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Review

Status of clean gene (selection marker-free) technology

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The ever-increasing world population has created two major problems: more mouths to feed and less land to farm. While classical breeding has enormously helped in providing more food, a lot still needs to be done. Transformation biotechnology can also help especially where classical breeding lacks solution (e.g. limited availability of stable and durable genetic source of resistance). However, plant transformation technologies require selectable marker genes to produce transgenic plants but such genes are of no value thereafter; in fact, marker genes in transgenic plants are perceived to pose potential bio-safety problems. In all genetic transformation technologies based on direct gene transfer (electroporation of protoplasts, particle bombardment etc) the selectable marker genes generally co-integrate with the gene of interest(s) in one Mendelian locus in the plant genome; hence, their removal is highly desirable. This may also help in the acceptability of transgenic plants by society. Transgenic plants that contain the desired gene of interest but lack the selection marker gene used in its production are termed "clean" and the methods utilized in their production are referred to as "clean gene" technology. There are several proved methods of eliminating selectable marker genes and these include: (A) Marker gene excision consisting of (1) intra-genomic relocation of transgenes via transposable elements, (2) site-specific recombination systems and (3) intra-chromosomal recombination. (B) Gene replacement or Targeted gene replacement; and (C) transformation with multiple T-DNAs, which could result in linked and/or unlinked co-integration of transgenes. Unlinked transgenes are then segregated out during meiosis.

Key words: Clean gene, selection-marker free, transposable elements, site-specific recombination systems, intra-chromosomal recombination, multiple T-DNAs co-cultivation, linked / unlinked transgenes.

INTRODUCTION

As a routine, plant transformation protocols utilize various antibiotic regimens and herbicide as selection agents to identify transformation events in a wide range of crop species. With the exception of when selectable marker gene is used as a gene of interest e.g. herbicide tolerance (HT), the marker genes generally have little agronomic value after selection events. Moreover, once the HT trait has been introduced into germplasm of a plant species, retention of the HT gene in the genome may be problematic and an alternative marker system must be used to incorporate subsequent transgenes.

As a rule of thumb, superior cultivars usually contain several distinct traits, which make them commercially valuable, e.g. multiple disease resistance genes. For transgene technology to be successful commercially, future elite cultivars will contain multiple independent transgenes, which, as they become available, would need to be added in sequence. While crosses and hybridisation will still be useful and necessary to pyramid transgenes, in many cases, there will also be situation where repeated transformations into cultivars will be better and more effective. Unfortunately, the presence of a particular marker gene in a transgenic plant necessarily precludes the use of the same marker in subsequent transformation and the use of a different marker system is required for each transformation round or event. Thus, any technique that can remove or eliminate a selection marker gene in transgenic crops is highly desirable if for no other reason than that the same procedure can be used in subsequent transformations.

Moreover, consumer and environmental groups have expressed concern on the use of antibiotic- and herbicide-resistance genes from an ecological and food safety perspective. Some went to the extent of "labelling" genetically modified crops as "Frankenstein Food". While no scientific basis has been determined for these con-

cerns yet, generating marker-free transgenic plants would certainly contribute to the public acceptance of transgenic crops and alleviating these concerns. Some of these public concerns include the potential danger of selectable marker genes in transgenic plants on health and safety grounds. One of the major apprehensions with the commercialization of transgene products has been the concern that selectable marker genes or their products may be toxic or allergenic when consumed. When antibiotic selection markers having wide clinical and veterinary application are used, the marker gene could be transferred into microorganisms in the human and animal gut, which could, render such antibiotics functionally useless for both the clinical and veterinary application due to bacterial resistance to such antibiotics.

Environmentally, some safety issues that are raised include:

(a) A marker encoding herbicide resistance may change the transgenic plant into a weedy pest
(b) A horizontal transmission of the herbicide marker into wild relatives may transform them into a weedy pest and
(c) The spread of the antibiotic selection marker to other organisms may upset the balance of the ecosystem (Gressel, 1992; Dale, 1992; Nap et al., 1992).

However, risk assessment of the npt11 gene and its protein products carried out concluded that, there is no human or animal health risks associated with this particular marker gene in transgenic crops (FDA, 1994; Flavell et al., 1992; Fuchs et al., 1993; Nap et al., 1992; Redenbaugh et al., 1993, 1995); but complete removal of the marker gene would be a better alternative. It would remove the need for regulatory work for additional marker genes, allow sequential transformation using the same selectable marker gene and allow the greater probability of public acceptance of transgenic plants.

STRATEGIES AVAILABLE FOR REMOVING/ELIMINATING SELECTION MAKER GENE

There are a few major approaches in the literature that offer the possibility of removing or eliminating selection marker genes in transgenic plants (Hare and Chua, 2002; Jaiwal et al., 2002; Ebinuma et al., 2001; Puchta, 2000; Yoder and Goldsbrough, 1994), and these include the following.

Selection marker gene excision

Intra-genomic relocation of transgenes via transposable elements

This was the first reported genetic system in which removal of a marker gene event was successfully attempted. It involves the incorporation of transgenes within *Ds* elements and its maize *Ac* transposon and inserting this within the T- DNA (Goldsbrough et al., 1993). There are two essential characteristics possessed by the autonomous *Ac* element for transposition that can be physically separated: a transposase coding gene (Ac) and the inverted repeat termini (Ds elements). The *Ds* elements lack the transposase function and are stable in the absence of *Ac* but can be activated by the introduction of *Ac* transposase coding sequences *in trans*. Very important also, is the fact that sequences cloned between the inverted repeats of a *Ds* element can also be mobilized to new genomic locations in the presence of a transposase gene (Lassner et al., 1989; Masterson et al., 1989). These observations led to the development of a series of novel transformation vectors that incorporated transposable elements to eliminate selectable marker genes and other ancillary sequences from transformed plants (Goldsbrough et al., 1993; Fedoroff, 1989).

Transposition of at least two of the best-characterized maize element families, the *Ac/Ds* and *Spm/dSpm* family resulted in excision of trangenes in transposable elements from one locus prior to reinsertion into a second (Fedoroff, 1989). Transpositions, in general, insert into linked and unlinked sites in roughly equal frequencies in maize and transformed tomato (Greenblatt, 1984; Jones et al., 1990). In situations where trangenes are inserted at unlinked sites, sexual crossing can separate genes of interest and selectable marker genes.

Many maize transposable elements can also maintain their transposition competence when transformed into other plant species (Baker et al., 1986; Yoder et al., 1988). In tomato, it is estimated that about 10% of the elements that excise do not reinsert, or reinsert into a sister chromatid and are subsequently lost by somatic segregation (Belzile et al., 1989). However, Jones et al. (1990) reported preferential transposition of genes in tobacco to linked chromosomal locations.

Two types of vector systems have been developed for transgene elimination. In the first type (Figure 1), the gene of interest is inserted between the *Ds* inverted repeats. The advantage of this is that by relocating the gene of interest, different levels of expression, both qualitative and quantitative, can be achieved. This change in expression pattern reflecting the genomic location, at which the element is inserted, is referred to as a “position effect” (Yoder and Goldsbrough, 1994). In the second type, the selectable marker gene is flanked by the *Ds* repeats. In the presence of an active transposase, which can be introduced into the plant as an additional component of the T-DNA, or by a secondary transformation, or by crossing to a plant containing transposase, the *Ds* element containing the marker gene will transpose to a new genomic location. The *Ds* element either flanking the gene of interest or the selectable marker gene will reinsert in about 90% of the events, and about 50% of these insertion events will be genetically unlinked

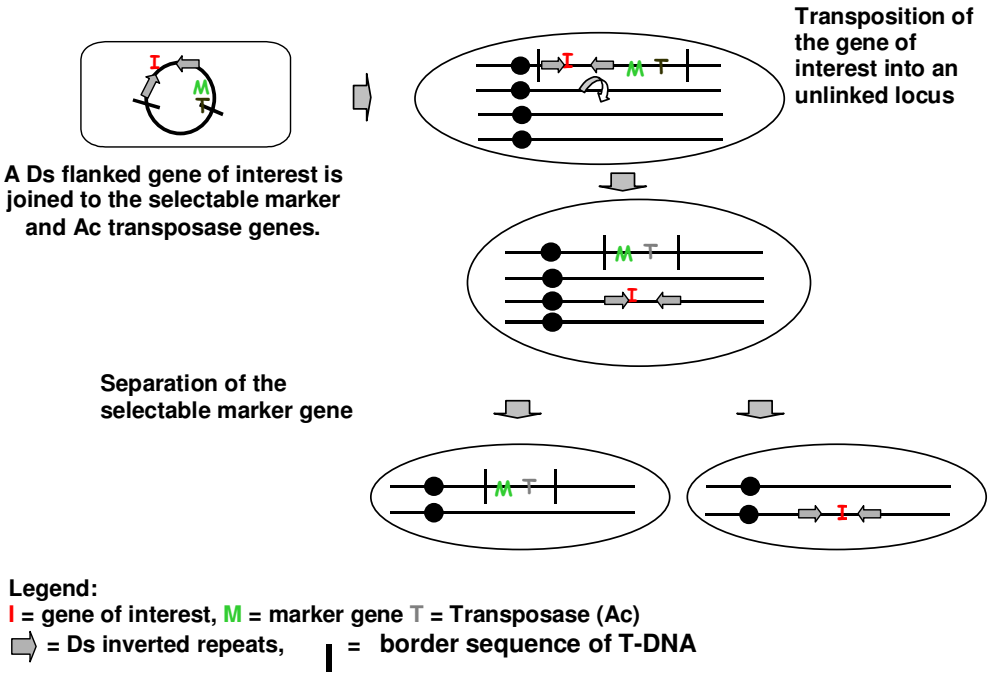

Figure 1. A Ds flanked gene of interest is joined to the selectable marker and Ac transposase genes. After transposition of the gene of interest into an unlinked locus, it is segregated from the selectable marker gene by sexual crossing.

to the primary site (Belzile et al., 1989). Genetic recombination between the new insertion and original site will yield progeny plants that segregate for the presence and absence of the gene of interest and marker genes. Consequently, plants which contain a stable transgene either internal to the *Ds* sequences (type 1) or linked to the T-DNA (type 2), and from which selectable marker has been removed, can be recovered.

Transposon- mediated intra-genomic re-localization of the transgene provides a very useful alternative to carrying out multiple independent transformations to achieve optimal transgene expression. Marker-free transgenic plants containing insect resistant *Cry1b* gene, between the Ds elements has been produced recently (Cotsaftis et al., 2002).

Ebinuma et al. (1999) developed a new vector system for the production of marker-free transgenic plants. It is named multi-auto transformation (MAT) because it can be used for repetitive transformation without sexual crossing as an added advantage. The *Agrobacterium* isopentyl transferase (*ipt*) gene, used as a positive selectable marker gene, was cloned into an *Ac* element within the T-DNA borders while *gusA* reporter gene (used as the gene of interest) carried in the T-DNA was outside the transposon. The *ipt* gene affects cytokinin metabolism by catalyzing the condensation of isopentyl pyrophosphate with adenosine mono phosphate (AMP) to produce isopentyl AMP, a precursor of several cytokinins (Akiyoshi et al., 1984; Barry et al., 1984). Cytokinins stimulate organogenesis in many cultured plant tissues and are widely used to regenerate transgenic plants from cultured cells after transformation.

The effect of the *ipt* gene on transgenic tobacco plants is an extreme “shooty” phenotype by which they can be selected. In this (MAT) vector system, where a chimeric *ipt* gene was inserted (as a selection marker gene) into *Ac*, the chimeric gene may transpose or become lost along with *Ac* in transgenic cells. Consequently, phenoltypically normal transgenic plants that had lost the *ipt* gene were recovered in both tobacco and hybrid aspen. The frequency of marker-free transgenic tobacco plants from which Ac has disappeared was 4.8% of the initial number of transformation events. However, when the *ipt* gene was introduced into tobacco plants, transgenic plants developed very many shoots and lost apical dominance. The frequency of marker free transgenic tobacco plants was 0.32% and the estimated frequency of somatic elimination of the Ac, involving cells in the apical meristem that can give rise to a shoot, was about 0.1 – 0.5% (Ebinuma et al. 1997). Although it can produce marker free transgenic plants without sexual crossing, the frequency was very low as most modified transposable elements containing the *ipt* gene reinserted elsewhere in the plant genome shortly after excision. Only cells with transposable errors would regenerate phenotypically normal shoots. Sugita et al. (1999) described a new practical MAT-vector in which the Ac for removing the ipt gene was replaced by the site-specific recombination system R/RS from *Zygosaccharomyces rouxii* (Arakiet et al., 1987). This method is discussed fur-

ther under site-specific recombination systems below.

Site- specific recombination systems

Procedures for introducing large-scale rearrangements of DNA of a eukaryotic chromosome was developed by the use of the bacteriophage *P1* derived *lox P - Cre* site-specific recombination system (Sauer, 1987, 1993; Dale and Ow, 1990; Odell et al., 1990). Briefly, the *Cre* enzyme (a 38-kDa product of the *cre* gene) catalyses recombination between two 34 base pair *loxP* sequences resulting in the excisions of internal sequences, if the lox repeats is in direct orientation.

Dale and Ow (1991) introduced luciferase (*luc*) gene into the tobacco genome by using the hygromycin phosphotransferase (*aphIV*) gene, flanked by two *lox P* repeats, as a linked selection marker in *Agrobacterium*–mediated transformation. Transgenic T_0 plants containing both *luc* and *aphIV* genes (Luc+ $Hygo^R$) were produced. To remove the chimeric *aphIV* selection marker gene from the T_0 plants, two types of independent procedures were carried out. Second round of *Agrobacterium*–mediated transformation with a plasmid containing the *cre* and *nptII* (kanamycin resistance) genes was carried out.

The kanamycin resistant and Luc + transgenic plants produced were screened for hygromycin resistant phenotypes (presence of *aphIV gene*). Ten of the 11 (91%) of kanamycin resistant plants were found not to contain the *aphIV* gene.

The *cre* gene was introduced into *luc* + *aphIV* + plants by pollinating them with pollen from a transgenic plant carrying the *cre* and *nptII* genes. Forty-two plants out of the 78 Luc + and kan^R transgenic plants were not carrying the *aphIV* gene. This indicated that about a 50 – 90% efficiency of selection marker excision occurred. Two Luc + Hyg^S kan^R transgenic plants were allowed to self-pollinate to allow segregation of the *luc* gene from the *cre* gene locus, which also harboured the linked *nptII* selectable marker. Of the approximately 100 T_2 germinated seedlings scored for luciferase activity, about ¾ (75%) was positive and ¼ (25%) of this was Kan^S indicating unlinkage and hence marker free. When the *Cre* gene was introduced by transformation, a marker gene cloned between two *lox* sites was eliminated from about 95% of the secondary transformants.

Gleave et al. (1999) reported the production of transgenic tobacco plants that contained a single copy T-DNA carrying a 35S–*gusA* expression unit linked to lox P flanked by the kanamycin resistance (*nptII*) and cytosine deaminase (*CodA*) genes. Retransforming these plants with a plasmid carrying *CreI* (containing 35S transcribed *cre* recombinase and hygromycin (*aphIV*) resistant gene) resulted in excision of the loxP- flanked genes from the genome. Phenotypic analysis of the progenies of the self-plants confirmed *nptII* and *codA* excision and integration of the *cre*–linked *aphIV* gene. To avoid the integration of the *aphIV* gene and thereby generate transgenic plants completely free of selection marker gene, transient expression of the *cre* gene was carried out by co-cultivating the doubly transformed tobacco plants with *Agrobacterium tumefaciens* carrying the p*CreI*. Shoots were regenerated in the absence of hygromycin but in the presence of 5- fluorocytosine which, when converted to the toxic 5-fluoro uracil by cytosine deaminase (enzyme coded for by *CodA* gene), killed plantlets with the *CodA* gene. Nineteen of 773 (0.25%) plantlets were tolerant. The absence of selectable marker genes was confirmed by the phenotype of the T_1 progeny.

The yeast's two -site specific- recombination systems, the *FLP* recombination system derived from the 2-µm plasmids of *Saccharomyces cerevisiae* are also available for the development of the same technologies (Broach, 1981). The efficacy of this *FLP* site-specific recombinetion system was demonstrated in transgenic *Drosophila* (Golic and Lindquist, 1989), mammalian cells (O'Gorman et al., 1991), but with not so promising results in maize and rice protoplasts (Lyznik et al., 1993). The *S. cerevisiae ARG4* gene cloned between the asymmetric inverted repeat sequences (*FRTs*) are substrates for the site-specific recombinase *FLP* (Cregg and Madden, 1989).

Like other site-specific recombinase systems, the R recombinase encoded by the *R* gene of the pSR1 of *Zygosaccharomyces rouxii* catalyses the excision of a DNA fragment between two directly oriented RS recombination sites in plant cells (Craig, 1988; Dale and Ow, 1990, 1991; Onouchi et al., 1991, 1995). Sugita et al. (1999) improved on the multi-auto-transformation (MAT) vector using the *gusA* and neomycin phosphotransferase (*nptII*) as reporter genes in tobacco plants and *ipt* gene as a selection marker gene cloned within two directly oriented RS sequences. After a month on hormone free MS medium, 134 adventitious buds were removed from the leaf segments and transferred to a fresh hormone free medium. Sixty-eight phenotypically normal, 18 *ipt*- shooty and 48 extreme *ipt*-shooty transgenic plants were recovered after a further month of culture. Molecular analysis showed that marker free transgenic plants were recovered from seven of 18 (39%) moderate *ipt*-shooty lines and 32 (70%) of 48 extreme *ipt*-shooty lines. This is considerable improvement to the Ac/Dc transposon system of Ebinuma et al. (1997). It also works without sexual crossing. However, 67% of the transgenic plant lines had three or more T-DNA copies in them. The method has also been improved to produce marker free transgenic rice plants without the *ipt* intermediate in a single step transformation with marker free plants produced at 25.5% efficiency (Endo et al., 2002a).

An improvement of this system is the GST-MAT vector in which the R/RS recombinase system was fused with the chemical inducible promoter of the gluthathione S-transferase (GS-T 11-27) from *Zea mays* (Sugita et al.,

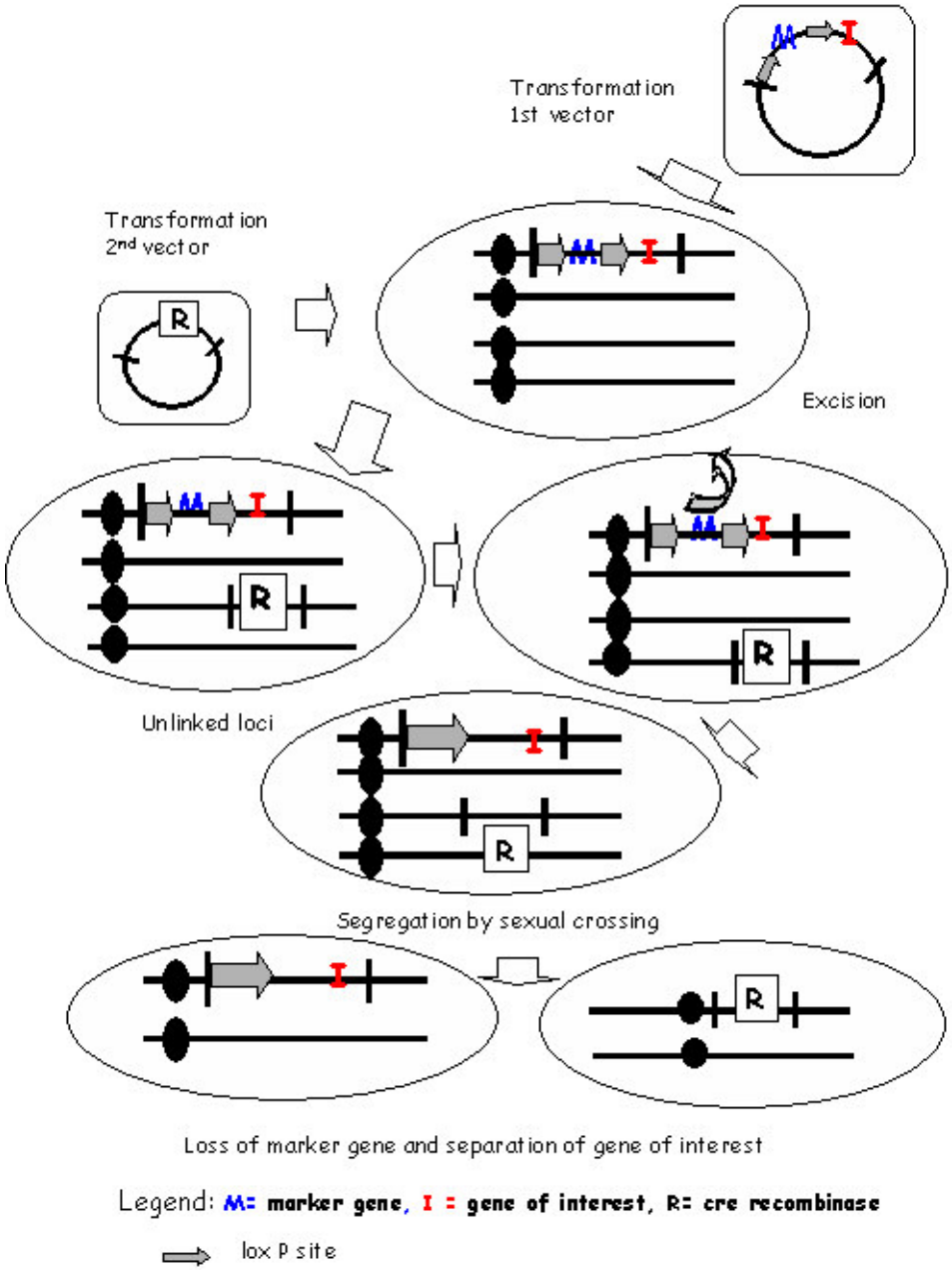

Figure 2. Site-specific recombination system (*Cre/ lox*) of removing a selectable marker gene. The selectable marker gene, joined to the gene of interest, is flanked by a *lox* sites. To remove the selection marker gene, the *cre* gene is introduced by re-transformation or cross -pollination. The gene of interest is segregated from the *cre* by sexual crossing.

2000a). Transformation efficiency was 88% (37/ 42 adventitious buds) and marker free transgenic tobacco plants at 19% frequency (7/37 T_0 transgenic plant). Six out of 7 (86%) of the marker- free transgenic tobacco plants had only one copy of the T-DNA inserted. The system has also been used to add (transgene stacking) another gene (*gfp*) into a previously generated marker free tobacco transgenic plant (Sugita et al., 2000b). The recombinase system is pictorially shown in Figure 2.

Intra-chromosomal homologous recombination

A less complicated approach of inducing DNA deletions is based on intra-chromosomal homology recombination (ICR) between two homologous sequences. Although ICR can be enhanced by stimulation of repair systems, the frequencies are currently too low for an efficient application of this system to produce deletions of transgene regions. In tobacco, for example, on average, less than

10 ICR events were detectable among all cells of a six-week old plant (Puchta et al., 1995). However, if recombination substrates, that will provide a more efficient target for the recombination machinery, could be found, the low ICR frequencies could be increased.

Zubko et al. (2000) developed a novel ICR strategy based on the recombination of the *attP* region of bacteriophage λ to generate deletions, followed by the identification of ICR products among a relatively small numbers of transformants, and subsequently providing a feasible procedure to remove undesirable transgene regions. This system was used to delete a 5.9 kb region from a recombinant vector that had been inserted into two different genomic regions in tobacco. This plant transformation vector called p*attP*-ICR contained *nptII*, *gfp*, and *tms2* genes inserted between two 352bp *attP* regions. Next to the left of the *attP* site was positioned the transformation booster sequence (TBS) which enhances homologous and illegitimate recombination, and an *oryzacystatine–I* gene that served as an example of the gene that will be transferred into the genome of the tobacco by the *attP* system.

Molecular analysis carried out on some of the independent transgenic tobacco plants lines (showing mixture of white and green shoots), with primers specific for *nptII* and *oryzacystatin* genes and the region flanking the *attpII* dimer cassette, showed that the *oryzacystatin* gene had been retained while the *nptII* gene had been lost, and that the region between the two attP fragments had been reduced by about 6 kb (as expected if the two *attP* sites had recombined). However, most plantlets that had lost the *nptII*/*tms2* region had also lost transgene regions outside the attP cassette. This clearly indicated that the ICR system is not always associated with precise homologous recombination between the attP regions, and that it can generate larger deletions, probably because of illegitimate recombination.

Targeted gene replacement

Targeted gene disruption by homologous recombination in rice has also been developed (Terada et al., 2002) using the *Waxy* gene, which encodes granule-bound starch synthase, as a target. About 1% of selected calli and their regenerated fertile plants were heterozygous at the targeted locus, and only one copy of the selective marker used was found at the targeted site in their genomes.

Co-cultivation with multiple T-DNA

Multiple copies of transfer-DNA (T-DNAs) can be transferred into a plant cell and integrated in the plant genome through *Agrobacterium*–mediated transformation. During investigation of whether the efficiency of transformation of plant cells with *A. tumefaciens* is limited by the infecting bacteria or the properties of the plant cell, tobacco protoplasts were co-cultivated with two different *Agrobacteria* strains carrying Ti plasmids with distinguishable T-DNAs.

The two types of T-DNA were found to co-transform (and express) in the produced transgenic calli at co-transformation frequency varying from 20-40%, and confirmed by repetition of the experiments. Although, no regeneration of plants was attempted, molecular analysis on the transgenic calli revealed the presence of the two different T-DNAs (Depicker et al., 1985).

In the process of assessing the use of a new T-DNA in which the *onc* gene had been deleted (and cloned into plasmid pRK290) to transform tobacco plants, it was accidentally observed that multiple unlinked T-DNAs did co-transform into tobacco calli, producing transgenic plants that segregated in the progeny (de Framond et al., 1986). Plasmid pRK290 was transformed into an *Agrobacterium* strain carrying an octopine TiB6-806 as a helper plasmid that could supply *vir*gene *in trans.* Two independent transgenic plant lines were found to produce both nopaline and octopine and were hormone dependent and fertile. One of the plants was self-pollinated and it was found that the two opine markers segregated in the T_2 progeny in a Mendelian fashion and, the presence of the respective T-DNA was confirmed by molecular analysis (de Framond et al., 1986). No co-transformation frequency was determined because one of the plasmid used was a wild type carrying the tumor gene. In addition, only one progeny plant was used for segregation analysis.

Integration of a T-DNA, containing the gene of interest at a different locus to another T-DNA carrying the selection marker gene, opens the possibility of producing transgenic progenies free of the selection marker genes. To achieve this, however, two conditions must be met: The efficiency of co-transformation of both T-DNAs (one carrying the gene of interest and the other selection marker gene) needs to be reasonably high, and the co – transformed genes must integrate at genomic locations sufficiently apart from each other (unlinked) to allow effective segregation during meiosis.

Agrobacterium - mediated transformation is the only technology that has been reported to date, that allows integration of different transgenes at different Mendelian loci with little transgene rearrangements in plants (Komari et al., 1996; Daley et al., 1998). The frequency of segregation of the transgenes is determined by their location in the plant genome relative to each other (Figure 3).

Komari et al. (1996) found that more than 50% of the co-transformed rice and tobacco plants segregated independently for the transgenes using a single octopine derived *Agrobacterium* strain. In addition, using an octopine–derived *Agrobacterium* strain, Daley et al. (1998), reported that progeny from 50% of the co–transformed *Brassica napus* and tobacco plants exhibited independent transgene segregation. Afolabi et al. (2005) found 50% unlinked TDAs

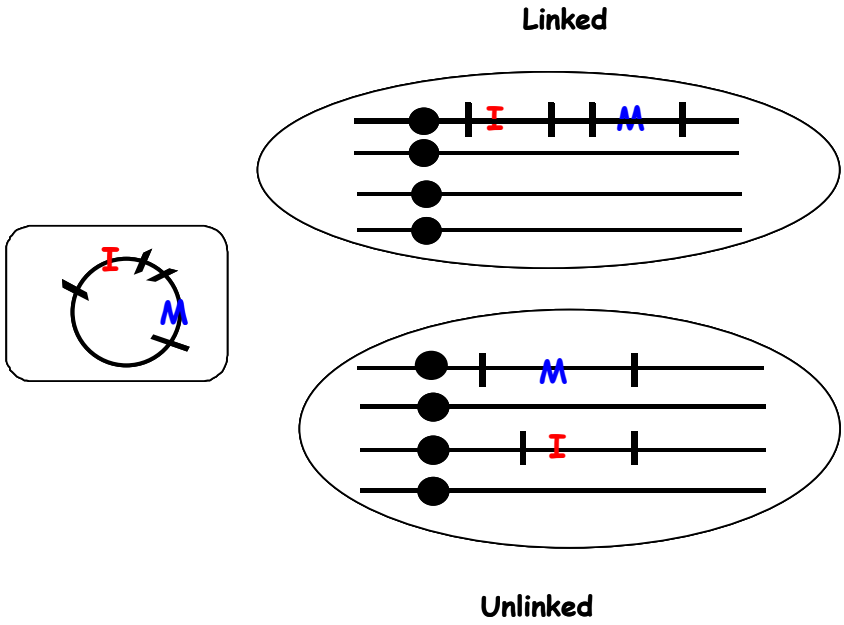

Figure 3. Linked and unlinked co- integrated genes in *Agrobacterium*-mediated transformation with multiple T-DNAs. *Agrobacterium* strain carrying two different T-DNAs. One T-DNA contains the gene of interest and the other T-DNA the selectable marker gene. T-DNAs can be integrated at either genetically linked or unlinked locations.

in rice using pGreen / pSoup dual binary vectors. Matthew et al. (2001) reported 16.5% of unlinked integration in barley using adjacent "twin T-DNA" in *Agrobacterium* strain (AGLO or AGL1). In soybean, the percentage of unlinked integration was found to be 40% (4 of 10 plant) Xing et al. (2000).

Agrobacterium-mediated co-transformation with multiple T-DNAs has been accomplished using: (a) one vector carrying multiple T- DNAs in one *Agrobacterium* strain (Depicker et al., 1985; Komari et al., 1996), (b) Two separate vectors, each carrying different T-DNAs contained in (i) one *Agrobacterium* (de Framond et al., 1986; Daley et al., 1998; Afolabi et al., 2005) or (ii) two or more *Agrobacterium* strains (Depicker et al., 1985; McKnight et al., 1987; De Block and Debrouwer, 1991; Komari et al., 1996).

ADVANTAGES AND DISADVANTAGES OF EACH OF THE STRATEGIES

Each of the systems discussed above for the production of marker free plants has its merits and demerits. Table 1 show the strategies utilized in producing "clean gene" (marker-free) technology of crops. The necessary factors to consider are the frequency and time- frame in which marker free plants are produced; the ease with which the transgenes can be cloned into vectors; the number of genes that can be introduced into plants at one time; and, the number of times a given system could be used for sequential transformation. While the excision frequency is very high (>80%) in the transposon system, it does not correlate with the percent of unlinked loci, especially when some of the excised genes can re-integrate back into the genome at unpredictable loci. The *cre/lox* site specific recombination and its transposable element system requires the cloning of flanking sequences, and an additional gene that encodes an enzyme, which must be active in the target plant.

The system also requires re-transformation or sexual crossing with the recombinase *cre* gene. This increases the production time for marker free plants. Furthermore, a lox site is usually left behind after recombination, posing problems later.

The use of a transposable element system to remove a selectable marker could also allow the recovery of

Table 1. Strategies utilized in producing "clean gene" (marker-free) technology of crops

Strategy	Plant/Crop	Segregated genes	Transformation efficiency and % Unlinkage	Reference & Comment
A	Tomato	*Ds*-35SP *gusA*-*Ds* - *Ac trans* -35SP *nptII* Truncated 35S CaMV promoter used. Ac/ Ds system	Progenies from 2 T_0 plants analysed. Excision frequency estimated to be 81% (71/87 plants) in T_1 progeny plants with single insertion (*gusA* gene moved to different locations). 8 different insertional types (locations) observed. Segregation of progeny plants with 2 T-DNA insertions: 56% (60/106) plants had 2 T-DNAs (I and J), 12% (13/106) had one (I), 21 had the other (J), and 11% (12/106) had neither (indicating independent assortment of two unlinked loci).	Goldsbrough *et al.* 1993. One transgenic primary plant each had single and multiple T-DNA insertions with 87 and 106 progeny seedling characterised respectively. Southern analysis done on progeny plants
A	Tobacco	NP – *nptII* –T– 35SP-*ipt* -T - Ac-35S- *gusA* –NT Ac/ Ds-MAT system	Frequency of excision 0.32%. Estimated frequency of somatic elimination of the Ac 0.1 – 0.5%. Multiple-auto-transformation (MAT) with positive selection.	Ebinuma et al. 1997. Does not require sexual crossing. (MAT vector pNI2106 with Ac/Ds system)
A	Rice	I-RS/dAc-IRS- Ds-*aphIV*- Ds RS / Ds system	Excision frequency 0-70%. Frequency of transposition event was 44% and 38% in two cases. Transposition was 20% (10 of 49) F_2 plant tested.	Nakagawa *et al*. 2000. Technique also useful for functional genomics. Copy number and T-DNA insertion by Southern analysis. T_1 analysis by PCR.
A	Rice	35SP –*aphIV* –35T Ds-*Crylb gene* –Ds-nosT 35SP–*aphIV* –35T-5S-Ped -*gfp* –nosT Ac/ Ds system	Excision of *Crylb* in 37% (27/68 T_0 plants); reinsertion in 25% (19/68T_0 plants). In F_2 plants, 20% (10/49 plants) showed segregation of *Crylb* gene. Frequency of transposition in T_1 plants around 0.2 –1.4%. Southern analysis done for genotyping of T_0 and T_1	Cotsaftis et al. 2002. T_0 analysis by GFP phenotyping. Segregation analysis by hygromycin inoculation of expanded leaves. *Analysis of Cry Ib* expression in leaf tissues by immunoblot assay. Bioassay done by insect feeding.
B	Tobacco	35SP- *luc* nosT-loxP-35SP *aphIV*-nosT-loxP 35SP- *creI*- nosT Cre-lox system	25% of unlinkage (Luc^+) in progeny. Excision frequency 50–90%. 91% (Ten of 11) kan^R plants and 54% (42 of 78) Luc^+ & kan^R were Hyg^-. 95% of maker gene eliminated from somatic cell. Expression of *cre* led to Hyg^S phenotype.	Dale and Ow (1991). Evidence of precise recombination event obtained by nucleotide sequencing of sites surrounding the lox sites of both parental and progeny genomes.

many insertion events from one transformation event, and the study of positional effects on expression of the gene of interest, provided such genes are flanked by the Ds inverted repeats (Yoder and Goldsbrough, 1994). Such an arrangement, however, would preclude a second round of transformation using this system, since the position of the Ds-flanked genes of interest from the first event, would be destabilized. If the marker gene is inserted between the Ds repeats, then the Ds sequences will be removed with the marker, allowing marker free plants produced by this method to be re-transformable using the same system.

Table 1. Strategies utilized in producing "clean gene" (marker-free) technology of crops (Cont.')

Strategy	Plant/Crop	Segregated genes	Transformation efficiency and % Unlinkage	Reference & Comment
B	Tobacco Arabidopsis	35SP-*gusA*-nosT-35SP-ALS^r alsT-loxP 35SP-*cre*-35ST- Cre-lox system	95% excision frequency in T_0 plants. Excision demonstrated phenotypically by the loss of sulfonylurea resistance (ability of calli to grow on Closulfuron supplemented medium). Southern analysis confirmed excision. 99% (2261/2262) inherited clorsulfuron sensitivity. F_1 progeny screened by spraying plants with sulfonylurea	Russell et al. 1992. Two methods of gene removal (re-transformation and cross–pollination) were utilised. Cab/22 leader sequence used with *gusA* and ALS^r promoters
B	Tobacco	35SP –*gusA*-ocsT-loxP-nosP-*nptII* –nosT-ocsT–*CodA*-35SP 35SP-*CreI*-ocsT-35SP *aphIV*- ocsT. Cre-lox system	0.25% (19 /773) plantlets were selection marker free. 2 T_1 progeny plants determined to be selection marker free by phenotyping.	Gleave et al. (1999). Use of *codA* gene as negative selection. Frequency of selection marker-free plants very low and procedure was complicated
B	Rice	35SP-loxP-*aphIV* –nosT –loxP nosT–gusA-nosP Ubi-*CreI* –nosT-35SP-aphIV- nosP Cre-lox system	26% (19 of 73 transgenic plants) were GUS+. 58% (77of 132) were GUS+ in T_2 plants	Hoa et al. 2002. Transpostion of 35S promoter to a promoter-less *gusA* gene with consequent *gusA* expression.
B	Tobacco	35S-FLP-polyA nosT-nosT-*kan* –35SP 35SP-FRT- 2.8-kb spacer- FRT- *aphIV* –*kan* –35SP FLP – FTS system	17.5% of crossed T_1 progeny plants were Hyg^R .Low value showed inefficiency of system. PCR analysis used to show recombination. Loss of 2.8-kb spacer sequences within the FRT indicated excision.	Lloyd and Davis (1994). Progenies of 2 T_0 plants well characterised
B	Tobacco	NosP-*nptII*-T-35SP-R-nosT- P-*ipt-nosT-RS*-35SP-gusA-nosT. R/RS – MAT system	Frequency of selection marker free plants was 39% (7/18) of moderate ipt-shooty, 70% (32/48) of extreme ipt-shooty plant lines.	Sugita et al. (1999). Improvement of MAT system. 67% of transgenic plant lines had three or more T-DNA inserts.
B	Tobacco	dGST-P-gusA-T-RS –P ipt t-NP-*Km*-T-RS 35SP-gusA-T-RS-GSTP-R-ipt-*t* -RS GST– MAT system	Transformation efficiency 88% (37/42 adventitious buds). 19% (7/37) marker-free transgenic plant GST-MAT (inducible promoter)	Sugita *et al.* 2000a. Use of herbicide "Safener" in growth medium to activate inducible promoter GST-II-27. 86% (6/7) of marker free transgenic plants had only one copy T-DNA.

The development of alternative selection markers that must also work with reasonable efficiency in the target plant takes additional time and effort, aside from the regulatory issues that must be considered. Additionally, sequential transformation would only be possible if the marker used in the previous transformation was different.

There are several advantages to the use of the various types of site-specific recombination systems. Recombination takes place only between specific sequences, usually several dozen base pairs in length, and this make the system highly specific. The system is very simple because single recombinase protein catalyses recombination events and no other proteins are required. The frequency is also remarkably high (Sugita et al., 1999, 2000a,b; Endo et al., 2002a, 2000b) unlike that of homologous recombination that is extremely low (Ebinuma et al., 1997). However, it has also been postulated that recombinase and transposase proteins might cause

Table 1. Strategies utilized in producing "clean gene" (marker-free) technology of crops (Cont.')

Strategy	Plant/Crop	Segregated genes	Transformation efficiency and % Unlinkage	Reference & Comment
B	Tobacco	NP- *Km*-T-RS-T-R-35SP-t-ipt–35SP-RS-35SP-*gusA*-T NP-*gfp*-T-RS-GSTP-R-T-P-ipt-t-RS	94% transformation efficiency (34/36 adventitious buds). MAT vector with *gfp* replacing *gusA* in the second transformation event	Sugita *et al.* 2000b. Transgene stacking in tobacco plant using R/RS –MAT system
B	Rice	*NptII*-RS-T-R-35SP-t-*ipt*-35SP-RS-35SP *gusA*-T. *NptII*-RS-T-R-35SP- *gfp*—T-T-ipt-35SP-RS 35SP-*gusA*-T-35SP-*aphIV*-t R/RS – MAT system	Transformation efficiencies were 34.1% (29/85); 48.8% (40/82); 56.6% (60/106) plants from scutellum tissues in 5, 8 and12 days on regeneration medium respectively. 25.5% marker-free plants without *ipt*-intermediates.	Endo et al, 2002a. Marker-free transgenic To rice using the MAT Vector. No Mendelian inheritance of gene(s) of interest observed.
B	Tobacco	*NptII*-RS-iaaM/H-ipt-T-RS-35SP- gusA-T *NptII*-RS-*ipt*-*TgusA* 35SP-*gusA*-T NosP- *gusA* –T-RS-GSTR-T-*ipt*-iaaM/H-RS GST – MAT system	Efficiency of marker–free plant generation was 41.6% (5/12 ipt- shoots) forming plants.	Endo et al, 2002b. Hormone-free medium used for culture. Significant proliferation of transformed calli. Novel inducible vector improved GST-MAT system
B	Hybrid Aspen	NP-Km-T-RS-T-ipt-rbcS-P- GST-P-R-T-RS 35SP-*gusA*-T GST – MAT system	38% GUS+ plants after infection of plant stems, 21.4% (3/14 ipt-shooty plants) were marker free upon inducible excision of the *ipt*- gene within 2 months after cutting induction	Matsunaga *et al.* 2002 Asexual production of marker-free transgenic vegetatively propagated woody plant
C	Tobacco	*nptII, gfp*, and *tms2* inserted between two 352 bp attP TBS and Oryzacystatine – I	*nptII / tms2* lost and *Oryzacystatine* I retained in genome.	Zubko et al. 2000. Loss of region outside the attP site is possible and problematical.
D_2 D_3	Tobacco Tobacco	*nos (*on one T-DNA) *oct* (on 2nd T-DNA) (nopaline and octopine) *nos* (one T-DNA) chimeric *nos/nptII* (on 2nd T-DNA)	Co-transformation frequency not determined. Segregation of nopaline and octopine genes in progeny of transgenic plants Only 3 co-transformed T_0 plants analysed. Co-transformation frequency was 19% (3/16 plants). In T_1 progeny 14% (28/195) plants had nos+ Kan-; 27% (53/195) plants had nos- Kan+	de Framond et al. 1986. Only twoT_0 plants produced, and segregation analysis done on one plant only. McKnight et al. (1987). Use of *Agrobacterium rhizogenes*

undesirable secondary effects in plants (Zubko et al., 2000). The MAT-based site-specific recombinase systems also have the advantage of producing marker free transgenic plants without sexual-crossing; hence, they can be potentially used for any crop.

Agrobacterium co-transformation as a method for marker removal does not require the development or application of additional selectable marker genes or DNA excision, but whether the one or two *Agrobacterium* strains method provides a higher frequency of marker-free plant recovery is still unclear. This comparison of a single and a double strain methods using the same species, transformation protocols, and similar plasmids, showed higher co-transformation frequencies but similar

Table 1. Strategies utilized in producing "clean gene" (marker-free) technology of crops (Cont.')

Strategy	Plant/Crop	Segregated genes	Transformation efficiency and % Unlinkage	Reference & Comment
D_3	*Brassica Napus*	*bar* (on one T-DNA) *nptII* (on 2nd T-DNA)	Transformation frequency of 39–85%. 43% (27/63) plants showed unlinked T-DNA integration.	De Block and Debrower (1991). In linked progeny, most were in head-to-head inverse repeat.
D_3	B. Napus	*bar* (on one T-DNA) *nptII* (on 2nd T-DNA)	33% (3/9) plants with unlinked T-DNA integration	Denis et al. 1995. Hypocotyl was used as explants.
D_1 D_3	Rice Tobacco	*gusA* (on one T-DNA) *aphIV* (on 2nd T-DNA)	Co-transformation efficiency: Tobacco 52% (61/118) Rice: 47% (259/549) Unlinkage in rice progeny: 65% (13/20) and 100% (2/2) had GUS+ aphIV-; In tobacco: 55% (5/9) and 71% (10/14) had GUS+ aphIV-.	Komari et al. (1996). Use of super-binary plasmid vector.
D_3	Arabido-psis Tobacco	*nptII* (on one T-DNA) *aphIV* (on 2nd T-DNA) "K" and "H" type T-DNA	Arabidopsis- 31% (7/22) plants were unlinked; 68% (15/22) plants had one linked locus. Tobacco- 42% (6/14) plants had MIXED linked and unlinked loci and 50% (7/14) plants were linked with one locus.	De Neve et al. 1997 Integration pattern suggested that T-DNA repeats originated from multiple co-integration of separate T-DNA.
D_2	*Brassica napus* Tobacco	*gusA* (on one T-DNA) *nptII* (on 2nd T-DNA)	Co-transformation efficiency: *B. napus* 61% (21/34) plants Tobacco 52% (52/100) plants. Unlinkage in progeny: In *B. napus,* 40% (8/20) plants were unlinked (GUS+ Kan-). Tobacco: 51% (24/41) plants were Kan+ GUS-	Daley et al. (1998).
D_1	Soybean	*bar* (on one T-DNA) *gusA* (on 2nd T-DNA)	Transformation efficiency very low (0.5-3.2%). Co-expression frequency was 70% (7/10 plants). Percent unlinkage was 40% (4/10 plants) determined by painting plants with PPT and GUS assay.	Xing et al. (2000). Only Phenotypic analysis done. No data on silent genes although they may appear unlinked.
D_1 mod	Barley	*aphIV* (on one T-DNA) cDNA coding for α-amylase (high and low ISEP) α- glycosidase (on 2nd T-DNA)	Co-transformation efficiency 66%. 16.5% unlinked integration.	Matthew et al. (2001). Use of "twin T-DNA" (additional right and left border on a single T-DNA
D_1 mod	Rice	Testing of procedure: *bar* and *gus.* For Clean gene in 2 rice varieties (Jara & Xiu-shui) *aphIV* (on one T-DNA) RRSVS5 (on 2nd T-DNA)	Co-transformation (for testing) was 100% (10/10plants). Unlinkage frequency was 70% (7/10 plants). 14 and 25 independent lines Jara and Xiu-shui respectively produced. 64% (9/14) of Jara and 36% (9/25) of Xiu had RRSVS5 alone. 57% (8/14) of Jara had *aphIV* only. No data available for Xiu –shui.	Lu et al. 2001. Use of double right border single T-DNA. Recovery of marker free transgenic rice plant containing rice ragged stunt virus (RRSV) derived synthetic gene.

frequencies of unlinked insertions only with the single strain system (Daley et al., 1998; Komari et al., 1996). In addition, the two-strain method of De Block and Debrouwer (1991) showed a similar co-transformation frequency, but with a lower unlinked insertion frequency, compared to the one strain method of Komari et al. (1996). However, it is also possible that De Block and Debrouwer could have obtained the same recovery frequencies (Komari et al.) if an octopine- instead of a nopaline - derived strain was used.

There have been suggestions that the type of *Agro-bacterium* strain used may influence the arrangement of T-DNAs in the plant genome: nopaline derived *Agro-bacterium* strains favour insertion of multiple T-DNAs at genetically linked loci, while octopine - derived strains favour integration at unlinked loci. De Block and Debrouwer (1991) obtained 22% independent segrega-tion of the transgenes in co-transformed progeny of *Brassica napus* plants when two nopaline - derived strains were utilized. Komari et al. (1996) reported that more than 50% of the co-transformants segregated independently for the transgenes when a single octopine

Table 1. Strategies utilized in producing "clean gene" (marker-free) technology of crops (Cont.').

Strategy	Plant/Crop	Segregated genes	Transformation efficiency and % Unlinkage	Reference & Comment
D_1, D_3 and Control (one T-DNA)	Maize	*gusA* (on one T-DNA) *bar* (on 2nd T-DNA)	For D_1 unlinkage percentages were 19.5% (17/87) plants; 28.7% (25/87) linked plants and 51.7% (45/87) plants (not determined). For D_3, 28.6% 2/7 plants linked, 28.6% (2/7) plants unlinked and 48.2% (3/7) plants not determined. 71.3% (62/87) plants (of D_1 segregated while 71.4 (5/7) plants of D_3, segregated the 2 genes	Miller et al. 2002. For D_1 and D_3, transformation efficiency was 52% and 57% respectively. Co-trans-formation efficiency was 55% and (11% respectively.
D_2	Rice	*bar and ppt on pGreen gfp and aphIV on pSoup*	Co transformation frequencies were 71% and 80% for the hygromycin only and herbicide (PPT) only selection, respectively. About half (50%) of the independent transgenic plant lines contained at least one unlinked T-DNA integration.	Vain et al. 2003 ; Afolabi *et al*. 2005

A; Intra-genomic relocation of transgenes via transposable elements; B; Site-specific recombination systems; C; Intra-chromosomal homologous recombination D_1; Co-cultivation with multiple T-DNAs on one plasmid vector in one *Agrobacterium* strain. $D_{1\ mod}$; Co-cultivation with only one modified T-DNA in one *Agrobacterium* strain. D_2; Co-cultivation with one *Agrobacterium* strain carrying two T-DNAs on separate plasmid vector. D_3; Co-cultivation with two *Agrobacterium* strains each carrying a T-DNA on one plasmid vector. NP, nopaline synthase promoter; NT, nopaline synthase terminator; P, isopentyl gene promoter; T, isopentyl transferase terminator. T; polyadenylation sequence of *ipt* gene; 35SPed double enhancer 35S CaMV promoter with a leader sequence, *ocs,* octopine synthase terminator;; ALS^r gene; acetolactate synthase gene (herbicide resistant gene- sulfonylureas is an example of one of its substrates; alsT; terminator of ALS gene; *bar*, phosphinothricin acetyl transferase gene; *gusA*, β- glucuronidase gene;; *aphIV* (*hph*), hygromycin phosphotransferase gene; *i*, intron; *nos*, nopaline synthase gene; *Km* or *nptII*, neomycin phosphotransferase gene; RRSV5S; rice ragged stunt virus coding sequence; *luc*, luciferase gene; *onc, Agrobacterium* tumor producing gene; GOI, gene of interest; ISEP; iso electric point electrophoresis; *oct*, octopine syntase gene; *gfp,* green fluorescent protein gene; tm2; TBS, transformation booster sequence; *creI* ; cre recombinase gene; *codA*, cytosine deaminase gene; MAT, multiple- auto transformation; *GST*, Gluthathione – S transferase gene; dGST-P, deleted promoter of Gluthathione–S-transferase gene; *iaaM/H*, Indoleacetamide hydrolase and tryptophan monooxygenase; *ipt*, iso pentenyl transferase gene; *Ac/Ds* transposon system of maize. R/RS site specific recombination of *Zygosaccharomyces rouxii;* FLP/FRT. Site specific recombination of the Saccharomyces cerevisae; rbcS-P, promoter of the rbcS-3B gene.

- derived strain was used. Similar data revealed that progeny from about half of the co-transformants exhibited independent transgene segregation using the octopine-derived strain (Daley et al., 1998). The genotyping of additional progeny could also increase the percentage of lines classified as segregating independently for both transgenes, especially in co-transformed lines with multi-ple insertions of one or both genes (Daley et al., 1998). However, the use of two plasmids in one octopine - derived strain has several advantages and desirable attributes and these include simplicity, easy cloning ability, and high co-transformation efficiency, high frequency of unlinked integration, reasonable production time line and unlimited use of re-transformation.

DEDICATION

This paper is dedicated to (1) George Thottappilly, Ph.D., D.Sc, who introduced me to Plant Sciences and (2) Prof. John W. Snape who gave me the opportunity to train in his excellent facility at the John Innes Centre, Norwich, UK.

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