Chapter 1
Plant Genetic Transformation and Transgenic Crops: Methods and Applications

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Abstract The combined use of recombinant DNA technology, gene transfer methods, and tissue culture techniques has led to the efficient transformation and production of transgenics in a wide variety of crop plants. In fact, transgenesis has emerged as an additional tool to carry out single-gene breeding or transgenic breeding of crops. Unlike conventional breeding, only the cloned gene(s) of agronomic importance is/are being introduced without cotransfer of undesirable genes from the donor. The recipient genotype is least disturbed, which eliminates the need for repeated backcrosses. Above all, the transformation methods provide access to a large gene pool, as the gene(s) may come from viruses, bacteria, fungi, insects, animals, human beings, unrelated plants, and even from chemical synthesis in the laboratory. Various gene transfer methods such as *Agrobacterium*, physicochemical uptake of DNA, liposome encapsulation, electroporation of protoplasts, microinjection, DNA injection into intact plants, incubation of seeds with DNA, pollen tube pathway, use of laser microbeam, electroporation into tissues/embryos, silicon carbide fiber method, particle bombardment, and "in planta" transformation have been developed. Among these, *Agrobacterium* and "particle gun" methods are being widely used. Recently RNAi and CRISPR/Cas9 systems have further expanded the scope for genome engineering. Using different gene transfer methods and strategies, transgenics carrying useful agronomic traits have been developed and released. Attempts are being made to develop transgenic varieties resistant to abiotic stresses, such as drought, low and high temperature, salts, and heavy metals, and also to develop transgenic varieties possessing better nutrient-use efficiency and better keeping and nutritional and processing qualities. Genetically modified foods, such as tomato containing high lycopene, tomato with high flavonols as antioxidants, edible vaccines, are leading examples of genetically engineered crops. Several genes of agronomic importance have been isolated from various organisms; cloned and suitable constructs have been developed for plant transformation. *Agrobacterium* and "particle gun" methods have been refined and

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now being used for genetic transformation of a wide variety of field, fruit, vegetable, forest crops, and ornamental plant species. Transgenic crops such as cotton, maize, papaya, potato, rice, soybean, and tomato, carrying mainly insect resistance, herbicide resistance, or both, are now being grown over an area of 185 million hectares spread over 28 countries of the world.

Keywords Genetic transformation · GM crops · GMOs · Recombinant DNA technology · Transgenesis · Transgenic breeding · Transgenic crops

1.1 Introduction

Plant genetic transformation leads to the production of transgenic plants (transgenics) which carry additional, stably integrated, and expressed foreign gene(s) usually from trans species. Such plants are commonly called genetically modified organisms (GMOs) or living modified organisms (LMOs). The whole process involving introduction, integration, and expression of foreign gene(s) in the host is called genetic transformation or transgenesis. The combined use of recombinant DNA technology, gene transfer methods, and tissue culture techniques has led to the efficient transformation and production of transgenics in a wide variety of crop plants (Yang and Christou 1994; Mathews et al. 1995; Hilder and Boulter 1999; Gosal and Gosal 2000; Chahal and Gosal 2002; Altman 2003; Grewal et al. 2006; Kerr 2011; Nayak et al. 2011; Bakshi and Dewan 2013; Kamthan et al. 2016; Arora and Narula 2017; Cardi et al. 2017; Tanuja and Kumar 2017). In fact, transgenesis has emerged as an additional tool to carry out single-gene breeding or transgenic breeding of crops. Unlike conventional breeding, only the cloned gene(s) of agronomic importance is/are being introduced without cotransfer of undesirable genes from the donor. The recipient genotype is least disturbed, which eliminates the need for repeated backcrosses. Above all, the transformation method provides access to a large gene pool, as the gene(s) may come from viruses, bacteria, fungi, insects, animals, human beings, unrelated plants, and even from chemical synthesis in the laboratory. Various gene transfer methods such as *Agrobacterium*, physicochemical uptake of DNA, liposome encapsulation, electroporation of protoplasts, microinjection, DNA injection into intact plants, incubation of seeds with DNA, pollen tube pathway, the use of laser microbeam, electroporation into tissues/embryos, silicon carbide fiber method, particle bombardment, and "in planta" transformation have been developed. Among these, *Agrobacterium* and "particle gun" methods are being widely used for plant genetic transformation.

1.2 Making Transgenic Plants

The appropriate gene construct carrying gene of interest, selectable marker/reporter gene, promoter, and terminator sequences are introduced into plants using standard procedures and suitable gene transfer method, and the resulting plants are characterized following phenotypic assays and various molecular techniques (Zhu et al. 2010).

1.2.1 Gene Transfer Methods in Plants

Various gene transfer methods (Ledoux 1965; Fraley et al. 1980; Herrera-Estrella 1983; Paszkowski et al. 1984; Tepfer 1984; Fromm et al. 1985; Lörz et al. 1985; Sanford et al. 1985; Uchimiya et al. 1986; Feldmann and Marks 1987; Grimsley et al. 1987; Klein et al. 1987; Sanford 1988; Sanford 1990; Weber et al. 1989; Kaeppler et al. 1990; Gunther and Spangenberg 1990; Saul and Potrykus 1990; Hooykaas and Schilperoort 1992; Bechtold et al. 1993; Kloti et al. 1993; Frame et al. 1994; Christou 1994; Hiei et al. 1994; Pescitelli and Sukhpinda 1995; Rhodes et al. 1995; Christou 1996; Trick and Finer 1997; Sanford 1988; Leelavati et al. 2004; Junjie et al. 2006; Keshamma et al. 2008; Takahashi et al. 2008; Rasul et al. 2014) are being used for developing transgenic plants (Table 1.1). Recently RNA interference (RNAi) in which RNA molecules inhibit gene expression or translation by neutralizing targeted mRNA molecules (Kim and Rossi 2008; Gupta et al. 2013; Younis et al. 2014) and CRISPR/Cas9 system (Wang et al. 2016; Arora and Narula 2017) have further expanded the scope for genome engineering.

Following the introduction of transgenes, the resulting putative transgenics are screened using various screenable markers (Rakosy Tican et al. 2007; Shimada et al. 2010; Shimada et al. 2011). Among the scorable markers/reporters genes (Table 1.2), GUS expression is the easiest way of assessing transformation. Transformed tissues are kept in X-gluc solution at 37 °C (in dark) for 1–12 h. Appearance of blue spots/sectors indicates their transgenic nature. Transgenic tissues are selected by growing them on medium containing selective agents (antibiotics/herbicides) at appropriate concentrations for at least two cycles of selection of 2 weeks each. Thus, selected tissues are cultured on suitable medium to regenerate the entire plants in the presence of respective selective agent. Regenerated plants are subjected to phenotypic assays, molecular analysis (Deom et al. 1990; Guttikonda et al. 2016), and insect bioassays using the following methods:

Table 1.1 Various methods for genetic transformation of plants

Transformation method	Remarks	Reference
DNA uptake	Uptake of DNA by living cells	Ledoux (1965)
Liposome encapsulation	Introduction of liposome-encapsulated SV40 DNA into cells	Fraley et al. (1980)
Agrobacterium tumefaciens (dicot plant)	First record on transgenic tobacco plant expressing foreign genes	Herrera-Estrella (1983)
Agrobacterium rhizogenes	Transformation of several species of higher plants by *Agrobacterium rhizogenes* and sexual transmission of the transformed genotype and phenotype	Tepfer (1984)
Electroporation	Expression of genes transferred into monocot and dicot plant cells by electroporation	Fromm et al. (1985).
Pollen-mediated transformation	Pollen-mediated plant transformation employing genomic donor DNA	Sanford et al. (1985)
PEG-mediated DNA uptake by protoplasts	Expression of a foreign gene in callus derived from DNA-treated protoplasts of rice (*Oryza sativa*)	Uchimiya et al. (1986)
Electroporation into protoplasts	Electroporation of DNA and RNA into plant protoplasts	Fromm et al. (1987)
Agrobacterium-mediated virus transfer	*Agrobacterium*-mediated delivery of infectious maize streak virus into maize plants	Grimsley et al. (1987)
Microprojectiles	High-velocity microprojectiles for delivering nucleic acids into living cells	Klein et al. (1987)
Microinjection	Transgenic rapeseed plants obtained by the microinjection of DNA into microspore-derived embryoids	Neuhaus et al. (1987)
Laser microbeam	A laser microbeam as a tool to introduce genes into cells and organelles of higher plants	Weber et al. (1989)
Silicon carbide fiber method	Silicon carbide fiber-mediated DNA delivery into plant cells	Kaeppler et al. (1990)
In planta transformation	In planta *Agrobacterium*-mediated gene transfer by infiltration of adult *Arabidopsis thaliana* plants	Bechtold et al. (1993)
Whiskers method	Production of fertile transgenic maize plants by silicon carbide whisker-mediated transformation	Frame et al. (1994)
Agrobacterium tumefaciens (monocot plant)	Efficient transformation of rice (*Oryza sativa*) mediated by *Agrobacterium* and sequence analysis of the boundaries of the T-DNA	Hiei et al. (1994)
Particle bombardment	Plant transformation using particle gun	Christou (1996)
Agrobacterium-based virus-induced gene silencing (VIGS)	Virus-induced gene silencing (VIGS) is a method that takes advantage of the plant RNAi-mediated antiviral defense mechanism	Lu et al. (2003)
SAAT(sonication-assisted *Agrobacterium* transformation)	Sonication-assisted *Agrobacterium*-mediated transformation	Trick and Finer (1997)
Agrobacterium based CRISPR/cas genome editing	Genome editing with CRISPR/Cas9 system	Gaj et al. (2013), and Gao et al. (2015)

Table 1.2 Reporter genes used in plant transformation

Reporter gene	Substrate and assay	Identification
UidA, GUS gene (β-glucuronidase)	X- GLUC	Histochemical assay
Chloramphenicol acetyl transferase (CAT)	^{14}Chloramphenicol + acetyl Co-A, TLC separation	Detection of acetyl chloramphenicol by autoradiography
Octopine synthase	Arginine pyruvate + NADH	Electrophoresis
Nopaline synthase	Arginine + ketoglutaric acid + NADH	Electrophoresis
β-Galactosidase (Lac Z)	β-Galactoside (X-gal)	Color of cells
Luciferase (LUC)	Decanal and $FMNH_2$ ATP + O2 + luciferin	Bioluminescence (exposure of X-ray films)
GFP	Green fluorescent protein	Fluorescent

Table 1.3 Selectable marker genes used in plant transformation

Marker gene	Enzyme	Selectable marker
Antibiotics		
Npt-II	Neomycin phosphotransferase	Kanamycin, neomycin, G418, Geneticin
Aad A	Aminoglycoside-3-adenyl transferase	Streptomycin, spectinomycin
hpt	Hygromycin phosphotransferase	Hygromycin B
ble	Bleomycin resistance	Bleomycin
Herbicides		
Aro A	5-enolpyruvylshikimate-3-phosphate synthase	Glyphosate
Bar	Phosphinothricin acetyltransferase	Phosphinothricin
bxn	Bromoxynil nitrilase	Bromoxynil
DHFR	Dihydrofolate reductase	Methotrexate
als	Acetolactate synthase	Chlorsulfuron
Epsps/aroa	Enolpyruvylshikimate phosphate synthase	Glyphosate

1.2.2 Characterization of Putative Transgenic Plants

1.2.2.1 Phenotypic Assay

A large number of selectable marker genes (Table 1.3) have become available which include antibiotics, herbicide-resistant genes, antimetabolites, hormone biosynthetic genes, and genes conferring resistance to toxic levels of amino acids or their analogs (Perl et al. 1993). The selection agent should fully inhibit the growth of untransformed cells. In general, the lowest concentration of the selection agent that suppresses growth of untransformed cells is used. The sensitivity of plant cells to the selection agent depends on the nature of explants, the plant genotype, the developmental stage, and the tissue culture conditions. Finally, the level of resistance also depends on the transcriptional and translational control signals to which the resistance gene is fused. It thus may be necessary to test several gene constructs. A plant is transformed if it grows in the presence of elevated concentration of selective compounds such as antibiotics and herbicides. Transgenic plants exhibit profuse hairy roots, lack of geotropism, and wrinkled leaves when a wild-type *Agrobacterium rhizogenes* is used for transformation.

1.2.2.2 Enzyme Assays

Enzyme assay of a genetic marker (nos, cat) is done to check the expression of the foreign DNA in the transformed tissue. Different genes express at different levels in different tissues, but enzyme assays are generally done using rapidly expanding tissues.

1.2.2.3 PCR Analysis

The polymerase chain reaction (PCR) amplifies DNA sequences between defined synthetic primers (Waters and Shapter 2014). A set of primers (forward primer and reverse primer) which is specific for the transgene is used to selectively amplify the transgene sequence from the total genomic DNA isolated from putative transgenic tissues/plant. PCR product can indicate the presence or absence of the transgene, but PCR usually amplifies a part of the gene and not the whole cassette. It is good for preliminary screening, but due to DNA contamination, it may lead to false positives. The PCR fails to tell anything about the transgene copy number, the integration sites and intactness of the cassette, and the expression level of the transgene.

1.2.2.4 Southern Blot Analysis

Southern blot hybridization (Southern 1975) is an efficient method for transferring DNA from agarose gels onto membranes prior to hybridization (using either radioactive or nonradioactive probes). It is a very sensitive technique which is used to detect the transgene in the genomic DNA even without any amplification. Southern analysis tells about the (1) stable integration of transgene into the genome, (2) copy number of the transgene, and (3) number of integration sites. However, it does not tell anything about the expression of transgene.

1.2.2.5 Western Blot Analysis

This method involves the detection of proteins produced by transgene in transgenic plant and is a reliable technique for analyzing the expression of transgenes (Burnette 1981). The level of gene expression is estimated by calculating the amount of protein produced by the transgene and its proportion in the total soluble plant protein.

1.2.2.6 Next-Generation Sequencing (NGS) Technologies

The emergence of next-generation sequencing (NGS) technologies has provided highly sensitive and cost- and labor-effective alternative for molecular characterization compared to traditional Southern blot analysis. This technique helps to determine the copy number, integrity, and stability of a transgene; characterize the integration site within a host genome; and confirm the absence of vector DNA. It has become a robust approach to characterize the transgenic crops (Guttikonda et al. 2016).

1.2.2.7 Progeny Analysis

The heritability of introduced gene can be easily determined by selfing or backcrossing of the putative transgenics. Transgene segregation can be studied by analyzing T_1 and subsequent generations.

1.2.2.8 Bioassay

Finally the bioassay is performed using greenhouse-/field-grown transgenic plants. For instance, if insect-resistant gene(s) has been introduced, then the larvae of target insect are allowed to feed on transgenic tissues/plants, and the extent of mortality is recorded. The transgenic lines with better transgene expression and causing higher mortality of larvae are selected, tested, and released for commercial cultivation.

We have produced transgenic sugarcane using *Agrobacterium* method (Fig. 1.1) and “particle gun” method (Figs. 1.2 and 1.3). PCR analysis (Fig. 1.4) has confirmed the presence of *Cry1Ac* gene in some of the regenerated plants which are being maintained for further studies.

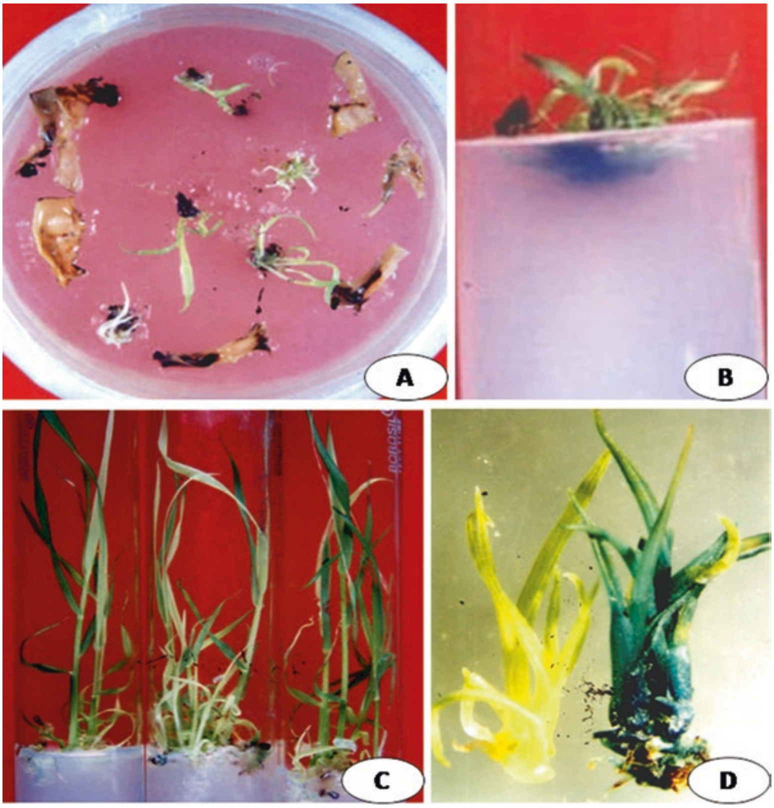

Fig. 1.1 (**a**–**d**) *Agrobacterium*-mediated genetic transformation of sugarcane (**a**) Direct plant regeneration from young leaves after cocultivation (**b**) Shoot proliferation (**c**) Shoot elongation and rooting (**d**) GUS assay of regenerated shoots

1.3 Engineering Crops for Agronomic Traits

The production of first transgenic plant of tobacco (*Nicotiana plumbaginifolia* L.) using *Agrobacterium tumefaciens* strain containing a tumor-inducing plasmid with a chimeric gene for kanamycin resistance (De Block et al. 1984; Horsch et al. 1984) generated lot of interest using this technique for crop improvement (Gasser and Fraley 1989). Rapid and remarkable achievements have been made in the production, characterization, and field evaluation of transgenic plants in several field, fruit, and forest plant species, the world over (Dale et al. 1993; Sharfudeen et al. 2014; ISAAA 2016; Kamthan et al. 2016). However, the major interest has been in the introduction of cloned gene(s) into the commercial cultivars for their incremental improvement. Using different gene transfer methods and strategies, transgenics in several crops carrying useful agronomic traits have been developed (Table 1.4).

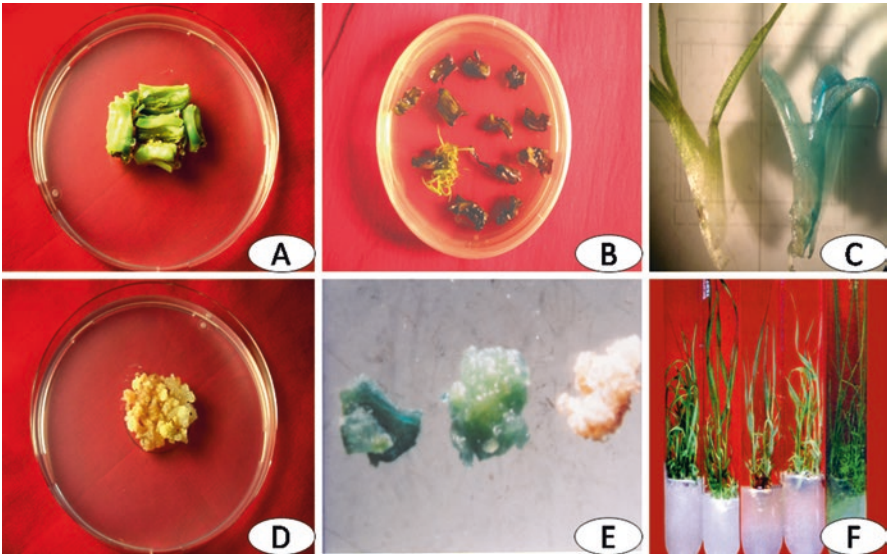

Fig. 1.2 (**a–f**) Particle gun-mediated genetic transformation of sugarcane (**a**) Cultured young leaf segments (target tissue) (**b**) Direct shoot regeneration from bombarded leaf segments (**c**) GUS assay of regenerated shoots (**d**) Embryogenic callus (target tissue) (**e**) GUS assay of bombarded embryogenic callus (**f**) Shoot regeneration from bombarded and selected calli

Fig. 1.3 T0 sugarcane plants in the glasshouse

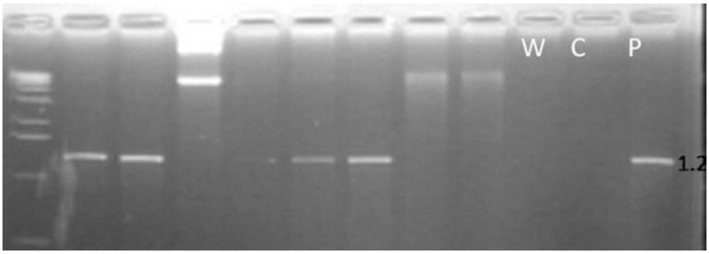

Fig. 1.4 PCR analysis of putative sugarcane transgenic plants showing amplification of Cry1Ac gene in some of the plants

1.3.1 Development of Insect-Resistant Plants

There have been two approaches to develop insect-resistant transgenic plants by transferring insect control protein genes.

1.3.1.1 Introduction of Bacterial Gene(s)

Bacillus thuringiensis synthesizes an insecticidal crystal protein, which resides in the inclusion bodies produced by the *Bacillus* during sporulation. This crystal protein when ingested by insect larvae is solubilized in the alkaline conditions of the midgut of insect and processed by midgut proteases to produce a protease-resistant polypeptide which is toxic to the insect. Lepidopteran-specific Bt gene from *Bacillus thuringiensis subsp. Kurstaki* has been widely and successfully used in tobacco, tomato, potato, maize, cotton, and rice (Gosal et al. 2001; Ahmad et al. 2002; Gómez et al. 2010; Sawardekar et al. 2012; Bakhsh et al. 2015; Abbas et al. 2016) for developing resistance against several lepidopteran insect pests. The use of redesigned synthetic Bt gene has also been used in some of these crops, and in several instances, the synthetic versions have exhibited up to 500-fold increase in the Bt gene expression.

1.3.1.2 Introduction of Plant Gene(s) for Insecticidal Proteins

Several insecticidal proteins of plant origin such as lectins, amylase inhibitors, and protease inhibitors can retard insect growth and development when ingested at high doses. Some genes like CpTi, PIN-1, PIN 11, ά A-1, and GNA have been cloned and are being used in the transformation programs aiming at insect resistance (Xu et al. 2005; Gao et al. 2006; Zhang and Pang 2009; Yu et al. 2007; McCafferty et al. 2008; Ismail et al. 2010; Wang et al. 2011; Yue et al. 2011; Mi et al. 2017).

Table 1.4 Engineering crops for agronomic traits

Crop	Remarks	Reference
Maize	*Agrobacterium*-mediated delivery of infectious maize streak virus into maize plants	Grimsley et al. (1987)
Citrus	Production of transgenic citrus plants expressing the citrus tristeza virus coat protein gene	Moore et al. (1993)
Basmati rice	Transgenic basmati rice carrying genes for stem borer and bacterial leaf blight resistance	Gosal et al. (2001)
Basmati rice variety 370	Expression of synthetic *Cry*1AB and *Cry*1AC genes in basmati rice (*Oryza sativa* L.) variety 370 via *Agrobacterium*-mediated transformation for the control of the European corn borer (*Ostrinia nubilalis*)	Ahmad et al. (2002)
Potato	PVY-resistant transgenic plants of cv. Claustar expressing the viral coat protein	Gargouri-Bouzid et al. (2005)
Tomato	Evaluation of agronomic traits and environmental biosafety of a transgenic tomato plant expressing satellite RNA of cucumber mosaic virus	Iwasaki et al. (2005)
Wheat	Molecular test and aphid resistance identification of a new transgenic wheat line with the GNA gene	Xu et al. (2005)
Wheat	Expression of synthesized snowdrop lectin(gna) gene in transgenic wheat and its resistance analysis against aphid	Gao et al. (2006)
Oryza sativa (rice)	Genetic engineering of *Oryza sativa* by particle bombardment	Grewal et al. (2006)
Brassica rapa subsp. chinensis	Vacuum infiltration transformation of pakchoi (*B. rapa* subsp. chinensis) with gene *pin* II and the bioassay for *Plutella xylostella*	Zhang and Pang (2009)
Lemon	Enhanced resistance to *Phoma tracheiphila* and *Botrytis cinerea* in transgenic lemon plants expressing a *Trichoderma harzianum* chitinase gene.	Gentile et al. (2007)
Wine grape	*Agrobacterium*-mediated transformation and regeneration of transgenic "chancellor" plants expressing the *tfdA* gene	Mulwa et al. (2007)
Rice	Breeding of transgenic rice lines with GNA and *Bar* genes resistance to both brown planthopper and herbicide	Yu et al. (2007)
Papaya	Papaya transformed with the *Galanthus nivalis* GNA gene produces a biologically active lectin with spider mite control activity	McCafferty et al. (2008)
Rice	Expression of a bacterial flagellin gene triggers plant immune responses and confers disease resistance in transgenic plants	Takakura et al. (2008)
Capsicum annuum L. (pepper)	Transformation of a trivalent antifungal recombinant into pepper (*Capsicum annuum* L.)	Jing et al. (2009)
Maize	Transformation of the salt tolerance gene BIGST into Egyptian maize inbred lines	Assem et al. (2010)
Elaeis guineensis	Molecular and expression analysis of cowpea trypsin inhibitor (CpTI) gene in transgenic *Elaeis guineensis* Jacq leaves	Ismail et al. (2010)

(continued)

Table 1.4 (continued)

Crop	Remarks	Reference
Grapevine (*Vitis vinifera* L.)	Expression of a rice chitinase gene enhances antifungal potential in transgenics	Nirala et al. (2010)
Papaya	Developing transgenic papaya with improved fungal disease resistance	Zhu et al. (2010)
Tomato	*Agrobacterium*-mediated transformation of tomato plants expressing defensin gene	El-Siddig et al. (2011)
Pea (*Pisum sativum* L.)	Enhancing transgenic pea (*Pisum sativum* L.) resistance against fungal diseases through stacking of two antifungal genes (chitinase and glucanase)	Amian et al. (2011)
Chinese cabbage	Inheritance and expression of *pin* II gene in DH transgenic lines and F_1 hybrids	Yue et al. (2011)
Brassica napus (spring rape)	Response of transgenic rape plants bearing the *Osmyb*4 gene from rice encoding a trans-factor to low above-zero temperature	Gomaa et al. (2012)
Chinese cabbage	Overexpression of rice leucine-rich repeat protein results in activation of defense response, thereby enhancing resistance to bacterial soft rot in Chinese cabbage	Park et al. (2012)
Pigeonpea	*Agrobacterium*-mediated genetic transformation of pigeonpea [*Cajanus cajan* (L.) Millsp] for pod borer resistance: optimization of protocol	Sawardekar et al. (2012)
Rice	Transgenic rice with inducible ethylene production exhibits broad-spectrum disease resistance to the fungal pathogens *Magnaporthe oryzae* and *Rhizoctonia solani*	Helliwell et al. (2013)
Peanut	Coat protein-mediated transgenic resistance of peanut (*Arachis hypogaea* L.) to peanut stem necrosis disease through *Agrobacterium*-mediated genetic transformation	Mehta et al. (2013)
Tomato	Heterologous expression of the yeast *HAL*5 gene in tomato enhances salt tolerance by reducing shoot Na + accumulation in the long term	GarcíaAbellan et al. (2014)
Eggplant	Enhancing salt tolerance in eggplant by introduction of foreign halotolerance gene, *HAL*1 isolated from yeast	Kumar et al. (2014)
Brassica juncea	Chitinase gene conferring resistance against fungal infections	Bashir et al. (2015)
Potato	Analysis of drought tolerance and herbicide resistance in transgenic potato plants overexpressing *DREB*1A/*Bar*	Jia et al. (2015)
Wheat	*Arabidopsis* EFTu receptor enhances bacterial disease resistance in transgenic wheat	Schoonbeek et al. (2015)
Maize	Breeding of transgenic maize with resistance to the Asian corn borer (*Ostrinia furnacalis*) and tolerance to glyphosate	Sun et al. (2015)
Cotton	Transgenic expression of translational fusion of synthetic *Cry*1Ac and *Hvt* genes in tobacco confers resistance to *Helicoverpa armigera* and *Spodoptera littoralis* larvae	Abbas et al. (2016)
Cucumber	Development of broad virus resistance in non-transgenic cucumber using CRISPR/Cas9 technology	Chandrasekaran et al. (2016)

(continued)

Table 1.4 (continued)

Crop	Remarks	Reference
Potato	Transgenic potato plants expressing the cold inducible transcription factor SCOF1 display enhanced tolerance to freezing stress	Kim et al. (2016)
Carrot	Transgenic approaches to enhance disease resistance in carrot plants to fungal pathogens	Punja et al. (2016)
Cotton	Transgenic upland cotton lines of *Gastrodia* antifungal protein gene and their performance of resistance to *Verticillium* wilt	Xiao et al. (2016)
Potato	Expression of the *Galanthus nivalis* agglutinin (GNA) gene in transgenic potato plants confers resistance to aphids	Mi et al. (2017)

1.3.2 Development of Disease-Resistant Plants

1.3.2.1 Virus Resistance

Genetic engineering for developing virus-resistant plants has exploited new genes derived from viruses themselves in a concept referred to as pathogen-derived resistance (PDR).

Coat Protein-Mediated Resistance (CP-MR)

Introduction of viral coat protein gene into the plant makes the plant resistant to virus from which the gene for the CP was derived (Shah et al. 1995). It was first demonstrated for tobacco mosaic virus (TMV) in tobacco. Subsequently, virus-resistant transgenics have been developed in tomato, melon, rice, papaya, potato, sugar beet, and some other plants (Gargouri-Bouzid et al. 2005; Pratap et al. 2012; Mehta et al. 2013). A variety of yellow squash called Freedom II has been released in the USA. Likewise, transgenic papaya resistant to papaya ringspot virus has been released for general cultivation in the USA. Several CP-MR varieties of potato, cucumber, and tomato are under field evaluation.

Satellite RNA-Mediated Resistance

Satellite RNAs are molecules which show little, if any, sequence homologies with the virus to which they are associated, yet are replicated by the virus polymerase and appear to affect 70 of the infections produced by the virus. It has been demonstrated that engineering cucumber, using cucumber mosaic virus (CMV) satellite RNA, leads to transgenics resistant to CMV. This approach has been extended to several other crops (Iwasaki et al. 2005).

Antisense-Mediated Protection

It is now established that gene expression can be controlled by antisense RNA. It has been proposed that antisense RNA technology can also play a role in cross protection. cDNAs representing viral RNA genomes were cloned in an antisense orientation to a promoter and transferred to plants. This approach has been effective against TMV although the protection was not as effective as with coat protein gene (Tang et al. 2005; Araújo et al. 2011).

Development of Resistance Using CRISPR/Cas9 Technology

Genome editing in plants has been boosted tremendously by the development of clustered regularly interspaced short palindromic repeats (CRISPR/Cas9) technology. This powerful tool allows substantial improvement in plant traits in addition to those provided by classical breeding. The development of virus resistance in cucumber (*Cucumis sativus* L.) using Cas9/subgenomic RNA (sgRNA) technology disrupts the function of the recessive eIF4E (eukaryotic translation initiation factor 4E) gene. Cas9/sgRNA constructs were targeted to the N′ and C′ termini of the eIF4E gene. Small deletions and single nucleotide polymorphisms (SNPs) were observed in the eIF4E gene-targeted sites of transformed T1 generation cucumber plants (Chandrasekaran et al. 2016).

1.3.2.2 Fungal Resistance

Genetic engineering for fungal resistance has been limited. But several new advances in this area now present an optimistic outlook.

Antifungal Protein-Mediated Resistance

Introduction of chitinase gene in tobacco and rice has been shown to enhance fungal resistance in plants. Chitinase enzymes degrade the major constituents of the fungal cell wall (chitin and α-1, 3 glucan). Co-expression of chitinase and glucanase genes in tobacco and tomato plants confers higher level of resistance than either gene alone. Use of genes for ribosome-inactivating proteins (RIP) along with chitinase has also shown synergistic effects. A radish gene encoding antifungal protein 2 (Rs-AFP2) was expressed in transgenic tobacco, and resistance to *Alternaria longipes* was observed. Other pathogenesis-related proteins/peptides include osmotin, thionins, lectins, etc. (Gentile et al. 2007; Jing et al. 2009; Nirala et al. 2010; Amian et al. 2011; El-Siddig et al. 2011; Fang et al. 2012; Bashir et al. 2015; Punja et al. 2016; Xiao et al. 2016).

Antifungal Compound-Mediated Resistance

Low-molecular-weight compounds such as phytoalexins possess antimicrobial properties and have been postulated to play an important role in plant resistance to fungal and bacterial pathogens. Expression of a stilbene synthase gene from grapevine in tobacco resulted in the production of new phytoalexin (resveratrol) and enhanced resistance to infection by *Botrytis cinerea*. Active oxygen species (AOS) including hydrogen peroxide also play an important role in plant defense responses to pathogen infection. Transgenic potato plants expressing an H_2O_2-generating fungal gene for glucose oxidase were found to have elevated levels of H_2O_2 and enhanced levels of resistance both to fungal and bacterial pathogens particularly to *Verticillium* wilt (Zhu et al. 2010; Helliwell et al. 2013).

1.3.2.3 Bacterial Resistance

Genetic engineering for bacterial resistance has relatively met with little success. The expression of a bacteriophage T4 lysozyme in transgenic potato tubers led to increased resistance to *Erwinia carotovora*. Besides, the expression of barley ά-thionin gene significantly enhanced the resistance of transgenic tobacco to bacteria *Pseudomonas syringae*. Advances in the cloning of several new bacterial resistance genes such as the *Arabidopsis* RPS2 gene, tomato *Cf9*, and tomato *Pto* gene may provide better understanding in the area of plant-bacteria interactions (Takakura et al. 2008; Schoonbeek et al. 2015).

1.3.3 Development of Herbicide-Resistant Plants

There have been two approaches to develop herbicide-resistant transgenic plants (Sun et al. 2015).

1.3.3.1 Transfer of Gene Whose Enzyme Product Detoxifies the Herbicide (Detoxification)

Using this approach, the introduced gene produces an enzyme which degrades the herbicide sprayed on the plant. For instance, introduction of *bar* gene cloned from bacteria *Streptomyces hygroscopicus* into plants makes them resistant to herbicides based on phosphinothricin (ppt). *Bar* gene produces an enzyme, phosphinothricin acetyltransferase (PAT), which degrades phosphinothricin into a nontoxic acetylated form. Plants engineered with *bar* gene were found to grow in phosphinothricin (ppt) at levels four to ten times higher than normal field application. Likewise, *bxn* gene of *Klebsiella ozaenae* which produces nitrilase enzyme imparts resistance to plants against herbicide bromoxynil. Other genes including *tfdA* for 2-, 4-D tolerance and

GST gene for atrazine tolerance have also been used. Among these, bar gene has been successfully introduced to develop herbicide-resistant soybean and cotton that have been commercially released in the USA (Hérouet et al. 2005; Mulwa et al. 2007).

1.3.3.2 Transfer of Gene Whose Enzyme Product Becomes Insensitive to Herbicide (Target Modification)

Using this approach, a mutated gene is introduced which produces modified enzyme in the plant which is not recognized by the herbicide; hence, the herbicide cannot kill the plant. For instance, a mutant *aroA* gene from bacteria *Salmonella typhimurium* has been used for developing tolerance to herbicide, glyphosate. The target site of glyphosate is a chloroplast enzyme 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS). Introduction of mutant *aroA* gene produces modified EPSPS, not recognizable to glyphosate. Likewise, sulphonylurea and imidazolinone herbicides inhibit acetolactate synthase (ALS) chloroplast protein. Tolerance to these herbicides has been achieved by engineering the expression of the mutant herbicide ALS gene derived from plant (Sato and Takamizo 2009; Cao et al. 2012).

1.3.4 Development of Plants Resistant to Various Abiotic Stresses

Transfer of cloned genes has resulted in the transgenics which are tolerant to some abiotic stresses. For instance, for frost protection, an antifreeze protein gene from fish has been transferred into tomato and tobacco. Likewise, a gene coding for glycerol-3-phosphate acyltransferase from *Arabidopsis* has been transferred to tobacco for enhancing cold tolerance. *Hal*2 gene is being tried for developing salt tolerance in rice (Assem et al. 2010; Gomaa et al. 2012; Duman et al. 2014; García Abellan et al. 2014; Kumar et al. 2014; Jia et al. 2015; Kim et al. 2016).

1.3.5 Development of Male Sterile and Restorer Lines for Hybrid Seed Production

The introduction of bacterial barnase gene results into male sterility, whereas the introduction of the bacterial barstar gene into another plant results into the development of restorer line. The resulting hybrid is fully fertile. This system has been commercially exploited in maize and oilseed rape. Thus, produced hybrids of *Brassica napus* are under field evaluation in India. Likewise, it can be exploited for production of hybrid wheat and rice (Ray et al. 2007).

1.3.6 Improvement in Nutritional Quality and Molecular Farming/Pharming

High protein "phaseolin" and *AmA-1* genes have been introduced to heterologous systems. Introduction of *AmA-1* gene into potato has caused improvement in the yield and protein content. Introduction of provitamin A and carotene genes has resulted into the production of "golden rice." Vitamin-producing transgenic plants have also been developed (Herbers 2003), and more emphasis is given to multigene engineering (Daniell and Dhingra 2002). Besides, transgenic plants producing specialty chemicals and biopharmaceuticals have been produced for molecular farming/pharming (Fischer and Emans 2000). The main objective of these crops is to add value to foods, such as tomato containing high lycopene, flavonols as antioxidants, cavity-fighting apples, rice enriched with carotene and vitamin A (golden rice), iron-pumping rice, canola rich in vitamin E (golden brassica), proteinaceous potatoes, edible vaccines, and decaffeinated coffee, which are some leading examples of genetically modified foods for the future (Doshi et al. 2013).

Thus, several genes of agronomic importance have been isolated from various organisms; cloned and suitable constructs have been developed for plant transformation. *Agrobacterium* and "particle gun" methods have been refined and now being used for genetic transformation of a wide variety of field, fruit, vegetable, forest crops, and ornamental plant species. Transgenic crops such as cotton, maize, papaya, potato, rice, soybean, and tomato, carrying mainly insect resistance, herbicide resistance, or both, are now being grown over an area of 185 million hectares spread over 28 countries of the world.

1.3.7 Biosafety Concerns of Transgenic Plants

The potential risks from the use of transgenics and their products fall under three categories including human health, environmental concerns, and social and ethical grounds. Risk to human health is related mainly to toxicity, allergenicity, and antibiotic resistance, whereas ecological risks include the gene flow to other plants, development of resistance in insects/pathogens, unintended secondary effects on nontarget organisms, and potential effects on biodiversity. In order to address these concerns, there are standard biosafety guidelines, and issues are addressed through deregulation of transgenic varieties for commercial cultivation. BCIL-DBT (2004).

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