**Botanical Studies (2010) 51: 277-292.**

**REVIEW PAPER**

# An update on the progress towards the development of marker-free transgenic plants

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(Received June 19, 2009; Accepted December 16, 2009)

**ABSTRACT.** Selection marker genes (SMGs) have been commonly used in genetic transformation of plants for efficient selection of transformed cells, tissue, or regenerated shoots. In the majority of cases, the selection is based on antibiotic or herbicide resistance. The presence of such genes within the environment or in the food supply might pose an unpredictable hazard to the ecosystem and to human health; therefore research has been initiated to develop an efficient marker-free transgenic system. Various techniques have been developed in recent years to generate marker free transgenic plants and to eliminate marker genes from transgenics. These include site-specific recombination, homologous recombination, transposition, transient co-integration of the marker gene, and a co-transformation-segregation approach, but success has been limited to only a few plant species. Transgenic technology could become more reliable with the improvement of existing marker gene removal strategies and the development of novel approaches for plant genome manipulation. This review describes the contemporary strategies deployed to generate marker-free transgenic plants and marker gene removal, the merits and shortcomings of different approaches, and possible directions for future research programmes.

**Keywords:** Bio-safety; Intra-chromosomal recombination; Marker-free; Positive selection; Selection marker gene; Site-specific recombination.

**Abbreviations: SMG,** Selection marker gene; **GOI,** Gene of interest; **Lox,** Locus of crossing-over; **Cre,** Cyclization recombination; **HR,** Homologous recombination; **RB,** Right border of T-DNA; **LB,** Left border of T-DNA; **IGT,** Identical gene terminator; **TBS,** Transformation booster sequence; **ptDNA**, Plastid DNA.

## CONTENTS



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## INTRODUCTION

The advent of recombinant DNA technology provided an impetus to plant genetic transformation research. Significant progress has been made using genetic engineering methods to produce transgenic plants with useful agronomic traits. Plant transformation techniques are based on the delivery, integration, and expression of defined genes in the plant cells, which can be grown to generate transgenic plants (Liu et al., 2006; Chen et al., 2008). In all plant transformation systems, to generate a substantial number of non-chimeric transformants, genes conferring resistance to selective agents such as antibiotics or herbicides are widely employed to selected transformants. With a few exceptions in which the selectable marker genes are used as the genes of interest, the marker genes generally have no agronomic value after the selection events. Moreover, once the selection marker trait has been introduced into the germplasm of a plant species, retention of this trait in the genome may be dubious. Hence, an alternative marker system must be used to incorporate subsequent transgenes. Consumer and environmental groups have expressed concern over the use of antibiotic/herbicide resistance genes from ecological and food safety perspectives. The horizontal and vertical transfer of the marker gene through the pollen and pollinators is the major environmental concern raised by the Europeans and several other communities. In order to answer these issues, one has to introduce as few foreign sequences, in addition to the gene of interest, as possible. The elimination of marker genes will not only appease some potential environmental and consumer concerns, but also remove technical barriers to plant genetic transformation (Bevan et al., 1983; Herrera-Estrella et al., 1983). Therefore, studies to avoid marker genes or to eliminate them after use have been initiated and a growing number of methods are under development (Lutz et al., 2007; Sundar et al., 2008; Rukavtsova et al., 2009). Marker-free transgenic research relies on either complete avoidance of using any marker gene or using screenable markers (genes which do not possess any potential harmful activities) to develop marker-free transgenic plants or to get rid of the selectable marker genes before transgenic plants are introduced into the fields. The elimination strategy is based on the excision of the selection marker gene out of the integrated transgene after successful selection, using several approaches like site-specific recombination, transposition, or homologous recombination (HR). However, the plastid genome (plastome or ptDNA) is an established target for plant genetic engineering for enhanced expression of foreign proteins. Excision of the marker gene from the plastid genome is particularly important, as these are present as several thousands of copies per cell. These recombination strategies are also being used to develop marker-free transcriptomic plants. In the following discussion, these strategies are described in detail.

## STRATEGIES TO DEVELOP MARKER-FREE TRANSGENICS

### Avoiding the use of selectable marker genes

The simplest way to eliminate marker genes from transgenics is to avoid their use in the transformation of plants. De Vetten et al. (2003) first reported the transformation of potato *cv.* Kanico using *Agrobacterium tumefaciens* strain AGL0 and no selection marker genes. The maximum transformation efficiency obtained was 4.5% as confirmed by PCR. The only problem observed with this system was the presence of vector backbone sequences in most of the transformants, which is a character as undesirable as selection marker genes. The backbone integration study is most desirable in this method. The same strategy was successfully used by Jia et al. (2006) for producing marker-free transgenic tobacco plants at a frequency of 15%. Kim et al. (2007) reported transgenic potato plants without any selection marker gene with chloroplast targeted *SOD* and *APx* genes under oxidative stress inducible promoter with an average transformation efficiency of 2.2%. Recently, Li et al. (2009) reported marker-free transgenic tobacco with a transformation efficiency of 4%, among which 65% plants were found to be stable transformants in the $T_1$ generation. They further described that the transgenic plants generated through no antibiotic or herbicidal selection were segregated in a Mendelian 3:1 ratio and that one-third of them were chimeric or escapes. Recently, marker-free transgenic potato plants resistant to oxidative stress (Ahmad et al., 2008) and to *Potato virus X* and *Potato virus Y* (Bai et al., 2009) were developed using this approach. In both these cases, the transgenic event was confirmed by PCR and by challenging the putative transformants to oxidative stress and to *Potato virus X* and *Y*, respectively. Similarly, Rukavtsova et al. (2009) constructed a novel plant transformation vector by removing the *nptII* marker gene conferring resistance to kanamycin from the vector together with its promoter and polyadenylation signal of the *Agrobacterial* nopaline synthase gene.

In spite of several advantages, some disadvantages are associated with this method. There is no control over

selective growth of the transformants, and the researcher has to screen several putative transformants to confirm the integration of the transgene(s), which is expensive and time consuming. Uncontrolled regeneration of a large number of chimeric plants is another drawback in the case of selection without the use of antibiotics as observed in tobacco (Li et al., 2009).

## Positive selection system

Some biochemical marker genes enable the identification and selection of transformed cells without injury or death of the non-transformed cells. In this method, the selection marker genes code for enzymes that give the transformed cells a capacity to metabolize specific nutrient substances not usually metabolized by normal plants (Figure 1). This fact will provide the transformed cells an advantage over the non-transformed ones. Addition of this substance in the culture medium, as nutrient source during the regeneration process, allows normal to enhanced growth and differentiation of transformed cells while non-transformed cells will not be able to grow and regenerate. Use of *GUS*, *manA*, and *xylA* genes are some of the popular positive selection systems.

*The GUS gene.* The *β-glucuronidase (GUS)* gene isolated from *E. coli* (Jefferson et al., 1986) is to date the most widely used reporter gene in genetically modified plants. Agronomically, the *E. coli GUS* gene has no value to crop plants. In this system of positive selection, a glucuronide derivative of benzyladenine (benzyladenine N-3-glucuronide) is used as selection agent. The glucuronide supplemented in the selection medium is hydrolyzed by the GUS enzyme produced in the transformed cells, forming active cytokinin (benzyladenine). The released cytokinin acts as a stimulator for transformed cell regeneration, while non-transformed cell development is slow as the inactive form of the selective agent does not influence the non-transformed cells. This system was successfully used to effectively recover transgenic plants of tobacco (Joersbo and Okkels, 1996; Okkels et al., 1997).

*The manA gene.* Phosphomannose isomerase (PMI, EC 5.3.1.8) is an enzyme that converts mannose-6-phosphate to fructose-6-phosphate, an intermediate of glycolysis that positively supports the growth of plant cells. This gene helps the transformed plant cells to utilize mannose as a carbon source to grow and differentiate on media containing mannose as a selection agent. In 1984, the gene coding for phosphomannose isomerase (manA or pmi) was first isolated from *E. coli* by Miles and Guest. However, its first application in plants as a selection marker gene was reported by Joersbo et al. (1998). Though most plant species are sensitive to mannose, a few species—especially dicots like carrot, tobacco, sweet potato and legumes—have shown a considerable insensitivity towards this sugar. Plants like sugar beet, maize, wheat, oat, barley, tomato, potato, sunflower, rapeseed, pea, onion, and rice are extremely sensitive and have been successfully transformed using *pmi* as a positive selection marker

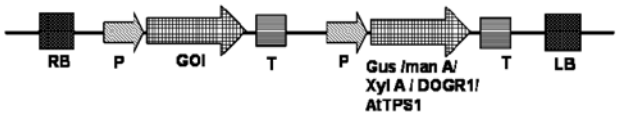


**Figure 1.** T-DNA of plant transformation vector harboring positive section marker genes, *GUS*, β-glucuronidase; *pmi*, phosphomannoisomerase; *xyl*A, Xylose isomerase A; *DOG$^R$1,* 2-deoxyglucose-6-phosphate phosphatase; *AtTPS1*, trehalose-6-phophate synthase; used for producing marker-free transgenic plants.

(Joersbo et al., 1998, 2000; Negrotto et al., 2000; Wang et al., 2000; Bhalla-Sarin et al., 2004; Gadaleta et al., 2006; Ku et al., 2006; Aswath et al., 2006; Penna et al., 2008). Some plant transformation protocols that use the positive selection with *pmi* were found to be at least 10 times more efficient than the traditional protocols based on kanamycin selection (Wright et al., 2001). Jain et al. (2007) reported a maximum of 56% efficiency for stable transformants using mannose selection in sugarcane (*Saccharum* spp.).

*The xylA, DOG$^R$1 and AtTPS1 genes.* The *xylA* gene isolated from *Thermoanaerobacterium thermosulfurogenes* or *Streptomyces rubiginosus* (Haldrup et al., 1998a) encodes for enzyme xylose isomerase, which catalyzes the isomerization of D-xylose to D-xylulose. It also catalyzes the isomerization of glucose to fructose, and is thus also termed glucose isomerase. Transgenic cells expressing the *xylA* gene can utilize xylose as a sole carbohydrate source and proliferate while non-transgenic cells starve. Using this system, transgenic plants of potato, tobacco, and tomato were successfully selected in xylose-containing media (Haldrup et al., 1998b; 2001). However, a problem initially encountered when xylose was used in the selection medium was the induction of callus, which resembled the auxin effect. This problem can be solved by adding auxin efflux inhibitor, triiodobenzoic acid (TIBA) (Nissen and Minocha, 1993), an antiauxin, p-chlorophenoxyisobutyric acid (PCIB) (Kasai and Bayer, 1995), or an ethylene inhibitor, aminoethoxyvinylglycine (AVG) (Hall et al., 1985), to the selection medium.

In another system, the *DOG$^R$1* gene isolated from yeast encoding 2-deoxyglucose-6-phosphate phosphatase (2-DOG-6-P) gives resistance to 2-deoxyglucose (2-DOG). In plant cells 2-DOG is converted into 2-DOG-6-phosphate (2-DOG-6-P), which is a competitor of glucose-6-phosphate. When over-expressed in transgenic plants, it was used as a positive selection system for tobacco and potato plants (Kunze et al., 2001). Recently, a new positive selection marker gene *AtTPS1*, encoding trehalose-6-phophate synthase, has been developed (Leyman et al., 2008). In this system, the selection agent used is the nontoxic and common sugar glucose. Wild-type non-transformed *Arabidopsis thaliana* plantlets germinated on glucose had small white cotyledons and remained petite due to stoppage of the photosynthetic mechanism by external sugar while transgenic plants expressing *AtTPS1* became insensitive to glucose. The selectable marker gene

which encodes trehalose-6-phophate synthase catalyzes the first reaction of the two-step trehalose synthesis. With glucose (6%) as selection agent, it was possible to distinguish green and normal-sized transgenic plants from the non-transformed ones.

### Negative selection system

The negative selection method places negative selectable marker genes next to a positive marker gene in the same construct and is a powerful means of creating marker-free transgenic plants. In this method, transformed plants are selected for the absence of negative selection marker genes under the selection pressure of a negative selection marker (Figure 2). This allows for the selection of marker-free transgenic plants without performing a large number of PCRs (Jeongmoo et al., 2004). Erikson et al. (2004) characterized a novel marker gene, *dao1* encoding D-amino acid oxidase. It can be used as either positive or negative marker, depending on the substrate. Therefore, it is possible to apply negative selection after a positive selection using same marker gene, *dao1*, by changing substrates D-alanine or D-serine to D-isoleucine or D-valine.

Indoleacetic acid hydrolase (IAAH) encoded by the *tms2* gene converts naphthaleneacetamide (NAM) to a potent auxin, naphthaleneacetic acid (NAA), which inhibits seedling growth. The *tms2* gene was first used as conditional selection marker gene in tobacco (Depicker et al., 1988) and in *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 (*CPMO*) 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). So far, the *cytochrome P450* and *codA* are the only genes used as conditional negative selectors in monocots. Both have been proven effective in barley (Koprek et al., 1999), and only *cytochrome P450* was found effective in rice (Chin et al., 1999).

### Abiotic stress related genes as selection markers

This strategy is based on the fact that various genes encode proteins that protect plants from environmental stresses like drought, salt, and oxidative stress. Many such genes characterized in *Arabidopsis* and in several agronomically important crops, can be used for the development of marker-free transgenic plants. The gene for salt tolerance can be incorporated into the plant cells without the selection marker gene. After transformation, the tissue grows under the pressure of salt stress, and the explants growing well without any deformities are selected. Hence, use of the selection marker is unnecessary as the gene itself can be used as selection marker to select the transformants.

Bouchabke-Coussa et al. (2008) reported the *ESKIMO1* gene involved in plant water economy, cold acclimation, and salt tolerance as a potential target for plant transformation in an effort to increase abiotic stress resistance. Attempts were made to introduce yeast *ENAl* gene encoding $Na^+$-ATPase under the control of the CaMV35S promoter into BY2 cells, which were able to grow in modified LS medium containing 120 mM of LiCl (Yoshida and Shinmyo, 2000). Recently, an *Arabidopsis AtHOL1* gene with high S-adenosyl-L-methionine-dependent methyltransferase activity towards the thiocyanate ion ($NCS^-$) was used as positive selection gene for an efficient selectable marker for screening the transformed seedlings of *Arabidopsis* (Midorikawa et al., 2009). Transgenic plants could show normal growth and development on a medium containing 3.0-5.0 mM potassium thiocyanate, which is toxic to non-transgenic plants. Veena et al. (1999) and Sanan-Mishra et al. (2005) explored the potential role of *glyI* gene (*glyoxalase I*) and *PDH45* gene (pea DNA helicase45), respectively, in overcoming salinity stress. The *glyI* or *PDH45* mRNA induced in response to high salt was over-expressed in transgenic tobacco plants that conferred salinity tolerance. These genes can be used as potential agents for the selection of transgenic plants on the medium supplemented with salts.

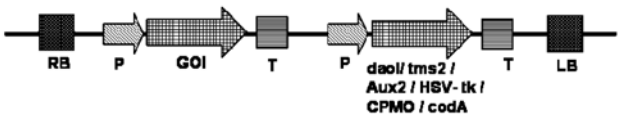


**Figure 2.** T-DNA of plant transformation vector harboring negative section marker genes, *dao1,* D-amino acid oxidase; *aux2, auxin synthase 2; HSV-tk, Herpes Simplex Virus thymidine kinase*; *CPMO, cytochrome P450 mono-oxygenase* and *coda, Cytosine deaminase* used for producing marker-free transgenic plants.

## STRATEGIES TO ELIMINATE MARKER GENES FROM THE NUCLEAR GENOME

### Co-transformation of two transgenes

Co-transformation is a simple and highly effective method to eliminate marker genes from the nuclear genome of transgenic plants. Co-transformation involves the transformation of plant cells with two plasmids that target insertion at two different loci in the plant genome. One plasmid carries a selection marker gene while the other carries a gene of interest. *Agrobacterium* mediated co-transformation is achieved in three ways (Figure 3). In the first method, two different vectors are carried in two separate *Agrobacterium* strains (McKnight et al., 1987; Deblock and Debrouwer, 1991; De Neve et al., 1997); the second method involves two different vectors placed in the same *Agrobacterium* cell (De Framond et al., 1986; Daley et al., 1998; Sripriya and Veluthambi, 2008); while the third method uses two T-DNAs that can be borne by a single binary vector (two T-DNA system) (Komari et al., 1996; Xing et al., 2000; Matthew et al., 2001; McCormac et al., 2001; Miller et al., 2002). The selection marker gene

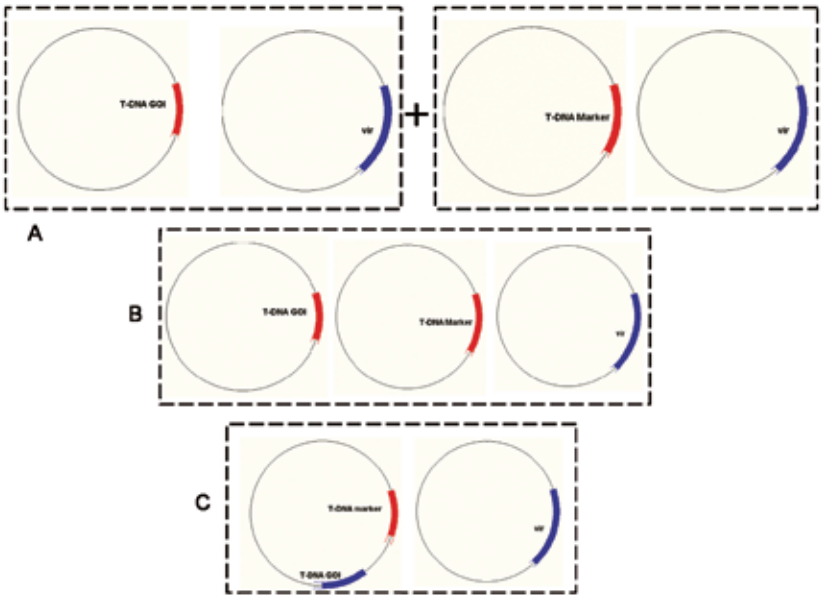


**Figure 3.** Schematic representation of the strategy for marker gene removal via *Agrobacterium*-mediated co-transformation with T-DNAs carrying marker and gene of interest (GOI). A, Marker and GOI are in separate vectors in separate *Agrobacterium* strains; B, Marker and GOI in separate vectors in single *Agrobacterium* strain; C, Marker and GOI borne by two T-DNAs in the same vector.

can be eliminated from the nuclear genome of the transgenic plants at the time of segregation and recombination that occurs during sexual reproduction by selecting the transgenic lines carrying the gene of interest. Xue et al. (2003) generated transgenic barley plants producing high levels of cellulase (1,4-β-glucanase using a binary plasmid containing *hph* and *cel-hyb1* in separate T-DNAs), where the selection marker gene (*hph*) was subsequently eliminated from transgenic lines through segregation of *hph* from synthetic thermo-tolerant hybrid cellulose (*cel-hyb1*) in $T_1$ progeny. Marker-free double haploid transgenic rice plants were generated via *Agrobacterium*-mediated transformation with a "double-T-DNA" binary vector followed by anther culture, with an efficiency of 12.2% (Zhu et al., 2007). Recently, Shiva Prakash et al. (2009) generated marker-free transgenic plants of maize by co-bombardment of immature embryos with TDNAs containing a gene of interest, *GUS*, and an antibiotic selection marker, *nptII*, separately. Marker-free transgenic *GUS* positive lines were selected in the $T_1$ generation after excision of the selection marker gene by recombination.

Though simple and effective, this method carries several unavoidable limitations. It is time consuming and compatible only for sexually propagated fertile plants. Secondly, the tight linkage between co-integrated DNAs may limit the efficiency of co-transformation. Indeed, the integration of marker gene and transgene is an indiscriminate event. Both may integrate in the same loci and only a proportion of plants carrying the selectable marker will also carry the desired gene at an unlinked site (Scutt et al., 2002). Also, this method may not be suitable for species with very low transformation efficiency.

## Site specific recombination systems

Recombination is a well known phenomenon in biological systems that takes place between the two homologous DNA molecules. In site specific recombination, DNA strand exchange takes place between segments possessing only a limited degree of sequence homology (Coates et al., 2005). Three site specific recombination systems are well known and described for the elimination of selection marker genes. These are the *Cre*/*lox* site specific recombination system (Dale and Ow, 1991), the *FLP*/*FRT* recombination system from *Saccharomyces cerevisiae* (Landy, 1989; Kilby et al., 1995; Lyznik et al., 1996), and the *R*/*RS* recombination system from *Zygosaccharomyces rouxii* (Onouchi et al., 1995; Sugita et al., 2000).

The recombination sites are typically between 30 to 200 nucleotides in length and consist of two motifs with a partial inverted repeat symmetry. The recombinase binds to these motifs, which flank a central crossover sequence at which the recombination takes place (Figure 4). In a pioneering work, Dale and Ow (1991) and Qin et al. (1994) used the *Cre* recombinase of the *E. coli* bacteriophage P1 to remove a selection marker gene flanked by *lox* target sites from a transgenic locus in transformed tobacco. The unique ability of Cre to catalyze a crossover between directly repeated *lox* sites flanking any fragment of DNA has been exploited to remove selectable marker genes from a number of transgenic plants. Basically, the strategy involves the generation of plants that express the *cre* gene and crossing them with plants in which the selection marker gene is flanked by *lox* sites. The marker gene is excised in the $F_1$ generation, and the *cre* gene is segregated away in the subsequent generations. In this system, the selection marker gene can be eliminated either by re-transformation (Odell et al., 1990; Dale and Ow, 1991; Russell et al., 1992) or by crossing over (Bayley et al., 1992; Russell et al., 1992; Gleave et al., 1999; Hoff et al., 2001; Arumugam et al., 2007; Chakraborti et al., 2008) (Figure 5A). This system has been very well studied for a generation of marker-free transgenic plants of *Arabiodopsis*, maize, to-

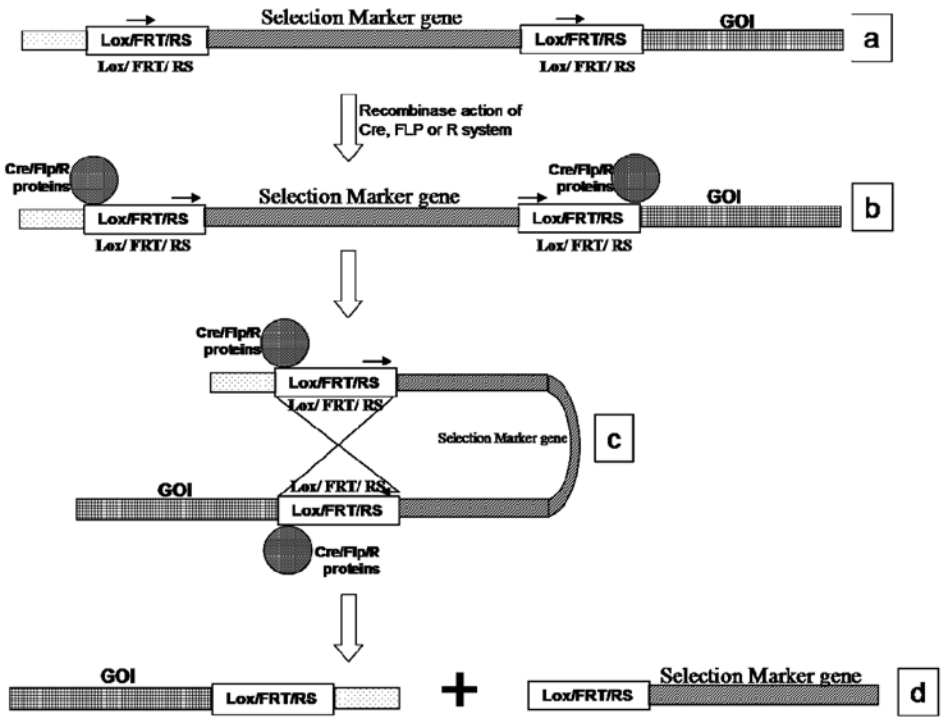


**Figure 4.** Schematic representation of the mechanism of action of microbial recombinase systems, Cre-lox, Flp/Frt and RS/R. The gene construct contains the Lox/Flp/R recombinase sequences flanked by the selection marker gene while the gene of interest lies outside the recombinase sequences (a). When the transgenics containing the Cre/FRT/RS is crossed with the other transgenic containing the Cre, FLP or R recombinase gene sequences (b), the recombination process starts (c) and the marker genes are removed (d) from chromosome. Remaining Cre recombinase sequence in chromosome is segregated out during the advancement of the generation. SMG; Selection marker gene, GOI; Gene of interest. Symbol cycle in the figure represent the recombinase (Cre, FLP or R) proteins.

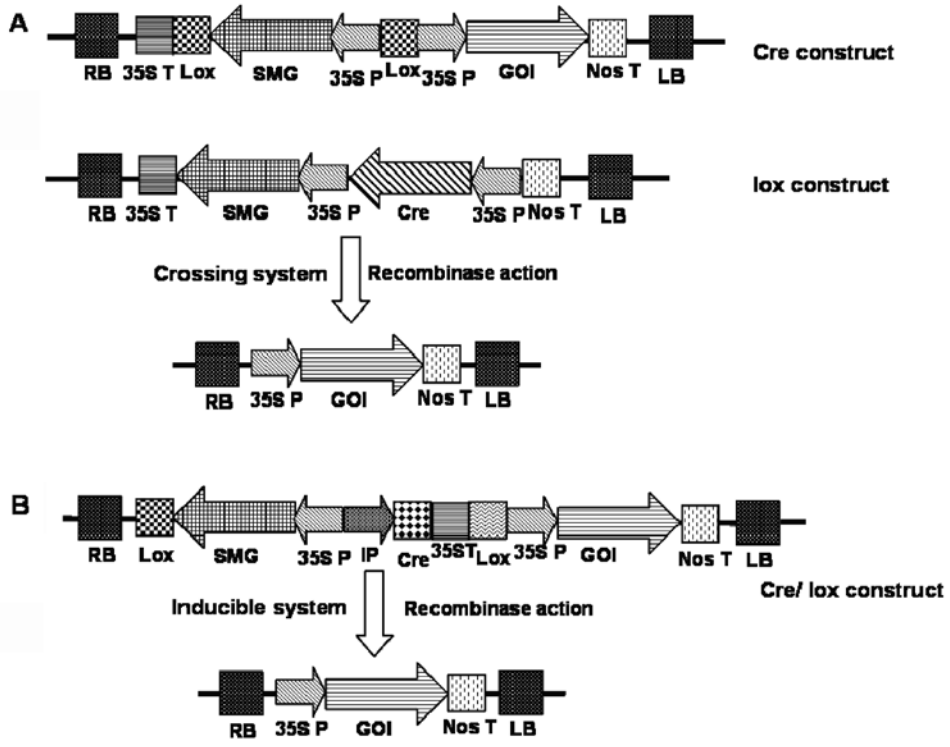


**Figure 5.** Strategy of *Cre-lox* site-specific recombination system for excision of selection marker gene from transgenic plants. A, The *lox* gene construct and the *Cre* gene construct to be crossed with lox transgenics; B, The inducible system where the *Cre* gene is under the control of inducible promoter (IP).

bacco, and rice (Zuo et al., 2001; Zhang et al., 2003; Yuan et al., 2004; Sreekala et al., 2005; Song et al., 2008). Jia et al. (2006) reported a self operating *Cre-lox* recombination system which uses a movement function-improved Tobacco Mosaic Virus (TMV) vector, m30B, to express Cre recombinase for elimination of the selection marker gene *nptII* from transgenic tobacco plants. Recently, Zhang et al. (2009) generated marker-free transgenic tomato plants with improved abiotic stress tolerance, employing a Cre/*loxP* DNA recombination system in which *AtIpk2b*, an inositol polyphosphate 6-/3-kinase gene from *Arabidopsis thaliana* was constitutively overexpressed. The expression of *AtIpk2b* conferred improved resistance to drought, cold, and oxidative stress in both sets of transgenic tomato plants.

*Inducible system for Cre-lox recombination.* Marker gene removal from transgenic plants using the *Cre-lox* recombination system of bacteriophage P1 requires re-transformation and out-crossing approaches which are laborious and time consuming (Dale and Ow, 1991). In order to initiate the *Cre-lox* recombination for removal of the marker gene, other novel inducible site-specific recombination systems have been applied (Figure 5B). However, several approaches were developed to overcome these shortcomings, including the use of some chemical inducers (Schaart et al., 2004; Yuan et al., 2004; Zhang et al., 2006) and heat shock (Wang et al., 2005; Cuellar et al., 2006). Marker-free transgenic tomato plants expressing *cry1Ac* were obtained, using a chemically regulated *Cre-lox*-mediated site specific recombination system. The marker gene *nptII* was eliminated by two directly oriented *loxP* sites located between the CaMV35S promoter and a *cry1Ac* without promoter. Upon induction by β-estradiol (2 μM), the selection marker gene fused with *Cre recombinase* and flanked by two *lox* sites was auto excised from the plant genome, thus generating marker-free transgenic plants (Zuo et al., 2001; Zhang et al., 2006). Recently, Lin et al. (2008) developed marker-free transgenic rice plants using a chemically-induced site specific recombinase system. They 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) reported on a marker-free transgenic tomato using a salicyclic acid-inducible *Cre/loxP* recombination system.

A *Cre/loxP* recombination system was used for marker elimination by Kuroda et al. (2003). Under it, a caseinolytic protease P1 (*clpP1*) was deleted, resulting in removal of the tobacco plant shoot system. Shan et al. (2006) used the heat-inducible system in a *FLP/frt* site-specific recombinase system. Under this, the expression of *FLP* was tightly under the control of the heat shock protein, hsp. Two different constructs were used, the *frt*-containing vector (pCAMBIA1300-betA-frt-als-*frt*) and the *FLP* expression vector (pCAMBIA1300-hsp-*FLP*-hpt). Through the process of re-transformation, the *FLP recombinase* gene was introduced into transgenic (betA-frt-als-*frt*) tobacco. In the re-transgenic plants after heat shock treatment, the marker gene *als*, flanked by two *frt* sites, could be excised by the inducible expression of *FLP recombinase* under the control of *hsp* promoter. A heat inducible strategy for the elimination of selection marker genes was also reported in vegetatively propagated plants like potato (Cuellar et al., 2006). Recently, Deb Roy et al. (2008) reported a heat inducible *Cre/loxP* site specific recombination system to remove *nptII* gene from *A. thaliana* transgenic plants transformed with *glyI* gene. The *cre* gene was driven by the heat-inducible promoter (*hsp*), and the *nptII* gene is flanked by *lox* sequences. These inducible site-specific recombination systems can also be applied in vegetatively propagated crop plants for marker gene excision.

In spite of several advantages, these methods carry some limitations. Due to the prolonged presence of bacterial recombinases in the plants, there may be unwanted changes in the plant genome at the sites of transgene excision.

*Auto Excision strategy to eliminate marker gene.* The earlier methods of excision like heat- and chemical-inducible systems are time consuming, and the marker gene is eliminated in the generation following segregation. A novel and ideal method to eliminate the selection marker gene in a single generation has been developed. This method is referred to as the "auto excision strategy" in which the marker gene is easily eliminated in the $T_1$ seeds of the transgenic plants. and the next generation of the transgenic plants becomes marker-free. An auto excision system is controlled by pollen and/or seed specific promoters. It relies on floral specific promoters to regulate the expression of Cre recombinase to generate marker-free transgenic plants. Functionally characterized promoters are used in the strategy, and the system was successfully demonstrated in rice (Bai et al., 2008). This approach was mediated by the *Cre/loxP* recombination system, and the *cre* gene was under the control of the floral specific promoter *OsMADS45*. Using this system, the marker gene *nptII* was completely removed from the $T_1$ progeny of the rice with 37.5% efficiency (Bai et al., 2008). Verweire et al. (2007) developed homologous marker-free transgenic plants of *Arabidopsis thaliana* introducing a germline-specific auto-excision vector containing a *cre* gene under the control of a germline-specific promoter derived from *APETALA1* and *SOLO DANCERS* genes from *Arabidopsis thaliana*. Using this method, the frequency of regeneration of marker-free transgenic lines obtained in *Arabidopsis* was 83-100%. Highly efficient auto-excision of selective markers has been successfully achieved in tobacco (Mlynarova et al., 2006; Luo et al., 2007; Kopertekh et al., 2009). Kopertekh et al. (2009) reported a developmentally regulated *Cre-lox* site-specific recombination system for excision of a *bar* marker gene by using seed-specific *napin* promoter.

In spite of all these advantages, an auto excision strategy has its limitations. It is, for example, successful only in flowering plants, and it is not useful for vegetatively

propagated plants like grapes, potato, or banana.

*Intrachromosomal homologous recombination (ICR).* This is based on the use of homologous recombination to eliminate selectable marker genes after insertion. A 352 bp *attP* region of bacteriophage λ is a target for three specific proteins that mediate integration and excision of the phage genome within the *E. coli* (Figure 6). The process of bacteriophage integration involves a phage-encoded λ integrase and a bacterially encoded integration host factor (IHF). This strategy was successfully used in transgenic tobacco to remove selection marker genes like *gfp, nptII*, and *tms2* flanked by the *attP* region (Zubco et al., 2000). The marker gene excision was achieved by selecting transformed tobacco calli initially on kanamycin-containing media and subsequently culturing on kanamycin-free media to allow for the loss of the *nptII* gene by ICR. The detection of ICR events was based on the acquisition of sensitivity to kanamycin and confirmed by the loss of a negative selection *tms2* marker gene. ICR events in plants were found to be very rare, with only ten such events detectable in all of the cells of a 6-week-old tobacco plant (Puchta et al., 1995).

The ICR method of marker gene removal is relatively simple as it does not require the expression of a heterologous recombinase. In addition, this technique does not require any sexual reproduction steps and could therefore be applied for vegetatively propagated plants too. This method requires transfer of transformed tissues to a selection medium followed by transfer to a non-selection medium to allow the loss of the selection marker gene. Such lengthy propagation may increase the risk of somaclonal mutations (Ow, 2001). Further, this method has to be tested on a large number of plant species before its large scale implementation.

*Multi-auto transformation (MAT) vector system.* The MAT vector system represents a sophisticated approach for the removal of marker genes from the nuclear genome of the transgenic plants (Ebinuma et al., 1997). This system involves placing a chosen transgene adjacent to a multigenic element flanked by reciprocal recombination (*RS*) sites. A copy of the selectable *isopentyl transferase* (*ipt*) gene from *A. tumefaciens* is inserted between these *RS* recombinase sites together with the yeast *R* recombinase gene, and this entire assembly is placed in T-DNA region. The MAT vector system allows the removal of the *R* recombinase gene along with the *ipt* gene. The system does not require sexual crosses for the removal of marker or recombinase genes and can also be used in vegetatively propagated plants. The only limitation is that the prolonged expression of recombinase in plant tissues may cause unwanted recombinase effects. This limitation can be overcome by the use of more recent version of the MAT vector system that allows a delay in the excision of the *ipt* and *R* recombinase genes by using the chemically-inducible *glutathione S-transferase* promoter from maize to drive *R* recombinase gene expression (Sugita et al., 2000). The one-step MAT vector system for removal of the selection marker gene has been successfully demonstrated in tobacco and hybrid aspen (Zuo et al., 2001), rice (Endo et al., 2002), and apricot (Noguera et al., 2006).

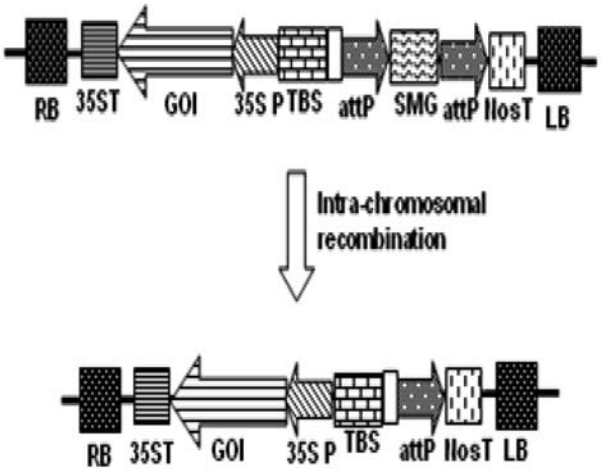


**Figure 6.** The intrachromosomal recombination system for marker gene removal from transgenic plants involving a phage-encoded λ integrase and a bacterially encoded Integration Host Factor (IHF). TBS; Transformation booster sequence, SMG, Selection marker gene, GOI; Gene of interest, attP; attachment P region of bacteriophage.

## Transposon-based marker excision methods

Transposons or the "jumping genes" have been used as a tool to excise the marker sequence from the gene of interest. The strategy makes use of the *Ac/Ds* transposition system and is primarily based on the fact that the DNA sequences located in the *Ds* repeats can be translocated to excise along with the *Ds* element (Figure 7). This method involves *Agrobacterium*-mediated transformation followed by intragenomic relocation of the transgene of interest, and its subsequent segregation from the selectable marker in the progeny (Goldsbrough et al., 1993) or direct excision of the marker gene from the genome (Ebinuma et al., 1997). Both strategies were developed using the maize *Ac/Ds* transposable element, and the system could be adapted successfully to use similar autonomous transposable elements. The *Ac* transposase activity requires the expression of plant promoters (Honma et al., 1993). Ebinuma et al. (1997) reported the feasibility of this strategy in tobacco and eliminated the *ipt* marker gene from transgenic tobacco plants. Transgenic plants constitutively expressing the *ipt* gene elevated cytokinin to auxin ratios resulting in a loss of apical dominance and suppression of root formation, referred to as shooty phenotype. Transformed tobacco leaf discs with a T-DNA containing *nptII* and *gus* gene and a chimeric *Ac* element, which included a *35S-ipt* gene, showed an extremely shooty phenotype. Upon sub-culturing these phenotypic distinct shoots, normal shoots were developed, which indicated the deletion of *ipt* gene expression. Jin et al. (2003) reported on an *Ac/Ds* transposon system for removal of the *hpt* selection marker gene to obtain marker-free transgenic plants in rice (*Oryza sativa* L.). The *Ds* element containing the gene of interest, *bar*, was constructed next to the selection marker

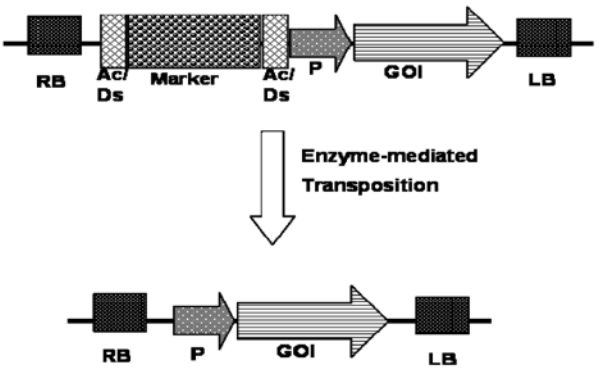


**Figure 7.** An Ac/Ds Transposon-based method for the removal of marker gene from nuclear genome of transgenic plants. The method is based on intragenomic relocation of GOI and its subsequent segregation from the selection marker gene in the progeny. GOI: Gene of interest.

gene *hpt* to get *Ds*-T-DNA. Rice plants were transformed by *A. tumefaciens* containing *Ac*-T-DNA and *Ds*-T-DNA, respectively. These two transformants crossed with each other to produce the $F_1$ plant containing both *Ac* and *Ds* elements. $F_1$ plant was then selfed to produce $F_2$ progeny in which the T-DNA insert and transposed *Ds* element became segregated. Recently, a maize transposon *Ac*-based system was utilized for excision of marker gene from rice transformants. The transposon system was induced by salicylic acid to excise transposon along with marker gene (Charng et al., 2008). A T-DNA bearing the *cry1B* endotoxin gene flanked by *Ac-Ds* transposon termini and cloned in the 5' untranslated region of a *gfp* gene cassette along with the gene encoding AcTpase was successfully transferred to a *japonica* cultivar of rice, generating 68 independent transformation events (Cotsaftis et al., 2002).

The major advantage of this strategy is that, the marker-free transgenic plants can easily be screened at the $T_0$ generation, avoiding the need for sexual reproduction and indicating the applicability of the strategy to the vegetatively propagated crops also. In spite of all the advantages, a few limitations are inevitable, like the very low regeneration frequency of marker-free transgenic plants and the genomic instability of transgenic plants because of the continued presence of heterologous transposons (Scutt et al., 2002). The requirement of genetic crossing or segregation for separating the transgene and the marker gene is a time consuming process and can thus be counted as one of the drawbacks of this method.

## STRATEGIES TO ELIMINATE MARKER GENES FROM THE PLASTID GENOME

The plastid genome is present in multiple copies in each cell, and this multiplies up to large numbers like $10^4$ copies per cell, particularly in leaf tissues. This gives a considerably high level of transgene expression, which may be useful for applications requiring high concentrations of proteins like the engineering of drought resistance or the production of pharmaceuticals in plants (Daniell et al., 1998, 2002). Modification of the plastid genome represents an attractive alternative to engineering of the plant nuclear genome for some applications. As the chloroplast genome is maternally inherited, it reduces—but does not eliminate—the possibility of transgene escape via pollination into its wild relatives. Removal of marker genes from the engineered chloroplast genome is particularly important as their very high copy number could lead to high levels of unwanted marker gene products. Some strategies for removing selection marker genes from the chloroplast genome of transplastomic plants have been suggested.

### Homologous recombination system

In the case of chloroplast transformation, transgene integration occurs through homologous recombination. Removal of the marker gene is possible through homologous recombination between identical gene promoters or terminators flanking the gene of interest and selection marker gene (Figure 8). This technique was successfully demonstrated for the first time in the unicellular green alga *Chlamydomonas reinhardtii* (Fischer et al., 1996) and in higher plants (Iamtham and Day, 2000) using recombinative excision between the similar sets of promoter or terminator sequences in the transplastomic plants. After the removal of antibiotic selection, these excision events accumulated to a high frequency, leading to a homoplastic, marker-free state in approximately 25% of the transgenic lines in the subsequent generation (Iamtham and Day, 2000).

Dufourmantel et al. (2007) applied a variant of the homology-based marker excision strategy to produce a marker-free soybean and tobacco plants with strong herbicide tolerance. A plastid transformation vector carrying an *aadA* that disrupts an herbicide resistance gene was used. Initial selection was done using spectinomycin,

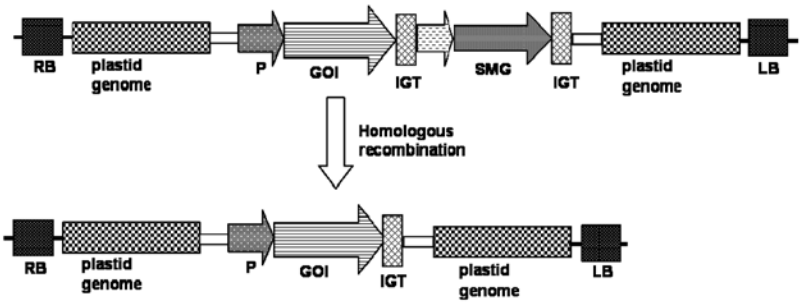


**Figure 8.** Schematic representation showing the mechanism of marker gene removal from chloroplast by homologous recombination. Selection marker gene is excised through homologous recombination between identical gene promoters or terminators flanking GOI and marker gene GOI; Gene of interest, IGT; Identical gene terminator, SMG; selection marker gene.

and the *aadA* marker gene was excised by homologous recombination, yielding a transplastomic plant carrying a complete herbicide resistance gene, 4-*hydroxyphenylpyruvate dioxygenase* (*HPPD*).

## Site specific recombination system

This system utilizes a two-step protocol. In the first step, construction of transplastomic plants carrying a marker gene flanked by two directly oriented recombinase target sites will be developed. In the second step, the marker will be removed by introducing a plastid-targeted recombinase in to the plant genome (Lutz and Maliga, 2007). So far, two recombinases (Cre and Int) were reported for excision of the marker gene from the plastid genome. The first method, the *Cre-lox* system, works similarly to nuclear transgenics. This system was successfully demonstrated in tobacco (Corneille et al., 2001; Hajdukiewicz et al., 2001). The system eliminates the selection marker gene flanked by two directly oriented *lox* sites. The nuclear *cre* gene can subsequently be removed by segregation in the seed progeny. A modified *Cre-lox* system is a highly efficient tool for obtaining marker-free transplastomic plants. Plastid transgene constructions contain a selection marker gene flanked by *lox* recombination sites. This method was found to be dependant on whether the *cre* gene was introduced into the nuclear genome of a transplastomic line by direct transformation or by sexual crossing (Corneille et al., 2001). In cases where *cre* was introduced by re-transformation, excision homologous recombination events between similar genetic elements present in the transgene construction were observed at efficiencies comparable to those of the Cre-mediated excision events observed by Iamtham and Day (2000). However, excision events by homologous recombination were not observed when the *cre* gene was introduced through sexual crosses. The only disadvantage of a Cre recombinase-mediated marker gene removal from the chloroplast genome is that it requires crossing the transformed plants to remove the nuclear-encoded *recombinase* gene and its associated genetic marker. This system is thus applicable only to sexually propagated plants.

In the second method, the phiC31 phage integrase enzyme Int is used to insert the gene of interest into the ptDNA (Lutz et al., 2004). The marker gene will be flanked with directly oriented nonidentical phage *attP* (215 bp) and bacterial *attB* (54 bp) attachment sites, which are recognized by Int recombinase. The excision of the marker gene will be achieved after transformation of the nucleus with an *int* gene encoding plastid-targeted Int (Kittiwongwattana et al., 2007).

## Transient co-integration of the marker gene

This method involves incorporation of the marker gene and gene of interest in the tDNA together by two homologous recombination events via the targeting sequences. Placing the marker gene outside the targeting region enables selection of a cointegrated structure that forms by recombination via only one of the targeting sequences. When selection for antibiotic resistance is stopped, the second recombination event can take place, and the marker gene is lost (Figure 9). This strategy was first demonstrated by Klaus et al. (2004) by linking gene of interest to a missing photosynthetic genes (*petA*, *rpoA*). This method is more convenient if repeated transformation is done with variants of the same plastid gene, and this approach requires an initial investment, obtaining a knockout mutant to use in the visually-aided complementation assay (Klaus et al., 2003).

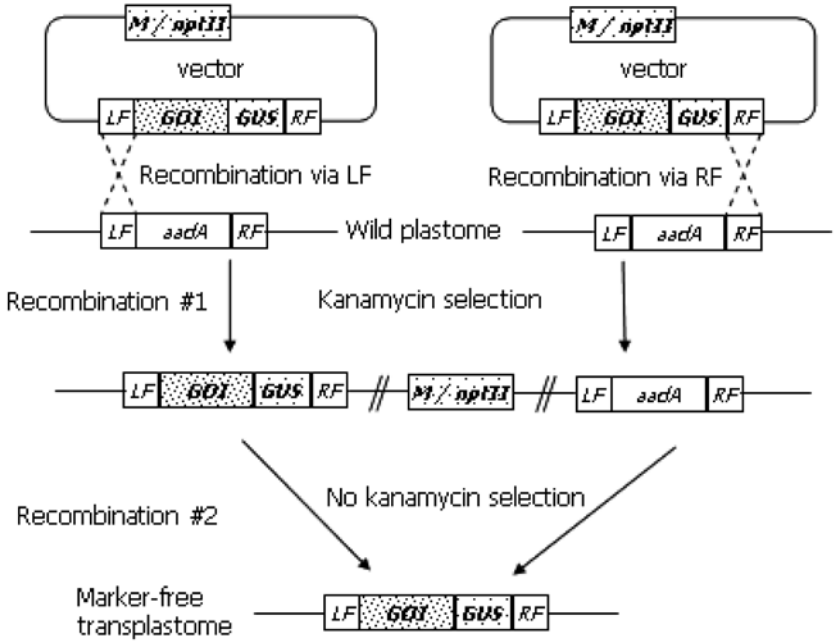


**Figure 9.** Transient co-integration based marker removal from chloroplast genome of transplastomic plants. In this system the marker gene is placed outside the targeting region that enables selection for a cointegrate structure and is later lost upon removal of antibiotic selection through second recombination. M/*nptII*: Marker, *neomycin phosphotransferase-II*, LF and RF: Left and right homologous fragments, GOI: Gene of interest, *GUS*: Reporter gene encoding β-glucuronidase, *aadA*: Gene for Spectinomycin resistance.

### Co-transformation-segregation

This method of co-transformation of two transgenes works similarly to the way nuclear transgenics does. This strategy involves transformation with two plasmids that carry target insertions at two different ptDNA locations. Selection for the marker yields transplastomic clones that also carry an insertion of the non-selected gene. This approach relies on the heteroplastomic ptDNA population obtained by cotransformation with two independently targeted genes. The feasibility of the approach was first shown in a unicellular alga, *C. reinhardtii*, with a single chloroplast (Kindle et al., 1991; Newman et al., 1991; Roffey et al., 1991). Later this approach was tried in tobacco to obtain marker-free plants that lack the antibiotic resistance gene and are resistant to the glyphosate herbicide (Carrer and Maliga, 1995; Ye et al., 2003).

## SPATIAL AND TEMPORAL EXPRESSION OF SELECTION MARKER GENE

This strategy involves the regulation of selection marker gene expression through an inducible promoter, which can be preferentially expressed, either temporarily or spatially at the time/site of transformation. This system allows the expression of a selection marker gene for only a limited period of time such as when transformants are selected without the expression of the markers in mature plant. This system was first successfully employed in tobacco for suppression of *nptII* gene driven by the wound-inducible promoter *AoPRi* (Özcan et al., 1993). This promoter was expressed at the wound site of tobacco leaf discs, to allow efficient selection of transformants. Mature leaf tissue showed very little expression of *nptII* and in some transformants it was virtually negligible.

## CONCLUDING REMARKS

The ever-increasing demand for producing marker-free transgenic crops arose from various public concerns regarding the bio-safety of genetically modified (GM) crops. Many GM crops contain selection marker genes which provide resistance against commonly used antibiotics or herbicides, and a common public perception about such crops is that, these genes could be passed from food to bacteria in the guts of humans and animals. Several legislative attempts to address these issues have already been made by the governing bodies of some European and American nations. These issues led to an extensive interest among researchers for the development of systems to produce marker-free transgenic plants, for their wider consumer acceptance. The range of options used is wide, ranging from attempts to transform without using selection markers to highly sophisticated transposon-based gene removal systems. Although, co-transformation of transgenes with selection marker genes is a widely popular technique to eliminate the undesired marker genes, it also has a few constraints. Among them is its restricted application, as it can not be used for vegetatively propagated plants. Another novel technique to eliminate marker genes from the transgenic plants is site-specific recombination under the control of inducible promoters, which seems quite promising. Integration of foreign genes into the plastid genome prevents the spread of transgenes, as plastids are maternally inherited. However, because of the high copy number and the prokaryotic expression signals of the selection markers, it is advisable to remove the markers after the generation of transplastomic plants (Iamtham and Day, 2000). The thrust of future research will be developing the most efficient systems for developing marker-free transgenic plants to address the concerns of ecologists and environmentalists regarding the bio-safety of genetically engineered plants. This research is in its infancy, and it is sure to expedite the refined study of plant systems, which will consequently increase the purview of biotechnology-based research in the near future.

**Acknowledgements.** The authors wish to thank the anonymous reviewers for their critical comments on the manuscript. Work in our laboratory focusing on the topic of this review is funded by a research grant from Konkuk University.

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