Aditya Pratap · Jitendra Kumar *Editors*

Alien Gene Transfer in Crop Plants, Volume 1

Innovations, Methods and Risk Assessment

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Editors
Aditya Pratap
Senior Scientist (Plant Breeding)
Crop Improvement Division
Indian Institute of Pulses Research
Kanpur, India

Jitendra Kumar
Senior Scientist (Plant Breeding)
Crop Improvement Division
Indian Institute of Pulses Research
Kanpur, India

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Chapter 5
Horizontal Gene Transfer Through Genetic Transformation

Pooja Bhatnagar-Mathur, Paramita Palit, and K.K. Sharma

Abstract Gene transfer technology in crop plants has tremendous potential to introduce newer and better traits through development of transgenics and broaden the genetic base of crop plants by transferring genes from novel sources overcoming the species and genus barriers. Nevertheless, development of efficient transformation systems remains a prerequisite and might involve many years of exhaustive research. This chapter overviews the different methods of alien gene transfer through genetic transformation and factors affecting efficient transformation across different crop species. A comparative study on *Agrobacterium* and biolistics-mediated transformation including methods for production of marker-free transgenics are described in detail. Addressing the growing concerns over the biosafety issue constraining wider application of GM products in agriculture this chapter also focuses on improved methods of choice with respect to a crop family and also deals with future strategies which can help in further exploiting the existing technologies to develop improved crop varieties which can help to combat poverty, hunger and global agro-climatic changes.

Keywords *Agrobacterium*-mediated transformation • Biolisitcs • Co-transformation • Gene transfer • Marker-free transgenics • Selectable marker • Transgene escape

P. Bhatnagar-Mathur, Ph.D. (✉) • P. Palit • K.K. Sharma
Genetic Transformation Laboratory, International Crops Research Institute for the Semi-Arid Tropics (ICRISAT), Patancheru, Andhra Pradesh 502 324, India
e-mail: p.bhatnagar@cgiar.org

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5.1 Introduction

The recent advancement of horizontal gene transfer technology enabled scientists to find a better way to answer problems related to stress response, disease or herbicide resistance or development of tolerance against climate change. It is now feasible to introduce into crop plants genes that have previously been inaccessible to the plant breeder or which did not exist in the crop of interest.

Transgenic technologies have enormous potential to improve crops in a relatively precise way (Barampuram and Zhang 2011). Genes of interest are introduced, often by *Agrobacterium*-mediated transformation, and become integrated at random positions in the genome. Initial experiments involved gene transfer by using *Agrobacterium tumefaciens* (Herrera-Estrella et al. 1983). The development of sophisticated methods later opened the way for an alternative procedure for engineering plants using direct DNA transfer. The protocols for this transfer include particle bombardment (Gan 1989), chemical treatments and electroporation (Bates 1994). However, the unavailibility of efficient transformation methods to introduce foreign DNA (alien gene) can be a substantial barrier to the application of recombinant DNA methods in some crop plants (Bhatnagar et al. 2010).

Despite significant advances over the past decade, the development of efficient transformation methods can take many years of painstaking research (Sharma et al. 2005a, b). The major components for the development of transgenic plants are (1) the development of reliable tissue culture regeneration systems; (2) preparation of gene constructs and transformation with suitable vectors; (3) efficient transformation techniques for the introduction of genes into the crop plants; (4) recovery and multiplication of transgenic plants; (5) molecular and genetic characterisation of transgenic plants for stable and efficient gene expression; (6) transfer of genes to elite cultivars by conventional breeding methods if required; and (7) evaluation of transgenic plants for their effectiveness in alleviating the biotic and abiotic stresses without being an environmental biohazard (Birch 1997). Some of the key characteristics of these components are discussed in this chapter.

5.2 Plant Regeneration in Tissue Cultures

Transformation of plants involves the stable introduction of DNA sequences usually into the nuclear genome of cells capable of giving rise to a whole transformed plant. Transformation without regeneration and regeneration without transformation are of limited value (Bhatnagar et al. 2010). The very basis of regeneration in tissue cultures is the recognition that somatic plant cells are totipotent (i.e., capable of giving rise to a whole plant) and can be stimulated to regenerate into whole plants in vitro, via organogenesis (shoot formation) or somatic embryogenesis, provided they

are given the optimum hormonal and nutritional conditions (Skoog and Miller 1957). Adventitious shoots or embryos are thought to arise from single cells and, thus, provide totipotent cells that can be identified which are both competent and accessible for gene transfer and will give rise directly to nonchimeric transformed plants. Transformation techniques reliant on plant regeneration from in vitro-cultured tissues have been described for many crop species (Lindsey and Jones 1989; Birch 1997).

5.3 Transformation Vectors

Most vectors used for the genetic transformation of plants carry 'marker' genes that allow the recognition of transformed cells, by either selection or screening. These genes are dominant, usually of microbial origin, and placed under the control of strong and constitutive, eukaryotic promoters, often of viral origin (Birch 1997). The most popular selectable marker genes used in plant transformation vectors include constructs providing resistance to antibiotics such as kanamycin, chloramphenicol and hygromycin and genes that allow growth in the presence of herbicides such as phosphinothricin, glyphosate, bialaphos and several other chemicals (Wilmink and Dons 1993).

For successful selection, the target plant cells must be susceptible to relatively low concentrations of the antibiotic or the herbicide in a non-leaky manner. Screenable marker 'reporter genes' have also been developed from bacterial genes coding for easily assayed enzymes, such as chloramphenicol acetyl transferase (CAT), b-galactosidase, b-glucuronidase (GUS), luciferase (LUX), green fluorescent protein (GFP), nopaline synthase and octopine synthase (Herrera-Estrella et al. 1983, Reichel et al. 1996). The utility of any particular marker gene construct as a transformation marker varies depending on the plant species and explant involved. To date kanamycin resistance (Reiss et al. 1984) is the most widely used selectable marker phenotype, and b-glucuronidase (Jefferson et al. 1987) is the most widely used screenable marker.

Most commonly used plant transformation vectors have features required for various recombinant DNA manipulations that include multiple unique restriction sites, bacterial origins of replication and prokaryotic selectable markers for plasmid selection and maintenance in *Escherichia coli* (e.g. antibiotic resistance). In addition, these vectors contain specific selectable marker genes engineered for expression in plants that may be used directly as transformation vectors in physical DNA delivery strategies such as particle bombardment. However, for *Agrobacterium*-mediated gene transfer, these vectors need additional features such as wide host range replication and transfer functions to allow conjugation from *E. coli* to *Agrobacterium* and plasmid maintenance in both bacterial hosts (Klee et al. 1987).

5.4 Methods of Plant Gene Transfer

5.4.1 Agrobacterium-Mediated Gene Transfer

Agrobacterium tumefaciens is a soil bacterium that leads to gall formation at the wound sites of many dicotyledonous plants. The tumour inducing capability is due to the presence of a large Ti (tumour inducing) plasmid in virulent strains of *Agrobacterium*. Likewise, Ri (root-inducing) megaplasmids are found in virulent strains of *A. rhizogenes*, the causative agent of 'hairy root' disease. The molecular biology of Ti and Ri plasmids and of crown gall and hairy root induction have been studied in great detail (Klee et al. 1987; Zambryski 1992). The number of plant species transformed by *Agrobacterium* vectors has increased steadily over the past few years, and representatives of many taxonomically diverse genera have proved amenable to transformation (Dale et al. 1993). This success can mainly be ascribed to the improvements in tissue culture technology, particularly adventitious shoot regeneration in the crop plants concerned. *Agrobacterium*-mediated transformation in plants has been carried out across a vast range of plant species by using both tissue culture-dependent transformation as well as tissue culture-independent transformation (non-tissue culture-based) techniques (Keshamma et al. 2008; Rao et al. 2012).

The important requirements for *Agrobacterium*-mediated transformation firstly include the production of some active compounds like acetosyringone by the explants in order to induce the *vir* genes present on the Ti plasmid and then the induced *Agrobacteria* must have access to competent plant cells that are capable of regenerating adventitious shoots or somatic embryos at a reasonable frequency (Barghchi 1995). There is evidence to suggest that for gene transfer to occur, cells must be replicating DNA or undergoing mitosis (Moloney et al. 1989; Sharma et al. 1990). The majority of transformation experiments utilise either freshly explanted tissue sections or protoplasts in the process of reforming a cell wall and entering cell division or callus and suspension-cultured cell clumps wounded by chopping or pipetting and stimulated into rapid cell division by the use of nurse cultures (Draper et al. 1988). The adventitious shoot production in vitro is most commonly employed in most systems of genetic transformation.

5.4.1.1 Role of *Agrobacterium*-Related Factors in Alien Gene Transfer

Plant-specific factors, such as compounds (phenolics) that induce the expression of *Agrobacterium vir* genes, are necessary for efficient transformation (Stachel and Zambryski 1989). Virulence-inducing phenolic compounds were first described by Bolton et al. (1986) and are limited to dicolyledonous plants (Smith and Hood 1995). Although these have been comprehensively reviewed (Gheysen et al. 1998; Gelvin 2000), transfer and integration process of T-DNA is still not fully understood.

Till date, several key factors involved in *Agrobacterium*-mediated transfer have been described (Pradhan et al. 2012; Guo et al. 2012). Impact of these factors on

transformation efficiency was modified by using a large number of *Agrobacterium* strains (Klee 2000), binary vectors (Hoekema et al. 1983), disarmed plasmids such as disarmed version of p*TiBo542* (Hood et al. 1986) and use of super-binary vector with a fragment containing the *virB*, *virC* and *virG* genes (from p*TiBo542*). Multiple T-DNAs were delivered to plant cells either from a mixture of strains or from a single strain and segregation of one T-DNA from others observed in various occasions (McKnight et al. 1987, De Block and Debrouwer, 1991). In another approach Komari et al. (1996) co-transformed tobacco and rice with unique plasmids carrying two separate T-DNAs and were able to separate them in successive generations by Mendelian segregation.

Addition of phenolic compounds, particularly acetosyringone, enhances the induction of the *Agrobacterium vir* genes, during bacteria/plant co-cultivation (Vijayachandra et al. 1995). Hence it was recognised as a key for successful transformation in rice (Hiei et al. 1994). Other inducing factors are low pH (Godwin et al. 1991), temperature (Dillen et al. 1997) and high osmotic pressure (Usami et al. 1988). It has been observed that certain carbohydrates in the presence of 100 μM acetosyringone did not have any significant synergistic effect (Hiei et al. 1997). Wounding of targeted tissue prior to co-cultivation enhanced *Agrobacterium* transformation frequencies by microprojectile bombardment (Bidney et al. 1992). However, inoculation of *Agrobacterium* after plasmolysis alone gave an even better transient expression compared to the combination of plasmolysis and bombardment (Uzé et al. 1997). Genotype and type of tissue to be transformed, composition of culture media and elimination of *Agrobacterium* after co-cultivation further influence the efficient production of stable transformants in plants (Nauerby et al. 1997).

5.4.1.2 Factors Affecting *Agrobacterium*-Mediated Transformations

The transfer of T-DNA and its integration into the plant genome are influenced by several *A. tumefaciens* and plant tissue-specific factors. These include plant genotype, explant, vectors-plasmid, bacteria strain, addition of *vir*-gene-inducing synthetic phenolic compounds, culture media composition, tissue damage, suppression and elimination of *A. tumefaciens* infection after co-cultivation (Nauerby et al. 1997; Klee 2000).

Osmotic Treatment

Osmotic treatment for enhancement of *Agrobacterium*-mediated transformation largely depends upon the species. However, plasmolysis with sucrose (292 mM) improved T-DNA delivery into precultured immature embryos of rice (Uzé et al. 1997) and was later used extensively later on in development of transgenic plants (Lucca et al. 2001). Extensive use of sucrose and glucose transformation did not describe any effect of osmotic medium on T-DNA delivery and stable transformation in rice and maize (Hiei et al. 1994; Zhao et al. 2001; Frame et al. 2002) and in wheat (Cheng et al. 2003).

Preconditioning and Co-cultivation Time

Co-cultivation of explants with *A. tumefaciens* has made possible the use of some explants, which were hitherto recalcitrant for transformation. Optimising the preconditioning time (72 h) and co-cultivation time with *A. tumefaciens* (48 h) increased the transformation efficiency in canola (Cadoza and Stewart 2003) and in Chinese cabbage (Zhang et al. 2000).

Desiccation of Explants

A significant factor that enhances transformation of crop species is desiccation of explants prior to or post *A. tumefaciens* infection. Arencibia et al. (1998) reported that air-drying sugarcane suspension cells prior to inoculation under laminar flow for 15–60 min slightly improved T-DNA delivery and subsequently increased transformation efficiency. Similarly in rice, air-drying calli derived from suspension cultures for 10–15 min increased the transformation efficiency by tenfold or more (Urushibara et al. 2001). Desiccation of precultured immature embryos, suspension culture cells and embryonic calluses of wheat and embrogenic calluses of maize greatly enhanced T-DNA delivery and plant tissue recovery after co-culture, leading to increased stable transformation frequency (Cheng et al. 2003). This treatment has also improved T-DNA delivery in dicot species such as soybean suspension cells based on preliminary study (Cheng and Fry 2000). Although the molecular mechanism of desiccation during co-culture remains unclear, it is known that desiccation suppresses the growth of *Agrobacterium* similar to the effect observed with silver nitrate (Opabode 2006).

Anti-necrotic Treatments

Anti-necrotic mixtures for pre-induction have shown to be important for reducing oxidative burst. Treatment of meristem explants of sugarcane and rice with medium containing 15 mg/l ascorbic acid, 40 mg/l cysteine and 2 mg/l silver nitrate improved the transformation efficiency and explant viability (Enriquez-Obregon et al. 1999). Inclusion of silver nitrate in co-culture medium enhanced stable transformation in maize (Armstrong and Rout 2001; Zhao et al. 2001). It significantly suppresses the *Agrobacterium* growth during co-culture without compromising T-DNA delivery and subsequent T-DNA integration, facilitating plant cell recovery and increased efficiency of transformation (Cheng et al. 2003). Inclusion of cysteine in the co-culture medium led to an improvement in both transient GUS expression in target cells and a significant increase in stable transformation frequency in maize (Somers et al. 2003).

Temperature

The effect of temperature during co-culture on T-DNA delivery was first reported in dicot species (Dillen et al. 1997). The optimal temperature for stable transformation

should be evaluated with each specific explant and *Agrobacterium* strain involved (Salas et al. 2001; Alimohammadi and Bagherieh-Najjar 2009). A temperature of 22 °C was found to be optimal for T-DNA delivery in tobacco leaves (Dillen et al. 1997). However, in another study the highest number of transformed plants were obtained in tobacco at 25 °C, even though 19 °C was optimal for T-DNA delivery (Salas et al. 2001). In monocots, the co-culture temperature for most of the crops ranged from 24 to 25 °C, and in some cases, 28 °C was used for co-culture (Rashid et al. 1996; Enriquez-Obregon et al. 1999; Hashizume et al. 1999). The effect of lower temperature (23 °C) on T-DNA delivery and stable transformation was also evaluated, and highest transient GUS expression (64 %) was observed at 22 °C in garlic (Kondo et al. 2000). In maize, higher transformation frequency was observed at 20 °C than at 23 °C (Frame et al. 2002). In another study, transgenic maize plants were obtained by co-culture of the immature embryos at 20 °C followed by 28 °C subculture (Gordon-Kamm et al. 1990). The optimal temperature for both T-DNA delivery and stable transformation was 23–25 °C for wheat and 23 °C for maize (Frame et al. 2002).

Surfactants

Including surfactants such as Silwet L77 and Pluronic acid F68 in inoculation medium greatly enhanced T-DNA delivery in immature embryos of wheat (Cheng et al. 1997). These surfactants may enhance T-DNA delivery by aiding *A. tumefaciens* attachment and/or by elimination of certain substances that inhibit this attachment. Their addition in the inoculation medium plays a role similar to vacuum infiltration, i.e. facilitating the delivery of *A. tumefaciens* cells to closed ovules (primary target for transformation of *A. thaliana* (Ye et al. 1999; Desfeux et al. 2000)). The surfactant Silwet L77 was shown to be useful to the success of the floral dip method of *Arabidopsis thaliana* transformation (Dehestani et al. 2010).

Inoculation and Co-culture Medium

Culture medium components like sugar, plant growth regulators and *vir* induction chemicals are important factors that affect the transformation frequency. MS (Murashige and Skoog 1962) or a modified MS-based medium has shown to be suitable for inoculation and co-culture (Dong et al. 1996; Enriquez-Obregon et al. 1999; Lucca et al. 2001). The modified N6 medium (Chu et al. 1995) containing 2,4-dichlorophenoxyacetic acid (2,4-D) was shown to be suitable for co-culture in rice. Transformation of maize immature embryo using LS-based (Linsmaier and Skoog 1965) medium and N6-based medium failed to generate transformed plants (Ishida et al. 1996). However, addition of silver nitrate in N6-based medium for inoculation and co-culture of immature embryos resulted in regeneration of transgenic plants in maize (Zhao et al. 2001). Similarly, addition of $CaCl_2$ in the medium has increased transformation efficiency in barley (Kumlehn et al. 2006). One-tenth MS salt

strength enhanced transient GUS expression tenfold over full-strength salts in barley (Ke et al. 2002). Furthermore, the distribution of cells expressing the GUS gene within each set of immature embryos was clearly altered, showing significantly more cells on the scutellar surface expressing GUS. Reduction in the salt strength of the inoculation and co-culture media was shown to be useful in development of transgenics of canola (Fry et al. 1987), wheat (Cheng et al. 1997) and maize (Armstrong and Rout 2001; Khanna and Daggard 2003). Use of *vir* induction chemicals improved the transformation efficiency in most of the crops (Cheng et al. 1997; Zhao et al. 2000; Kumlehn et al. 2006). However in some other cases, explants of monocot species could be efficiently transformed without the aid of external *vir* induction chemicals for special treatment (Enriquez-Obregon et al. 1999; Cheng et al. 2003).

Antibiotics

Antibiotics such as cefotaxime, carbenecillin and timentin have been used regularly in *Agrobacterium*-mediated transformation (Cheng et al. 1997; Bottinger et al. 2001; Sunikumar and Rathore 2001). Though initially cefotaxime worked well in rice and maize, later on it was observed that its use had a detrimental effect to maize Hi II callus (Ishida et al. 1996). Hence the use of carbenicillin has become the antibiotic of choice in reports of *Agrobacterium*-mediated transformation of wheat and maize in subsequent studies (Cheng et al. 2003, Zhang et al. 2003). On the other hand, 100 mg/l kanamycin was economical and improved the transformation efficiency in white spruce by enrichment of transformed tissue in budforming callus (Le et al. 2001) and increased the proportion of positively transformed shoots during subculture on kanamycin-containing medium in peanut and pigeonpea (Sharma and Anjaiah 2000; Thu et al. 2003).

Selectable Marker

Genes encoding for hygromycin phosphotransferase (*hpt*), phosphinothricin acetyltransferase (*pat* or *bar*) and neomycin phosphotransferase (*npt*II) are most widely used selectable markers for transformation. These marker genes work well under the control of constitutive promoters such as the *35S* promoter from cauliflower mosaic virus and the ubiquitin promoter from maize for selection of transformed cells. In *Asparagus* and banana, the *npt*II gene under the control of the nopaline synthase promoter has been used successfully to select stable transformants with kanamycin (May et al. 1995; Limanton-Grevet and Jullien 2001). The positive selectable marker phosphomannose isomerase (PMI) was first used for *Agrobacterium*-mediated transformation of sugar beet and was recently used to enhance transformation of sorghum (Joersbo et al. 1998; Gao et al. 2005). Introns were inserted into the coding region of *hpt* for enhancing transgene expression in monocot species (Simpson and Filipowcz 1996). Besides improving transformation frequency in rice, this modification in the selectable marker reduced copy numbers

of the marker gene, enabled better control of *Agrobacterium* growth during the transformation (Wang et al. 1997) and enhanced stable transformation (Wang et al. 2001). Glyphosate-insensitive plant 3-enolpyruvylshikimate-5-phosphate synthase (EPSPS) genes, the bacterial CP4 gene or a bacterial gene that degrades glyphosate, i.e. glyphosate oxidoreductase (GOX) gene, have also been used as selectable marker genes to generate transgenic plants in wheat and maize (Armstrong and Rout 2001; Howe et al. 2002; Hu et al. 2003).

5.4.2 *Modified Methods* of *Agrobacterium-Mediated Gene Transfer*

5.4.2.1 Sonication-Assisted *Agrobacterium*-Mediated Transformation

An important modification in *Agrobacterium*-mediated transformation involves subjecting the plant tissue to brief periods of ultrasound in the presence of *Agrobacterium*. Sonication-assisted *Agrobacterium*-mediated transformation (SAAT) treatment produces a large number of small and uniform wounds throughout the tissue, allowing easy access to the *Agrobacterium*, resulting in improved transformation efficiency in several different plant tissues including immature cotyledons, leaf tissue, suspension cultures and somatic and zygotic embryos. It was reported to increase transformation rates in those species which are more recalcitrant to *Agrobacterium*-mediated transformation (Trick and Finer 1997).

Tissue culture-independent transformation systems have also been demonstrated in various crops such as soybean (Chee et al. 1989), Arabidopsis (Feldmann and Marks 1987), sunflower (Rao and Rohini 1999), safflower (Rohini and Rao 2000a) and peanut (Rohini and Rao 2000b). Arabidopsis seeds infected with *Agrobacterium* and allowed to grow into mature plants in vivo resulted in about 1 % transformation frequency. Inoculation of *Agrobacterium* onto wounded sites arising from cutting away inflorescences of *Arabidopsis* yielded transformed seeds from newly emerging inflorescences (Chang et al. 1994; Katavic et al. 1994). This has also been used to generate transgenics in groundnut (Rohini and Rao 2000b).

5.4.2.2 Floral Dip Method

In this method, *Agrobacterium* is directly applied to floral tissues and thus eliminates possibility of generation of somaclonal variations due to the bypass of tissue culture techniques (Clough et al. 1998). In *Arabidopsis*, studies demonstrated the use of female gametophytes of immature flowers as targets of floral-dip transformation (Ye et al. 1999; Desfeux et al. 2000). This method requires considerably less time and effort than vacuum infiltration and is greater in yield. *Agrobacterium*-based floral dip transformation method, requiring no vacuum infiltration step, reported transformation efficiencies up to 0.8 % (Liu et al. 2012).

5.4.2.3 Vacuum Infiltration Method

The vacuum infiltration method of transformation has been applied mostly in monocot crops in order to avoid both in vitro culture and regeneration steps during transformation. The cells of a plant when subjected to a vacuum environment establish more intimate contact with *Agrobacterium*. This method was used to obtain stable transgenics in *Medicago truncatula* (a model legume plant) (Trieu et al. 2000).

5.4.2.4 Agrolistics

The agrolistics approach combines the advantages of efficient biolistic delivery and the precision of the *Agrobacterium* T-DNA insertion mechanism, minimising the regions of homology contributing to genetic and/or epigenetic instability (Hansen and Chilton 1996). By combining features of *Agrobacterium*-mediated transformation it is possible to achieve relatively predictable inserts in plants that are not normally transformable using *Agrobacterium*. Agrolistic transformation allows integration of the gene of interest without the undesired vector sequence, using plant expression cassettes for *virD1* and *virD2* genes co-delivered with a vector containing T-DNA border sequences flanking a gene of interest, resulting in production of transformants without the extraneous vector DNA as a result of T-DNA border cleavage by *virD1* and *virD2* gene products (Sharma et al. 2005a, b).

5.4.3 Biolistics-Mediated Gene Transfer

The invention of the particle bombardment technique (Sanford et al. 1987) was a major breakthrough in plant transformation as it has enabled the genetic engineering of species not amenable to *Agrobacterium* or protoplast-based transfer techniques. Based on acceleration, microscopic tungsten (Russel et al. 1992) or gold particles coated with DNA can be propulsed into practically all kinds of tissues (Tomes et al. 1990; Ritala et al. 1994; Zhong et al. 1996). It has been used to develop the transgenic cereal plants in wheat (Vasil et al. 1992), oat (Somers et al. 1992), barley (Wan and Lemaux 1994) and rye (Castillo et al. 1994).

5.4.3.1 Factors Affecting Biolistics-Mediated Gene Transfer

Several factors have been found to influence the applicability and efficiency of biolistic gene transfer. The factors related with tissue culture techniques include genotype (Koprek et al. 1996), type and age of bombarded explants (Armaleo et al. 1990), culture period prior to and after gene transfer (Rasco-Gaunt et al. 1999), culture medium composition (Barro et al. 1998) and osmotic pretreatment (Vain et al. 1993). Concerning the biolistic device, the applied acceleration pressure

(Rasco-Gaunt et al. 1999); the adjustable distances between rupture disc, macrocarrier, stopping screen and target plate (Koprek et al. 1996; Rasco-Gaunt et al. 1999); the vacuum pressure in the bombardment chamber (Rasco-Gaunt et al. 1999); number of bombardments (Lonsdale et al. 1990) as well as size and density of microparticles (Altpeter et al. 1996; Rasco-Gaunt et al. 1999); DNA/micro-particle mixing protocols (Perl et al. 1992) and physical configuration of transforming DNA (Nandadeva et al. 1999; Fu et al. 2000) are factors to be optimised. Several attempts to establish or improve transformation protocols focused on transient GUS expression without consideration of the regeneration response of the bombarded tissues (Chibbar et al. 1991; Bilang et al. 1993). However, particle bombardment, especially of recalcitrant species, can have severe effects on the regeneration capability of cultures. Optimised protocols for generating transgenic plants should therefore not be based exclusively on transient gene expression assays (Nandadeva et al. 1999); adjustment of bombardment parameters to maintain the shoot regeneration ability and allow the recovery of stable transformants is recommended (Altpeter et al. 1996).

5.4.4 Other Methods of Gene Transfer

Other DNA delivery protocols like macroinjection (Soyfer 1980; Zhou et al. 1983), the use of silicon carbide whiskers (Wang et al. 1995; Petolino et al. 2000) and ultrasound- (Joersbo and Brunstedt 1990) or laser-mediated gene transfer (Weber et al. 1988) are of rather theoretical importance and have been extensively reviewed by Rasco-Gaunt et al. (1999).

5.5 Marker-Free Plants: The Most Relevant Answers to Biosafety-Related Issues

Selectable marker gene (SMG) and reporter genes play the most crucial role in transferring foreign genes and are almost always present in engineered DNA plasmids used for genetic transformation of plant tissue (Lee and Gelvin 2008). It is only the presence of SMG that serves as a weapon for transformed cells to tolerate a lethal exposure to antibiotic and herbicide, and the desired gene can safely grow and regenerate into the plants (Lee and Gelvin 2008). Selectable markers and visible marker reporter genes rarely affect the studied trait of interest, but provide a powerful tool in determining the success of the transformation events or identification of transformation events before the gene of interest can be identified in the culture (Sheen et al. 1995). However there is a need to free transgenic plants from these markers due to harmful effects to human. Therefore the following strategies have been used widely to remove such markers.

5.5.1 Co-transformation

The co-transformation method is used to eliminate the marker gene from the nuclear genome and involves transformation that targets insertion at two different plant genome loci. Co-transformation studies utilise the strategy to load the selectable and target genes between the same pair of borders or loaded into separate T-DNAs, which are expected to segregate independently in a Mendelian fashion (Ramana Rao et al. 2011). The three methods used in the co-transformation system include (1) two different vectors carried by different *Agrobacterium* strains (De Neve et al. 1997) and biolistic introduction of two plasmids in the same tissue (Shiva Prakash et al. 2009; Kumar et al. 2010); (2) two different vectors in the same *Agrobacterium* cell (Sripriya et al. 2008) and (3) two T-DNAs borne by a single binary vector (2 T-DNA system) (Miller et al. 2002). This has the following advantages.

1. The conventional unmodified *Agrobacterium*-mediated gene transfer methods have high adaptability and easier handling of the binary vectors (Tuteja et al. 2012).
2. This method depends on the co-transformation efficiency which ranges from 30 to 50 % and the independent integration of T-DNA into the plant genome, which is acceptable for practical applications (McCormac et al. 2001). Recently, in rice, high transformation frequency (86 %) was achieved through genetic separation in four out of ten primary co-transformants that were forwarded to the T1 generation (Sripriya et al. 2011).

5.5.2 Multi-Autotransformation Vector System

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 is based on morphological changes caused by oncogene [the isopentenyl transferase (*ipt*) gene] or rhizogene (the *rol* gene) of *A. tumefaciens* (Tuteja et al. 2012). The *ipt*-type MAT system has been considered better for successful generation of marker-free transgenic plants (Saelim et al. 2009). This system utilises the *ipt* gene as morphological marker for visual selection of transgenic lines. The extreme shooty phenotype (ESP) of transgenic lines is lost due to the removal of *ipt* gene mediated by the yeast recombinase/recognition sites R/RS system. As a result, phenotypically normal shoots, considered marker-free transgenic plants, could be obtained. The *ipt* marker gene has been efficiently used as selectable marker gene for obtaining marker-free plant in several crops (Khan et al. 2010a, b). Rol-type MAT vector (pMAT101) containing *lacZ* gene as a model gene and the removable cassette with *GUS* gene in the T-DNA region were used to produce morphologically normal transgenic *Kalanchoe blossfeldiana* pollen, employing *rol* gene as the selectable marker gene and *gus* gene as a reporter

gene (Thirukkumarana et al. 2010). This vector has been tried in *Antirrhinum majus* (Cui et al. 2001), tobacco (Ebinuma and Komamine 2001), white poplar (Zelasco et al. 2007) and *Petunia hybrida* (Khan et al. 2010c). Genetic transformation of an elite white poplar genotype (*Populus alba* L., cv. 'Villafranca') was performed with MAT vectors carrying the *ipt* and *rol* genes from *A. tumefaciens* spp. as morphological markers. The occurrence of abnormal *ipt* and *rol* phenotypes allowed the visual selection of transformants (Zelasco et al. 2007). *A. tumefaciens* strain EHA105 harbouring a Rol-type MAT vector, pMAT101, was used to produce morphologically normal transgenic *Petunia hybrida* 'Dainty Lady' employing *rol* gene as the selection marker gene. The *lacZ* gene was used as a model GOI (Khan et al. 2010c).

5.5.3 Site-Specific Recombination

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). The three well-known site-specific recombination systems discussed below are used for the elimination of selection marker gene.

5.5.4 Cre/Lox Site-Specific Recombination System

The Cre/loxP (CLX) 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. A number of studies describes the successful utilisation of CLX system including *Arabidopsis thaliana* (Zuo et al. 2001), *Nicotiana benthamiana* (Gleave et al. 1999), *Zea mays* (Zhang et al. 2003) and *Oryza sativa* (Hoa et al. 2002; Sreekala et al. 2005).

The specificity of the enzyme for its 34 bp recognition sequence is one of the major advantages of Cre/lox system because insertion and excision of genes with precision in the plant genome without a site-specific recombination system are difficult (Tuteja et al. 2012). However, use of this system for marker gene removal requires re-transformation and outcrossing approaches that are laborious and time consuming (Dale and David 1991). Several approaches were developed to overcome these shortcomings, including the use of some chemical inducers (Zhang et al. 2006) and heat shock (Cuellar et al. 2006). Marker-free transgenic tomato plants expressing *Cry1Ac* were obtained by using a chemically regulated Cre/lox-mediated site-specific recombination system (Zuo et al. 2001; Zhang et al. 2006).

Lin et al. (2008) reported a chemical induction method for creating selectively terminable transgenic rice using benzothiadiazole (bentazon), a herbicide used for weed control in major crops like rice, maize, wheat, cotton and soybean. Similarly, Ma et al. (2009) used salicyclic acid-inducible Cre–loxP recombination system to develop marker-free transgenic tomato.

5.5.5 *Flippase/Flippase Recombination Target Recombination System*

Nandy and Srivastava (2011) reported the use of flippase (FLP)/flippase recognition target (flp) system for efficient targeting of foreign gene into the engineered genomic site in rice. In the FLP/frt site-specific system, the FLP enzyme efficiently catalyses recombination between two directly repeated FLP recombination target (frt) sites, eliminating the sequence between them (Tuteja et al. 2012). By controlled expression of the FLP recombinase and specific allocation of the frt sites within transgenic constructs, the system can be applied to eliminate the marker genes after selection (Cho 2009). This system has been used to generate marker-free salt-tolerant transgenic maize plants constitutively expressing AtNHX1, a Na(+)/H(+) antiporter gene from *A. thaliana* (Li et al. 2010). An oxidative stress-inducible FLP gene was used successfully to excise antibiotic-resistance genes from transgenic plants (Woo et al. 2009). Two site-specific recombination systems, Cre/lox and FLP/frt, were tested for marker gene removal and targeted gene transfer in a *Populus* (Fladung et al. 2010) and observed to be useful for removal of marker genes. Combining both site-specific recombination systems, a strategy is suggested for targeted transgene transfer and removal of antibiotic marker genes.

5.5.6 *R/RS Recombination System*

The MAT vectors consist of yeast site-specific recombination R/RS system to excise the DNA fragment and the *ipt* gene positioned between two directly oriented recombination sites (Araki et al. 1987). The R/RS system comprises an R recombinase gene and two RS recombination site sequences. The *ipt* combined with the *'R'* gene is placed within two directly oriented recognition sites to remove it from transgenic cells after transformation. The improved MAT vector is used to generate marker-free transgenic plants efficiently. A new binary vector for *A. tumefaciens*-mediated transformation, pHUGE-Red, was developed (Untergasser et al. 2012). This vector enables modular cloning of large DNA fragments by employing Gateway technology and contains DsRED1 as visual selection marker. However, an R/Rs-inducible recombination system was included allowing subsequent removal

of the selection markers in the newly generated transgenic plants. This strategy allowed successful transfer of eight genes essential for *Medicago truncatula* (Untergasser et al. 2012).

5.5.7 Transposon-Based Marker Methods

Transposon-mediated repositioning of a transgene of interest has been proposed as an alternative for generating a wide range of expression levels in selectable marker gene-free transgenic plants (Yoder and Goldsbrough 1994). Two transposon-mediated strategies have been developed to generate marker-free transgenic plants. The first strategy involves intragenomic relocation of transgene of interest after *Agrobacterium*-mediated transformation and its subsequent segregation from the selectable marker in the progeny (Goldsbrough et al. 1993). The second involves excision of the marker gene from the genome (Ebinuma et al. 1997). Though maize Ac/Ds transposable element has been used in the above strategies, similar approach can be adopted to other autonomous transposable elements. In 2012, Li and Charng developed an improved strategy involving insertion of the end of the inducible transposon in an intron of a target gene for subsequent removal of its function in transgenic plants. Salicylic acid-induced transposition of COKC transposon, which led to both marker gene and transposase gene breakages in exons, was analysed in single-copy transgenic rice plants. It has been observed that the COKC element exhibits the potential as a tool to create 'marker-off' (marker free) transgenic plants (Li and Charng 2012). However, its application is limited for selectable marker gene without native introns, e.g. hygromycin- or kanamycin-resistant genes. Therefore in order to expand the application of the 'marker-off' transgenic system, an artificial intron containing one end of the transposon has been generated (Li and Charng 2012), and as the result successful transgenic plants were obtained after screening with the selection agent. Thus it indicated the use of an inducible transposon for 'cleaner' plant biotechnology (Li and Charng 2012).

5.5.8 Chemical-Inducible System

The chemical-inducible CLX vector system benefits from a particularly regulated system of chemical induction (Sreekala et al. 2005). It is used in vegetatively propagated and other crop species (Tuteja et al. 2012). The strategy utilises the CLX recombination system keeping it under the control of estrogen receptor-based transactivator XVE. Upon induction by β-estradiaol, the selection marker gene fused with Cre recombinase, flanked by two lox sites and autoexcised from the genome (Zuo et al. 2001). Marker-free transgenic tomato expressing *cry1AC* was obtained by using the above system. A chemical-induced method for creating

selectively terminable transgenic rice using benzothiadiazole herbicide (bentazon) has already been discussed in the section of Cre/lox site-specific recombination system (Lin et al. 2008).

5.5.9 Heat-Inducible System

Transgenic tobacco has been developed using FLP/frt recombinase system in which the expression of FLP was tightly under the control of heat-shock protein (HSP) (Shan et al. 2006). Heat-inducible strategy for the elimination of selection marker gene was also used in vegetatively propogated plants like potato (Cuellar et al. 2006) and seed-producing plants like tobacco (Wang et al. 2005). The disadvantage of this method is that when autoexcision constructs are used, the recombinase can be activated by a chemical compound or by a heat shock in the shoots and seeds or during a subculture step and an extra regeneration step. The latter possibility lengthens the time to obtain marker-free transgenic plants and can introduce (additional) somaclonal variation (Tuteja et al. 2012).

5.5.10 Positive Selection System

A better way to select and identify the genetically modified cell is through the positive selection system. The GUS gene is widely used as a reporter gene in transgenic plants. A glucuronide derivative of benzyladenine (benzyladenine N-3-glucuronide) is used as a selective agent and being in inactive form does not have any effect on the non-transformed cells. However, there are only a few reports concerning the successful use of this system in the effective recovery of transgenic plants (Joersbo and Okkels 1996; Okkels et al. 1997).

The *manA* gene codes for the PMI enzyme (EC 5.3.1.8) and is isolated from *Escherichia coli*. Because mannose is used as the sole carbohydrate source for the transformed cells this selection system is immediate and extremely efficient (Joersbo et al. 1998). Those species which are extremely sensitive to mannose such as sugar beet, maize, wheat, oat, barley, tomato, potato, sunflower, oilseed rape and pea have been successfully transformed by the use of mannose as a selective agent (Joersbo et al. 1998; Wang et al. 2000). Use of positive selection system was found at least ten times more efficient than the traditional protocols based on the use of kanamycin as selection agent (Wright et al. 2001). Similarly a positive selection system has also been developed using the xylose isomerase gene (xylA) isolated from *Thermoanaerobacterium thermosulfurogenes* or from *Streptomyces rubiginosus*, as selection marker gene (Haldrup et al. 1998), and used in development of transgenic plants of potato, tobacco and tomato. The DOG R1 gene encoding 2-deoxyglucose-6-phosphate phosphatase (2-DOG-6-P) was used to develop a positive selection system for tobacco and potato plants (Kunze et al. 2001).

5.5.11 Negative Selection System

Negative selectable markers are of two types: (a) conditional negative selectable marker and (b) non-conditional negative selectable marker (e.g. diphtheria toxin). MYMV TrAP is a good non-conditional negative selectable marker for developing marker-free transgenic plants (Ramana Rao and Veluthambi 2010). It is also possible to apply negative selection after a positive selection using one marker gene. Use of *tms2* gene is the first conditional selective marker gene, which is used in tobacco (Depicker et al. 1988) and *Arabidopsis* (Karlin-Neumann et al. 1991). Other conditional markers proven to be effective in dicots are *aux2* in cabbage (Beclin et al. 1993), the *HSV-tk* gene in tobacco (Czako and Marton 1994), a bacterial cytochrome P450 mono-oxygenase gene in tobacco (O'Keefe et al. 1994) and *Arabidopsis* (Tissier et al. 1999) and *codA* in *Arabidopsis* (Kobayashi et al. 1995) and tobacco (Schlaman and Hooykaas 1997).

5.5.12 Autoexcision Strategy

Autoexcision strategy is used to eliminate selection marker gene from the plant genome, controlled by pollen- and/or seed-specific promoters. Highly efficient autoexcision of selective markers has been reported to be successful in tobacco (Mlynarova et al. 2006; Luo et al. 2007) and in rice (Bai et al. 2008). The novel marker-free approach mediated by the *Cre–lox* recombination system and the *Cre* gene was under the control of floral specific promoter *OsMADS45*. The marker-free transgenic plants of *A. thaliana* were developed by using a germline-specific autoexcision vector containing a *Cre* recombinase gene under the control of a germline-specific promoter (*APETALA1* and *SOLO DANCERS* genes from *Arabidopsis*). However, this strategy is not useful in the vegetatively propagated plants (Verweire et al. 2007).

5.6 Gene Transfer for Stress Tolerance in Crop Species

Development of genetically engineered plants by the introduction and/or overexpression of selected genes seems to be a viable option to hasten the breeding of 'improved' plants against various biotic and abiotic stresses. It is a faster way to achieve transgenesis when genes of interest are originated from cross barrier species, distant relatives or non-plant sources. Several traits associated with resistance to various stresses have been introgressed and tested in transgenic plants for improving stress tolerance in plants (Bhatnagar et al. 2010).

5.6.1 Tolerance to Abiotic Stresses

Drought is one of the most significant environmental stresses on world agricultural production, and enormous efforts are being made by plant scientists to improve crop yields in the face of decreasing water availability. The genes that encode enzymes for the synthesis of selected osmolytes have been used to develop osmoprotection in plants (Bray 1993). This has resulted in a profusion of reports involving osmoprotectants such as glycine-betaine (Ishitani et al. 1997; Sakamoto et al. 2000; McNeil et al. 2000) and proline (Nanjo et al. 1999; Yamada et al. 2005). Also, a number of 'sugar alcohols' (mannitol, trehalose, myo-inositol and sorbitol) have been targeted for the engineering of compatible solute overproduction, thereby protecting the membrane and protein complexes during stress (Pilon-Smits et al. 1995; Garg et al. 2002; Cortina and Culianez-Macia 2005; Gao et al. 2000). Similarly, transgenics engineered for the overexpression of polyamines have also been developed (Waie and Rajam 2003; Capell et al. 2004).

LEA proteins are high-molecular-weight proteins found in abundance during late embryogenesis and accumulate during seed desiccation in response to water stress (Galau et al. 1987). Transgenic melon (Borda's et al. 1997) and tomato (Gisbert et al. 2000) plants expressing the *HAL1* gene showed a certain level of salt tolerance due to retention of more K+ under salinity stress. Overexpression of *AtCLCd* gene, which is involved in cation detoxification, and *AtNHXI* gene which is homologous to *NhxI* gene of yeast conferred salt tolerance in *Arabidopsis*. Transgenic *Arabidopsis* and tomato plants that overexpress *AtNHX1* accumulated abundant quantities of the transporter in the tonoplast and exhibited substantially enhanced salt tolerance (Zhang and Blumwald 2001). Transgenic groundnut plants expressing *AtNHX1* gene showed more resistance to high concentration of salt and water deprivation due to higher level of salt and proline (Asif et al. 2011).

It has been observed that transferring a single gene encoding a single specific stress protein may not be sufficient to reach the required tolerance levels (Bohnert et al. 1995). Therefore, use of gene encoding a stress-inducible transcription factor has been suggested as an alternative for enhancing tolerance towards multiple stresses (Yamaguchi-Shinozaki et al. 1994; Chinnusamy et al. 2005). Several studies showed that overexpression of drought-responsive transcription factors can enhance abiotic stress tolerance in plants (see review, Zhang et al. 2004). For example, overexpression of an ethylene response factor controlled by gene *Sub1A* in rice conferred enhanced submergence tolerance (Xu et al. 2006). Manipulating *CBF/DREB* genes confer improved drought tolerance in crop plants. Transgenic tomato plants expressing *CBF1*, containing three copies of an ABA-responsive complex (ABRC1) from the barley *HAV22* gene, exhibited enhanced tolerance to chilling, water deficit and salt stress (Lee et al. 2003). In another study, expression of *CBF/DREB* genes under stress-inducible promoters in transgenic plants is reported to minimise the growth retardation and other adverse effects (Al-Abed et al. 2007). Development of transgenic with the use of a single regulatory gene (*DREB1A* transcription factor) in groundnut regulated the expression of downstream genes leading

to the activation of many functional genes (Bhatnagar-Mathur et al. 2009). Another transcription factor that has been manipulated in order to increase plant drought tolerance is the *HARDY* (*HRD*) gene, which has been linked to increased transpiration efficiency related to stomatal adjustment. HRD is an AP2/ERF-like transcription factor isolated from hrd-dominant (*hrd-D*) *Arabidopsis* mutants, which displayed vigorous rooting and dark green leaves that were smaller and thicker than WT plants. Karaba et al. (2007) isolated the HRD gene and constitutively expressed it in *Arabidopsis* under the control of the cauliflower mosaic virus (CaMV) 35S promoter. Transgenic rice seedlings, expressing *OsWRKY11* under the control of a rice HSP promoter, HSP101, were shown to survive longer and lose less water under a short and severe drought treatment (Wu et al. 2008). Young transgenic rice plants overexpressing *ZFP252* survived longer, displayed less relative electrolyte leakage and accumulated more compatible osmolytes than WT plants during a 14-day period of drought stress (Xu et al. 2008). A salt- and drought-induced RING-finger protein, SDIR1, was found to confer enhanced drought tolerance to tobacco and rice (Zhang et al. 2008).

Prior to transcriptional activation of genes, drought stress signals are received and messages conveyed to the appropriate components of the downstream pathway (Xiong and Ishitani 2006). Receptor molecules that have been identified to date in plants include ROP10, a small G protein from the ROP family of Rho GTPases, that negatively regulates ABA response in *Arabidopsis* (Zheng et al. 2002); ATHK1, a putative homolog of the yeast SLN1, which is a functional histidine kinase feeding into the HOG MAPK pathway (Reiser et al. 2003); NtC7, a receptor-like membrane protein from tobacco (Tamura et al. 2003) and Cre1, a putative cytokinin sensor and histidine kinase from Arabidopsis (Reiser et al. 2003). The *ERECTA* gene from *Arabidopsis* is the first gene to be shown to act on the coordination between transpiration and photosynthesis (Masle et al. 2005).

Few known studies have focused on engineering heavy metal tolerance in plants. For example, Zhang et al. (2008) reported an aquaporin gene *BjPIP1* from the heavy metal hyperaccumulator Indian mustard, which is upregulated in leaves under drought, salt, low temperature and heavy metal stress. Constitutive expression of *BjPIP1* in tobacco decreased water loss rate, transpiration rate and stomatal conductance of transgenic plants compared to WT under osmotic stress.

5.6.2 Tolerance to Biotic Stresses

Plants sense and respond to environmental cues by a repertoire of mechanisms that regulate gene expression in order to maximise chances of survival in hostile environments (Dorantes-Acosta et al. 2012). In addition to preformed defence traits, plants have evolved inducible defences against microbial pathogens, herbivores and even other plants that involve the regulation of gene expression for the synthesis of defensive secondary metabolites and specific proteins (Walling 2000; Mithofer and Boland 2012).

5.6.2.1 Insect Resistance

Bt technology has emerged as a powerful modality for battling some of the important insect pests. It is a chemical free and economically viable approach for insect pest control in plants (Gatehouse 2008; Pratap and Gupta 2009; de Villiers and Hoisington 2011). Transgenic Bt crops are used worldwide to control major pests (caterpillars and rootworms) of cotton, corn and soybean. The first widely planted Bt crop cultivars were corn producing Bt toxin Cry1Ab and cotton producing Bt toxin *Cry1Ac* (Tabashnik et al. 2009). However, resistance in five lepidopteran pests against the Bt crops carrying the different genes (*Cry1Ab, Cry1F, Cry1Ac* and *Cry2Ab*) has also been reported in South Africa, Puerto Rico, India, the USA and Australia (Kruger et al. 2009; Tabashnik et al. 2008, 2009; Downes et al. 2010). In spite of this, the area under the Bt crops has been increasing since 1996 and in 2011, and biotech crops were grown on 160 million hectares (James 2011). The USA had the largest share of global biotech crop plantings in 2011 (69 million ha), followed by Brazil (30.3 M ha). The other main countries planting biotech crops in 2011 were Argentina (23.7 M ha), India (10.6 M ha) and Canada (10.4 M ha). Brookes and Barfoot (2005) reported that 725 approvals for commercial cultivation had been granted for 155 events in 24 crops, and 57 countries globally have granted regulatory approvals for biotech crops for import for food and feed use and for release into the environment since 1996 (Karthikeyan et al. 2012).

5.6.2.2 Virus Resistance

Plant viruses constitute one of the major problems of the agricultural production worldwide. To date, there are no therapeutical measures available for the control of plant-virus diseases in the field, and the main control strategy used in practice is based on prevention measures. Host plant resistance is by far the most effective way to control plant viruses. However, 'traditional' genetic sources of resistance to viruses are rare, and due to the high rate of mutation of the viral genomes this resistance even when applicable is frequently broken under field conditions. *Agrobacterium*-mediated genetic transformation technology (Thomashow et al. 1980) offered new promising prospects for engineered genetic resistance to viruses with numerous following studies reporting a successful use of the transgenic technology against almost all genera of plant viruses or even viroids (Prins et al. 2008, Schwind et al. 2009). The breakthrough for the creation of transgenic resistance to plant viruses came by Beachy's group which showed that the expression of the coat protein gene of tobacco mosaic virus (TMV) in transgenic plants confers resistance to TMV (Abel et al. 1986).

RNA silencing-based resistance against viruses was first reported by Lindbo et al. (1993) and was shown to be related to the previously observed co-suppression mechanism (Van der Krol et al. 1990). It has been reported that short genome incomplete sequences can be used, and efficiencies of up to 90 % of all transgenic plants produced to be resistant to the homologous virus were achieved (Lin et al.

2007; Tenllado et al. 2004). In order to overcome the weakness of RNA silencing-based resistance Bucher et al. (2006) fused 150-nt fragments of viral sequences of four tospoviruses in a single small chimeric IR construct. This strategy resulted in a high frequency of resistant plants. A most recent approach used modified plant miRNA cistrons to produce a range of antiviral artificial miRNAs (amiRNAs) (Niu et al. 2006; Qu et al. 2007; Zhang et al. 2011). Vasillakos (2012) reviewed that recent advances like the construction of chimeric IR constructs incorporating sequences derived from different virus species if combined with epidemiological data and pest risk analyses could reduce the effect of mixed virus infections on the resistance (Dafny-Yelin and Tzfira 2007; Kung et al. 2009). Recently, virus resistance was achieved through the expression of amiRNAS against viral coding sequences (Ding and Voinnet 2007; Duan et al. 2008; Zhang et al. 2011). Although there was evidence that amiRNA-mediated virus resistance may not be inhibited by low temperature (Niu et al. 2006) this possibly depends on the plant species examined (Qu et al. 2007). Moreover, the durability of this approach, which resulted in relatively few antiviral small RNAs compared with those of the long dsRNA approach, needs to be further demonstrated (Duan et al. 2008; Simon-Mateo and Antonio Garcia 2006).

5.6.2.3 Fungus and Bacteria Resistance

Significant yield losses due to fungal attacks occur in most of the agricultural and horticultural species. In Indian context, fungal diseases are rated either the most important or second most important factor contributing to yield losses in our major cereals, pulses and oilseed crops. The most significant development in the area of varietal development for disease resistance is the use of the techniques of gene isolation and genetic transformation to develop transgenics resistant to fungal diseases.

Genetic engineering allows for introduction of resistance genes from unrelated plant species, which often remain functional in the new host plant (Collinge et al. 2008). The R-gene *Rxo1* from maize was successfully introduced into rice and conferred resistance against bacterial streak disease caused by *Xanthomonas oryzae* pv. *oryzicola* (Zhao et al. 2005). Additional examples of this strategy involve the R-gene *RCT1* from *Medicago truncatula* that was expressed in alfalfa and conferred resistance to *Colletotrichum trifolii* (Yang et al. 1996) and *RPI-BLB2* from wild potato, *Solanum bulbocastanum,* conferring resistance to *Phytophthora infestans* in cultivated potato (Van der Vossen et al. 2005). Chitinase (PR) originating from mycoparasitic biocontrol agents, most notably *Trichoderma harzianum*, that can exhibit higher anti-fungal activity than plant chitinases, has been proven to be a more effective source for enhancing fungal disease resistance in transgenic plants (Dana et al. 2006; Kumar et al. 2009).

In contrast to biotrophic pathogens, necrotrophs produce copious amounts of pathogenicity factors, including toxins and cell wall-degrading enzymes, as a means of successfully establishing infections. Mutants lacking these pathogenicity factors often have reduced virulence or in some instances are completely avirulent.

Overexpression of PGIPs in transgenic plants has successfully reduced disease symptoms due to *B. cinerea* (Joubert et al. 2007; Manfredini et al. 2005) and *Bipolaris sorokiniana* (Janni et al. 2008). Similarly, antisense suppression of PGIPs in *Arabidopsis* increased susceptibility towards *B. cinerea* (Ferrari et al. 2006). The main strategy to resist *Sclerotinia sclerotiorum* infection includes wheat oxalate oxidase and oxalate decarboxylase, converting oxalic acid to CO2 and hydrogen peroxide or CO_2 and formate, respectively. Overexpression of these enzymes in lettuce (Dias et al. 2006), sunflower (Hu et al. 2003), soybean (Cober et al. 2003), rapeseed (Dong et al. 2008) and tomato (Walz et al. 2008) demonstrated at least partial resistance to *S. sclerotiorum*. Adaptation of these technologies will only progress once the costs associated with growing, developing and registering the transgenic technologies are balanced by the gains observed by the producers and ultimately with the consumers of the plants. Once the economic threshold is passed and the plants can be proven safe to be consumed, large-scale adoption of these technologies may become a reality (Wally and Punja 2010).

5.7 Regulations and Biosafety Concerns

Biosafety issue has already become a crucial factor in constraining the further development of transgenic biotechnology and wider application of GM products in agriculture. The most important concerns can be summarized as follows: (1) direct and indirect effects of toxic transgenes (e.g. the Bt insect-resistance gene) to non-target organisms (O'Callaghan et al. 2005; Oliveira et al. 2007); (2) influences of transgenes and GM plants on biodiversity, ecosystem functions and soil microbes (Giovannetti et al. 2005; Oliveira et al. 2007); (3) transgene escape to crop landraces and wild relatives through gene flow and its potential ecological consequences (Mercer et al. 2007) and (4) potential risks associated with the development of resistance to biotic-resistance transgenes in the target organisms (Wu 2007). 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 (Ellstrand 2001, 2003). Transgene escape may result in potential ecological consequences if significant amounts of transgenes constantly move to non-GM crops and wild relative species.

The development of marker-free transgenic plants could solve the issues of biological and biosafety in the genetically engineered (GE) crops, besides supporting multiple transformation cycles for transgene pyramiding (Vaucheret et al. 1998). The presence of SMG is undesirable as per the European regulatory agencies' norms. Also trasngene integration at random positions in the genome leads to possible unwanted side effects (mutation) and unpredictable expression patterns. In addition to the risk of HGT, there is also a 'vertical cross-species' transfer risk that could potentially create enhanced weediness problems (Dale et al. 2002). The production of marker-free transgenic crops eliminates the risk of HGT and could mitigate vertical gene transfer. In view of the biosafety requirements, it is recommended

to phase out the SMG since these are unnecessary once an intact transgenic plant has been identified and established (Darbani et al. 2007). Besides, there are public concerns about the widespread occurrence of SMG in novel ecosystems as these are integrated into the plant genome along with the gene of interest (Daniell et al. 2001). 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(s) from the plant genome, probably as linear fragments; (2) protection of the gene(s) from nuclease degradation in the plant as well as animal gastrointestinal tract; (3) uptake of the gene(s) with dietary DNA; (4) transformation of bacteria or competent mammalian cells; (5) insertion of the gene(s) 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; Tuteja et al. 2012).

5.8 Conclusions and Future Prospects

Genetic transformation of crop plants has emerged as a remarkable achievement in modern biotechnology. Transgenic plant varieties hold great promise for augmenting agricultural production and productivity when properly integrated into traditional agricultural research systems. From the recent advances in genetics and genomics it is clear that gene transfer is emerging as a major player in the understanding of gene function and its validation and also that it has a potential to play an important role in generating genetic novelties that, once traits are introduced in the field, should find their way into the breeding strategies for a number of crops. Owing to the utility of this technique, the use of transgenic crop varieties having resistance to a wider range of biotic and abiotic stresses is expected to gain more popularity. However, at the same time, the concerns of general public regarding the safety issues as well as their impact on environment need to be properly addressed. Advancements in removal of selectable markers from the transgenics once they are identified and detailed and unbiased studies on transgene escape to the environments and their real ecological impact may help to a great extent in tackling public scepticism about the development and use of transgenics.

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