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Review

Techniques for the removal of marker genes from transgenic plants

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Abstract

The presence of marker genes encoding antibiotic or herbicide resistances in genetically modified plants poses a number of problems. Various techniques are under development for the removal of unwanted marker genes, while leaving required transgenes in place. The aim of this brief review is to describe the principal methods used for marker gene removal, concentrating on the most recent and promising innovations in this technology.

Keywords: Marker-gene; Antibiotic; Herbicide; Genetically modified organism

1. Introduction

The addition of genes conferring desired traits to plants also requires the inclusion of marker genes that enable the selection of transformed plant cells and tissues. These selectable markers are conditionally dominant genes that confer the ability to grow in the presence of applied selective agents that are toxic to plant cells, or inhibitory to plant growth, such as antibiotics and herbicides. Following transformation, the continued presence of marker genes in genetically modified plants usually becomes unnecessary and may also be undesirable. Herbicide resistance marker genes in transgenic crop plants, for example, could escape to wild relatives of the crop through the transfer of pollen, potentially leading to the spread of herbicide resistance in wild plant populations [1,2]. The presence of antibiotic resistance markers in transgenic plants intended for human or animal consumption may also be a cause for concern. Fears have been expressed that such genes may be transferred horizontally to microorganisms of the gut flora of man or animals and lead to the spread of antibiotic resistances in pathogenic microorganisms. Though extensive studies have failed to detect a quantifiable risk of this occurrence [3], many biotechnologists view the negative publicity related to the presence of unnecessary marker genes as sufficient reason to warrant their removal.

In addition to environmental and health concerns, there are also practical reasons for the removal of unnecessary marker genes from plants. Both in fundamental and applied research, there is frequently a need to add two or more transgenes to the same plant line. One method for the serial transformation of plants involves the use of two or more different selectable markers. However, the number of marker genes available is limited and not all of these are well adapted to all transformable plant species. The combination of several nuclear transgenes can also be achieved through sexual crosses following the transformation of independent plant lines. However, this is not possible in plants that must be propagated by vegetative means. These include non-sexually reproducing plants and highly heterozygous varieties whose genetic backgrounds would be greatly changed by sexual reproduction. Examples of such vegetatively propagated plants include: apple, hybrid aspen, banana, cassava, eucalyptus, grapevine, potato and strawberries [4]. In addition, the combination of transgenes by sexual crosses may be slow, particularly in trees species. The possibility of removing unwanted marker genes following plant transformation allows the same marker to be used for the sequential addition of further transgenes. A second practical reason for the removal of marker genes relates to the greater possibility of instability of transgene expression if several homologous marker gene

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Table 1
Marker genes and selective agents used for plant transformation. [cp] Can be used for chloroplast transformation [26]

Gene	Gene product	Selective agents	Gene sources	References
aadA[cp]	Aminoglycoside-3-adenyltransferase	Streptomycin, spectinomycin	*Shigella flexneri*	[9]
accC3/accC4	Gentamycin-3-N-acetyltransferase	Gentamycin	*Serratia marcescens*, *Klebsiella pneumoniae*	[33]
AK	Aspartate kinase	High concentration lysine and threonine	*E. coli*	[33]
als	Acetolactate synthase	Sulfonyl ureas, imidazolinones, thiazolopyrimidines	*Arabidopsis thaliana*, *Nicotiana tabacum*	[9]
BADH[cp]	Betaine aldehyde dehydrogenase	Betaine aldehyde	*Spinacea oleracea*	[9]
bar[cp]	Phosphinothricin acetyltransferase	Glufosinolate, L-phosphinthrin, bialaphos	*Streptomyces hygroscopicus*	[9]
bla	β-Lactamase	Penicillin, ampicillin	*E. coli*	[9]
Ble	Bleomycin resistance protein	Bleomycin, phleomycin	*E. coli* TN5, *Streptoalloteichus hindustanus*	[33]
bxn	Bromoxynil nitrilase	Bromoxynil	*Klebsiella pneumoniae var. iozaenae*	[9]
cat[cp]	Chloramphenicol acetyltransferase	Chloramphenicol	Bacteriophage P1 Cm [R]	[9]
dhfr	Dihydrofolate reductase	Methotrexate	Plasmid R67	[33]
DHPS	Dihydrodipicolinate sythase	S-aminethyl L-cysteine	*E. coli*	[33]
epsps/aroA[cp]	5-Enoylpyruvate shikimate-3-phosphate	Glyphosate	*Agrobacterium* CP4, maize, *Petunia hybrida*	[9]
gox	Glyphosate oxidoreductase	Glyphosate	*Achromobacter* LBAA	[9]
hpt	Hygromycin phosphotransferase	Hygomycin B	*E. coli*	[9]
manA	Phosphomannose isomerase	Mannose-6-phosphate	*E. coli*	[9]
nptII[cp]	Neomycin phosphtransferase II	Kanamycin, neomycin, geneticin (G418), paromommycin, amikacin	*E. coli* Tn5	[9]
nptIII	Neomycin phosphotransferase III	Kanamycin, neomycin, geneticin (G418), paromommycin, amikacin	*Streptococcus faecalis* R plasmid	[9]
pat[cp]	Phosphinothricin acetyltransferase	Glufosinolate, L-phosphinthrin, bialaphos	*Streptomyces viridochromogenes*	[9]
SPT	Streptomycin phosphotransferase	Streptomycin	*E. coli* Tn5	[33]
sul	Dihydropteroate synthase	Sulfonamide	Plasmid R46	[33]
TDC	Tryptophan decarboxylase	4-Methyltryptophan	*Catharanthus roseus*	[9]
tfdA	2,4-D Monooxygenase	2,4-Dichlorophenoxyacetic acid	*Alcaligenes eutrophus*	[33]
uidA/GUS[cp]	β-Glucuronidase	Cytokinin glucuronides	*E. coli*	[9]
xylA	Xylulose isomerase	D-Xylose	*Thermoanaerobacterium thermosulfurogenes*	[9]

copies are present in the same plant [5]. Multiple copies of marker genes could potentially lead to the silencing of the required transgenes through homology-dependent gene silencing mechanisms.

The problems associated with the presence of marker genes in transgenic plants have been known for quite some time and various studies over the last decade have demonstrated methods for the removal of these, while leaving the desired transgenes in place. All of the methods published up until recently have suffered from various drawbacks limiting their efficiency or widespread applicability. Recently, however, a number of studies have presented methods that seem to offer advantages over earlier techniques. These include methods for the removal of nuclear marker genes by intrachromosomal recombination, or using inducible heterologous recombinases, in addition to novel methods for the removal of chloroplast marker genes. The aim of this brief review is to describe and compare the different techniques that have been tested for the removal of marker genes from transgenic plants, concentrating particularly on the more recent and promising innovations in the field.

2. Selectable marker genes used for plant transformation

Approximately 25 marker genes, mostly conferring resistance to antibiotics or herbicides, have been successfully used for plant transformation (Table 1). In addition, a number of so-called marker gene-free approaches to plant transformation have been developed [6]. Selection of transformed tissues in these systems is based on genes that confer the ability to proliferate or differentiate in the absence of some otherwise essential factor, such as a necessary exogenous plant hormone used in tissue culture. The gene that has so far been most widely used in such an approach is the *ipt* gene from the Ti plasmid of *Agrobacterium tumefaciens*, encoding the enzyme isopentyl transferase [4,7]. This enzyme cata-

lyzes the synthesis of isopentyl AMP, a precursor of cytokinins. The excessive level of cytokinins produced in plant tissues constitutively expressing the *ipt* gene leads to a proliferation of these tissues on hormone-free media. Plant tissues over-expressing the *ipt* gene exhibit an "extreme shooty phenotype" characterized by a loss of apical dominance and an inability to produce roots. The removal of the *ipt* gene can be accomplished using one of the forms of technology for marker gene removal discussed in this review. Recently, *ipt* genes of plant origin that also produce elevated cytokinin levels when over-expressed have been identified through activation tagging screens [8].

A further type of gene that can be specifically of use in advanced strategies for marker gene elimination acts as a dominant negative selective marker [9]. The proteins encoded by such genes act to inhibit the growth of plant tissues in the presence of appropriate selective agents. Under selective conditions, these dominant negative markers may be used to identify plant tissues that have lost their marker genes through a recombination event brought about by one of the techniques discussed in this review. The *tms2* gene of *Agrobacterium tumefaciens*, e.g., has been used as such a dominant negative marker [10]. This gene encodes the enzyme indolacetamide amidohydrolase that converts napthaline acetemide (NAM) into the auxin NAA. Plants expressing *tms2* are unable to root on media containing NAM due to elevated levels of auxin.

3. The removal of marker genes from the plant nuclear genome

3.1. Simple microbial recombinase-based systems

One of the earliest techniques tested for marker gene removal involved the heterologous expression of microbial recombinase enzymes in plants to excise marker transgenes that were flanked by microbial recombination sequences. The general method employed for this is illustrated in Fig. 1A . For example, the Cre recombinase enzyme of bacteriophage P1 has been used to excise marker genes cloned between pairs of 34 bp directly repeated *loxP* recombination sites [11]. Such excision events are precise and leave one *loxP* site in place. Other microbial recombinase enzymes that have been similarly used to remove marker genes from transformed plants include the yeast FLP and R recombinases [9]. The FLP recombinase, encoded by a gene of the *Saccharomyces cerevisiae* 2μ plasmid, catalyzes the recombinatorial excision of sequences flanked by directly repeated *FRT* sites. The R recombinase of *Zygosaccharomyces rouxii*, acts similarly to catalyze recombinatorial excision between directly repeated *RS* sites.

In early studies, the introduction of microbial recombinase genes into plant lines carrying desired trait genes was achieved by re-transformation of these either with a recombinase gene linked to a further selectable marker gene, or by sexual crosses with a recombinase-expressing transformant [9]. In either case, both the recombinase gene and its own associated marker gene must subsequently be separated from the desired trait gene by genetic segregation. Two major problems have been reported that limit the applications of these simple recombinase systems. Firstly, all of these systems require sexual crosses for the removal of recombinase genes and so cannot be used with vegetatively propagated plants. Secondly, the expression of microbial recombinases for prolonged periods in plant cells may result in unwanted changes to the genome at sites removed from transgene insertions. The use of microbial recombinases for marker gene removal, however, continues in more refined systems such as the MAT [12] and CLX [13] vector systems discussed below. A further advanced use of the Cre-*lox* recombination system exploits a transformation cassette designed to eliminate multiple tandem insertions of transgenes and to remove marker genes in one step [14].

3.2. Transposable element-based systems

Heterologous plant transposons have also been used for the removal of marker genes [15]. In one such system, the maize Ac transposable element was engineered to contain the *ipt* gene, conferring a selectable "extreme shooty phenotype" [4]. The Ac element encodes its own transposase and so its excision conveniently removes this gene along with the *ipt* marker gene (Fig. 1B), thereby obviating the need for sexual reproduction steps in the procedure. However, transposon-based systems of marker gene removal suffer from a number of disadvantages. Their efficiency is low, partly due to the tendency of transposable elements to reinsert elsewhere in the genome. Excision of transposons is frequently imprecise, and repeated cycles of insertion and excision may lead to the generation of mutations at numerous unknown loci. The continued presence of heterologous transposons may also lead to genomic instability in transgenic plants. For these reasons, transposon-based systems seem to be currently less favored as a means of the removal of marker genes.

3.3. Co-transformation systems

A further conceptually very simple method for marker gene removal is based on the co-transformation of plants using two distinct transgene constructs present in the same transformed line of *A. tumefaciens*[16]. One of these constructs contains the selectable marker transgene to be used, while the other includes the desired trait transgene, itself unlinked to any marker gene. In a variant of this technique, these two transgenes are inserted into two different T-DNA elements present in the same "super-binary" plant transformation vector [17]. Co-transformation methods for marker gene removal are based on the principle that a proportion of transformed plants carrying the selectable marker gene will also have integrated the required trait transgene at a second, unlinked insertion site. Marker genes can subsequently be

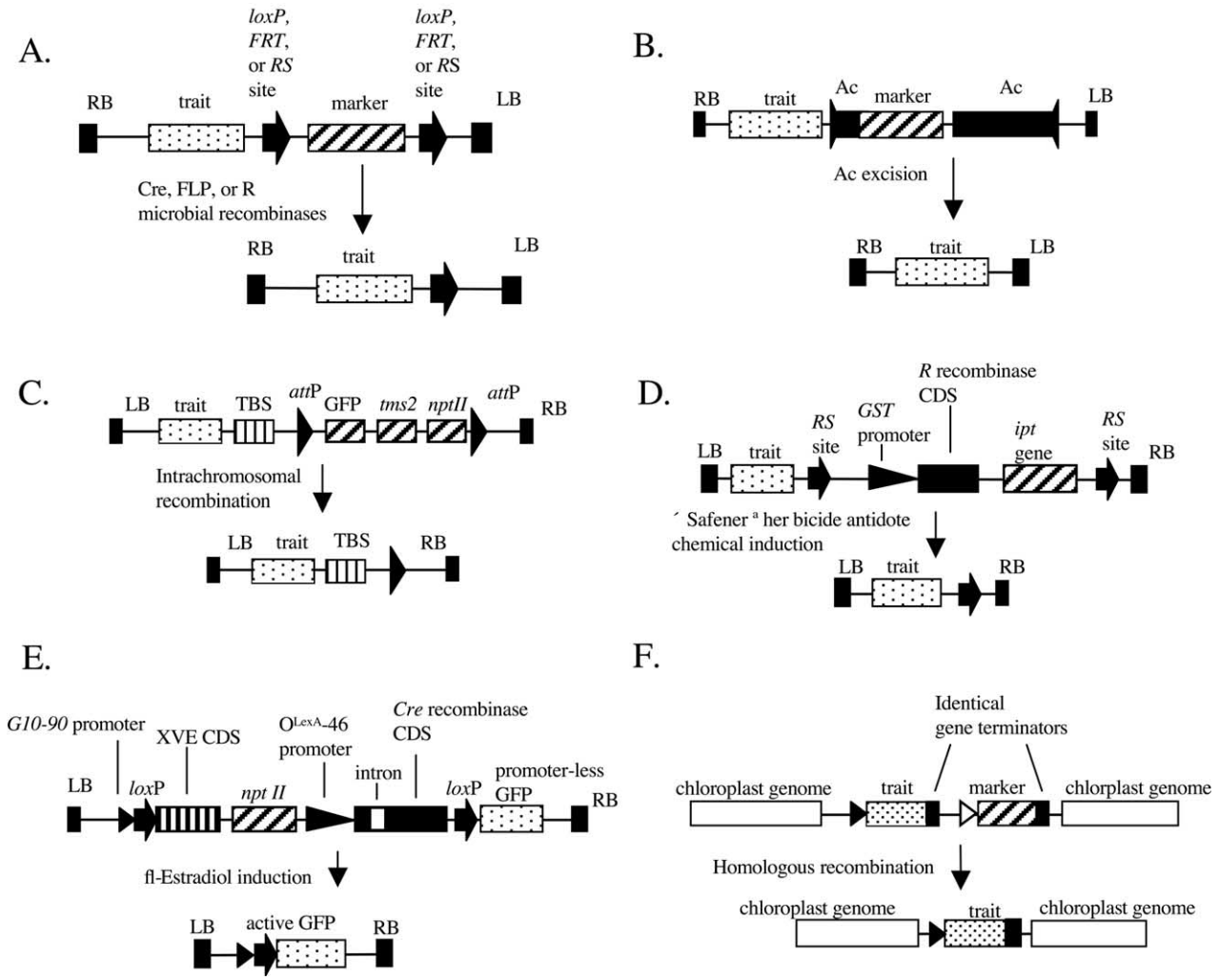

Fig. 1. Transgene constructions used for the removal of marker genes from transgenic plants. (A) The simple use of microbial recombinases such as Cre, FLP and R [9]. (B) An Ac Transposon-based method for the removal of nuclear marker genes [4]. (C) The intrachromosomal recombination method for nuclear marker gene removal [10]. (D) The GST-MAT vector system [12]. (E) The CRX vector system [13]. (F) Removal of chloroplast marker genes by homologous recombination [30]. Ac = maize Activator transposable element, CDS = coding sequence, GFP = gene coding Green Fluorescent Protein. GST promoter = glutathione S-tranferase promoter, *ipt* = isopentyl transferase gene, LB and RB = left and right *Agrobacterium tumefaciens* T-DNA border sequences, *nptII* = neomycin phosphotransferase II gene, TBS = transformation booster sequence, *tms2* = indolacetamide amidohydrolase gene, XVE = estrogen-activated hybrid transcriptional regulator gene [25].

removed from such plants by genetic segregation. Co-transformation methods suffer from the obvious inefficiency that only a proportion of plants carrying the selectable marker will also carry the desired trait gene at an unlinked site. Furthermore, as for the simple use of heterologous recombination systems, co-transformation methods cannot be used for vegetatively propagated plants.

3.4. An intrachromosomal recombination (ICR) system

A more recently devised alternative approach to the removal of nuclear transgene markers exploits the natural nuclear recombination systems present in plants [10]. Removal of marker genes by this approach is based on intrachromosomal recombination (ICR) between two directly repeated sequences flanking the marker gene to be excised. Zubko et al. [10] tested the efficiency of a pair of 352 bp *attP* regions from bacteriophage λ as substrates for ICR in plants. During the integration of the λ genome into the *E. coli* chromosome, the phage λ *attP* region recombines with a bacterial *attB* site over a pair of homologous core sequences. The process of bacteriophage integration involves a phage-encoded λ integrase and a bacterially encoded Integration Host Factor (IHF). The construction used for plant transformation in the studies of Zubko et al. [10] (Fig. 1C) contained a group of three marker and reporter genes flanked by a pair of directly repeated *attP* sites. This entire element was situated adjacent to a copy of the transformation booster sequence (TBS) from *Petunia hybrida* and a test transgene conferring a desired trait. The TBS has been found to increase the frequency of both ICR and illegitimate recombination events in *Petunia*, *Nicotiana* and maize [18]. In this

study, transformed tobacco calli were initially selected on kanamycin-containing media and subsequently cultured 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* gene marker. Two identical excision events from 11 initial transformed callus cultures were recovered, in which a 5.9 kb region containing the three marker genes and precisely one of the two *attP* sites had been lost by ICR. Some illegitimate recombination events in sister lines of calli were also noted.

ICR events in plants have previously been found to be very rare, with only 10 such events detectable in all of the cells of a 6-week old tobacco plant [19]. In the studies of Zubko et al. [10], however, the use of *attP* sequences and the TBS seems to have greatly increased the frequency of ICR events, despite the absence of the enzymes and co-factors necessary for the recombination of *attP* sites in the phage λ system. The structure of the *attP* site may partially explain its apparent recombination-stimulating activity, as sequences containing a high A + T base composition have been found to favor both ICR and illegitimate recombination events in plants [20]. However, a further possible explanation for these results has recently emerged from a study of repair to double-stranded DNA breaks in plants. Such DNA breaks were previously known to be repaired by recombination events, though this was thought to occur predominantly by illegitimate, rather than by homologous recombination. Siebert and Puchta [21], however, devised a system capable of measuring the relative frequencies of repairs to double-stranded breaks by homologous and illegitimate recombination mechanisms. In this study, pairs of double-stranded DNA breaks were generated in a plant transgene insertion by the transient expression of a rare-cutting restriction enzyme. In the transgene insertion used, a pair of rare restriction sites to be cut was flanked by partial sequences of the *uidA* (*GUS*) reporter gene, of which the central portion formed a pair of direct repeats. The induction of pairs of double-stranded DNA breaks led to the loss of a marker gene situated between the two rare restriction sites. In cases where these breaks were then repaired by homologous recombination of the repeated *GUS* sequences, rather than by non-homologous end-joining, an active *GUS* gene was reconstituted. This study found that double-stranded breaks could be repaired either by homologous recombination or by non-homologus end-joining, and that both of these events occurred at very high frequencies. The high incidence of ICR events noted in the studies of Zubko et al. [10], therefore, might be explained by invoking the involvement of a double-stranded break repair mechanism whose activity was in some way stimulated by the presence of *attP* sequences and/or the TBS.

The ICR method of marker gene removal has the advantage of relative simplicity 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 used for vegetatively propagated plants. However, in its present form, it does involve a two-stage procedure to select transgenic calli. Calli are transferred from selective to non-selective media for propagation and then re-transferred to a selective shoot-inducing medium to detect (white) tissue that has lost the marker gene. Such lengthy propagation may increase the risk of somaclonal mutations [22]. In addition, it has been pointed out that the activity of *attP* sequences as recombination substrates has yet to be demonstrated in a large range of plant species, and the mechanism by which the recombination of these sequences occurs in plants is not yet fully understood [9], though the recent results of Siebert and Puchta [21] may go some way towards finding an explanation.

3.5. The MAT vector system

The MAT (multiautotransformation) vector system represents a highly sophisticated approach for the removal of nuclear marker genes [23]. In this system, a chosen trait transgene is placed adjacent to a multigenic element flanked by *RS* recombination sites (Fig. 1D). A copy of the selectable *ipt* gene from *A. tumefaciens* is inserted between these recombinase sites, together with the yeast *R* recombinase gene and this entire assembly is situated within a T-DNA element for the *Agrobacterium*-mediated transformation of plant tissues. The MAT vector system allows the removal of the *R* recombinase gene along with the *ipt* gene. The system does not, therefore, require any sexual crosses for the removal of marker or recombinase genes, and recombinase expression in plant tissues is limited to a minimal period of time, thereby reducing the possibility of any unwanted recombination effects. In an earlier version of the MAT vector, *R* recombinase activity was constitutively up-regulated by the action of the CaMV 35S promoter. This system was found to incur a risk of marker gene excision before the selection of transformed plant tissues could take place. To circumvent this problem, a more recent version of the MAT vector [12] allows for a delay in the excision of the *ipt* and *R* recombinase genes. This is made possible by the use of a chemically inducible glutathione S-transferase promoter from maize to drive *R* recombinase gene expression. Once the positive selection of transformed plant tissues showing an “extreme shooty phenotype” has occurred, the excisive recombination of *RS* sites, leading to a loss of the recombinase and marker genes, is induced by treatment with the herbicide antidote “Safener”. This two-step procedure using MAT vectors has been successfully demonstrated for tobacco and hybrid aspen transformation, both of which are accomplished using organogenesis for plant regeneration. Plant species for which current transformation techniques require regeneration of transformed embryos from embryogenic cultures were thought to be potentially not amenable to selection using the *ipt* gene [13]. However, it has recently been demonstrated that transformed rice plants can be regenerated from embryogenic cultures by the use of the MAT vector system [24]. In this case, transformed embryos that had lost the *ipt* marker gene

but retained the desired transgene were selected directly in a one-step procedure without the occurrence of an "extreme shooty phenotype".

3.6. The CLX chemically inducible system

In a further highly sophisticated approach to nuclear marker gene removal, the Cre-*lox* recombination system has been engineered to be chemically inducible [13]. Antibiotic selection using the CLX vector system for plant transformation and marker gene removal is based on an *nptII* gene (Table 1) driven by a constitutive promoter. This *nptII* gene is positioned adjacent to a *Cre*-recombinase gene driven by the hybrid, chemically inducible O^{LexA}-46 promoter, and a hybrid XVE gene [25], encoding the binding protein necessary for the induction of *Cre* gene transcription (Fig. 1E). These three transcription units, with the exception of the constitutive promoter driving XVE gene expression, are flanked by a pair of directly repeated *loxP* sites. Background expression of the *Cre* gene in *Agrobacterium* is avoided by the incorporation of a plant intron. Following the *Agrobacterium*-mediated transformation of *Arabidopsis* root tissues using the CLX vector system and the selection of transformed tissues on kanamycin-containing media, Cre recombinase activity was induced by exogenous application of β-estradiol. As a result of Cre recombinase activity, the XVE coding sequence and the *Cre* and *nptII* genes were lost by precise excisive recombination between the *loxP* sites. This excision led to the close juxtaposition of the promoter previously driving XVE expression, and a previously promoterless Green Fluorescent Protein (GFP) coding sequence, with concomitant activation of GFP expression. As with the MAT vector system, the use of the CLX vector for transformation and marker gene removal exposes plants to recombinase activity for the minimum possible time period, and does so after an adequate period of time has elapsed to permit transformant selection. The CLX vector system benefits also from a particularly tightly regulated system of chemical induction [22]. The procedure could be used for vegetatively propagated species and may be particularly well adapted to crop species requiring transformation by the regeneration of embryo cultures.

4. The removal of marker genes from the chloroplast genome

The genetic modification of chloroplasts can represent an attractive alternative to engineering of the plant nuclear genome for some applications [26]. Unlike the nuclear transformation of higher plants, chloroplast transformation takes place almost invariably by homologous recombination, resulting in precise and predictable genetic modifications. The plastid genome is present in multiple copies in each organelle, and these can multiply to large numbers, particularly in leaf tissues, such that a chloroplast transgene can exist in up to 10^4 copies per cell [26]. This considerable amplification can give a very high level of transgene expression, which may be useful for applications requiring high concentrations of proteins. Examples of these include the engineering of drought-resistance, or the production of pharmaceuticals *in planta* by molecular farming. The chloroplast genome is uniquely transmitted through the female germ line in many crops, reducing the possibility of transgene escape via pollination into local wild populations of plants. Examples of chloroplast transgenes used to date include: the Cry gene, encoding *Bacillus thuringiensis* (*Bt*) toxin to confer insect resistance [27] and the *hST* gene encoding human somatatrophin [28].

Transformation of chloroplasts is currently performed by biolistic methods. Following the integration of transgenes into the chloroplast genome, a heterogeneous population of plastids will exist in transformed tissues, and selection using a marker gene is required to produce homoplasmic plants in which the modified plastid genome has completely replaced the unmodified one. Transformation cassettes used for chloroplast transformation contain sequences homologous to two adjacent regions of the chloroplast genome to allow the integration of the transgenes by homologous recombination. Selectable marker genes and desired trait transgenes are placed between these homologous recombination sequences. The removal of marker genes from the chloroplast genome is particularly important as their very high copy numbers could otherwise lead to high levels of unwanted marker gene products. A further argument for the removal of chloroplast genetic markers relates to the conservation of activity that often exists between chloroplast and bacterial promoters. This could increase the risk of the horizontal transfer of functional marker genes from plants to bacteria. Fewer resistance genes are available for chloroplast than for nuclear transformation, with most of the published studies based on the use of the *aadA* gene (Table 1). The paucity of available selection methods for chloroplast transformation further increases the value of technology that enables the recycling of marker genes for the serial re-modification of a single transgenic plant line.

4.1. Homologous recombination systems

As the integration of foreign transgenes into the chloroplast genome takes place by homologous recombination, it was entirely logical to test this native plant mechanism as a means for the removal of marker genes from the chloroplast (Fig. 1F). The first demonstration of this technique was performed on the unicellular green alga, *Chlamydomonas reinhardtii*[29]. Iamtham and Day [30] then demonstrated its applicability to higher plants using a construction of three marker genes which shared two identical promoter sequences of 174 bp and three identical terminator sequences of 418 bp. Several different recombinative excision events were detected between the similar sets of promoter or terminator sequences in the series of transgenic plants analyzed in

these studies. After the removal of antibiotic selection, these excision events accumulated to high frequency, leading to a homoplastic, marker-free state in approximately 25% of transgenic lines in the next generation. Homoplastic marker-free plants may be identified in this technique by PCR or by Southern blot analysis.

4.2. Cre-lox *recombination-based systems*

Two recent studies have demonstrated that the Cre-*lox* system can also be used for the removal of plastid transgene markers [31,32]. These systems function essentially as for the removal of nuclear transgenes by Cre-*lox* recombination. A *Cre*-recombinase gene is expressed from a plant transformation cassette integrated into the nuclear genome, while an N-terminal chloroplast-directing signal sequence routes the Cre recombinase protein that is produced to the plastids. Plastid transgene constructions for use with these methods of marker gene removal contain selectable marker genes flanked by *loxP* recombination sites. In one study [31], different results were noted depending on whether the *Cre*-recombinase gene was introduced into the nuclear genome of a chloroplast transformed line by direct transformation or by sexual crossing to a Cre recombinase transformant. In cases where Cre was introduced by re-transformation, excisive homologous recombination events between similar genetic elements present in the transgene construction, as noted in the studies of Iamtham and Day [30], were observed at an efficiency approximately equivalent to that of Cre-mediated excision events. However, excision events by homologous recombination were not observed when the *Cre* gene was introduced though sexual crosses. It is not currently clear whether Cre-*lox*-based systems represent a considerable increase in efficiency over homologous recombination for the removal of chloroplast marker genes. One disadvantage of the current Cre recombinase-mediated methods for chloroplast transgene removal is that they require the sexual crossing of transformed plant lines to remove the nuclear-encoded recombinase gene and its associated genetic marker and so cannot be used for vegetatively propagated plants.

5. Conclusions

The field of marker gene removal continues to produce new innovations. For example, the possibilities of increasing the number of different heterologous recombinase systems available by molecular evolution approaches have been discussed, and new marker gene and marker-free strategies are under development [9]. Work in progress aims to devise systems that are efficient, rapid, precise, applicable to many plant species and transformation methods, do not require sexual reproduction steps, minimize the risk of introducing unwanted genetic changes, and leave the genome in a stable condition. Though research continues, it is clear that several viable methods for the removal of unwanted marker genes already exist. 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 for marker gene removal under development will also facilitate the more precise and subtle engineering of the plant genome, with widespread applications in both fundamental research and biotechnology.

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