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REVIEW PAPER

Strategies for Developing Marker-free Transgenic Plants

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Abstract The development of marker-free transgenic plants has responded to public concerns over the safety of biotechnology crops. It seems that continued work in this area will soon remove the question of unwanted marker genes from the debate concerning the public acceptability of transgenic crop plants. Selectable marker genes are co-introduced with genes of interest to identify those cells that have integrated the DNA into their genome. Despite the large number of different selection systems, marker genes that confer resistance to the antibiotics, hygromycin (*hpt*) and kanamycin (*nptII*) or herbicide phosphinothricin (*bar*), have been used in most transgenic research and crop development techniques. The techniques that remove marker gene are under development and will eventually facilitate more precise and subtle engineering of the plant genome, with widespread applications in both fundamental research and biotechnology. In addition to allaying public concerns, the absence of resistance genes in transgenic plants could reduce the costs of developing biotechnology crops and lessen the need for time-consuming safety evaluations, thereby speeding up the commercial production of biotechnology crops. Many research results and various techniques have been developed to produce marker-free transgenic plants. This review describes the strategies for eliminating selectable marker genes to generate marker-free transgenic plants, focusing on the three significant marker-free technologies, co-transformation, site-specific recombinase-mediated excision, and non-selected transformation.

Keywords: genetically modified (GM), selectable marker gene, marker-free, plant transformation, biotechnology, biosafety

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1. Introduction

Plant transformation is a genetic engineering tool for introducing transgenes into plant genomes. Since the first introductions of successful plant transformation in the early 1980s, plant transformation technology has become an adaptable platform for cultivar improvement as well as for studying gene function in plants. Transgenic plants, plants in which transgenes have been introduced, have impacts in agricultural fields (BT crops) and non-agricultural fields (pharmaceutical farming, oral vaccines *etc*). The process of the transformation of the plant consists of several steps. After introduction of the transgene into the genome, one of the essential steps is the selection process of separating transformed calli from non-transformed calli, the majority, because of the low transformation efficiency, $10^{-3} \sim 10^{-6}$ [1]. Usually, selectable marker genes linked to the transgene have been used, they permit the preferential growth of transformed cells.

Although antibiotic or herbicide-resistant genes have traditionally been used as selectable marker genes, several types of such genes exist. They can be separated into four groups, antibiotic-resistant, herbicide-resistant, metabolic pathway-based, and negative selectable marker genes. The most commonly used selectable marker genes are neomycin phosphotransferase gene (*npt*II), from *Escherichia coli* (*E. coli*) transposon Tn5 (the first-used selectable marker gene in plants), the hygromycin phosphotransferase gene (*hpt*) from *E. coli*, and the phosphinotricin acetyl

transferase gene (*bar*) from *Streptomyces*, which confers resistance to the antibiotics kanamycin, hygromycin, and the herbicide glufosinate, respectively. Since the introduction of biotechnology (BT) crops in 1996, rapid increases in commercialized BT crops have led to concerns that the presence of selectable marker genes in the environment or the food supply might be an unpredictable hazard to the ecosystem or to human health. The main environmental concern with herbicide-resistant genes is that these might be transferred by out-crossing into weeds [2]. There are also some concerns from a health perspective that antibiotic-resistant gene products in transgenic crops might theoretically lead to the spread of resistance *via* intestinal bacteria in human populations, although there is no evidence supporting this proposition.

All transgenic plants are required to undergo thorough and rigorous safety and risk assessments before commercialization. Assessments of transgenic plants include assessment of the selectable marker gene and its products. Marker genes that encode resistance to clinically important antibiotics should not be used in transgenic plants. Based on these considerations, the kanamycin-resistant gene *npt*II used in transgenic crops was approved by the FDA and became the common antibiotic-resistant gene occurring in commercial transgenic crops [3]. There is no scientific basis to argue against the use and presence of selectable marker genes. Regardless of scientific assurances about selectable marker genes, consumer acceptance is ultimately the important element for successful commercialization of transgenic plants and produce. Consumers would naturally be directed towards alleviating perceived problems with selectable marker genes by eliminating them from transgenic crops.

The elimination of the selectable marker gene has several potential merits. The absence of resistance genes in transgenic plants could lower the costs for developing and marketing BT products and might speed up the commercial release of new products [4-6]. Moreover, many of the new transgenic crops such as those with broad pathogen resistance or tolerance to abiotic stress, need the introduction of multiple desired transgenes. Currently, transformation technique limits the number of genes that can be introduced simultaneously. Therefore, development of BT crops with multiple genes may require re-transformation of a single line, sequential introduction of genes into a cultivar, and multiple genes brought together through conventional genetic crossing. These solutions would be time consuming due to long generation time and vegetative propagation of plants. Co-transformation of different markers with each transgene is available for plant transformation, use of markers for transformation selection must be repeatedly optimized empirically, and certain plant species have proven the limited use of some marker genes due to the species' degrees of tolerance to the selective agent even in the absence of the marker gene. The constitutive promoters for efficient expression of marker genes in BT crops could activate gene silencing mechanisms with negative effects on the expression of one or more transgenes of interest. After establishing a transgenic plant, the transgene elimination mechanisms permit the repeated use of a single marker by its removal after each transformation step. A number of research articles have already discussed several methods for producing marker-free transgenic plants. This paper focused on the major strategies, methodologies, and practical approaches developed to generate marker-free transgenic plants.

2. Co-transformation

Multiple copies of T-DNA can be transferred into a plant cell and integrated in the plant genome through biolistic-mediated transformation or *Agrobacterium*-mediated transformation. *Agrobacterium* can also co-transform or integrate two different T-DNAs into a plant cell. The principle of the co-transformation strategy to generate marker-free transgenic plants is the introduction of a selectable marker gene and a gene of interest from different T-DNAs. If the two genes are integrated into unlinked loci, subsequent crossing can separate the gene of interest from the selectable marker gene. The marker-free transgenic plants are yielded at the progeny level. Co-transformation provides unique advantages for the production of transgenic plants. Because of its high frequencies, it allows the simultaneous insertion of a large number of genes into a plant with a limited number of selectable marker genes. For example, Wu *et al.* [7] observed that the frequency of co-transformation in rice using biolistic-mediated transformation to deliver nine different T-DNAs was about 70%. The transgenic cell lines in rice carried at least three genes and 17% (11/66) of the cell lines carried all nine genes. Here, multiple transgenes were integrated into the same locus of the genome *via* biolistic-mediated transformation [7,8]. The high incidence of linkage using biolistic-mediated transformation would allow it to be an alternative transformation method for the manipulation of multiple trait genes. However, it would be impractical for the elimination of marker genes from transgenic plants, because the multiple copies integrated into the same loci of the genome were not segregated in the progeny of T_0 plants.

An advantage of *Agrobacterium*-mediated co-transformation technologies over biolistic-transformation is that co-transformed genes are often integrated into different loci in the plant genome. Thereafter, unlinked selectable marker

genes can be segregated from the genes of interest and allow the production of marker-free transgenic plants [9]. Three approaches were sequentially used for co-transformation with *Agrobacterium*: (i) the introduction of two T-DNAs within different *Agrobacterium* strains (Fig. 1A), (ii) the introduction of two T-DNAs carried by different replicons within the same *Agrobacterium* strain (Fig. 1B), and (iii) the introduction of two T-DNAs located on the same replicon within one *Agrobacterium* strain (Fig. 1C).

Agrobacterium-mediated co-transformation of non-selected genes with selectable marker genes has had relatively high frequencies in early experiments. An [10] showed that tobacco cells could be co-transformed by a single *Agrobacterium* strain containing both a Ti plasmid (phytohormone-independent growth) and a T-DNA binary vector (kanamycin-resistant growth). When the transformed cells were selected with kanamycin, 10 ~ 20% of kanamycin-resistant calli displayed phytohormone-independent growth. Conversely, when the transformed cells were selected with phytohormone, 60% of the resulting calli were kanamycin-resistant. These different frequencies can be explained by the higher copy number (5 ~ 10) of the binary vector in the bacterium relative to the single-copy Ti plasmid. De Frammond *et al*. [11] expanded on these experiments. They transformed tobacco tissue with one *Agrobacterium* strain, C58 that contained T-DNA from a Ti plasmid and from a micro-Ti. The two T-DNAs of the co-transformed line were segregated in progeny plants, indicating that the T-DNAs had integrated into genetically separable loci. In another single-strain approach utilizing *Agrobacterium tumefaciens* containing an *npt*II gene and a GUS gene on two different binary vector plasmids with compatible replicons [12], the frequency of co-transformation was 62% (21 GUS+/34 kanamycin-resistant lines) for rapeseed and 52% (52 GUS+/100 kanamycin-resistant lines) for tobacco. Of the co-transformed plants that expressed only one of the transgenes, self-pollinating progeny, were observed for 40% of the rapeseed and 58% of the tobacco. Similarly, when Depicker *et al*. [13] co-transformed tobacco protoplasts with a mixture of *Agrobacterium* strains to deliver different T-DNA, the frequency of co-transformed calli was nearly identical to the frequency of single transformation events, indicating that co-transformation was the result of independent transformation events. However, co-transformation of hypocotyl explants of rapeseed (*Brassica napus*) with mixtures of *Agrobacterium tumefaciens* strains to deliver different T-DNAs results in a higher occurrence of two independent transformation events than expected, indicating that variations in plants and strains were able alter linkage relationships and co-transformation frequencies [14].

Komari *et al*. [15] produced tobacco and rice co-transformants with *Agrobacterium tumefaciens* LBA4404 carrying two T-DNAs from the same replicon. They demonstrated that single-strain approach utilizing an *Agrobacterium* strain carrying one binary plasmid that contained a selectable marker gene and a gene of interest on different T-DNAs yielded higher co-transformation frequencies (about 50%) than results of mixtures of *Agrobacterium tumefaciens* strains carrying separate vectors. A wide range of variation can be found in frequencies of co-transformation. It has been reported that the relative size of the co-transforming T-DNA has a major impact [16], and that co-transformation frequency with an octopine-derived strain carrying a binary vector with T-DNAs was dramatically higher (93%) than with mixed *Agrobacterium* strains in maize (11.7%) [17].

In co-transformation strategies for marker-free plants, the selectable marker genes have to be eliminated in the progeny of co-transformants by crossing. The integration of two different T-DNAs into unlinked loci is needed for this segregation. However, despite the high co-transformation frequencies in various plants, De Neve *et al*. [18] showed that the ligation of co-transformed T-DNAs frequently occurred before integration and that the two distinct T-DNAs were mainly integrated at the same genomic locus. The frequency of linked T-DNAs can be influenced by the type of *Agrobacterium* strain used in co-transformation procedure [19,20]. The nopaline-derived *Agrobacterium* strains favor the insertion of linked T-DNAs, while the octopine-derived *Agrobacterium* strains favor integration at unlinked loci. McKnight *et al*. [21] co-transformed tobacco cells with two separate octopine-derived *Agrobacterium tumefaciens* strains, each carrying a different T-DNA on an identical binary vector plasmid. In this work, although the co-transformation frequencies were relatively low, the progeny of self-pollinating co-transformed tobacco showed the independent segregation indicating that the T-DNAs were genetically unlinked. In contrast, De Neve *et al*. [18] found at least one linked copy of two different T-DNAs in 72% of co-transformed plants using octopine-derived strains. In that report, authors inferred that the copy number of the T-DNA replicon, the physiology and competence of the plant tissue, and the number of bacteria per plant cell all constitute as main determinants influencing the frequency of linked T-DNAs. Although the frequency of linked loci could be decreased by lowering the number of transferred T-DNAs that would result in a reduction in the transformation frequency [22].

The co-transformation strategy has demonstrated that marker-free plants can be generated using *Agrobacterium*-mediated co-transformation followed by segregation of the genes in the subsequent sexual generation. However, this strategy is not suitable for all plant species and its efficiency is clearly dependent on a number of variables

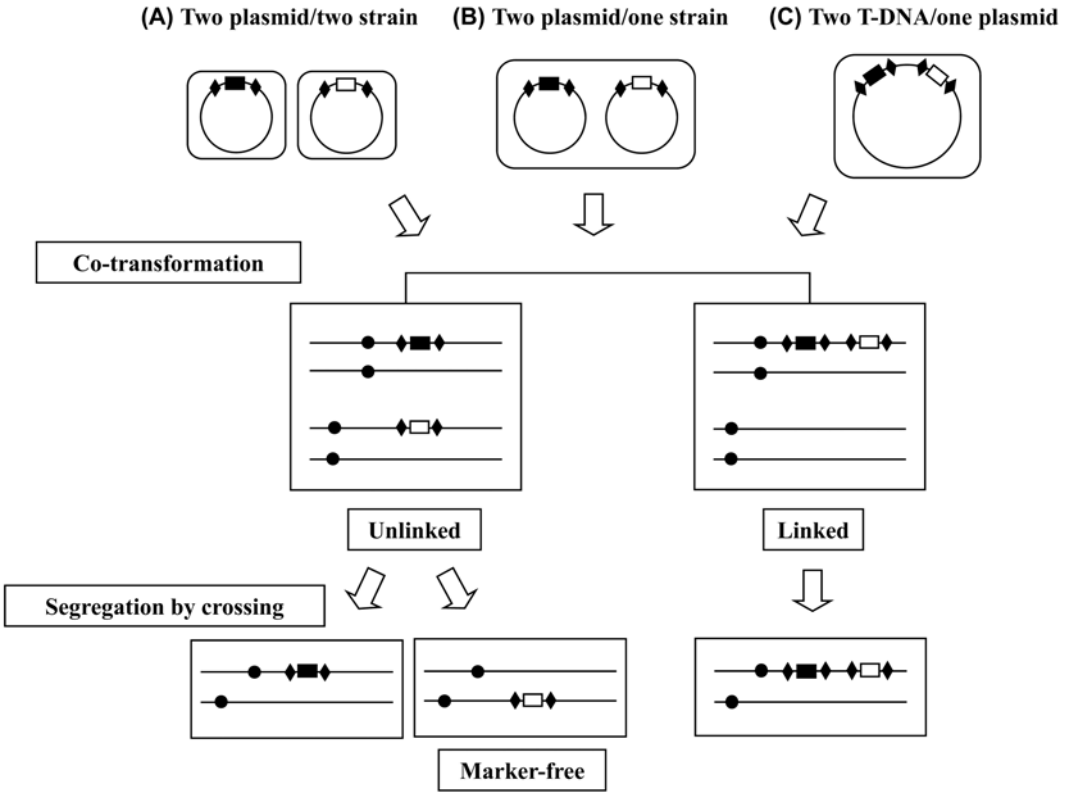

Fig. 1. Generation of marker free transgenic plants by the co-transformation method. ■ selectable marker gene, □ gene of interest, ◆ border sequence of T-DNA.

[9]. Furthermore, when compared to methods that produce plants in which the marker gene is linked to the gene of interest, this method requires about a four-fold greater production of transgenic lines to recover a comparable number of marker-free plants [12].

3. Transposon-mediated Approaches

In the maize *Ac*/*Ds* transposable element system, the non-autonomous *Ds* element can excise itself from its location and transpose to other sites within a genome only in the presence of the autonomous *Ac* element [23]. Many maize-transposable elements can maintain their transposition competence when transformed into other plant species [24,25]. Transposable elements can also be used to produce marker-free transgenic plants. The use of transposable elements for marker gene removal involves *Agrobacterium*-mediated transformation. Two types of vector systems have been developed for selectable marker gene elimination. The first type of vector system uses relocation *via* the *Ac*/*Ds* system (Fig. 2A) and the second uses excision *via Ac*/*Ds* system for production of marker-free transgenic plants (Fig. 2B). In the first type, the gene of interest is inserted between the *Ds* inverted repeats to facilitate the physical separation from the selectable marker by transposition to a new locus of the chromosome after transformation. If the new locus by transposon-mediated relocation is sufficiently distant from the original site, the marker-free progeny with the transgene can be produced *via* self-pollinating or out-crossing. Goldsbrough *et al*. [26] transformed tomato plants with *Agrobacterium* containing binary vector composed of a *gus* gene flanked by the *Ds*-inverted repeat sequences, an *Ac* transposase gene, and an *npt*II marker gene. After two transformants containing one or two copies of T-DNA were produced, they self-crossed to produce progeny in which the T-DNA insert and transposed chimeric *Ds*/*gus* element segregated independently. The segregation frequencies of identified progeny containing the *Ds*/*gus* element but not an *npt*II gene were 2.3% (2/87) in a primary transformant with one T-DNA insert and 6.6% (7/106) in a primary transformant with two T-DNA inserts. The different frequencies for the progeny from the two T-DNA transformant and the single T-DNA transformant were probably a consequence of a higher level of transposase activity in the transformant with two T-DNA inserts. These results indicate that the *Ac*/*Ds* system is capable of relocating a gene of interest and producing marker-free transgenic plants. This change in the expression pattern reflecting the genomic location is referred to as a 'position

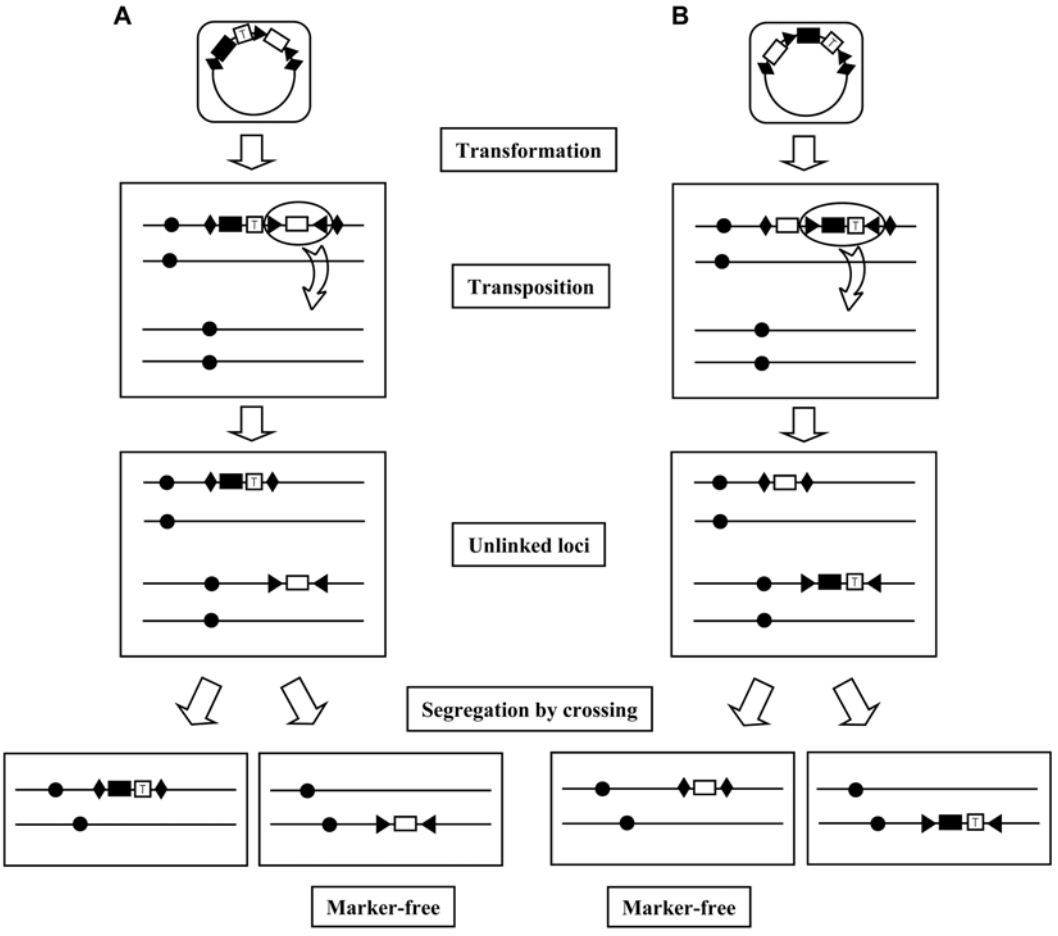

Fig. 2. Removal of a selectable marker gene by the *Ac*/*Ds* system. (A) *Ds*-flanked gene of interest is joined to the selectable marker and *Ac* transposase gene. After transposition of the gene of interest into an unlinked locus, it is segregated from the selectable marker gene by sexual crossing. (B) *Ds*-flanked genes of selectable marker and *Ac* transposase is joined to the interesting gene. After transposition of the genes of the selectable and transposase into unlinked locus, that can be eliminated from the interesting gene by sexual crossing. ■: selectable marker gene, □: gene of interest, ◆: border sequence of T-DNA, and ▶: Ds inverted repeat, square with T: transposase.

effect' [27]. The advantages of this type of vector system are that marker-free transformants can be produced from primary transformant containing multiple T-DNA inserts and that marker-free transgenic progeny by relocation can achieve different levels of expression of a gene of interest. In the second type of vector system, the selectable marker gene and the *Ac* element gene are inserted between the *Ds* inverted repeats. The *Ds* element with a marker gene will be reinserted in a location unlinked to the primary site in primary transformants. Consequently, the selectable marker gene in the transgenic plant containing the gene of interest can be removed in progeny *via* self-pollinating or outcrossing. Ebinuma *et al*. [28] demonstrated the feasibility of this strategy by eliminating the isopentenyl transferase (*ipt*) marker gene in plants.

In this research-generated *ipt*-type MAT (multi-auto-transformation) vector system, the chimeric *ipt* gene with a 35S promoter is inserted into the *Ac*-transposable element to remove it from transgenic cells after transformation, while a β-glucuronidase gene (*gusA*) is located outside of the *Ac* element. The *ipt* gene affects cytokinin metabolism by catalyzing the condensation of isopentyl pyrophosphate with adenosine monophosphate (AMP) to produce isopentyl AMP, a precursor of several cytokinins [29,30]. Cytokinins stimulate organogenesis in many cultured plant tissues and are widely used to regenerate transgenic plants from cultured cells after transformation. Of 100 adventitious tobacco shoots regenerated in a hormone-free kanamycin medium, 63 lines exhibited the *ipt*-shooty phenotype that had lost apical dominance and rooting ability due to the overproduction of cytokinin. Within six months after infection, several normal shoots with normal apical dominance appeared from three of the 63 *ipt*-shooty lines (4.8%). However, when the *ipt* gene was introduced into tobacco plants, transgenic plants developed 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% [28]. A significant advantage of this *ipt*-strategy is that marker-free transgenic plants can be selected at the T_0 generation, obviating the need to sexually crop plants. The strategy can therefore be applicable to vegetative propagating crops (*e.g.* potato, grape) and plants with long reproductive cycles (*e.g.* forest trees). However, the co-transformation frequencies with the *ipt*-type *Ac*/*Ds* system have generally been as low as those of previous results with the transposable element system.

4. Site-specific Recombination

Site-specific recombination systems are common in prokaryotes and lower in eukaryotes. Natural functions of such systems include integration of a bacteriophage into the host genome, maintenance of the copy number, and switching of the host range. Site-specific recombinases are enzymes that recognize short DNA sequences, and in the presence of two recombination sites, they catalyze the recombination of DNA strands. With some of these recombinases, the orientation of the recognition sites relative to each other (direct or inverted) affects whether the recombination results in the intervening DNA strand is either excised or inverted. Some site-specific recombination systems do not require other factors for their function. Thus, they represent relatively simple systems that are capable of functioning accurately within heterologous systems. Several kinds of site-specific recombination systems have been shown to function in plants: the Cre/*lox* from *Escherichia coli* phage P1 (where the Cre recombinase recognizes *lox* sites), the FLP/*FRT* from the 2- μm plasmid of *Saccharomyces cerevisiae* (where the FLP recombinase acts on the FLP recombination target, *FRT* sites), the R/*RS* from *Zygosaccharomyces rouxii* (where R and *RS* are the recombinase and recombinase site, respectively), a modified Gin/gix from the enteric bacteriophage Mu.

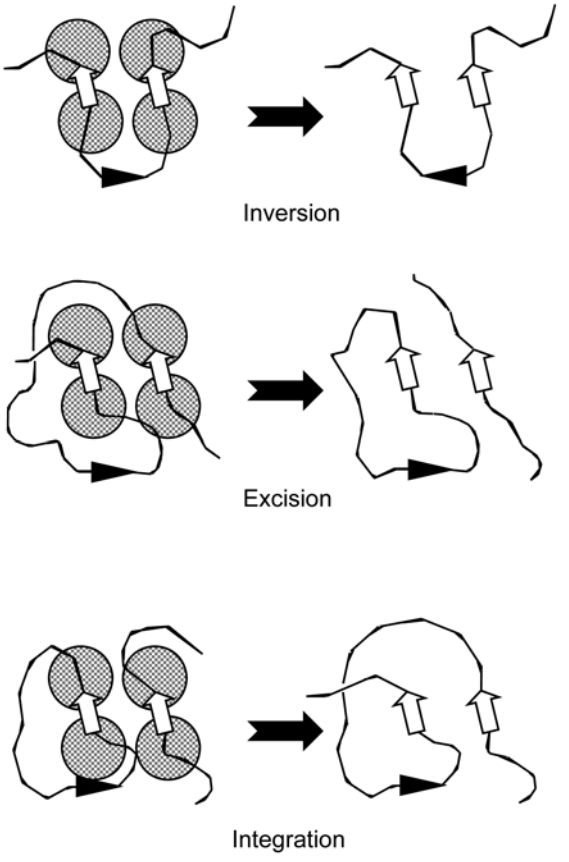

Fig. 3. Diagram depicting recombinase-mediated rearrangements catalyzed by site-specific recombination system. Depending on the orientation and location of the recombination sites, excision, inversion, or integration of DNA fragment occurs.

The recombination sites of simple two-component systems such as FLP/*FRT* and Cre/*lox* are similar in that short oligonucleotides surrounded by short inverted repeats determine the orientation of the recombination site. Recombinase-mediated rearrangements can be determined by the orientation of recombination sites relative to each other. A direct orientation of the two recombination sites in one DNA molecule leads to intramolecular recombination or excision, leaving behind a recombination site in the parent molecule and a circularized DNA fragment containing the other recombination site (Fig. 3). When the recombination sites are located on two DNA molecules, this produces an intermolecular recombination or integration. When a pair of recombination sites remains on each of two DNA molecules, the double recombination events lead to exchange of DNA fragment delimited by recombination sites.

4.1. Cre/lox system

The Cre/*lox* system consists of the 38.5 kDa Cre recombinase and the 34-bp *lox* site, which includes two 13-bp inverted repeats flanking an 8-bp core (Fig. 4). The wild-type lox site is called *lox*P. Cre binds to inverted repeats of 13-bp in the *lox* site and catalyzes a crossover in the 8-bp spacer regions of two lox sites. The Cre/*lox* system has been the most extensively used in the generation of marker-free plants. Dale and Ow [31] reported the use of the Cre/*lox*P system to eliminate the marker gene from transgenic tobacco plants. The T-DNA vector carrying a P35S-luciferase (*luc*) gene was constructed with *lox*P sites flanking the P35S-*hpt* selectable marker gene and inserted into tobacco. The *Cre* recombinase gene with an *npt*II gene was then introduced by a second round of transformation to achieve precise excision of the marker gene. The Kan^R Luc^+ transgenic plants were screened for hygromycin-

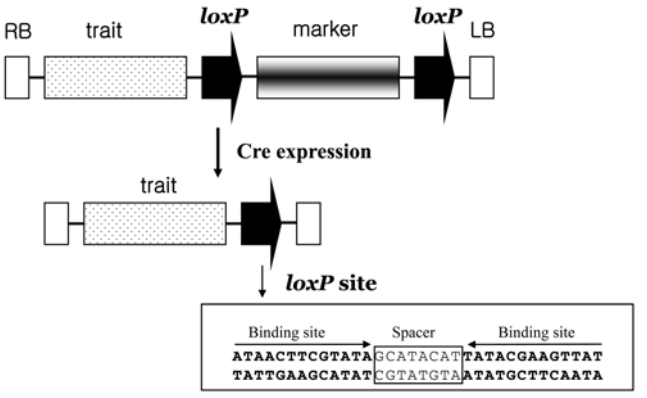

Fig. 4. Basic strategy using Cre-mediated site-specific recombination to marker gene removal and nucleotide sequences of *lox*P site.

resistant phenotypes. Of the 11 of kanamycin-resistant plants, ten (91%) were found susceptible to hygromycin due to Cre-mediated recombination between the *lox*P sites and excision of the *hpt* gene from the genome. Two Luc^+ Hyg^S transgenic plants were allowed to self-pollinate to achieve segregation of the *luc* gene from the *cre* gene locus. Of the approximately 100 T_2 germinated seedlings, about 1/4 (25%) showed luciferase expression and the absence of both the *npt*II and *hpt* marker genes. This was subsequently confirmed with other plants and other marker genes. Gleave *et al*. [32] demonstrated that transient expression of Cre, combined with a conditional negative marker, could be used to excise a marker gene in the absence of sexual crosses. To avoid integration of the marker gene when using the re-transformated T-DNA vector for Cre expression, they produced T-DNA vector carrying a P35S-*gus* gene linked to *lox*P-flanked *npt*II and cytosine deaminase (*cod*A) genes. The leaf discs of transformed lines were co-cultivated with *Agrobacterium* that express the Cre recombinase. The shoots were regenerated in the absence of antibiotics. Of 773 shoots, 19 (0.25%) showed tolerance to 5-fluorocytosine (5-fc) which was converted to the toxic 5-fluorouracil by cytosine deaminase. The absence of selectable marker genes was confirmed by the phenotype of the T_1 progeny.

The simple strategies to generate marker-free plants *via* site-specific recombination systems (*e.g.*, Cre/*lox*) are re-transformation or crossing of the recombinase gene into transgenic plants in which the selectable marker gene is flanked by recombination sites. However, these strategies require at least one instance of sexual crossing due to elimination of the recombinase-linked selectable marker gene. This is somewhat problematic for plants which are vegetatively propagated or have long reproductive cycles. An alternative that obviates the crossing is an auto-excision strategy for marker-gene removal. Here, the recombinase gene is part of the same insert DNA as the marker gene, and both genes are flanked by a single pair of recombination sites. Expression of the recombinase gene can be controlled by a tissue-specific or chemically inducible promoter that drives auto-excision at fixed stages of the transformation process or plant development. Zuo *et al*. [33] used a chemically inducible promoter to perform auto-excision in *Arabidopsis*. In this system, the gene of interest was separated from its promoter by a fragment containing the genes coding for the XVE transcription factor, the *npt*II selectable marker, and the Cre recombinase (under the control of the inducible promoter) surrounded by *lox* sites. Transformation of *Arabidopsis* was achieved by selection for kanamycin resistance. Subsequent induction of the Cre recombinase with β-estradiol resulted in the excision of the *cre* gene and selectable marker genes. The final product was the reconstituted gene of interest, in this case GFP. In *Arabidopsis*, excision occurred in all of the plants with high efficiency in the germline cells (29 ~ 66%) using a single transformation. In rice and tomato the same induction system showed 30 and 15% efficiencies, respectively [34,35]. Some transformants demonstrated incomplete removal of multicopy loci. Application of heat shock inducible Cre/*lox* systems in tobacco and potato resulted in 30 ~ 80% and 4.7% excision rates, respectively [36,37]. In recent publications it was shown that the Cre recombinase in this self-excision unit can be activated by developmentally regulated promoters in different tissues such as microspores, inflorescences and seeds [38-40].

4.2. FLP/FRT system

The FLP enzyme is a 48-kDa protein that covalently binds as four monomeric units to two *FRT* sites and catalyzes the cleavage and ligation of these sites. FLP mediated DNA excision or inversion occurs depending on the orientation of the *FRT* sites. The *FRT* site consists of three repeated DNA sequences of 13 bp each; two repeats in a direct orientation and one repeat inverted relative to the other two [41]. In addition, there is an 8-bp spacer region between the repeats that determines the overall orientation of the *FRT* recombination site. The FLP/*FRT* was first reported to function in rice and maize cells by Lyznik *et al*. [42] and then in tobacco [43] and in *Arabidopsis* [44,45]. Interestingly, Luo *et al*. [46] demonstrated that the *loxP-FRT* fusion sequences, as recognition sites, dramatically enhance the FLP- or Cre-mediated excision efficiency. They constructed a new binary vector, named the 'genetically modified (GM)-gene-deletor', to excise a selectable marker gene from transgenic plants. In this vector system, *loxP-FRT* fusion sequences were used as recognition sites and simultaneous expression of both FLP and Cre reduced the average excision efficiency. However, the expression of

FLP or Cre alone increased the average excision efficiency, with many transgenic events being 100% efficient based on more than 25,000 T_1 progeny examined per event. Recently, the oxidative stress-inducible peroxidase gene promoter from the sweet potato was used to control the expression of the *FLP* gene. The oxidative stress-inducible auto-excision system was able to eliminate the selection marker with chemical (hydrogen peroxide) treatment or without treatment, by spontaneous induced stress, in the callus stage of plant transformation [47].

4.3. R/RS system

The R/*RS* system is a site-specific recombination system that has been isolated from the circular plasmid pSR1 of *Zygosaccharomyces rouxii* [48]. The 56-kDa R recombinase excises the DNA fragment between two directly adjacent *RS* recognition sites comprised of 12 bp inverted repeats on either side of a 7 bp asymmetric core. Onouchi *et al*. [49,50] established that the R/*RS* system was functional in tobacco and *Arabidopsis*. The R/*RS* system has been used with MAT vectors as an alternative to the *Ac* transposase-mediated transposition of the genes as described above. Ebinuma and Komamine [51] reported a single-step transformation method for generating marker-free transgenic rice plants using the *ipt*-type MAT vector. The MAT vector consisted of *npt*II, *gus*, and *hpt* genes outside of the *RS*-flanked 35S-*ipt*, 35S-*R*, and *gfp* genes. Several shoots regenerated from 29 (34.1%) of 85 infected tissues after four weeks. The regenerated shoots from 18 (75.0%) of 24 tissues contained the *gus* gene but had lost the *ipt* gene and the other shoots had neither gene. The results indicated that marker-free transgenic rice plants had directly regenerated from 25.5% of the infected scutellum tissues without forming *ipt*-shooty intermediates.

5. Intra-chromosomal Homologous Recombination for Sequence Excision

Homologous recombination (HR) involves the exchange of genetic information between highly similar DNA sequences. Studies have been done on the use of HR in plants to eliminate selectable marker genes after insertion. Although intra-chromosomal recombination (ICR) can be enhanced up to nine-fold by genotoxic stress factors such as irradiation (*e.g.*, ultraviolet light or X-rays) or radio-mimicking chemicals (*e.g.*, methyl methanesulfonate), the basal frequency in plants is low, varying between 10^{-5} and 10^{-6} of cells containing a recombination cassette [52,53].

Zubko *et al*. [54] reported the development of a transformation system that uses intrachromosomal homologous recombination between two *att*P sites to remove a selectable marker gene without the two additional proteins, integrase (int) and integration host factor (IHF). They constructed binary vectors in which the oryzacystatin-I gene and the transformation booster sequence (TBS), which enhances homologous and illegitimate recombination [55], are linked to the *att*P-flanked *gfp*, *tms*2, and *npt*II genes. The negative marker gene, *tms*2, is used to identify deletion events in the presence of naphthalene acetamide (NAM). The construct was introduced into tobacco plants by *Agrobacterium*-mediated transformation, and 11 kanamycin-resistant tobacco calli were obtained. After their cultivation in the absence of kanamycin, a mixture of green and white shoots was induced from two (18.2%) of 11 clones on the kanamycin-containing shoot-regeneration medium. Of 52 white shoots, 23 (44.2%) showed the ability to form roots by NAM selection. This implies excision of the tms2 gene. PCR analysis confirmed that only three (13.0%) of the 23 transgenic plants had lost the *tms*2 and *npt*II genes containing the oryzacystatin-I gene. These results indicate that excision had occurred beyond the *att*P sequences and that marker-free transgenic plants can be produced by intrachromosomal recombination. However, the overall efficiency of this process appears to be low, and many deletions in genes of interest occur through illegitimate recombination [54]. Furthermore, the success of this approach in tobacco was not dependent on the expression of the λ proteins int and IHF normally required for recombination at these sites. Therefore, the mechanistic basis of the phenomenon is not yet understood, and it is not yet known how applicable the system may be in other plants [56].

6. Non-selected Transformation

A less simple approach developed for generation of marker-free transgenic plants is to select transformants without the use of marker genes. In initial research, marker-free transgenic plants were obtained using a non-selected approach in vegetative propagating crop potato and tobacco [57,58]. In these studies, explants were infected with *A. tumefaciens* harboring a binary vector without a selectable marker gene, either by soaking in the *Agrobacterium* solution for a few minutes as in potato or by agroinfiltration as in tobacco. The regenerated shoots were analyzed by polymerase chain reaction (PCR) for the presence of the transgene, and PCR-positive shoots were identified. De Vetten *et al*. [57] reported that, for the potato variety Karnico, transgenic lines were obtained using the *A. tumefaciens* strain AGL0 with an average frequency of 4.5% (228/5027). However, undesirable vector backbone sequences were transferred along with the gene of interest in 60 out of the 99 trans-

genic lines, and only ten vector-free lines contained a single T-DNA copy. Moreover, these analyses were only performed on the T_0 generation, and the study did not provide evidence on stable transmission of the transgene into progeny, a critical step for confirming the stable integration of a foreign gene. Li *et al*. [59] analyzed the transgene inheritance for the selectable marker-free transgenic tobacco plants produced without selection by *A. tumefaciens*-mediated transformation. Twenty-five to 75% of the transformants were able to transmit transgene activity to the T_1 generation in a Mendelian 3:1 ratio, and a transformation efficiency of 2.2 ~ 2.8% was achieved.

In similar experiments using non-selected transformation methods for producing transgenic plants, the efficiency obtained from these studies has been very low where the frequency of marker-free transgenic plants was 1.55% in wheat and 0.93% in triticale [60]. Recently, marker-free peanut transgenic plants were developed with 75% transformation efficiency using a binary vector pCAMBIA2300 devoid of selection marker gene [61], but most evidence was from PCR only, requiring confirmation of this promising result. In another studies, transgenic apples and alfalfa by transforming without any selective marker gene were obtained high efficiently. However, some transgenic plants appeared to be chimeric as shown by nonuniform GUS staining of the tissues or lower segregation rations than the Mendelian 3:1 ratio expected [62,63]. Non-selected transformation method is capable to generate marker-free plants, but screening for selection of transformed plants is very laborious and time-consuming. This method should be much improved to be practically application for efficiently transformation.

7. Conclusion

A large number of transgenic plants with desired agricultural traits will become the main consumer preferences in the near future. People are mainly concerned with environmental impact and have concerns about the widespread release of organisms expressing genes that confer resistance to antibiotics or herbicides. The selection process for separating transformed cells from the majority of non-transformed cells because of low transformation efficiency is the essential step in transforming the transgene into a genome. A selectable marker gene is co-introduced with the gene of interest to identify those cells that have integrated the DNA into their genome. Despite the large number of different selection systems, marker genes that confer resistance to the antibiotics hygromycin (*hpt*) and kanamycin (*nptII*) or the herbicide phosphinothricin (*bar*) have been used in most plant research and crop development techniques.

The European Union suggests avoiding the use of selectable markers in genetically engineered crops, and the ultimate goal is to introduce as few foreign sequences as possible, along with the gene of interest. Moreover, the generation of marker-free transgenic plants has not only to respond to public concerns over the safety of biotechnology (BT) crops, but also to support multiple transformation cycles for transgene pyramiding. It seems highly likely that continued work in this area will soon remove the question of unwanted marker genes from the debate concerning the public acceptability of transgenic crop plants. The techniques that have been under development for marker gene removal will also facilitate more precise and subtle engineering of the plant genome, with widespread applications in both fundamental research and biotechnology. In addition to allaying public concerns, the absence of resistance genes in transgenic plants could also reduce the costs of developing BT products and lessen the need for time-consuming safety evaluations, thereby speeding up the commercial release of new products.

A lot of research effort has been directed towards the development of marker-free transformation methods and selectable-marker elimination strategies. Site-specific recombination systems have been used successfully to excise marker genes in plants, including the economically important crops rice and maize [64,65]. Rice plants were transformed with insecticidal genes using Cre/*lox* in excision of selectable marker gene [66]. The marker-free transgenic technology based on the CLX system has been applied in developing rice and tomato. The MAT system has also been used in developing rice and tobacco [51,64,67] and cup flowers and oranges [68,69]. The auto-excision system, mediated by the oxidative stress-inducible FLP/*FRT* system for eliminating a selectable marker gene, has been adapted for efficient generation of marker-free transgenic tobacco plants [47,70]. The biotechnology crops based on marker-free technologies have been actively developed and recently commercialized. The maize biotechnology crops, MON89034 and LY038 were developed by removing *nptII* antibiotics using marker-free technology in the progeny of transgenic plants after gene transformation. In the case of MON89034, a co-transformation system was used that transformed two vectors at the same time and the marker was separated in the segregating generation by selfing. In LY038, the selection marker was eliminated by the Cre/*lox* system which was based on site-specific recombinase.

MON89034 was developed by *Agrobacterium*-mediated transformation of corn with PV-ZMIR245, a binary vector containing two T-DNAs. The first T-DNA, designated as T-DNA I, contains the *cry 1A.105* and the *cry2ab2* expression cassettes. The second T-DNA, designated as T-DNA

II, contains the *nptII* expression cassettes. During transformation, both T-DNAs were inserted into the genome. The *nptII* was used as the selectable marker needed for selection of the transformed cells. Once the transgenic cells were identified, the selectable marker gene was no longer needed. Therefore, traditional breeding was used to isolate plants only containing the *cry 1A.105* and the *cry2ab2* expression cassettes (T-DNA I) and not containing the *nptII* expression cassette (T-DNA II), thereby to produce marker-free corn MON89034 [71].

Corn LY038 was produced by a particle acceleration methodology, with a plasmid vector PV-ZMPQ76, which contained the *cordapA* gene as well as the *nptII* gene and was used as a selectable marker. Positive plants underwent a series of conventional crosses. Although the original transformants contained the *nptII* gene, it was subsequently removed from the plant genome using the Cre/*lox* site-specific recombination system [65]. Health Canada's review of the information presented in support of the food use of corn lines containing event LY038 concluded that it does not raise concerns related to safety [72]. Health Canada is of the opinion that LY038 is similar to non-transgenic parental strains of corn in acceptability as a food source. The marker-free transgenic technology would accelerate the commercialization of agricultural products using biotechnology crops. It would simplify the commercialization processes for biotechnology crops, reduce the cost of development and marketing, and eventually meet consumer preferences. Although the initial cost of introducing a marker-free mechanism may be high, the long-term benefit of such a technology would be sufficient to justify commercialization.

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