu et al., 2000). Plants such as different leafy crops, cereals, legumes, oilseeds, fruits, vegetables, cell cultures, algae, etc. have been used for the production of biopharmaceutical proteins (Fischer et al., 2004). Some vegetables such as potato, tomato, and carrot have been reported to express vaccines (Walmsley and Arntzen, 2000). Potato has been used as a model plant for the production of oral vaccines (Polkinghorne et al., 2005). Tomato is the new system used as such an expression system. Proplastids of cultured carrot cells have been shown to express recombinant proteins (Daniell et al., 2005). Lettuce, celery cabbage, and cauliflower are among other plants that are being used as production system for the vaccines (Koprowski, 2005). Several plant-made vaccines for veterinary purposes, including avian influenza, Newcastle disease, foot-and-mouth disease, and enterotoxigenic *E. coli*, have been expressed in the plant (Lentz et al., 2010; Ling et al., 2010). Pigeon pea and peanut have been used to express the hemagglutinin protein of rinder pest (Satyavathi et al., 2003). The vaccine has already been commercialized (World Health Organization, 2007) against human papilloma virus (HPV), which is the causal organism of cervical cancer in women.

HPV virus and L1 proteins were generated in transgenic potato and tobacco plants (Santi et al., 2006). Others such as the L1 protein of human papillomavirus types 11 and 16 (Giorgi et al., 2010; Maclean et al., 2007), the Norwalk virus capsid protein (Mason et al., 1996), and the H5N1 pandemic vaccine candidate (D'Aoust et al., 2010) have been expressed in one or two of the plants like tobacco, potato, and carrots. Among fruit crops, expression of foreign proteins (vaccines) in banana with the help of promoter has been demonstrated (Trivedi and Nath, 2004). Vaccine production in papaya has also been demonstrated through expression of novel synthetic vaccine SPvac (Carter and Langridge, 2002; Sciutto et al., 2002). It is known that human immunodeficiency virus type 1 (HIV-1) is a dreadful disease worldwide. Effective vaccination will be much useful to control this virus. The expression of HIV-1 antigens expression in plants has been reported by a number of scientists (Bogers et al., 2004) and the production of HIV-1 subtype G Gag-derived proteins in *Nicotiana* spp. has also been demonstrated (Meyers et al., 2008). Thus, it is possible that vaccine production is done using all these systems on a large scale for systemic and oral immunization. Antigenic F1-V fusion protein from *Yersinia pestis*, the causal organism of bubonic and pneumonic plague, was expressed in lettuce for plant-based vaccine production (Rosales-Mendoza et al., 2010).

10.19.2 ANTIBODIES PRODUCED IN PLANTS

Recombinant antibodies were expressed for the first time in plants (Hiatt et al., 1989). Then on, different moieties ranging from single chain Fv fragments (ScFvs, which contain the variable regions of the heavy and light chains joined by a flexible peptide linker) to Fab fragments (assembled light chains and shortened heavy chains), small immune proteins (SIP), IgGs, and chimeric secretory IgA and single-domain antibodies have been expressed in plants (Ismaili et al., 2007; Xu et al., 2007). There are two main approaches that are being employed to produce biologically active whole antibodies in plants. One approach is crosspollination of individually transformed plants expressing light or heavy chains, resulting in high yield which reaches 1–5% of total plant protein (Hiatt et al., 1989; Ma et al., 1994). The other one involves cotransformation of the heavy and light chain genes on a single- (Düring et al., 1990), two- (Villani et al., 2009), or more expression cassettes (Nicholson et al., 2005). One of such plant derived antibodies; a secretory antibody against a surface antigen of *Streptococcus mutans* was actually found to be as effective as the original mouse IgG, in protecting against

S. mutans colonization on teeth (Ma et al., 1998). Recently, a HIV-specific monoclonal antibody produced in maize seeds was found to be as active as its Chinese hamster ovary-derived counterpart (Ramessar et al., 2008a, 2008b). Similar to antigenic vaccines, there are several different plant produced antibodies that are being tested in the clinical trials.

Plants do not produce antibodies naturally. However, plants can correctly assemble functional antibody molecules encoded by mammalian antibody genes. Pathogen toxins cause many plant diseases. One such disease is the soybean sudden death syndrome (SDS) caused by the fungal pathogen *Fusarium virguliforme*. It has so far not been possible to isolate the pathogen from diseased foliar tissues. One or more toxins produced by the pathogen have been considered to cause this foliar SDS. One of these possible toxins, FvTox1, was recently identified. Expression of anti-FvTox1-1 in stable transgenic soybean plants resulted in enhanced foliar SDS resistance compared to that in nontransgenic control plants (Brar and Bhattacharya, 2012).

10.19.3 NEUTRACEUTICAL AND THERAPEUTIC PROTEINS PRODUCED IN PLANTS

Antimicrobial nutraceuticals such as human lactoferrin and lysozymes have been successfully produced in several crops (Huang et al., 2008; Stefanova et al., 2008) and are also commercially available. There are other neutraceuticals that are under clinical trials.

A human growth hormone was the first therapeutic human protein to be expressed in plants (Barta et al., 1986). Human serumal bumin, which is normally isolated from blood, was produced in transgenic tobacco and potato (Sijmons et al., 1990). Since then, several human proteins have been expressed in the plants, which include epidermal growth factor (Bai et al., 2007; Wirth et al., 2004), α-, β-, and γ-interferons, which are used in treating hepatitis B and C (Arlen et al., 2007), erythropoietin, which promote red blood cell production (Musa et al., 2009; Weise et al., 2007), interleukin used in treating Crohn's disease (Elías-López et al., 2008; Fujiwara et al., 2010), insulin used for treating diabetes (Nykiforuk et al., 2006), human glucocerebrosidase used for the treatment of Gaucher's disease in GE carrot cells (Shaaltiel et al., 2007) and some other plants. Some of these therapeutics are at different stages of clinical trials or at the verge of commercialization.

10.19.4 NONPHARMACEUTICAL PROTEIN DERIVED FROM PLANTS

The nonpharmaceutical plant-derived proteins or industrial proteins such as avidin, trypsin, aprotinin, β-glucuronidase (GUS), peroxidase, laccase, cellulase are available in the market. Molecular farming of cell-wall degrading enzymes such as cellulases, hemicellulases, xylanases, and ligninases are of great importance for the biofuel industry required for production of cellulosic ethanol (Chatterjee et al., 2010; Mei et al., 2009; Sticklen, 2008). Other nonhydrolytic proteins such as cell wall disintegrating carbohydrate-binding modules of cell wall degrading enzymes, and the cell wall loosening proteins like the expansins that are useful in enhancing the efficiency of cell wall degradation by disrupting the different polysaccharide networks and thereby allowing increase accessibility of the hydrolytic enzymes to the substrate are potential candidates for molecular farming (Obeme et al., 2011).

Other potential nonpharmaceutical plant-derived technical proteins that are being explored and optimized for production include polyhydroxyalkanoate (PHA) copolymers, and poly(3-hydroxybutyrate) (PHB), which are biodegradable plastic-like compounds (Conrad, 2005; Matsumoto et al., 2009). However, it should be noted that only a few plant-derived pharmaceuticals have been approved for molecular farming so far (Obeme et al., 2011).

10.20 GENETIC IMPROVEMENT OF BIOFUEL CROP PLANTS

Major obstacles in biofuel production include lack of biofuel crop domestication; low oil yields of relevant crop plants as well as recalcitrance of lignocellulose to chemical and enzymatic breakdown. Research and development efforts for biofuel production are targeted at obtaining renewable liquid fuels from plant biomass. Researchers have gathered quite some knowledge on the genetic and genomic resources available for improvement biofuel crops. Biofuel production from various crop plants has already been demonstrated. This knowledge will be used to produce the next generation of biofuel crops by increasing lipid content with respect to some specific FAs and by optimizing the hydrolysis of plant cell walls to release fermentable sugars.

Commercially, bioethanol is derived from corn and biodiesel is obtained from plants with a high content in FAs such as soybean, canola, and sunflower. However, an alternative to these crops is required because corn and soybean are some of the major food crops and the yields of starch and plant oil in

these crops are too low to cover the huge demand of transportation fuels. This has prompted the development of alternative biofuel production based on lignocellulosic biomass (Schubert, 2006; Sticklen, 2008; Tilman et al., 2009). Lignocellulose, composed of the polysaccharides cellulose and hemicellulose, and lignin, a phenolic polymer, are some of the most abundant biomaterials on earth (Pauly and Keegstra, 2008). Biofuel crops are to be grown in a strategic manner. Biofuel crops should not take away arable land area, at least, for major crops, and at the same time, it is advisable to avoid application of any fertilizer or pesticide. Methods for efficient genetic transformation of switchgrass, Jatropha, poplar and *Brachypodium* using *Agrobacterium* have been developed. Some of the other achievements in this field also includes production of PHB in transgenic switchgrass (Somleva et al., 2008) and development of tissue culture techniques for the propagation of *Miscanthus* and Jatropha explants (Sujatha et al., 2008).

10.20.1 ENGINEERING OF PLANT OIL METABOLISM

Increasing seed oil production is a major goal for global agriculture to meet the high demand for oil consumption by humans and for biodiesel production. Overexpression of *ZmLEC1* (maize LEAFY COTYLEDON1) increased seed oil by as much as 48%, but it resulted in reduction in seed germination and leaf growth in maize, whereas overexpression of *ZmWRI1* (maize WRINKLED1) resulted in an oil increase similar to overexpression of *ZmLEC1* without affecting germination, seedling growth, or grain yield (Shen et al., 2010). Triacylglycerols (TAGs) from plant seed storage oils are excellent sources for the generation of biodiesel (Durett et al., 2008; Dyer et al., 2008). Trans-esterification of plant TAGs with methanol is done in the presence of acid or alkali to produce fatty-acid methyl esters (FAMEs). Biofuel crops such as soybean and Jatropha have either low or unpredictable oil yields. Redirecting the biosynthesis of specific types of FAs are needed to achieve optimal biodiesel production increasing oil content in plants. The strategies for optimal FAME production includes lowering of the levels of both saturated and polyunsaturated FAs, while increasing the amount of monounsaturated FAs, such as palmitoleate (C16:1) or oleate (C18:1) (Durett et al., 2008). Downreglation of FATB, an acyl-ACP thioesterase, in soybean caused accumulation of oleic acid up to 85% from 18% in the wild type and reduction in the levels of the saturated FA palmitate (Buhr et al., 2002). Expression levels of enzymes involved in synthesis of TAG have been engineered to increase oil content in seeds. Overexpression

of a fungal diacylglycerol acyltransferase (DAGT2) enzyme led to increase in oil content in soybean seeds and Arabidopsis (Lardizabal et al., 2008). Activation of the FA biosynthetic pathway has been an alternative means to increase seed oil content in plants. For example, total FA and lipid seed content increased in transgenic Arabidopsis overexpressing of two soybean transcription factors **Dof4** and **Dof11** (Wang et al., 2007). **Dof4** and **Dof11** activated lipid biosynthesis in Arabidopsis through activity of acetyl CoA carboxylase and long-acyl-CoA synthase, respectively. Similarly, the lipid content in transgenic canola seeds was increased by 40% by overexpression of the yeast glycerol 3-phosphate dehydrogenase (ghpd1) (Vigeolas et al., 2007). It is noteworthy that engineering of oil accumulation in vegetative tissues, such as leaves, is an attractive approach to increase overall yield of oils for biodiesel production.

10.20.2 ENGINEERING PLANT LIGNOCELLULOSE

One of the main areas of research and development is the study of synthesis of plant cell wall components and their degradation. The plant cell walls are composed of cellulose, hemicellulose, and lignin. The highly complex nature of lignocellulose requires costly and harsh pretreatments to gain access to monosaccharides. The ultimate goal is develop improved lignocellulosic characteristics for easier and more efficient breakdown. The lignin component has repeatedly been pointed out as the major factor contributing to cell wall recalcitrance for access to monosaccharides (Akin, 2007; Weng et al., 2008). Significant improvement in fermentable sugar release from lignocellulose was achieved by downregulating certain monolignol biosynthetic enzymes in transgenic alfalfa. Enhanced enzymatic cell wall hydrolysis was correlated with lower lignin amounts in alfalfa lines silenced for cinnamate 4-hydroxylase, hydroxycinnamoyl CoA:shikimate hydroxycinnamoyl transferase, and coumaroyl shikimate 3-hydroxylase (Chen and Dixon, 2007). The enzymes such as cinnamoyl CoA reductase (CCR) and cinnamyl alcohol dehydrogenase (CAD) function at later stages in monolignol biosynthesis. Downregulaiton of CCR and CAD in alfalfa lines led to significant improvements in enzymatic saccharification efficiency. This plasticity of lignin can be exploited for engineering of lignin compositions for improved lignin extraction from a given plant biomass. For example, maize cell walls with coniferyl ferulate as an additional component had improved enzymatic hydrolysis and sugar release (Grabber et al., 2008).

Still, the improvement of plant biomass characteristics for biofuel production is at infancy. Biofuel crops have been identified and are at various levels of domestication and cultivar selection, whereas genetic and genomic resources for these species, including draft genome sequences and transformation protocols, are constantly being developed. Major breakthroughs on the understanding of lipid metabolism and plant cell wall biosynthesis and structure are still needed to overcome low oil yields and the recalcitrance of lignocellulose, respectively, for efficient and cost-competitive conversion to biofuels (Vega-Sánchez and Ronald, 2010).

10.21 ENGINEERING MULTIPLE GENES IN PLANTS

Recently, it is seen that increasing number of researchers are transferring multiple genes in order to generate plants with ambitious phenotypes. This allows researchers to achieve objectives that were once thought almost impossible. The potentiality of this approach now appears limitless. The transformation methods used earlier for plants were developed with the implicit intention to introduce one or two genes, which mainly included a primary transgene and a selectable or screenable marker and corresponding protocols had been optimized accordingly (Twyman et al., 2002). Technical bottlenecks limiting the number of genes to be transferred to plants had introduced a serious limitation to the progress of plant biotechnology in past decades (Carpell and Christou, 2004; Dafny-Yelin and Tzfira, 2007; Halpin, 2005). Current ability to transfer multiple genes into plants enables researchers to study and manipulate entire metabolic pathways, express multimeric proteins or protein complexes, and study complex genetic and regulatory networks. However, various multiple gene transfer methods certainly still have some limitations. The methods may involve conventional gene stacking method such as crossing transgenic lines, sequential transformation of desired cultivars using either more than a single vector each containing a single transgene or a single vector consisting of more than a single transgene of interest, in addition to the necessary selectable or screenable markers. The more the number of transgenes, the lesser will be the chance that all of them get integrated in the genome and expressed. It would thus require larger populations of plants to be screened to identify rare transgenic lines with the most sought-after genotype. Essentially all these methods aim to achieve the creation of a SMART locus, that is, a locus containing stable multiple arrays of transgenes (Naqvi et al., 2009).

The major application of multiple gene transfer to plants has been in the analysis and modification of metabolic pathways, which requires a number of genes. For example (1) three genes were engineered each into potato and rice for carotenoid pathway, linseed, tobacco, and Arabidopsis for PUFA synthesis and canola for vitamin E synthesis; (2) up to four genes were engineered into Arabidopsis, canola, and soybean for vitamin E synthesis; (3) four genes were engineered into maize for carotenoid, ascorbate, and folate pathways; (4) up to five genes were engineered into maize for carotenoid pathway, (5) five genes were engineered into soybean for PUFA synthesis; (6) seven genes were engineered into canola for carotenoid pathway; and (7) up to nine genes were engineered into mustard for PUFA synthesis.

10.21.1 ENGINEERING FOR VITAMIN E SYNTHESIS

Arabidopsis ***pds1***, ***hpt1***, and ***vte1*** genes were introduced in canola using an *Agrobacterium*-linked cotransformation strategy. The tocochromanol content was doubled in best performing lines (Raclaru et al., 2006). The subsets of ***tyrA***, ***pds1***, ***hpt1***, and ***ggh*** genes were introduced into Arabidopsis, canola, and soybean using *Agrobacterium*-linked cotransformation strategy to increase tocochromanol levels. Best result obtained was 15-fold tocochromanol increase in soybean (94% tocotrienols) (Karunanandaa et al., 2005).

10.21.2 ENGINEERING FOR PUFA SYNTHESIS

Arabidopsis was transformed with three different combinations of two desaturases and one elongase using an *Agrobacterium*-linked cotransformation strategy. It resulted in increased EPA and ARA content (Hoffmann et al., 2009). Two desaturases and one elongase were again separately engineered into Arabidopsis using an *Agrobacterium*-linked cotransformation strategy. In this case, EPA and ARA content increased at the expense of ALA (Qi et al., 2004). Similarly, when linseed and tobacco were genetically transformed with paired combinations of six different FA desaturases and elongases increase in GLA and SDA levels were achieved (Abbadi et al., 2004). Introduction of five genes encoding FA desaturases and elongases from two microbial species and Arabidopsis into soybean using an *Agrobacterium*-linked cotransformation strategy achieved a 20% increase in EPA (Kinney et al., 2004). When nine genes encoding FA desaturases and elongases from five microbial species were engineered into mustard using

Agrobacterium-linked cotransformation strategy, a 25% increase in ARA and a 15% increase in EPA was achieved (Wu et al., 2005).

10.21.3 ENGINEERING FOR CAROTENOID PATHWAY

Introduction of the genes such as ***crtI***, ***crtB***, and ***crtY*** from two species into rice using a combo-linked/unlinked strategy (multiple genes on two T-DNAs), a 23-fold increase in β-carotene levels was achieved in rice grains (Ye et al., 2000). When the same set of genes was engineered into potato there was a 20-fold increase in carotenoid levels (Ravanello et al., 2003). Introduction of ***psy1***, ***crtI***, ***lycb***, ***bch***, and ***crtW*** genes from four species using an unlinked direct transfer cotransformation strategy in maize recovered transgenic plants with a range of phenotypes reflecting different carotenoid contents (Zhu et al., 2008). The genes ***idi***, ***crtE***, ***crtB***, ***crtI***, ***crtY***, ***crtZ***, and ***crtW*** from three species were introduced into canola using *Agrobacterium*-linked cotransformation strategy with the aim to increase carotenoid levels, particularly ketocarotenoids and resultant effect a tremendous enhancement in the level of carotenoids was observed (Fujisawa et al., 2009).

10.21.4 ENGINEERING CAROTENOID, ASCORBATE, AND FOLATE PATHWAYS

The genes such as ***Zmpsy1*** and ***PacrtI*** for carotenoid pathway, ***Dhar*** for ascorbate pathway, and ***folE*** for folate pathway were engineered into maize using an unlinked direct transfer cotransformation strategy to increase levels of β-carotene, folate, and ascorbate in the endosperm. Significant increases in all three nutrients were achieved producing "super-nutritious" cereals (Naqvi et al., 2009).

10.21.5 ENGINEERING ABIOTIC STRESS TOLERANCE

Abiotic stress tolerance has mainly been achieved in plants by the transfer of a single gene (Muthurajan and Balasubramanian, 2009). Since abiotic stress tolerance of plants is a very complex trait and involves multiple physiological and biochemical processes, it is thought that the improvement of plant stress tolerance should involve pyramiding of multiple genes. Therefore, the generation of transgenic plants by introducing two or more

foreign genes has become one of the important goals of plant genetic engineers to combat abiotic stresses (Gouiaa et al., 2012). A novel cultivar of maize expressing ***betA*** and **TsVP** (encoding $V^{-}H^{+}$-PPase from *Thellungiella halophila*) was developed using conventional cross hybridization technique (Wei et al., 2011). Development of GE maize plants expressing two genes ***ApGSMT2*** and ***ApDMT2*** from the bacterium *Aphanothece halophytica* with an enhanced ability to synthesize glycine betaine was reported (He et al., 2013). Effectiveness of co-expression of two heterologous abiotic stress tolerance genes ***HVA1*** and ***mtlD*** in maize (*Z. mays*) was demonstrated to confer drought and salt tolerance (Nguyen et al., 2013).

10.21.6 ENGINEERING BIOTIC STRESS TOLERANCE

In a few cases, the genes for chitinases and glucanases have been expressed together in a given host to attain even a higher degree of fungal disease resistance. Sometimes they have been also used together with some other antifungal genes. For example, transgenic potato-expressing chitinase and glucanase (Chang et al., 2002); rice-expressing chitinase and RIP (Kim et al., 2003); rice-expressing chitinase and glucanase (Mei et al., 2004); soybean-expressing chitinase and RIP (Li et al., 2004); tomato-expressing glucanase and an antifungal protein **(*alf*AFP)** (Chen et al., 2006); rice-expressing chitinase, glucanase, and RIP (Zhu et al., 2007b); barley-expressing chitinase and TLP (Tobias et al., 2007); and carrot-expressing chitinase, glucanase, and a cationic peroxidase (Wally et al., 2009) for enhanced fungal disease resistance were developed. When modified ***cry1Ab*** and ***cry1Ac*** genes from *Bt* were pyramided in transgenic chickpea (*C. arietinum* L.), it improved resistance to pod borer insect *H. armigera* (Mehrotra et al., 2011).

10.22 BIOSAFETY CONCERNS AND EXPERIMENTAL STRATEGIES

10.22.1 BIOSAFETY

Biosafety is all about ensuring safety and security of both, ecology and human health, so that biological integrity is maintained. It is about minimizing the perceived risks to environment and human health from the handling of genetically modified organisms (GMOs) developed through modern biotechnology. It is remarkable that convention on biological diversity addresses the conservation and sustainable use of biodiversity. Governments from 130

countries agreed the Cartagena Protocol on Biosafety in Montreal in January 2000. It sets out rules for risk assessment, risk management, Advance Informed Agreement (AIA), technology transfer, and capacity building. AIA procedures will take care of the transgenic plants introduced into the environment intentionally, which may threaten biodiversity.

10.22.2 BIOSAFETY CONCERNS

Transgenesis has been in use for over 20 years for genetic improvement of crop plants. Transgenic crops generally carry foreign genes inserted randomly in the genome, and their commercialization is frequently prevented by public concern over *health and environmental safety issues*. Transgenic crop products are the most highly regulated items in the world. In recent years, there have been calls in the United States to relax some of the rules for their oversight. But, controversies over the safety of transgenic food products continue to resonate, particularly in Europe, Africa, and recently in the Far East. Numerous national and international scientific panels have concluded that food derived through transgenic approaches is as safe as food produced otherwise. In fact, the foodborne pathogens pose a much greater threat to human health. However, scary stories continue to appear in the media and questions continue to be asked about the adequacy of current regulatory systems to determine the safety of our food, transgenic or otherwise (DeFrancesco, 2013). It is thought people would show more preference for GM foods if they were eco-friendly.

The great success of GM crops has had an enormous impact on world crop production and cultivation pattern of agricultural species (James, 2006). The extensive environmental release and cultivation of GM crop varieties have aroused tremendous biosafety concerns and debates worldwide (Stewart et al., 2000). Biosafety issue has already become a crucial factor in constraining the further development of transgenic biotechnology and wider application of GM products in agriculture. There are a number of ***biosafety-related concerns*** in general, but the most important ones envisaged as ***ecological risks*** can be summarized as follows:

1. Direct and indirect effects of toxic transgenes (e.g., the *Bt*-insect-resistance gene) to nontarget organisms (O'Callaghan et al., 2005; Oliveira et al., 2007); insect pests may develop resistance to crops with *Bt* toxin.

2. Influences of transgenes and GM plants on biodiversity, ecosystem functions, and soil microbes (Oliveira et al., 2007); it may lead to monoculture and threaten crop genetic diversity with a possible genetic erosion over a period of time.
3. Transgene escape to crop landraces and wild relatives through gene flow and its potential ecological consequences (Lu and Snow, 2005; Mercer et al., 2007); potential transfer of genes from herbicide-resistant crops to wild or weedy relatives thus creating "superweeds."
4. Potential risks associated with the development of resistance to biotic-resistance transgenes in the target organisms (Li et al., 2007a, 2007b; Wu, 2006).

Among the above environmental biosafety issues, transgene escape from a GM crop variety to its non-GM crop counterparts or wild relatives has aroused tremendous debates worldwide Lu and Snow, 2005). This is because transgene escape can easily occur via gene flow that may result in potential ecological consequences, if significant amounts of transgenes constantly move to non-GM crops and wild relative species. Despite the potential benefits of transgenic crops, there are also concerns regarding the possible environmental and agronomic impacts if the transgenes escape and get established in natural or agricultural ecosystems. From an agronomic point of view, the transfer of novel genes from one crop to another may have many implications, including depletion in the quality of seeds leading to a change in their performance and marketability. Concerns over the ecological impacts of transgenic crops largely depend upon whether or not a crop has wild relatives and the ability to cross pollinate them. If crops hybridize with wild relatives and gene introgression occurs, wild populations could incorporate transgenes that change their behavior and they could present a serious threat as weeds or competitors in natural communities. This is particularly true when these transgenes can bring evolutionary selective advantages or disadvantages to crop varieties or wild populations. It is therefore essential to properly address the most relevant questions relating to the transgene outflow and its potential environmental consequences on a science-based altitude. Some of these concerns have been discussed in detail in the following section.

10.22.3 GENE FLOW IN THE NATURE

Because transgene technology has profound effect on management of biotic stress, transgenic plants may have substantial impact in the coming years.

The only risk of transgenic crops to the environment that might be permanent is ***gene flow*** from the crop to the close relative. Darwin considered mutations to be the basis of species evolution, but the mutations need not have been in the genes of the species in question. Instead, mutations can also come from a related species, or even further afield. The genome of any species or even cultivar is constantly changing, and thus the term "genetic purity" of varieties is inappropriate, as varieties continually change through selection, breeding, or genetic engineering. However, genes regularly move within species, to and from crops, as well as to their con-specific progenitors, feral and weedy forms. This gene movement between sexually compatible individuals, known as "vertical gene flow," can occur between varieties and strains and among some readily interbreeding species. "Horizontal gene flow" can occur between the kingdoms or distantly related species. It is far more common in prokaryotic organisms, for example, phages move from plasmids with antibiotic resistance among bacteria. A third type of gene introgression called "diagonal gene flow" occurs between the crops and distantly related, hardly sexually interbreeding relatives, within a genus, or among closely related genera. The risks are quite different from genes flowing to natural ecosystems versus ruderal and agro-ecosystems. Transgenic herbicide resistance posses a major threat if introgressed into weedy relatives, whereas disease and insect resistance pose less so (Gressel, 2014).

Naturally, various marker traits such as AFLP, RAPD, SSLP, RFLP, chloroplast, etc. move from crops to weeds. Incorporation of crop genes into wild and weedy relative populations (i.e., introgression) has long been of interest to ecologists and weed scientists. Potential negative outcomes that result from crop transgene introgression (e.g., extinction of native wild relative populations; invasive spread by wild or weedy hosts) have not been documented, and few examples of transgene introgression exist. However, molecular evidence of introgression from nontransgenic crops to their relatives continues to emerge, even for crops considered as low-risk candidates for transgene introgression. Recently, there are reports of gene flow from crops to relatives via pollen for traits such as resistance to imidazolinone, chlorotoluron, difenzoquat, glufosinate, glyphosate, disease, etc.

10.22.4 IS THE TRANSGENE FLOW DELETERIOUS TO LANDRACES, WEEDS, AND IN GENERAL?

Some argue for preserving "genetic purity" of landraces, although others counter-argue that transgene flow of crop protection traits into landraces will

facilitate their continued cultivation. Farmers are less likely to abandon the landraces for higher yielding cultivars or hybrids if the landraces are resistant to most insects and pathogens, or if weeds can be cost-effectively controlled without hand labor. Thus, if the intention really is to preserve the cultivation of landraces, and not just have their presence in gene banks, then such gene flow is obligatory (Gressel, 2014). Crop protection traits have already introgressed from crops to related weeds. Here, the answer for crop protection traits is usually affirmative for herbicide resistance, but not so much for other traits such as disease or insect resistance, where data are more ambiguous owing to the sporadic nature of disease and insect incidences affecting weeds. Such genes would clearly increase weed fitness when microbial or insect biocontrol measures are used against the weed (Gressel, 2014). It is a basic assumption of many technology detractors that transgene flow to wild species is deleterious, and that transgenes will "takeover" local genes and "contaminate" natural populations, cause a loss of "genetic purity" and lead to a loss of biodiversity. Swamping is usually doubtful, as pollen must get from the crop to the distant wild ecosystem. Pollen loses vitality with time, and there will be a distance between the two ecosystems, and distance equals time. Whether the transgene becomes established in an ecosystem then depends on the nature of the transgene (Gressel, 2014). There are two potential risks following transgene introgression from crops to their wild or weedy relatives as depicted.

10.22.5 HOW TO DEAL WITH "TRANSGENE FLOW" WITHIN THE ECOSYSTEM?

Technologies have been proposed to contain genes within crops (chloroplast transformation, male sterility) that imperfectly prevent gene flow by pollen to the wild. Pollen that carries a transgene is required in almost all transgene introgression models. Hence, transgene introgression could be completely prevented if pollen does not develop, and multiple methods have been used to decrease pollen fertility via *genetic male sterility* or *cytoplasmic male sterility* (CMS). Conversely, since pollens contain no cytoplasm, it is probably safe if we can contain the selectable markers within the chloroplast for generation of transgenic plants. Chloroplasts do not enter into the male gamete and thus, the possibility of transgene escape via pollens is negated. On the other hand, containment does not prevent related weeds from pollinating crops. Repeated backcrossing with weeds as pollen parents results in gene establishment in the weeds. Transgenic

mitigation relies on coupling crop protection traits in a tandem construct with traits that lower the fitness of the related weeds. Mitigation traits can be morphological (dwarfing, no seed shatter) or chemical (sensitivity to a chemical used later in a rotation). Tandem mitigation traits are genetically linked and will move together. Mitigation traits can also be spread by inserting them in multicopy transposons, which disperse faster than the crop protection genes in related weeds. Thus, there are gene-flow risks mainly to weeds from some crop protection traits, and these risks will have to be dealt with (Gressel, 2014).

10.22.6 WHAT IS REQUIRED FOR SUCCESSFUL TRANSFER OF A TRANSGENE FROM PLANT TO EITHER A MICROBE OR MAMMALIAN CELL?

Transfer of plant DNA into microbial or mammalian cells under normal conditions of dietary exposure would require all of the following events to occur: (1) removal of the relevant gene from the plant genome, probably as linear fragments; (2) protection of the gene from nuclease degradation in the plant as well as animal gastrointestinal tract; (3) uptake of the gene with dietary DNA; (4) transformation of bacteria or competent mammalian cells; (5) insertion of the gene into the host DNA by rare repair or recombination events into a transcribable unit; and finally (6) continuous stabilization of the inserted gene (FAO/WHO, 2000).

10.22.7 REMOVAL OF SELECTABLE MARKERS FROM GM CROPS

During the efficient genetic transformation of plants with the gene of interest, some selectable marker genes are also used in order to identify the transgenic plant cells or tissues. Usually, antibiotic- or herbicide-selective agents and their corresponding resistance genes are used to introduce economically valuable genes into crop plants. From the biosafety authority and consumer viewpoints, the presence of selectable marker genes in transgenic crops released may be transferred to weeds or pathogenic microorganisms in the gastrointestinal tract or soil, making them resistant to treatment with herbicides or antibiotics, respectively. Sexual crossing also raises the problem of transgene expression because redundancy of transgenes in the genome may

trigger homology-dependent gene silencing. The future potential of transgenesis technologies for crop improvement depends greatly on our abilities to engineer stable expression of multiple transgenic traits in a predictable fashion and to prevent the transfer of undesirable transgenic material to nontransgenic crops and related species. Therefore, it is now essential to develop an efficient marker-free transgenesis system. These considerations underline the development of various approaches designed to facilitate timely elimination of transgenes when their function is no longer needed. Due to the limited availability of suitable selectable marker genes, the stacking of transgenes will be increasingly desirable in future. The production of marker-free transgenic plants is now a critical requisite for their commercial deployment and also for engineering multiple and complex trait. Here we describe the current technologies to eliminate the selectable marker genes in order to develop marker-free transgenic plants and also discuss the regulation and biosafety concern of GM crops.

The genetic markers developed for use for genetic transformation of plants have been derived from either bacterial or plant sources and can be divided into two types: selectable and screenable markers. *Selectable markers* are those that allow the selection of transformed cells, or tissue explants, by their ability to grow in the presence of an antibiotic such as hygromycin, and kanamycin or a herbicide like glyphosate. In addition to selecting for transformants, such markers can be used to follow the inheritance of a foreign gene in a segregating population of plants. The co-introduction of selectable marker genes, especially antibiotic-resistance genes, is required for the initial selection of plant cells that are complemented with a new trait. ***Screenable markers*** encode gene products whose enzyme activity can be easily assayed, allowing not only the detection of transformants but also an estimation of the levels of foreign gene expression in transgenic tissue. Markers such as GUS, luciferase, or β-galactosidase allow screening for enzyme activity by histochemical staining or fluorimetric assay of individual cells and can be used to study cell-specific as well as developmentally regulated gene expression.

A number of selectable marker genes, mostly conferring resistance to antibiotics or herbicides, have been used previously for plant transformation studies. However, the most commonly used selectable markers are (1) *nptII* and *hpt* genes (for resistance to the aminoglycoside antibiotics, kanamycin and hygromycin, respectively) and (2) *bar* gene (for resistance to herbicide phosphinothricin).

10.22.7.1 THE CAUSE OF CONCERN

The successful use of antibiotics in medicine has now become a problem. The presence of selectable marker genes, especially those which include genes coding for antibiotic resistance and which are essential for the initial selection of transgenic plants, is considered undesirable. This is because the transgenes integrate at random positions in the genome leading to possible unwanted mutations and unpredictable expression patterns. The drawbacks of traditional markers are well felt even in practical research, which includes the following.

1. There are only a few selectable markers available for each crop species. But, different marker gene systems are required for the retransformation of plants that have already been GM.
2. If several marker genes left over from various developmental phases accumulate in a plant, it is possible that the stability of the GE trait can be impaired.
3. The probability of unforeseen effects (pleiotropic effect) occurring in the plants increases with the number of transferred genes and marker genes because one gene may affect the functionality of the other.
4. In addition, there is a potential risk of *horizontal gene transfer* and *vertical gene transfer* that could create environmental problems.

However, the most confident way to overcome all the concerns is just to remove the cause of concern, that is, the selectable marker gene itself. Therefore, there is a need for the development of techniques for the efficient production of "clean" marker-free transgenic plants. Thus, the development of efficient techniques for the removal of selectable markers, as well as the directed integration of transgenes at safe locations in the genome, is of great interest to biotech companies. Furthermore, the removal of selectable marker genes will also have a technical advantage, since the number of available selectable marker genes is limiting, and stacking of transgenes will become more and more desirable in the near future. In the next generation of transgenic plants, antibiotic-resistance markers will be the exception rather than the rule. However, there is still a long way to go before sufficient new procedures and strategies are designed, optimized, and become available with the scientific community.

Selectable marker gene-free transgenic rice harboring the garlic leaf lectin gene exhibited resistance to sap-sucking planthoppers (Sengupta et al., 2010) and another set of marker-free transgenic plants had enhanced seed

tocopherol content (Woo et al., 2015). Chilling tolerance was improved in marker-free transgenic tomato plants through induced transgenic expression of ***At-CBF1*** (Singh et al., 2011). Selectable marker-free transgenic potato plants expressing ***cry3A*** against the Colorado potato beetle (*Leptinotarsa decemlineata* Say) were also developed (Guo et al., 2015).

10.23 TOOLS OF MODERN GENETIC ENGINEERING

10.23.1 METHODS OF ELIMINATION OF SELECTABLE MARKERS

There are several strategies to exclude the selectable genes from transgenic plants, such as cotransformation, site-specific recombination, multi-autotransformation vector, transposition system, and homologous recombination (HR).

10.23.1.1 COTRANSFORMATION

The cotransformation method is a very simple method to eliminate the marker gene from the nuclear genome. Cotransformation involves transformation with two plasmids that target insertion at two different plant genome loci. One plasmid carries a selective marker gene and the other carries the GOI. The following three methods are used in the cotransformation system: (1) Two different vectors carried by different *Agrobacterium* strains followed by ATMT (De Neve et al., 1997) or two plasmids are introduced in the same tissue by means of particle bombardment (Kumar et al., 2010); (2) two different vectors introduced into the same *Agrobacterium* cell for plant transformation (Sripriya et al., 2008); and (3) two T-DNAs can be introduced a single binary vector (two T-DNA system) for genetic transformation of plants (Miller et al., 2002). In these cotransformation systems, selectable marker genes and target genes are not placed between the same pair of T-DNA borders. Instead, they are placed into separate T-DNAs, which are expected to segregate independently in a Mendelian fashion. In this method, the selectable marker can be eliminated (Fig. 10.3) from the plant genome at the time of segregation and recombination that occurs during sexual reproduction by selecting on the transgene of interest and not the SMG in progeny.

The advantages of cotransformation methods include the high adaptability of conventional, unmodified *Agrobacterium*-mediated gene transfer methods and easier handling of the binary vectors because the two T-DNA are separated and, hence, target gene T-DNA can be handled independently of selectable marker T-DNA.

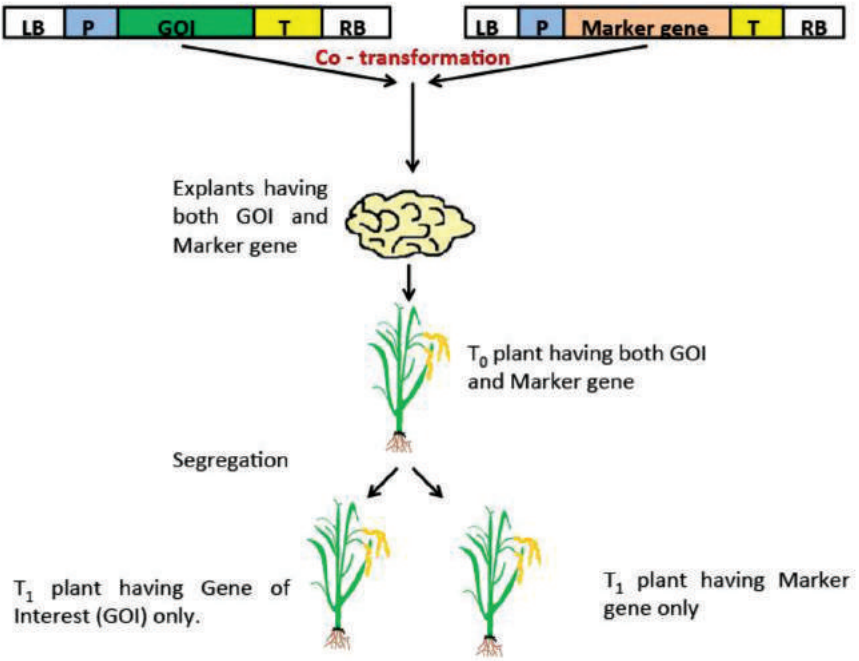

FIGURE 10.3 Diagram showing various steps of cotransformation method for generation of marker-free transgenic plants. (Adapted from Tuteja et al., 2012).

The limitations of the methods described above are very time consuming and compatible only for fertile plants. The tight linkage between co-integrated DNAs limits the efficiency of cotransformation. Indeed, integration of selectable marker and the transgene is at indiscriminate event: both the selectable marker and transgene may integrate in the same loci and that is not feasible for cotransformation.

10.23.1.2 MULTI-AUTOTRANSFORMATION

The multi-autotransformation (MAT) vector system represents a highly sophisticated approach for the removal of nuclear marker genes (Ebinuma et al., 1997). It is a unique transformation system that uses morphological changes caused by oncogene isopentenyltransferase (*ipt*) 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 (ESP), which subsequently reverts into normal shoots due to the excision of *ipt* gene by the function of "hit-and-run" cassette system (Ebinuma and Komamine, 2001). In this MAT system, a chosen GOI is placed adjacent to a multigenic element flanked by RS recombination sites (Fig. 10.4). 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). The MAT vector system is a positive selection system that gives the advantage of regeneration to the transgenic cells without killing the nontransgenic cells.

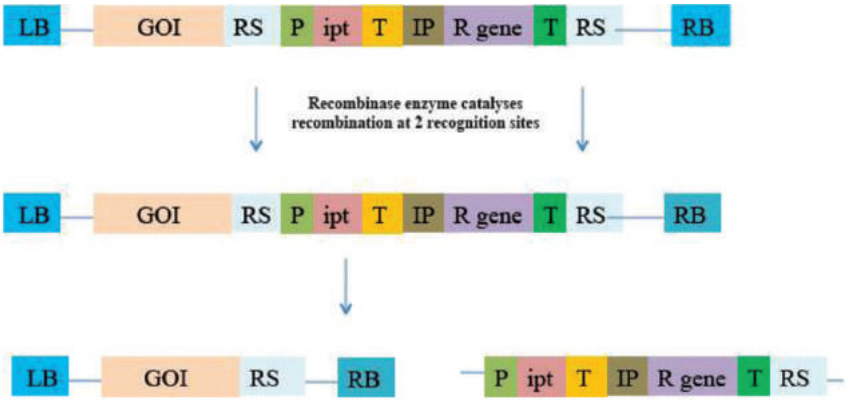

FIGURE 10.4 Multi-autotransformation (MAT). Oncogene (*ipt*) for selection of transgenic plants and a site-specific recombination system (R/Rs) are used in the principle of MAT. Recombinase (R) gene expression is under the chemically inducible promoter (IP) in order to avoid early removal of ipt gene. R catalyses recombination between two directly oriented recognition sites (RS) and removes a "hit-and-run" cassette from the plant genome (abbreviations: P, promoter; T, terminator; GOI, gene of interest; LB, left border; RB, right border). (Adapted from Tuteja et al., 2012).

10.23.1.3 SITE-SPECIFIC RECOMBINATION

Recombination is very clear phenomenon in biological systems: it occurs between two homologous DNA molecules. In bacteriophage, site-specific recombination takes place between defined excision sites in the phage and

in the bacterial chromosome. In site-specific recombination, DNA-strand exchange takes place between segments possessing only a limited degree of sequence homology (Coates et al., 2005). The site-specific recombination methods in plants have been developed to delete selection markers to produce marker-free transgenic plants or to integrate the transgene into a predetermined genomic location to produce site-specific transgenic plants (Nanto and Ebinuma, 2008). Basically, three site-specific recombination systems are well known and are described in the following sections for the elimination of selectable marker.

10.23.1.4 CRE/LOX RECOMBINATION SYSTEM

The Cre/loxP system consists of two components: (a) two loxP sites each consisting of 34-bp inverted repeats cloned in direct orientation flanking a DNA sequence and (b) 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 Arabidopsis (Zuo et al., 2001), *Nicotiana* (Gleave et al., 1999), *Z. mays* (Zhang et al., 2003) and *O. sativa* (Sreekala et al., 2005). *One of the greatest advantages* of the Cre/lox system is the specificity of the enzyme for its 34-bp recognition sequence. With a few exceptions, it is difficult to insert and excise genes with precision in the plant genome without a site-specific recombination system. One of the major limitations of this system is that marker gene removal from transgenic plants using the Cre/lox recombination system of bacteriophage P1 requires retransformation and out-crossing approaches that are laborious and time-consuming.

10.23.1.5 FLP/FRT RECOMBINATION SYSTEM

In the FLP/FRT site specific system of the 2-μm plasmid of *S. cerevisiae*, the FLP enzyme efficiently catalyses recombination between two directly repeated FLP recombination target (frt) sites, eliminating the intervening sequence. By controlled expression of the FLP recombinase and specific placement of the frt sites within transgenic constructs, the system can be applied to eliminate the marker genes following selection (Cho, 2009). It is possible to make an inducible FLP/frt site-specific recombinase system. However, one of the limitations of the process is it requires the process of retransformation to get both FLP and frt in the same system. A heat-inducible

strategy for the elimination of selection marker genes was also reported in vegetatively propagated plants like potato (Cuellar et al., 2006) (Fig. 10.5).

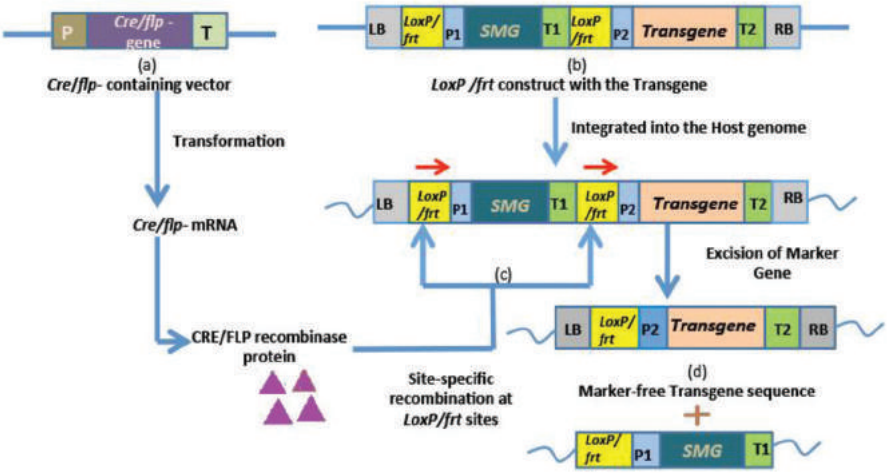

FIGURE 10.5 Development of selectable-marker-free transgenic plants using Cre/lox and/or FLP/FRT recombination system: (a) *cre/flp* encoding CRE-a Tyr recombinase protein or FLP is expressed under a constitutive or inducible promoter (chemical/heat shock). (b) *loxP/frt* flank the selectable marker gene on the T-DNA but not the transgene. These constructs can be introduced into the plant system via sequential transformation or cotransformation. Alternatively, genetic crossing can be used to bring both the constructs in the same plant. Following transformation, CRE/FLP gets access to *LoxP/frt* and causes site-specific recombination (c), thereby resulting into generation of selectable marker-free transgene sequence (d). Abbreviations: P, promoter; T, terminator; P1, promoter for marker gene; T1, terminator for marker gene; P2, promoter for transgene; T2, terminator for transgene; SMG, selectable marker gene; LB, left border; RB, right border sequence.

10.23.1.6 TRANSPOSITION-BASED METHODS

In general, all *Activator (Ac)* elements are identical, 4563 bp in length from maize. Transposase are the proteins that stimulate the movement of Ac. Deletions of Ac elements created *Dissociator (Ds)* elements in which all or part of this transposase was eliminated. This lack of transposase activity accounts for the inability of Ds elements to move in the absence of Ac. The transposase that is encoded by Ac elements can move throughout the cell and excise any Ds or Ac element. Thus, the Ac/Ds transposase is said to be transacting ability. Two transposon-mediated strategies have been developed

to generate marker-free transgenic plants. The Ac/Ds elements can be introduced into the plant genomes and can be very useful in removing the selectable marker gene as depicted (Fig. 10.6).

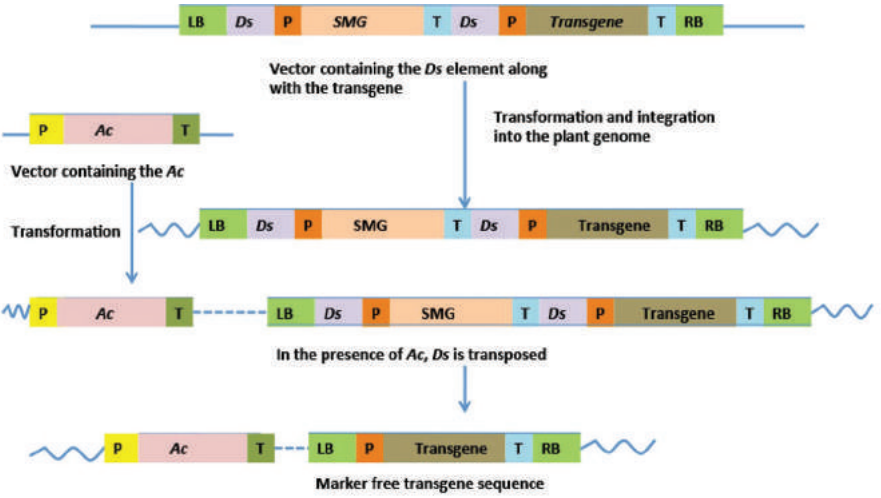

FIGURE 10.6 Development of selectable-marker-free transgenic plants using of Ac/Ds transposition system: Individual sequential transformation or cotransformation or genetic crossing can be used to introduce the constructs consisting of activator (*Ac*) and dissociator (*Ds*) elements in plants. An inducible promoter (chemical/heat shock) should regulate the expression of *Ac* gene in case of cotransformation or when both the expression cassettes are placed in a single vector backbone. *Ds* gets transposed (in the absence of *Ac*, *Ds* cannot get transposed) when *Ac* integrates into the genome and gets expressed, along with the selectable marker gene resulting in a marker-free transgene sequence. Abbreviations: P, promoter; T, terminator; SMG, selectable-marker gene; *Ac*, activator element; *Ds*, dissociation element; LB, left border; RB, right border.

10.23.1.7 POSITIVE SELECTION METHOD

In positive selection, GM cells are identified and selected without causing any injury or death to the nontransformed cell population (negative selection). In this case, the selectable marker gives the transformed cell the capacity to metabolize some compounds that are not usually metabolized. This fact 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. However, the nontransformed cells will not be able to grow and regenerate de novo plants.

10.23.1.7.1 The Gus Gene

The *gus* gene from *E. coli* codes for the GUS enzyme is widely used as a reporter gene in transgenic plants. In this system, GUS enzyme produced in the transformed cells hydrolyses benzyladenine *N*-3-glucuronide, glucuronide derivative of benzyladenine, which is an inactive form of the plant hormone cytokinin and releases benzyladenine, which is active cytokinin, in the medium. This cytokinin thus generated stimulates the transformed cell to regenerate, whereas the development of nontransformed cell is arrested. This marker system has been used for effective recovery of some transgenic plants (Okkels et al., 1997).

10.23.1.7.2 The manA Gene

The man gene from *E. coli* codes for the phosphomannose isomerase (PMI) enzyme. Mannose is converted into mannose-6-phosphate by endogenous hexokinase. Thus, when mannose is added to the culture medium, plant growth in the nontransfomed tissue may be minimized due to mannose-6-phosphate accumulation. PMI converts mannose-6-phosphate into fructose-6-phosphate, which in turn, is immediately channelized to glycolysis can be used as the sole carbohydrate source for the transformed cells. The mannose-6-phosphate cannot be metabolized the nontransformed cell and toxicity in plant cells was shown to be responsible for apoptosis, or programed cell death, through induction of an endonuclease, responsible for DNA laddering (Stein and Hansen, 1999). Mannose-6-phosphate accumulation also causes phosphate and ATP starvation, and thus, the critical functions such as cell division and elongation are retarded, giving rise to growth inhibition. Therefore, mannose turns out to be a very useful selection agent.

10.23.1.7.3 The xylA and DOGR1 Genes

Another positive selection system similar to PMI is the *xylA* encoding xylose isomerase isolated from *Thermoanaerobacterium thermosulfurogenes* or from *Streptomyces rubiginosus*. Transgenic plants of potato, tobacco, and tomato were successfully selected in xylose-containing media. Another gene *DOGR1*, from yeast, encoding 2-deoxyglucose-6-phosphate phosphatase (2-DOG-6-P) was has also been developed as a positive selection system. This marker confers resistance to 2-deoxyglucose (2-DOG) when overexpressed in transgenic plants. This system has been used to develop transgenic tobacco and potato plants (Kunze et al., 2001).

10.24 CISGENICS AND INTRAGENICS VERSUS TRANSGENICS

Although scientists add genes to crops via crop breeding, the breeding progeny is not considered as a GM crop because the introgressed genes and the regulatory sequences belong to the same host crop genus or in rare cases to the host's cross-breedable crop. One of the major concerns of the general public about transgenic crops relates to the mixing of genetic materials between species that cannot hybridize by natural means. To meet this concern, the two transformation concepts ***cisgenesis*** and ***intragenesis*** were developed as alternatives to transgenesis. Both concepts imply that plants must only be transformed with genetic material derived from the species itself or from closely related species capable of sexual hybridization. Furthermore, foreign sequences such as selection genes and vector-backbone sequences should be absent. If the donor gene and all of transgene's regulatory sequences belong to the same crop species or belong to the host's cross-breedable species, the resulting crop is called ***cisgenic***. In the ***cisgenic technology***, the cisgenic must be an identical copy of the host's native gene cassette, including its regulatory sequences integrated in the host plant in the normal sense orientation. In the ***intragenic technology***, gene cassettes containing specific gene sequences from crops are inserted into the crop that belongs to the same breedable gene pool. In this case, the promoters and terminators of different genes can regulate the gene-coding sequences. Cisgene and intragene constructs are depicted (Fig. 10.7). Cisgensis has been applied for improved baking quality of durum wheat using *1Dy10* (Gadaleta et al., 2008), late blight resistance in potato using *R* genes (Haverkort et al., 2009), scab resistance in apple using *HcrVf2* (Vanblaere et al., 2011), fungal disease resistance in grapevine using *VVTL-1* and *NtpII* (Dhekney et al., 2011), and improved grain phytase activity in barley using *HvPAPHY_a* (Holme et al., 2012). Intragenesis has been applied for high amylopectin content in potato using GBSS (de Vetten et al., 2003), scab resistance in apple using *HcrVf2* (Joshi et al., 2011), preventing black spot bruise in potato using *Ppo*, *R1*, and *PhL* (Rommens et al., 2006), gray mold resistance in strawberry using *PGIP*, limiting level of acrylamide in French fries from potato using *StAs1*, *StAs2* (Chawla et al., 2012), reducing lignin level in alfalfa using *Comt* (Weeks et al., 2008) and improving drought tolerance in perennial ryegrass using *Lpvp1* (Bajaj et al., 2008). Several surveys show higher public acceptance of intragenic/cisgenic crops compared to transgenic crops. The sexually compatible gene pool carries a high potential for generating plants with environmental, economic, and health benefits that may be essential for meeting the global need for a more efficient and sustainable crop production.

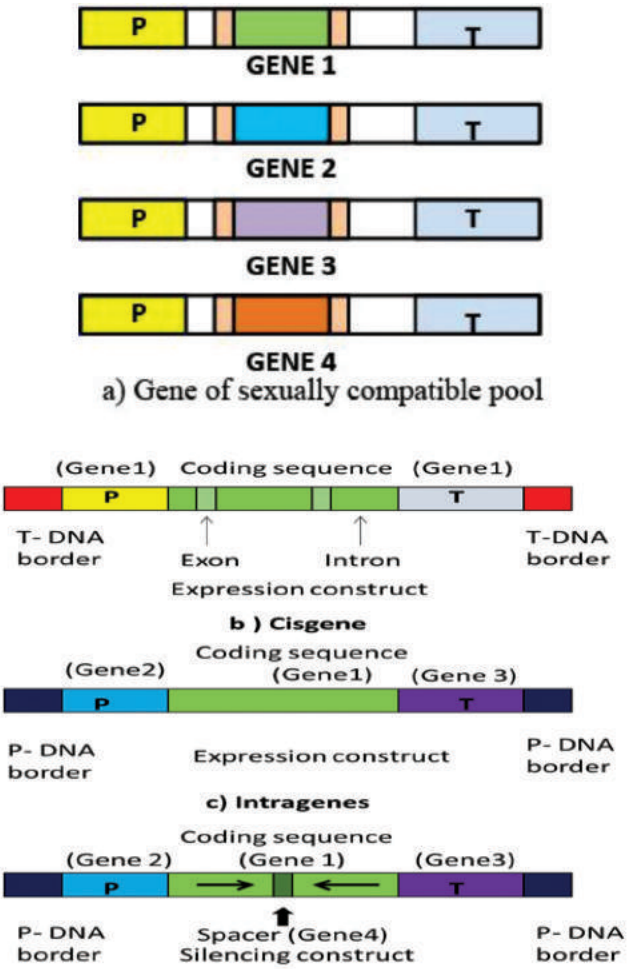

FIGURE 10.7 The cisgene is an identical copy of a gene from the sexually compatible pool including promoter, introns, and terminator. (a, b) The cisgene is inserted within *Agrobacterium*-derived T-DNA borders when following *Agrobacterium*-mediated transformation. Intragenesis allows in vitro recombination of elements isolated from different genes within the sexually compatible gene pool (a, c). (Adapted from Holme et al., 2013).

10.25 PLASTID GENETIC ENGINEERING

Plastid genetic engineering, with several unique advantages including transgene containment, has made significant progress in the last two decades in various biotechnology applications including development of crops with high

levels of resistance to insects, bacterial, fungal, and viral diseases, different types of herbicides, drought, salt and cold tolerance, CMS, metabolic engineering, phytoremediation of toxic metals and production of many vaccine antigens, biopharmaceuticals, and biofuels. However, useful traits should be engineered via chloroplast genomes of several major crops (Clarke and Daniell, 2011).

Plastid transformation was initially developed in *Chlamydomonas* and tobacco, but, it is now feasible in a broad range of species. It now is widely used in basic research and for biotechnological applications. Selection of transgenic lines where all copies of the polyploid plastid genome are transformed requires efficient markers. A number of traits have been used for selection such as photoautotrophy, resistance to antibiotics, and tolerance to herbicides or to other metabolic inhibitors. The most successful and widely used markers are derived from bacterial genes that inactivate antibiotics, such as *aadA* that confers resistance to spectinomycin and streptomycin, although the presence of a selectable marker that confers antibiotic resistance is not desirable for many biotechnological applications.

Selectable markers for plastid transformation routinely involve those for (1) photosynthesis such as *petA*, *ycf3*, and *rpoA* in tobacco (Klaus et al., 2003), and *rbcL* in tobacco (Kode et al., 2006); (2) antibiotic resistance such as *aphA-6* for kanamycin in tobacco (Huang et al., 2002) and cotton (Kumar et al., 2004) and *rrnS* for spectinomycin and spectromycin in tomato (Nugent et al., 2005); (3) herbicide resistance such as *bar* for phosphinothricin in tobacco, EPSP for glyphosate in tobacco (Ye et al., 2003), and *HPPD* for diketonitrile in tobacco (Dufourmantel et al., 2007), (4) metabolism such as *BADH* for betaine aldehyde in tobacco (Daniell et al., 2001b) and *ASA2* for Trp analogues in tobacco (Barone et al., 2009). One of the highly remarkable markers, *aadA*-encoding resistance to spectinomycin and spectromycin, has been routinely used over time for transformation of number of crop plants such as rice (Lee et al., 2006b), tomato (Ruf et al., 2001), oilseed rape (Cheng et al., 2005; Hou et al., 2003), carrot (Kumar et al., 2004), soybean (Dufourmantel et al., 2004), lettuce (Lelivelt et al., 2005; Ruhlman et al., 2010), cauliflower (Nugent et al., 2006), cabbage (Liu et al., 2007), sugarbeet (De Marchis et al., 2009), eggplant (Singh et al., 2010), etc.

10.26 SMALL RNA ENGINEERING

MicroRNA-based genetic modification technology (miRNA-based GM tech) can be used for increasing crop yields and quality. It is one of the most

promising solutions that contribute to agricultural productivity directly by developing superior crop cultivars with enhanced biotic and abiotic stress tolerance and increased biomass yields. Manipulating miRNAs and their targets in transgenic plants including constitutive, stress-induced, or tissue-specific expression of miRNAs or their targets, RNAi, expressing miRNA-resistant target genes, artificial target mimic, and artificial miRNAs are some of the useful strategies. In general, miRNAs and their targets not only provide an invaluable source of novel transgenes but also inspire the development of several new GM strategies, allowing advances in breeding novel crop cultivars with agronomically useful characteristics. Applications of microRNA-based gene regulation for crop improvement (Fig. 10.8) and strategies for developing miRNA-based GM crops (Fig. 10.9) are depicted. Specifically, RNA silencing has been a powerful tool that has been used to engineer various crop plants in last two decades. Based on the siRNAs-mediated RNA silencing (RNAi) mechanism, transgenic plants were designed to trigger RNA silencing by targeting pathogen genomes. Diverse targeting approaches have been developed based on the difference in precursor RNA for siRNA production, including sense/antisense RNA, small/long hairpin RNA and artificial miRNA precursors (Prins et al., 2008; Simón-Mateo and Garcia, 2011). Approaches to induce RNAi (Fig. 10.10) include (1) sense or antisense

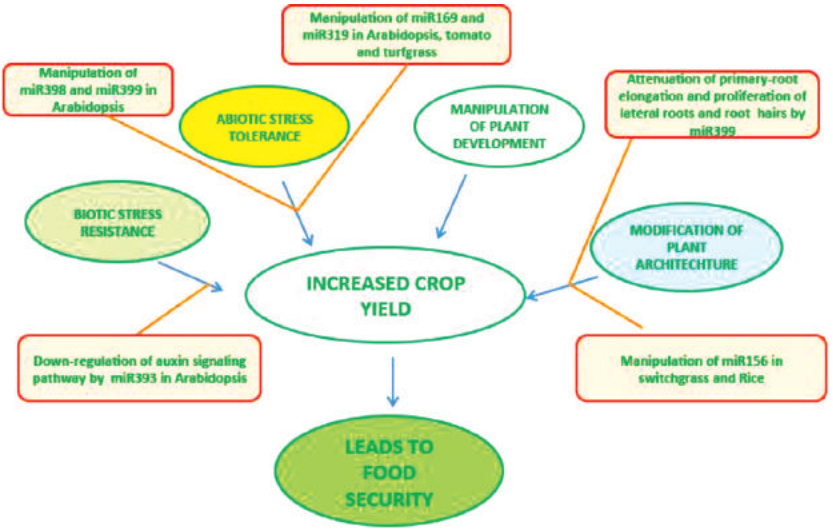

FIGURE 10.8 Applications of miRNA-based gene regulation in crop improvement. MicroRNA-based GM technology can help address food insecurity by either enhancing crop adaptations to extrinsic environmental stresses or increasing intrinsic yield potential in plants. (Adapted from Zhou and Luo 2013)

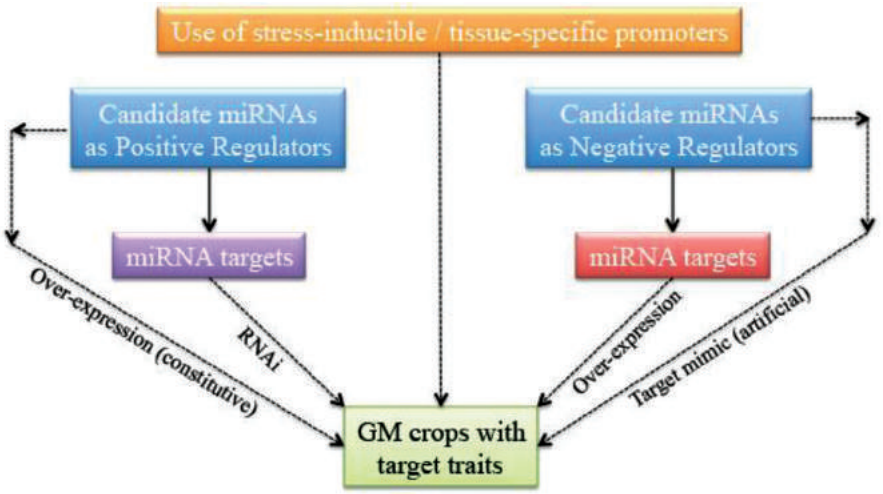

FIGURE 10.9 Strategies for development of genetically modified crop plants using miRNA approach. (Adapted from Zhou and Luo 2013).

viral sequences in transgene-mediated resistance; (2) virus-derived hpRNA transgene-mediated resistance; (3) artificial microRNA-mediated resistance, etc. RNAi of *JcFAD2-1* in transgenic Jatropha increased the proportion of oleic acid versus linoleic through genetic engineering, enhancing the quality of its oil (Qu et al., 2012). High-level resistance to banana bunchy top virus infection has been achieved in transgenic banana plants expressing small-interfering RNAs targeted against viral replication initiation (Shekhawat et al., 2012). RNAi-based resistance has been demonstrated in transgenic tomato plants against Tomato yellow leaf curl virus-Oman (Ammara et al., 2015). When transketolase activity was decreased by means of antisense technology in cucumber, it reduced the photosynthetic rate, seed germination, growth yield and tolerance to low temperature, and weak light stress (Bi et al., 2015). Silencing of both *FAD2* genes in stable transformants of flax, which was high in LA, led to high level of oleic acid (Chen et al., 2015). Cotton plants expressing CYP6AE14 dsRNA showed enhanced resistance to bollworms (Mao et al., 2011). RNAi-mediated silencing of *HaHR3* gene (Xiong et al., 2013) and *HaAK* gene (Liu et al., 2015b) in transgenic cotton also disrupted development of this insect pest. Transgenic plants overexpressing insect-specific microRNA, which is an effective alternative to *Bt*-toxin, acquired insecticidal activity against *H. armigera*. RNAi-mediated knockdown of midgut genes in transgenic rice has been a valuable tool to control the hemipteran insect *N. lugens* (Zha et al., 2011). Pest resistance was also increased

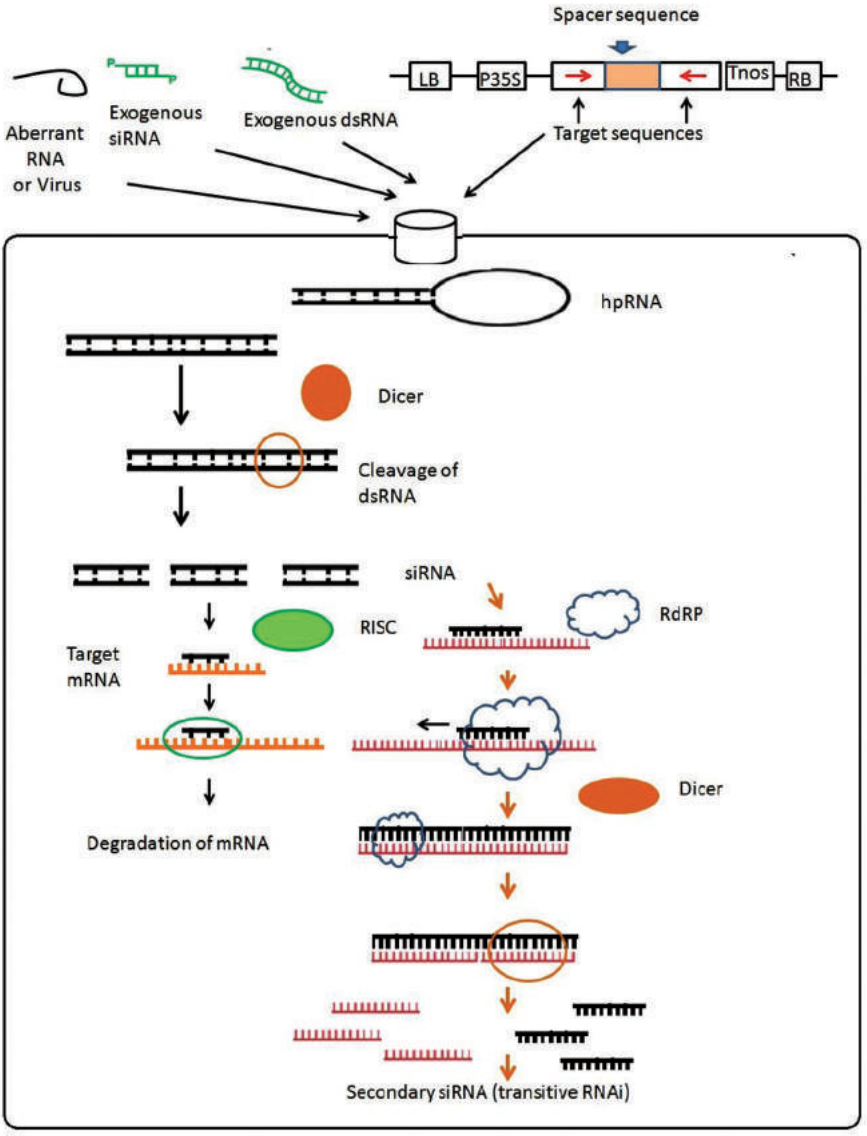

FIGURE 10.10 The RNA-mediated gene silencing in plants: Double-stranded RNA (dsRNA) generated through aberrant gene expression from a foreign gene, virus infection or tandem-repeat sequence due to insertion of a transposon/retrotransposon is digested into 21–25 nucleotide-long short interfering RNA (siRNA), by *Dicer* (an RNaseIII-like RNase), which functions as a template for the targeted degradation of mRNA in RISC (RNA-induced silencing complex) and also acts as the primer for RNA-dependent RNA polymerase (RdRp) to amplify the secondary dsRNA, although some differences between endogenous and foreign genes have been found for secondary RNAi.

in transgenic tobacco plants expressing dsRNA of an insect-associated gene ***EcR*** (Zhu et al., 2012). In addition, the transgenic tobacco plants expressing *H. armigera EcR* dsRNA were also resistant to another lepidopteran pest, the beet armyworm, *Spodoptera exigua*, due to the high similarity in the nucleotide sequences of their *EcR* genes. Transgenic maize plants with improved salt tolerance have been made free from selectable marker too (Li et al., 2010). Tomato plants resistant to Gemini viruses have been developed using artificial transacting small siRNA (Singh et al., 2015). RNAi-mediated resistance in transgenic cassava exhibited resistance to cassava brown streak Uganda virus (Yadav et al., 2011).

10.27 FUTURE PERSPECTIVES

Research on transgenic crops is expected to increase dramatically, given the whole world scenario. It is expected that there will be development and commercial release of several new abiotic and biotic stress-tolerant transgenic crop lines and biofuel plant platforms, coupled with vivid discussion at the public, academic, and government interface on biosafety of transgenic crops. These aspects will be the foci of future long-term monitoring programs because they have greater potential to alter plant fitness and to increase weedy or invasive tendencies, compared with traits in current commercial transgenic crops. Novel molecular strategies for monitoring and strategies for containment will also be foci of future studies. Monitoring approaches that survey transgenic crops and wild or weedy populations at crucial steps along the introgression process could also provide empirical data for enhancement, evaluation, and utilization of population models of transgene introgression (Kwit et al., 2011).

The growing demand for food is one of the major challenges to humankind. We have to safeguard both biodiversity and arable land for future agricultural food production, and we need to protect genetic diversity to safeguard ecosystem resilience. We must produce more food with less input, while deploying every effort to minimize risk. Agricultural sustainability is no longer optional but mandatory (Jacobsen et al., 2013). The traditional techniques are no longer sufficiently powerful to satisfy current and future needs for the three targets mentioned above. A combination of approaches will likely be needed to significantly improve the stress tolerance of crops in the field. These will include mechanistic understanding and subsequent utilization of stress response and stress acclimation networks, with careful attention to field growth conditions, extensive testing in the laboratory,

greenhouse, and the field; the use of innovative approaches that take into consideration the genetic background and physiology of different crops; the use of enzymes and proteins from other organisms; and the integration of QTL mapping and other genetic and breeding tools (Mittler and Blumwald, 2010). Understanding of genomics paradigms has advanced considerably in the past decade. This resulted in a more integrative and deeper comprehension of how genetic and epigenetic processes regulate plant growth and development and response to the environment. The era of omics, including genomics, transcriptomics, epigenomics, proteomics, and metabolomics, is poised to facilitate biotechnological improvement of crops, particularly for physiological phenotypes that are controlled by complex genetic and epigenetic mechanisms (Moshelion and Altman, 2015). Further advances in plant biotechnology and agriculture depend on the efficient combination and application of diverse scientific inputs.

10.27.1 GENOME EDITING

In the genome-editing era, the dissemination of plants developed by advanced genetic engineering is not hampered by technological aspects but by the understanding and acceptance of such technologies in society. Researchers, the public, and regulatory bodies should proactively discuss the socially acceptable integration of genome-editing crops, if they recognize that the agricultural use of genome-editing can satisfy the needs of breeders and consumers alike and improve global food security (Araki and Ishii, 2015). Genome editing tool such as sequence-specific nucleases (SSNs) harness DNA editing repair pathways. SSNs enable precise genome editing by introducing DNA double-strand breaks (DSBs) that subsequently trigger DNA repair by either nonhomologous end joining (NHEJ) or HR (Fig. 10.11). The NHEJ is error-prone and frequently introduces small deletions and insertions at the junction of the newly rejoined chromosome, some of which cause gene knockouts by generating frameshift mutations. In genome editing by HR, DNA templates bearing sequence similarity to the break site are used to introduce sequence changes at the target locus. HR can be used to change single amino acids or small stretches of amino acids in proteins, or single base pairs or groups of base pairs in control elements. Thus, DNA repair by HR is a precise gene-targeting method.

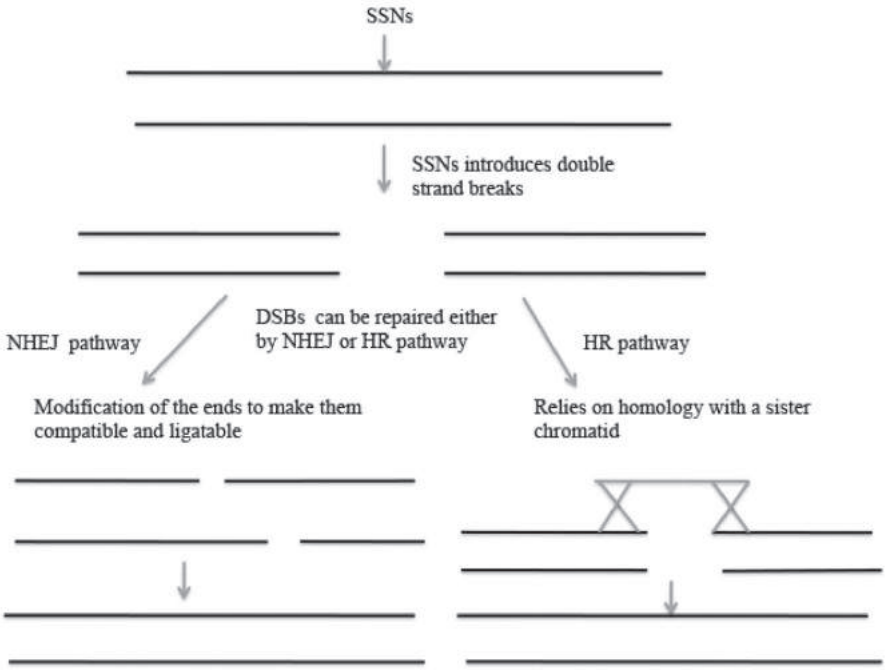

FIGURE 10.11 Genome repairing pathways after DSBs are induced by SSNs such as ZFNs, TALENs, and CRISPR/Cas system. (Adptered from Gao C, 2015).

Considering the regulatory and social hurdles associated with transgenic crops, novel and latest biotechnological tools like SSNs, namely, zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), and clustered regulatory interspaced short palindromic repeat (CRISPR)/Cas-based RNA-guided DNA endonucleases have emerged. These tools allow precise insertion of specific genes for modification or replacement of genes at their specific genomic location without involving any other source of DNA. Genome editing is an advanced genetic engineering tool that can more directly modify a gene within a plant genome. The absence of foreign DNA, most notably selectable markers in the final product, and introduction of genes derived from the same plant species, should help to increase consumer acceptance of novel GM plant products developed with these technologies. Thus, with the emergence of such technologies, the time is right to visit the benefits of genetic modification and to begin the development of novel, consumer-acceptable products. These tools can be used for precision genome engineering and agriculture. These novel biotechnological tools have been successfully demonstrated in Arabidopsis, tobacco,

rice, sorghum, and *Brachypodium* (Jiang et al., 2013; Townsend et al., 2009; Shan et al., 2013a, 2013b). Genome editing allows plant breeding without introducing a transgene, and this has led to new challenges for the regulation and social acceptance of GMOs. Genome editing is, thus, expected to generate many new crop varieties with traits that can satisfy the various demands for commercialization, by utilizing plant genomic information. This modern genome editing technology can produce novel plants that are similar or identical to plants generated by conventional breeding techniques, and therefore, creating distinct boundaries with regards to GMO regulations (Araki et al., 2014; Camacho et al., 2014; Hartung and Schiemann, 2014; Kanchiswamy et al., 2015; Voytas and Gao, 2014). Therefore, an appropriate regulatory response is urgently required toward the social acceptance of genome-edited crops. Recent reports regarding genome editing of major crops, including barley (*Hordeum vulgare*), maize (*Z. mays*), rice (*O. sativa*), soybean (*Glycine max*), sweet orange (*C. sinensis*), tomato (*S. lycopersicum*), and wheat (*Triticum*), have demonstrated a high efficiency of indels. Most notably, three homeoalleles of *TaMLO* were simultaneously edited in hexaploid bread wheat, resulting in heritable resistance to powdery mildew (Wang et al., 2014). Moreover, maize, which has indels in *ZmIPK1* is expected to have improved nutritional value as a result of decreased phosphorus content (Liang et al., 2014; Shukla et al., 2009). Furthermore, rice with indels in *OsBADH2* (Jiang et al., 2013; Shan et al., 2013) may appeal to consumers in view of its improved fragrance (Bradbury et al., 2008; Chen et al., 2008). Such results show that genome editing dramatically simplifies plant breeding even in major crops, with potential impact on the future of agriculture and human nutrition. However, most of these reports did not address potential off-target mutations. The occurrence of off-target mutations is one of the crucial issues in the agricultural use of genome editing. Some off-target mutations are likely to result in silent or loss-of-function mutations, others might lead to immunogenicity or toxicity in the food products by changing amino acids within a protein. However, there is no documented instance of any adverse effect resulting from foods produced from GM plants (Goodman and Tetteh, 2011).

Genome-editing tools are expected to become a method of choice, in addition to other novel technologies, for allelic modifications, gene replacement, structural characterization of the proteome, and posttranslational modifications. Multinational research is already taking into account the biology–agriculture crosstalk, paving the way to more effective and productive development of new cultivars. Recent studies have identified a large

number of genetic and molecular networks underlying plant adaptation to adverse environmental growth conditions. All of these studies emphasize the complexity of the various traits and their polygenic nature. All biotechnological applications should be scrutinized with respect to global food security, economic, sociological, legal, and ethical considerations, aiming at public acceptance.

Genome editing based on SSNs is one of the most promising novel plant breeding technologies for crop improvement. Gene knockouts are valuable for generating new genetic variants, and genome editing can be used to make knockout collections for agronomically important crop plants such as rice and maize. The plants created by SSN mutagenesis do not appear to have any foreign DNA in their genomes, and are often indistinguishable from natural variants or those produced by conventional mutagenesis. They may therefore fall outside the existing regulations affecting GM crops. Additionally, because SSNs can be used to introduce single nucleotides or long stretches of DNA at predefined genomic sites, the types of insertion they produce may avoid the position effects associated with random insertion by traditional transgenesis. Further, if multiple transgenes are inserted at the same site, such a gene stack will be inherited as a single Mendelian locus, allowing introduction of several different transgenes into the genome. We believe that progress in genome editing in plants promises to open exciting new avenues for crop improvement. Scientists must not slow down on advancing these promising technologies because such advancements may well lead to yet more powerful technologies in favor of public.

10.28 PRESENT AREAS OF EMPHASIS

1. Gene mining and genome editing, followed by integration of transgenic approach to conventional plant breeding will be very useful in developing “biotech crops.”
2. Utilization of “clean gene” or “marker-free” transgene technologies should be one of the main essences of the new transgene technologies.
3. Toxicity and allergenicity tests should be done on a case-by-case basis to assess the perceived risk of the transgenic food products.

4. Thorough implementation and supervision of biosafety guidelines via development of a network between agricultural universities/ institutes, other relevant laboratories, and biosafety committees is a must.
5. A comprehensive survey needs to be conducted for the degree of crossability between crop species and their wild relatives, existing wild and weedy relatives in an environment into which the transgenic crops are intended to be released so that we do not compromise with the health of the environment.
6. There is an urgent need for balanced risk assessment procedures with better models of monitoring system, on step-by-step and case-to-case basis, for studying the deleterious effects of herbicide, insect and disease resistant crops.
7. Sensible and realistic decisions should be taken by the policy-makers not to release these crops at the centers of origin, delicate ecological zones and the pockets rich in biodiversity, considering the potential impact of transgenic crops on genetic diversity.
8. Ecologists should be commissioned to comprehend the effect on biodiversity in the long run, after commercialization of transgenic crops, on regular basis. Additionally, monitoring and mapping of the biodiversity of hot spots should be regularly conducted.
9. There is an urgent need of an in-depth study to address the effects of transgenic plants on nontarget animals, plants and other organisms, etc., since not much scientific information is available in this area.
10. Public awareness program is a must regarding not only the benefits offered by transgenic crops, but also on their perceived risks and the need to protect valuable genetic resources. Literacy programs in schools/colleges/universities for basic understanding of modern genetics, molecular biology, etc. to make safe and responsible use of transgenic products is mandatory.
11. Government coordinated public–private partnership program in transgenic research and development will be very useful.

KEYWORDS

- **genetic engineering**
- ***Bt*-toxin**
- **protease inhibitors**
- **chitinases**
- **glucanases**
- **phosphinothricin acetyl transferase (PAT)**
- **RNAi**
- **Cre/lox recombination system**
- **biosafety**

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