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GENETIC TRANSFORMATION TECHNOLOGY: STATUS AND PROBLEMS

KIRAN K. SHARMA[1]*, POOJA BHATNAGAR-MATHUR[1], AND TREVOR A. THORPE[2]

[1]*Genetic Transformation Laboratory, International Crops Research Institute for the Semi-Arid Tropics (ICRISAT), Patancheru, Andhra Pradesh 502 324, India*
[2]*Plant Physiology Research Group, Department of Biological Sciences, University of Calgary, Calgary, AB T2N 1N4, Canada*

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Summary

Transfer of genes from heterologous species provides the means of selectively introducing new traits into crop plants and expanding the gene pool beyond what has been available to traditional breeding systems. With the recent advances in genetic engineering of plants, it is now feasible to introduce into crop plants, genes that have previously been inaccessible to the conventional plant breeder, or which did not exist in the crop of interest. This holds a tremendous potential for the genetic enhancement of important food crops. However, the availability of efficient transformation methods to introduce foreign DNA can be a substantial barrier to the application of recombinant DNA methods in some crop plants. Despite significant advances over the past decades, development of efficient transformation methods can take many years of painstaking research. The major components for the development of transgenic plants include the development of reliable tissue culture regeneration systems, preparation of gene constructs and efficient transformation techniques for the introduction of genes into the crop plants, recovery and multiplication of transgenic plants, molecular and genetic characterization of transgenic plants for stable and efficient gene expression, transfer of genes to elite cultivars by conventional breeding methods if required, and the evaluation of transgenic plants for their effectiveness in alleviating the biotic and abiotic stresses without being an environmental biohazard. Amongst these, protocols for the introduction of genes, including the efficient regeneration of shoots in tissue cultures, and transformation methods can be major bottlenecks to the application of genetic transformation technology. Some of the key constraints in transformation procedures and possible solutions for safe development and deployment of transgenic plants for crop improvement are discussed.

Key words: Agrobacterium tumefaciens; biolistics; gene silencing; genetic transformation; transgenics.

Introduction

Conventional plant breeding, combined with improved agricultural practices and modern technology, has contributed to dramatic crop improvements over the past 50 yr, and will continue to provide future benefits. However, there are strong pressures for further improvements in crop quality and quantity exerted from population growth, social demands, health requirements, environmental stresses, and ecological considerations. The world population is predicted to reach 8 billion by the year 2010. To feed 3 billion additional people in the next 20 yr will require dramatic increase in crop productivity, a formidable task by any standard. Conventional plant breeders and related scientists have worked diligently and skillfully to upgrade quality and raise the yields by employing various crop improvement techniques, with commendable results. Notwithstanding the impressive gains in productivity so far, there are limitations to conventional plant breeding technology, either due to the limited gene pool or to the restricted range of organisms between which genes can be transferred due to species barriers. Although these methods have proved to be useful, they depend ultimately upon reliable testing and proper selection, and do not involve the identification and manipulation of genetic targets as defined in molecular terms. Plant biotechnology continues the trend of improving crops with more precise methods, permitting the transfer of a single gene with a known function into existing crop varieties, in contrast to the cross-breeding techniques which transfer thousands of genes of unknown functions into crops. Genetic engineering has been used to complement traditional breeding methods in crop improvement. Transfer of genes from heterologous species provides the means of selectively introducing new traits into crop plants and expanding the gene pool beyond what has been available to traditional breeding systems. Hence, new biotechniques, in addition to conventional plant breeding, are needed to boost the yield of crops that feed the world (Borlaug, 1997). The newly acquired ability to transfer genes among organisms without sexual crossing provides breeders with new opportunities to improve the efficiency of production and to increase the utility of agricultural crops. Plants with new traits, such as resistance to herbicides, insect pests, and viruses, have been genetically engineered using genes from unrelated organisms. However, it should be emphasized that the biotechnology is not a substitute or replacement for conventional breeding methods. Rather, it can improve on past, conventional methods. The major

*Author to whom correspondence should be addressed: Email k.sharma@cgiar.org

differences between conventional breeding and biotechnology lie neither in goals nor processes, but rather in speed, precision, reliability, and scope.

Plant biotechnology offers new ideas and techniques applicable to agriculture. It uses the conceptual framework and the technical approaches of plant tissue culture and molecular biology to develop commercial processes and products. Hence, with the rapid development of biotechnology, agriculture has moved from a resource-based to a science-based industry. The technology required for engineering transgenic plants is considerably more sophisticated than the one for producing somatic hybrid plants, and much more so than that required for production of hybrid plants by cross-fertilization (Goodman et al., 1987; Kung, 1993; Birch, 1997).

Non-sexual DNA transfer techniques make possible manipulations that are outside the repertoire of breeding or cell fusion techniques. Genes can be accessed from exotic sources, plant, animal, bacterial, even viral, and introduced into a crop. Because the DNA elements that control gene expression can, and often must, be modified for proper function in the new host, it is possible to control timing, tissue specificity, and expression level of transferred genes. Endogenous plant genes may even be reprogrammed through the reintroduction of an engineered gene (Maniatis et al., 1987; Schibler and Sierra, 1987).

Genetic Engineering of Plants

With the advent of recombinant DNA methods and genetic transformation procedures, it is possible to transfer genes into crop plants from unrelated plants, microbes, and animals. Most of the modifications being carried out, or envisaged, are for disease, pest, or herbicide resistance. Because of these possibilities, it is now feasible to introduce into crop plants, genes that have previously been inaccessible to the conventional plant breeder or which did not exist in the crop of interest. However, the availability of efficient transformation methods to introduce foreign DNA can be a substantial barrier to the application of recombinant DNA methods in some crop plants. Despite significant advances over the past decade, the development of efficient transformation methods can take many years of painstaking research. 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 characterization 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 here.

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. 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 non-chimeric transformed plants. Transformation techniques reliant on plant regeneration from *in vitro*-cultured tissues have been described for many crop species (Draper et al., 1988; Lindsey and Jones, 1989; Dale et al., 1993; Birch, 1997).

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 phosphinotricin, 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 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), β-galactosidase, β-glucuronidase (GUS), luciferase (LUX), green fluorescent protein (GFP; Reichel et al., 1996) nopaline synthase, and octopine synthase (Herrera-Estrella et al., 1983). 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 β-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).

Efficient techniques for transformation. The unavailability of efficient transformation methods to introduce and express foreign genes can be a substantial barrier to the application of recombinant DNA methods in some crop plants. However, there have been significant advances over the past decade, but the development of efficient transformation methods is frequently not straightforward and it can take many years of painstaking research to test a range of different methods (Potrykus, 1990, 1991). Gene transfer methods in plants are mainly classified into direct and indirect transformation systems. The majority of gene transfer experiments have been focused on maximizing the efficiencies for the recovery of stably transformed plants, and also extending the range of genotypes that

could be engineered using a specific procedure. Although several approaches have been tried successfully for integrative transformation (Potrykus, 1991), only three are widely used that have enabled the introduction of genes into a wide range of crop plants (Dale et al., 1993). These include *Agrobacterium*-mediated gene transfer, microprojectile bombardment with DNA or biolistics, and direct DNA transfer into cells.

Agrobacterium-*mediated gene transfer*. *Agrobacterium tumefaciens* is a soil bacterium that has been implicated in 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; Lichtenstein and Fuller, 1987; Binns and Thomashow, 1988; Zambryski, 1988, 1992; Zambryski et al., 1989). 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. Notable by their absence from this list are the majority of the major seed legumes and monocotyledonous plants. 'Agroinfection' studies (Grimsley et al., 1987, 1988) indicate that although the T-DNA transfer to monocot cells occurs, the block to transformation by *Agrobacterium* may lie in the wound response of monocotyledonous cells and possibly a lack of competence for T-DNA transport to the nucleus or its integration. *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. 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. There is evidence to suggest that for gene transfer to occur cells must be replicating DNA or undergoing mitosis (Meyer et al., 1985; Okada et al., 1986; Binns and Thomashow, 1988; Moloney et al., 1989; Sharma et al., 1990). The majority of transformation experiments utilize 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.

Sonication-assisted Agrobacterium-*mediated transformation (SAAT)*. An important modification in *Agrobacterium*-mediated transformation involves subjecting the plant tissue to brief periods of ultrasound in the presence of *Agrobacterium*. 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, somatic and zygotic embryos. A 100–1400-fold increase in transient β-glucuronidase expression has been demonstrated in different plant species such as soybean, cowpea, white spruce, wheat, and maize (Trick and Finer, 1997), allowing increase in transformation rates in these species which are more recalcitrant to *Agrobacterium*-mediated transformation. The major problem in the development of transformation systems is providing induced *Agrobacterium* with access to cells capable of dedifferentiation followed by regeneration. In species such as tobacco and *Brassica napus*, this combination can be achieved with relative ease (Horsch et al., 1984; Moloney et al., 1989); however, it is often difficult to combine transformation competence with totipotency (Birch, 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).

Over the past decade and half, several tissue culture-independent methods for *Agrobacterium*-mediated transformation have been developed. *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). Progress has been made on non-tissue culture-based approaches for generating transgenic groundnut (Rohini and Rao, 2000b).

Floral-dip method. In this method the plants are transformed by direct application of *Agrobacterium* to floral tissues, bypassing the tissue culture technique and thereby eliminating the somaclonal variations (Clough et al., 1998). Subsequent studies demonstrated the use of female gametophytes of immature flowers as targets of floral-dip transformation in *Arabidopsis* (Ye et al., 1999; Desfeux et al., 2000). The floral-dip method requires considerably less time and effort than vacuum infiltration, resulting in greater yields.

Vacuum infiltration method. The vacuum infiltration method of transformation has been applied to a number of crops, particularly monocots, to avoid both *in vitro* culture and regeneration steps during transformation. The cells of a plant when subjected to a vacuum environment establish a more intimate contact with *Agrobacterium*. Stable transgenics of *Medicago truncatula* (a model legume plant) have been obtained by using this *in planta* method of transformation (Trieu et al., 2000).

Agrobacterium-mediated transformation has strong advantages, such as stable integration into genomic DNA and simple segregation pattern by low copy number, making it preferred by breeders more than direct transformation methods (Hiei and Komari, 1994). However, following the development of direct DNA transfer methods over the past several years, transgenic plants from many cultivated species have been recovered at high frequencies, often in a variety-independent fashion. Thus, constraints previously imposed by biological gene transfer systems, either in terms of vectors or cellular parameters influencing regeneration from dedifferentiated tissue, no longer limit the range of species that can be engineered.

Biolistics or microprojectile bombardment with DNA. Acceleration of heavy microprojectiles (0.5–5.0 μm diameter tungsten or gold particles) coated with DNA has been developed into a technique that carries genes into virtually every type of cell and

TABLE 1

KEY TECHNIQUES USED FOR THE TRANSFORMATION AND REGENERATION OF MAJOR CROPS

Crop species	Method of transformation	Explant	Reference
Arabidopsis thaliana	*A. tumefaciens*	Petiole pieces	Catlin et al., 1988
	Floral-dip method	Gametophytes	Ye et al., 1999; Desfeux et al., 2000
	Biolistics	Roots	Seki et al., 1991
Arachis hypogaea L.	*A. tumefaciens*	Cotyledons	Sharma and Anjaiah, 2000
	Biolistics	Leaflets	Livingstone and Birch, 1995
	ACCELL	Mature embryos	Brar and Cohen, 1994
Avena sativa	Biolistics	Mature zygotic embryos	Torbert et al., 1996
Beta vulgaris	*A. tumefaciens*	Shoot base tissues	Lindsey and Gallois, 1990
	SAAT	Protoplasts	Joersbo and Brunstedt, 1990
Brassica napus	*A. tumefaciens*	Petioles, cotyledonary nodes	Moloney et al., 1989; Boulter et al., 1990
Brassica oleracea	*A. tumefaciens*	Hypocotyl segments	De Block et al., 1989
Cajanus cajan	Biolistics	Leaflets	Dayal et al., 2003
Glycine max	Biolistics	Zygotic embryos	Christou et al., 1989
	A. tumefaciens	Immature embryos	Parrott et al., 1989
Hordeum sp.	Biolistics	Microspores	Jahne et al., 1994
Lycopersicon esculentum	*A. tumefaciens*	Leaf discs	Horsch et al., 1985
Medicago truncatula	Vaccum infiltration		Trieu et al., 2000
Nicotiana tabacum	SAAT	Protoplasts	Joersbo and Brunstedt, 1990
	Biolistics	Leaves, pollen grains	Tomes et al., 1990; Stoger et al., 1995
Oryza sativa	Electroporation	Embryos	Rao, 1995
		Inflorescence stalks	Boulter et al., 1990
Phaseolus vulgaris	Electroporation	Seedling tissue	Dillen et al., 1995
Triticum aestivum	*A. tumefaciens*	Pre-flowering spikelets	Hess et al., 1990
	Electroporation	Scutella and callus	Zaghmout, 1993; Kloeti et al., 1993
	Biolistics	Immature zygotic embryos	Becker et al., 1994
Vicia faba	SAAT	Roots	Miller et al., 1974
Zea mays	Electroporation	Embryos	Songstad et al., 1993
	Biolistics	Shoot apices	Zhong et al., 1996

A. tumefaciens, *Agrobacterium tumefaciens*; SAAT, sonication-assisted *Agrobacterium*-mediated transformation.

tissue (Klein et al., 1988; Sanford, 1990). Microprojectile-mediated DNA delivery has been a flexible method for stable genetic transformation with nuclear, mitochondrial, and chloroplast tissues (Miki et al., 1990), opening new possibilities in plant species such as monocots (angiosperms) and conifers (gymnosperms), where *Agrobacterium*-mediated transformation did not produce transgenics efficiently. This method allows the transport of genes into many cells at nearly any desired position in a plant without too much manual effort. The technology basically involves loading tiny tungsten or gold particles with vector DNA and then spreading the particles on the surface of a mobile plate. Then, under a partial vacuum, the 'microprojectile' is fired against a retaining plate or mesh by a shock wave caused by helium under pressure, achieving speeds of one to several hundred meters per second. The microprojectile decelerates instantly, whilst the momentum and small size of the dense microprojectiles cause them to be thrown from the surface of the microprojectile and to penetrate the target plant tissue. The particles are capable of penetrating through several layers of cells and allow the transformation of cells within tissue explants. By eliminating the need for passage through a protoplast stage, the particle gun method has the potential to allow direct transformation of commercial genotypes. This technique, although not as efficient as the *Agrobacterium*-mediated gene transfer, has a distinct advantage in that virtually any type of meristematic totipotent cells, tissues, organs, and monocots that are not readily amenable to agroinfection can be used with a reasonable success rate. The real advantage of the biolistic technique lies in its application in transient gene expression studies in differentiated tissues (Klein et al., 1992). Particle bombardment has worked generally not only for dicots, but also for monocots where it has given transgenic plants, among other crops; in maize and wheat however, the lack of good embryogenic cell culture systems in monocots has limited the use of bombardment methods, because bombardment of embryogenic tissue largely destroys the capacity for plant regeneration, sterility, and transgene inactivation (Christou, 1995). The transformants often show poor fertility and phenotypic abnormality. In addition, the regenerated plants often appear stressed, i.e., low seed production, premature senescence, poor reproductive development, and stunted growth.

Agrolistics. The agrolistics approach combines the advantages of efficient biolistic delivery and the precision of the *Agrobacterium* T-DNA insertion mechanism, minimizing the regions of homology contributing to genetic and/or epigenetic instability (Hansen and Chilton, 1996). Biolistic transformation is the method of choice for some plant species but many of the integration events resulting from these transformations are not desirable. 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.

TABLE 2

LIST OF SELECTABLE MARKERS GENES USED IN TRANSFORMATION SYSTEMS

Gene	Enzyme encoded	Selective agent(s)	Reference
als	Acetolactate synthase	Chlorosulphuron, imidazolinones	Haughn et al., 1988
Aro A	5-Enolpyruvylshikimate-3-phosphate synthase	Glyphosate	Shah et al., 1986
dhfr	Dihydrofolate reductase	Methtrexate	Herrera-Estrella et al., 1983
hpt	Hygromycin phosphotransferase	Hygromycin B	Van den Elzen et al., 1985
npt II	Neomycin phosphotransferase	Genticin (G418), kanamycin	Bevan and Chilton, 1983; Herrera-Estrella et al., 1983
ppt	Phosphinothricin acetyl transferase	Phosphinothricin (Bialophos)	De Block et al., 1987

Direct DNA transfer methods into the plant cells. Direct gene transfer by using isolated protoplasts is an alternative to the use of *Agrobacterium* because of the foreseeable difficulties with cereal crops. Virtually every protoplast system has proven transformable, though with different efficiency. Unfortunately, there are severe problems with the recovery of transgenic plants from protoplasts. Plant regeneration from protoplasts is a delicate process and depends upon parameters not under experimental control (e.g., species and genotype-dependent competence for wound response and regeneration; Potrykus and Shillito, 1989). DNA transfer into protoplasts can be successfully promoted by various treatments, including polyethylene glycol (PEG), electroporation, and microinjection (Potrykus, 1991). PEG transformation has generally led to relatively low frequencies of transformation (less than 1% of treated cells). Nevertheless, due to the availability of a large number of cells in such systems, a number of transgenic plants can be produced by using effective selection systems (Zhang and Wu, 1988). Besides, electroporation seems to be an efficient method of introduction of foreign DNA into protoplasts, where protoplasts are mixed in DNA solution and subjected to short electrical pulses, which reversibly make holes in the plasma membrane through which DNA invades the cell. Electroporation is generally much less harmful to protoplasts than PEG treatment and introduction of the foreign DNA is quite efficient (Shimamoto et al., 1989). Since the main drawback in employing electroporation lies in the ability to obtain fully developed plants from protoplasts, whole tissues have been used as explants, like zygotic intact embryos in cowpea (Akella and Lurquin, 1993), common bean (Dillen et al., 1995), and rice (Xu and Li, 1994; Rao, 1995).

Microinjection of DNA. There have been methods reported for several dicot and monocot plant species for direct injection of genetically engineered DNA into nuclei of embryogenic single cells. The microinjection technique demands relatively expensive technical equipment for micromanipulation of single cells or small colonies of cells under a microscope and precise injection of small amounts of DNA solution. Single cells or small colonies of cells are held fixed to the end of a glass tube by light pressure and DNA is injected into the nucleus with a very thin glass micropipette. Injected cells or clumps of cells are subsequently raised in *in vitro* culture systems, often with nurse systems, and regenerated into plants. Successful regeneration of these cells with incorporated DNA into their genome produced stable transformants (Neuhaus et al., 1987).

Microfiber 'whiskers'. This method involves the use of microscopic 'whiskers' that look like tiny needles with sharp ends. Tissue culture cells, hundreds of copies of the desired gene(s), and whiskers are suspended in a tube of solution and shaken vigorously. The tiny whiskers stab the plant cells, potentially delivering the desired gene into the nucleus of the cell without killing it (Wang et al., 1995).

TABLE 3

LIST OF SCREENABLE MARKER (REPORTER) GENES USED IN TRANSFORMATION SYSTEMS

Gene	Enzyme encoded	Reference
cat	Chloramphenicol acetyl transferase	Herrera-Estrella et al., 1993
lacZ	β-Galactosidase	Helmer et al., 1984
lux	Luciferase	Ow et al., 1986
npt II	Neomycin phosphotransferase	Reiss et al., 1984
*uid*A	β-Glucuronidase	Jefferson et al., 1987

Chloroplast transformation. Standard methods of genetic transformation in plants generally concentrate on the nuclear expression of foreign genes. Researchers have developed novel methods of transformation, enhancing the ease and efficiency of plastid transformation globally. Current methods for chloroplast transformation include biolistics and PEG-mediated transformation. Chloroplast-specific vectors have been developed to facilitate the incorporation of the transgenes into the chloroplast genome. Boynton et al. (1988) reported the first successful chloroplast transformation in *Chlamydomonas reinhardtii* using gene gun and biolistic technology, followed by introduction of spectinomycin resistance into tobacco (Svab et al., 1990). Knoblauch et al. (1999) demonstrated the microinjection of plasmid DNA using the heat-induced expansion of a liquid metal, galistan, within a glass syringe, forcing the plasmid DNA through a capillary tip, with a diameter of approximately 0.1 mm, into the chloroplast.

Stable chloroplast transformation depends on the integration of the foreign DNA into the chloroplast genome by homologous recombination and therefore must be flanked by sequences homologous to the chloroplast genome (Staub and Maliga, 1992). Recent advancements in the plastid transformation systems in *Arabidopsis* (Sikdar et al., 1998), potato (Sidorov et al., 1999), and rice (Khan and Maliga, 1999) come as a viable way forward, not only in the modification of a number of economically important crop plants, but also for a number of reasons such as high levels of protein expression, simultaneous expression of several genes as a polycistronic unit, and in the elimination of positional effects and gene silencing. Plastid expression of foreign genes also aims at eliminating environmental risks that arise due to the gene flow via pollen to other plants.

Selection of the transformants. Once the target cells have been transformed by one of the above methods, the transgenic cells or plants produced by them are chosen on a selection medium. A marker gene is necessary because only a low proportion of the cells exposed to the transformation process subsequently become stably transformed (Klee et al., 1987). Selecting on the selective medium gives an advantage to those cells that have stably incorporated the transgene construct, and are therefore resistant to the selective antibiotic in the selective medium. The use of a marker gene in a transformation process aims to give a selective advantage to the transformed cells, allowing them to grow faster and better, and to kill the non-transformed cells. These genes could be divided in two categories according to their mode of action: genes for positive and negative selection. Some marker genes for positive selection enable the identification and selection of genetically modified cells without injury or death of the non-transformed cell population (negative selection). In this case, the selection marker genes give the transformed cell the capacity to metabolize some compounds that are not usually metabolized.

Almost all the transformation methods require the incorporation of a selectable marker gene into the vector construct used to introduce the genes of interest. The selectable marker gene most commonly used is *npt* II (neomycin phosphotransferase) that confers resistance to kanamycin. The antibiotic resistance genes (ARGs) under control of prokaryotic promoters are used to select bacteria for direct plant transformation. In most cases, these prokaryotic ARGs are introduced in the vector along with the gene of interest. In addition, ARGs under the control of eukaryotic promoters are widely used as selection marker genes. There have been complex evaluations to study the potential impacts of ARGs present in transgenic plants on human health and the environment as these genes code for enzymes that are not natural food proteins. There have been constant efforts in developing strategies to remove the promoter used to drive the selectable gene, to reduce the chance of (transcriptional) gene silencing of the desired transgene linked to the same promoter.

Characterization of the transformed plants. After selection, the putative transgenic shoot is propagated *in vitro* followed by rooting and transfer to the containment glasshouse for further evaluation and production of seeds from subsequent sexual generations. Stable integration and the number of copies of the inserted DNA are confirmed by Southern hybridization while the gene expression (mRNA) is confirmed by Northern hybridization and protein synthesis by Western blotting (Sambrook et al., 1989). Periodically, the transgenic plants need to be confirmed for the presence and expression of the introduced gene by molecular methods followed by genetic characterization (see Birch, 1997). The number of copies of a transgene construct inserted is variable for all transformation methods. The integration of a single T-DNA copy is common, but high numbers are frequently observed. Data from several different transgenic dicotyledonous species showed an average of three T-DNA inserts, with occasionally up to 20–50 copies in some plants. In a segregation analysis of 161 transgenic plants, 55% segregated for one copy, 20% for two unlinked copies, 6% for three unlinked copies, and 1% for four unlinked copies. The remainder did not segregate in a simple Mendelian ratio (Zambryski, 1988). The position of the T-DNA insertion also appears to be random within the nuclear genome. The expression of transgenes can vary considerably between different independently transformed plants (Hobbs et al., 1990; Jefferson et al., 1990; Blundy et al., 1991). In some instances there is a positive association between transgene expression and copy number, but other studies have shown no association, or even a negative one (Hobbs et al., 1990). Transgene expression may sometimes be unstable or may decline over generations (Vaucheret et al., 1998). In *Arabidopsis*, among an allelic series of lines comprising a primary transformant and various recombinant progeny carrying different numbers of drug resistance gene copies at the same locus, gene silencing was found to depend strictly on repeated sequences and to correlate with an absence of steady-state mRNA (Ye and Singer, 1996).

There could be several reasons for non-expression or low expression of the transgene in a transgenic plant (Finnegan and McElroy, 1994; Matzke and Matzke, 1995; Meyer, 1995; Stam et al., 1997). These include pleiotropic effects from transgenes, somaclonal variations in the regenerated transgenic plants, or environmental effects on the promoters driving the transgenes. The practical way of avoiding problems associated with variation in transgene expression and stability, and somaclonal variation (if any), is to produce a large number of independently transformed plants (often >100) and to select those with a desirable phenotype (see Birch, 1997). Except for vegetatively propagated crop plants, it is usually desirable to identify genotypes with single inserts of the transgene construct, which will have simpler inheritance patterns, and are likely to have more predictable transgene expression levels in subsequent segregating populations.

Gene silencing. There is a wide range of expression levels for the transgene in independent transformation events. Some transgenes are inactivated or silenced in addition to the variable expression effects. Gene silencing is the phenomenon of non-expression/minimal expression of a transgene and/or a homologous gene in a transgenic plant (organism) (Hammond et al., 2001). Transgene expression may be blocked in the primary transformant, or the silencing may occur *de novo* in subsequent generations. Silencing is unpredictable and it tends to affect some plants but not others, even if all plants carry the same construct.

Gene silencing may occur at transcriptional (TGS) and post-transcriptional (PTGS) levels. TGS and PTGS operate differently but both involve DNA methylation, albeit of different regions (Wassenegger et al., 1994; Morel et al., 2000). These may involve homology between multiple transgene copies or between the transgene and an endogenous gene. Gene silencing works by interrupting or suppressing the activity of a targeted gene, preventing it from coordinating production of specific proteins. Short double-stranded RNA (dsRNA) molecules, 21–25 nucleotides in length, play an important role in both processes. In TGS, cytosine residues in promoter sequences are methylated, inactivating the promoter by hindering interactions with transcription factors or triggering formation of heterochromatin in the promoter region. In PTGS, dsRNA is reported to act as a trigger for sequence-specific RNA degradation (Bass, 2000; Zamore et al., 2000; Bernstein et al., 2001).

PTGS in plants is an RNA-degradation mechanism involving dsRNA along with the accumulation of small interfering RNA (Vaucheret et al., 2001). RNA-dependent RNA polymerase uses these RNAs as substrates to synthesize antisense RNAs. The pairing of both sense and antisense RNA leads to the formation of dsRNAs, which become the target for degradation (Fagard and Vaucheret, 2000), indicating the two major processes involved in

gene silencing in plants as RNA-directed RNA degradation and RNA-directed DNA methylation (RdDM) (Wang and Waterhouse, 2000).

Considerable attention has been given to homology-dependent gene silencing phenomena in plants, since the presence of homologous sequences not only affects the stability of transgene expression, but also the activity of endogenous genes could be altered after insertion of homologous transgenes into the genome. Recent studies indicate that a number of plants use PTGS to recover from viral infections and develop resistance to viruses as well (Ratcliff et al., 1997). *Arabidopsis* mutants that exhibit impaired PTGS are found to be susceptible to infection by the cucumovirus CMV, indicating the participation of PTGS in plant resistance mechanisms against the viruses (Vaucheret et al., 2001). However, many viruses have developed strategies to counteract PTGS and successfully infect plants.

Attempts have been made to minimize the risk of gene silencing in transgenes, such as use of a matrix attachment region (MAR) in the transformation vectors to prevent the influence of heterochromatin on the integrated genes (Allen et al., 1996). Besides, use of transformation vectors without duplicated sequences or segments that might trigger silencing/methylation prevent the risk of gene silencing in transgenes. Also, a calmodulin-like protein (rgs-CaM) has been identified in tobacco with the ability to suppress PTGS (Anandalakshmi et al., 2000). Exploitation of such proteins as tools to avoid transgene silencing is still in its infancy for investigative plant biology, further studies may reveal important information to elucidate the mechanisms of transgene silencing.

Field-testing of transgenic plants. The transgenic status of the transgenic plants is confirmed by assaying for expression of the transgenes inserted. The introduced transgene should follow the Mendelian inheritance pattern for its stable expression and inheritance. Following initial analysis, the transgenic plants need to be grown in a containment glasshouse for further phenotypic and genotypic analysis using the original non-transgenic genotype as a control. Further evaluation of the transgenic plants is done under agronomic conditions by carrying out field assessment studies. Risk assessment to study the effect of the transgene on the environment, livestock, and human health needs to be carried out before each novel type of transgenic plant is grown in small-scale open field trials, and before they are used in transgenic crop cultivars under a non-regulated status. The field evaluation and risk assessment have to be performed according to the biosafety guidelines of the host country under the immediate guidance and supervision of the Institute Biosafety Committee. Assessment procedures are being harmonized internationally by various organizations (Levin and Strauss, 1993).

Strategies to Produce Marker-free Transgenic Plants

Co-transformation. In this system, the transformation is achieved using two separate plasmid vectors: one containing the gene of interest and other the selective marker gene. The basic requirement to make this system functional involves high co-transformation efficiency along with vector integration in 'unlinked' loci in order to allow effective recovery of recombination events and/or gene segregation. In oilseed rape the co-transformation efficiency with two *Agrobacterium* strains ranged from 60 to 80% (De Block and Debrouwer, 1991). However, 78% of these events were in the same locus (linked sites). Different vectors integrate into unlinked sites at high frequency, depending on the transformation vector, transformation methodology, strains of *Agrobacterium*, plant species, etc. (Goldsbrough et al., 1993; Yoder and Goldsbrough, 1994; Daley et al., 1998; Lu et al., 2001; Matthews et al., 2001).

Multi-auto-transformation (MAT) system. The MAT system is primarily based on the visual selection of transgenic plants containing the *ipt* gene (encoding for the enzyme isopentenyl phosphotransferase), under control of the CaMV 35S promoter inserted into the transposable *Ac* element (Ebinuma et al., 1997). The transformed plant loses apical dominance and the ability to root, acquiring the abnormal phenotype called *extreme shooty phenotype* (ESP). The unsuitable *ipt* gene is removed subsequently from the transgenic plant through the transposition of the *Ac* transposable element from maize, resulting in marker-free transgenic plants with the normal phenotype restored and containing only the gene of interest.

Intra-genomic relocation of transgenes via transposable elements. In another system, the selection marker gene is flanked by the inverted and repeated sequences of the *Ds* element of the *Ac/Ds* maize transposable system. The *Ds* element and the marker gene is transferred to a new locus of the plant genome or eliminated when in the presence of the transposase, whereas the gene of interest is left in the first insertion locus (Goldsbrough et al., 1993). The advantage of this system is that the selective marker gene will be lost in some somatic tissues due to the failure of the *Ds* element reintegration, making the strategy suitable for removal of marker genes in the vegetatively propagated plants (Yoder and Goldsbrough, 1994).

Site-specific recombination system. The most common system used to mediate site-specific recombination in plants is the bacteriophage P1 *Cre/lox* (Yoder and Goldsbrough, 1994; Vergunst and Hooykaas, 1998, 1999; Vergunst et al., 1998; Gleave et al., 1999). In this recombination system, the plant is transformed with a selective marker gene cloned between two sequences of the gene *lox*, each with 34 bp repeats in direct orientation. In a second stage, the *Cre* gene is introduced in the same plant by a second transformation, by sexual crossing or by transient expression. Expression of the *Cre* gene makes the *Cre*-recombinase enzyme catalyse the recombination between the *lox* repeat sequences, thereby eliminating the marker gene in the subsequent progeny. However, in plants, the site-specific recombination rate is very low and the current knowledge of homologous recombination is still limited (Mengiste and Paszkowski, 1999; Vergunst and Hooykaas, 1999).

Two T-DNA system. The two T-DNA system (Komari et al., 1996; Xing et al., 2000; Matthews et al., 2001; McCormac et al., 2001; Miller et al., 2002) has been developed to produce marker-free plants by co-transformation of the vector harboring two T-DNAs each bearing a marker gene. The first T-DNA of the binary vector, delimited by *A. tumefaciens*, contains *hpt* and green fluorescent protein reporter gene (*gfp*) while the second T-DNA, delimited by *A. rhizogenes* borders, bears the phosphinothricin acetyl transferase (*bar*) gene. This system represents a valuable approach to generate selectable marker-free plants, with a consistent frequency seen among three elite cultivars of rice decreasing the plasmid backbone transfer, lowering the number of T-DNA copy integrations, and avoiding artifacts due to gene silencing.

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. Great advances have been made in the development of various components of transgenic technology including transformation techniques. However, there is a need to address specific issues linked to the development and application of strategies to generate marker-free transgenic plants, to gene silencing and sustained gene expression, and to their deployment under biosafety regimes. Several options to accomplish this are now available and have been demonstrated successfully. Moreover, genetic transformation technology is not yet routinely available for most crops of importance in developing countries, which can be a substantial barrier to its sustainable application to crop improvement.

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