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Review

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Marker Genes for *In Vitro* Selection of Transgenic Plants

Ana C. M. Brasileiro, Francisco J. L. Aragão*

Embrapa Recursos Geneticos e Biotecnologia, Parque Estacao Biologica (PqEB), Final Av. W5 Norte, 70.770-900 - Brasilia - DF, Brazil.

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Footnote (Abbreviations): AHAS = acetohydroxy acid synthase; ARGs = antibiotic resistance genes; EPSPS = 5-enolpyruvyl-shikimate-3-phosphate synthase; GUS = β-glucuronidase; HPPD = 4-hydroxyphenyl pyruvate dioxygenase; HPT = hygromycin phosphotransferase; PMI = phosphomannose isomerase; NPT II = neomycin phosphotransferase II; PAT = phosphinothricin-N-acetyltransferase

Abstract

The use of a marker gene in a transformation process aims to give a selective advantage to the transformed cells, allowing them to grow faster and better, and to kill the non-transformed cells. In general, the selective gene is introduced into plant genome along with the genes of interest. In some cases, the marker gene can be the gene of interest that will confer an agronomic characteristic, such as herbicide resistance. In this review we list and discuss the use of the most common selective marker genes on plant transformation and the effects of their respective selective agents. These genes could be divided in categories according their mode of action: genes that confer resistance to antibiotics and herbicides; and genes for positive selection. The contention of the marker gene flow through chloroplast transformation is further discussed. Moreover, strategies to recover marker-free transgenic plants, involving multi-auto-transformation (MAT), co-transformation, site specific recombination and intragenomic relocation of transgenes through transposable elements, are also reviewed.

Introduction

Selective marker genes are introduced into plant genome to express a protein with, generally, an enzymatic activity, allowing to distinguish transformed from non-transformed cells (Brasileiro and Dusi, 1999). The purpose of the use of a selective marker gene is (1) to give to the transformed cells a selective advantage, allowing them to growth faster and better, and (2) to kill the non-transformed cells. The ideal selective marker gene should be capable to express in any kind of cell and tissue, in a great number of plant species. This expression should be easily distinguished from any endogenous activity in the plant tissue, allowing differentiate the phenotype of transformed from non-transformed tissues. Therefore, the probability to recover transgenic plants in the presence of a selective agent is greater than in its absence, especially because the transformation rate is always low (10^{-3} to 10^{-6}). Moreover, only in rare cells the introduced gene is correctly integrated and expressed.

In general, the selective gene is introduced into plant genome along with the genes of interest. These genes could be separated or physically linked in the same DNA vector. The frequency of co-transformation (i.e. cells with both genes integrated into the genome) is about 100% when the genes are linked and 50% when they are separated (Aragão et al., 1996). The selective agents are generally used in the initial stages of transformation for an early selection of transgenic cells (Sawahel, 1994). During the further regeneration steps, the influence exerted by the dying of non-transformed cells on the transformed cells should be minimal on the selective medium. In most cases, the expression of selection marker genes is under the con-

* Corresponding author, E-mail; aragao@cenargen.embrapa.br
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trol of a constitutive promoter like the cauliflower mosaic virus (CaMV) 35S, nopaline and octopine synthase, actin or ubiquitin promoter genes. In some cases, the marker gene can confer an agronomic characteristic, such as herbicide resistance.

The aim of this review is to list and discuss the most common selective marker genes used in plant transformation and the effects of their respective selective agents. The strategies currently used to further eliminate the selective marker gene are also reviewed.

Genes that confer resistance to antibiotics

A first category of marker genes corresponds to those that confer to the plant resistance to an antibiotic. These genes correspond to the first genes successfully used to select transgenic plants.

A. npt II (or neo) gene

Origin: The *npt* II (or *neo*) gene was isolated from the transposon Tn5 of Escherichia coli and it codes for the enzyme neomycin phosphostransferase II (NPT II; EC 2.7.1.95), also known as aminoglycoside 3'-phosphotransferase II (APH[3]II) (Bevan et al., 1983; Fraley et al., 1983; Herrera-Estrella et al., 1983).

Mode of action: The NPT II transfers the γ-phosphate group of ATP to the 3'-hydroxyl group of the amino-hexose portion of aminoglycoside antibiotics that are consequently detoxificated. Due to this ATP-dependent phosphorylation, the bind of the antibiotic to the bacterial ribosome is prevented, allowing protein synthesis. Aminoglycoside antibiotics that contain the 3'-hydroxyl group, like the kanamycins A, B, and C, neomycin, paramomycin and geneticin (G-418), are substrates for NPT II (Norelli and Aldwinckle, 1993). Endogenous NPT II activity is very rare in plant tissues.

The selective agent's action: The active aminoglycoside antibiotic inhibits the protein synthesis in prokaryote cells, by binding to the 30S subunit of the ribosome, blocking the formation of initiation complexes and decreasing the fidelity of translation. In plant cells, these antibiotics exert its effect on mitochondria and chloroplasts, acting in the same manner by impairing protein synthesis. These organelles have ribosome that are similar to those found in bacteria and are also susceptible to aminoglycoside antibiotics. Therefore, in the presence of antibiotic, the plant tissue will show a chlorosis, caused by the lack of chlorophyll synthesis, and inhibition of the growth (Benveniste and Davies, 1973; Brasileiro, 1998).

Observation: Up to date, *npt* II is the most selective marker gene used in plant transformation and kanamycin is the antibiotic more frequently used for the selection of *npt* II-transgenic plants.

B. hpt (or aph IV) gene

Origin: The *hpt* gene codes for the enzyme hygromycin phosphotransferase (HPT; EC 2.7.1.119), also known as aminoglycoside 4'-phosphotransferase (APH[4]). This gene was isolated from *Escherichia coli* and it confers resistance to the antibiotic hygromycin B (van den Elzen et al., 1985).

Mode of action: HPT catalyses the phosphorylation of the hydroxyl group in the antibiotic hygromycin, inactivating it. Hygromicin is also an aminoglycoside antibiotic.

The selective agent's action: When active, hygromycin occupies the ribosomal binding site of the elongation factor 2 (EF-2) in prokaryote cells. Consequently, the elongation of polypeptide chain is inhibited and protein synthesis interrupted, causing the same symptoms described for the other aminoglycoside antibiotics (Benveniste and Davies, 1973).

Observation: This gene has been extensively used, especially when it is not possible the use the *neo* gene. This is the case of several monocotyledonous species that show high levels of natural resistance to kanamycin (Wilmink and Dons, 1993; Vasil, 1994). Hygromycin B is usually more toxic than kanamycin and kill sensitive cells more quickly.

Genes that confer resistance to herbicides

Another category of selective markers is the genes that confer resistance to herbicides. To be used as suitable selective agents, herbicides should have some characteristics, which allow its use *in vitro*. For example, herbicides that act blocking the photosynthesis are not ideal to be used on *in vitro* conditions. However, these herbicides could be used to select transformed green tissue when applied direct to the differentiated organs.

A. crs 1 (or ahas) genes

Origin: These genes code for mutated forms of the enzyme acetohydroxy acid synthase (AHAS; EC 4.1.3.18), also called acetolactate synthase (ALS). Each mutant gene *crs* 1 contains a single nucleotide change, resulting in a single amino acid substitution in the AHAS protein. The *crs* 1

genes were isolated from mutants of *Arabidopsis thaliana* resistant to sulfonylurea and imidazolinone herbicides, such as chlorsulfuron, imazapyr and imazaquin (Haughn et al., 1988; Mourad et al., 1995).

Mode of action: Transgenic plants containing one of these mutated *crs* 1 genes will produce an altered AHAS enzyme, which it is not recognized by the sulfonylurea and imidazolinone herbicides. Consequently, the enzymatic pathway will remain working, turning the transgenic plants resistant to the herbicide (Haughn et al., 1988; Brasileiro et al., 1992; Aragão et al., 2000).

The selective agent's action: The sulfonylurea and imidazolinone herbicides inhibit the endogenous AHAS enzyme in the plant tissue. AHAS is the first common enzyme in the metabolic pathway leading to the branched-chain amino acids (leucine, isoleucine and valine). Consequently, the deficiency of these amino acids, accumulation of the toxic substrate (α-ketobutyrate) and an eventual disturbing of the protein synthesis will lead to the plant cell death (Chaleff and Mauvais, 1984; Ray, 1984).

Observation: Mutated genes that code for an altered form of the AHAS enzyme were also isolated from (a) another plants, such as tobacco and sugar beet; (b) bacteria, such as *Escherichia coli* and *Salmonella typhimurium* and (c) yeast (Saccharomyces cerevisiae) (Wilmink and Dons, 1993).

B. aroA (or epsps) gene

Origin: This gene was isolated from *Salmonella typhimurium* treated with mutagenic agent and selected for resistance to the herbicide glyphosate.

Mode of action: The mutated *aro*A gene codes for a modified form of the enzyme 5-enolpyruvyl-shikimate-3-phosphate synthase (EPSPS; EC 2.5.1.19) that shows reduced affinity to glyphosate, that is the active compound in the commercial herbicide Roundup™ (Comai et al., 1983; 1985). A high level of the *aro*A gene constitutive expression, in transgenic plants, allows the achievement of resistance to glyphosate. The overproduction of this enzyme results in high enzymatic activities that turn possible to the plant cell survive even in the presence of the herbicide (Mazur and Falco, 1989).

The selective agent's action: The herbicide glyphosate inhibits, by competition, the enzyme EPSPS, which is involved in the enzymatic pathway of aromatic amino acids biosynthesis in bacteria and plants. The inhibition of EPSPS results in shikimate accumulation, inhibition of synthesis of aromatic amino acids and secondary metabolites, causing cell death.

Observation: Other *epsps* genes which code for an EPSPS with reduced affinity to glyphosate were also isolated from the line CP4 of *Agrobacterium* sp. and from *Petunia hybrida*.

In plants, endogenous EPSPS is mainly localized in the chloroplast. The bacterial *aro*A gene lacks a transit peptide sequence to address the protein to the chloroplast. Consequently, it originates a cytoplasmic form of the enzyme. Even so, transgenic plants expressing the *aro*A gene in the cytoplasm showed increased, but incomplete, tolerance to glyphosate. Higher transformation efficiency has been achieved by fusing the *aro*A gene to a chloroplast transit peptide to address the pro-protein into this organelle (Oxtoby and Hughes, 1990).

C. bar and pat genes

Origin: The *bar* and *pat* genes were isolated from *Streptomyces hygroscopicus* and *S. viridochromogenes*, respectively. These similar genes code for the enzyme phosphinothricin-N-acetyltransferase (PAT; EC 2.3.1.-) (Murakami et al., 1986).

Mode of action: The enzyme PAT inactivates herbicides that possess the phosphinothricin (PPT) as active compound, such as Basta™, Liberty™ and Herbiace™. The herbicide detoxification occurs through the acetylation of the free amino group of PPT, using acetyl coenzyme A as a cofactor, preventing its bind to the enzyme glutamine synthetase (GS).

The selective agent's action: PPT, also well known as ammonium glufosinate, is analogous to glutamate, the substrate of GS, and acts as a competitive inhibitor of GS. The enzyme GS catalyzes the conversion of glutamate to glutamine, removing the toxic ammonia from the cell. This enzyme plays as essential role in the regulation of nitrogen metabolism and ammonia assimilation. When the GS is inhibited, it results in ammonia accumulation and an associated disruption of chloroplast structure, that leads to inhibition of photosynthesis and to death of the plant cell (Lindsey, 1992).

Observation: The *bar* gene is found in strains of *Streptomyces hygroscopicus* that produce bialaphos, a tripeptide antibiotic that consists of PPT and two L-alanine residues (Murakami et al., 1986). The product of the *bar* gene (PAT) protects those strains from the action of its own antibiotic, metabolizing PPT in an inactive acetylated-derived compound and preventing autotoxicity (De Block et al., 1987; Mazur and Falco, 1989).

The *bar* gene is the most widely and successfully used selective marker gene for all of the major cereal species,

such as wheat, rice, maize, barley, sorghum, oats and rye (Vasil, 1994; Vain et al., 1995).

D. hppd gene

The use of a foliar bleaching herbicide, called isoxaflutole (5-cyclopropyl isoxazol-4-yl-2-mesyl-4-trifluoromethylphenyl ketone), was recently reported as a successful selective agent for soybean transformation (Murh et al., 2000). Isoxaflutole, the active ingredient of the herbicide Balance™, inhibits 4-hydroxyphenyl pyruvate dioxygenase (HPPD; EC 1.13.11.27), that is an enzyme involved in tyrosine degradation and plastoquinone biosynthesis (Pallett et al., 1998). A mutated *hppd* gene isolated from *Pseudomonas fluorescens* codes for an altered form of the enzyme, conferring tolerance to isoxaflutole. The greatest advantage of this selection system is that the transformed tissues become green among the white non-transformed tissues, allowing a simultaneous visual screening and chemical selection. Transgenic plants resistant to isoxaflutole were also obtained for *Arabidopsis thaliana* and tobacco.

Markers genes for positive selection

Some marker genes for positive selection make possible the identification and selection of genetically modified cells without injuries or death of the non-transformed population of cells (negative selection). In this case, the selection marker genes should confer, to the transformed cell, the capacity to metabolize some compounds that are not usually metabolized. This fact will give to the transformed cells an advantage over the non-transformed ones. The addition of this new compound in the culture medium, as nutrient source during the regeneration process, allow the normal growth and differentiation of transformed cells, while non-transformed will not be capable to growth and regenerate *de novo* plants.

A. manA gene

Origin: The *man* gene codes for the enzyme phosphomannose isomerase (PMI; EC 5.3.1.8) isolated from *Escherichia coli.*

Mode of action: In the presence of mannose in transformed cells, the PMI converts mannose-6-phosphate into fructose-6-phosphate that can be immediately incorporated in the plant metabolic pathway. Thus, the mannose can be used as sole source of carbohydrate for the transformed cells. This selection system is immediate and extremely efficient (Joersbo et al., 1998).

The selective agent's action: Mannose can not be usually metabolized by non-transformed cells and is converted into mannose-6-phosphate by endogenous hexokinase. Therefore, when mannose is added to the culture medium, could minimize the plant growth due to mannose-6-phosphate accumulation. The mannose-6-phosphate toxicity in plant cells showed to be responsible for the apoptosis, or programmed cellular death, through induction of an endonuclease, responsible for DNA laddering (Stein and Hansen, 1999). Mannose-6-phosphate accumulation also causes phosphate and ATP starvation that depletes cell energy from critical functions such as cell division and elongation, giving rise to growth inhibition. Therefore, mannose is a hexose that fills the desirable requirements for a good selection agent: it is (a) soluble in plant culture media; (b) absorbed by plant cells; (c) cheap; (d) easily available; and (e) safe.

Observation: Despite of most plant species be sensitive to the mannose, some species, especially dicotyledonous, have shown a considerable insensibility to this sugar, including carrot, tobacco, sweet potato and leguminous. Other species are extremely sensitive and were successfully transformed using mannose as selective agent, such as sugar beet, maize, wheat, oat, barley, tomato, potato, sunflower, oilseed rape and pea (Joersbo et al., 1998; 1999; 2000; Negrotto et al., 2000; Wang et al., 2000).

Some plant transformation protocols that utilize the positive selection system with PMI have shown at least 10 times more efficient than the traditional protocols based on the use of kanamycin as selection agent.

A similar positive selection system has been developed using the xylose isomerase gene (*xyl*A) isolated from *Thermoanaerobacterium thermosulfurogenes* or from *Streptomyces rubiginosus*, as selective marker gene (Haldrup et al., 1998a; 1998b). Transgenic plants of potato, tobacco and tomato were successfully selected on xylose-containing media.

B. gus gene

Origin: The gene gus codes for the enzyme β-glucuronidase (GUS; EC 3.2.1.31) and was isolated from *Escherichia coli.* This gene is widely used as reporter gene in transgenic plants.

Mode of action: In this system, the selective agent is a glucuronide derivative of benzyladenine (benzyladenine N-3-glucuronide), an inactive form of the plant hormone cytokinin. This glucuronide, present in the selection medium, can be hydrolyzed by the GUS enzyme produced in the transformed cells, releasing active cytokinin (benzyladenine) in the medium. This cytokinin will be a stimula-

tor for regeneration of transformed cells while non-transformed cells are arrested in development.

The selective agent's action: In the non-transformed cells, the selective agent (benzyladenine N-3-glucuronide) does not have any effect because the cytokinin is in its inactive form.

Observation: There is only one report concerning the successful use of this system in the effective recovery of transgenic plants (Joersbo and Okkels, 1996; Okkels et al., 1997).

C. Auxotrophic markers

In plants, auxotrophic mutations that request nutritional supplements are unusual and only few cases can be easily propagated as homozygous. Consequently, the complementation of auxotrophic mutants by transformation with a functional gene are rarely reported (Bowen, 1993). Two examples are well known. Mutants of *Nicotiana plumbaginifolia*, deficient in threonine dehydratase, require isoleucine for development. These mutants were complemented by transformation with the *ILV1* gene from yeast, which codes for the threonine dehydratase. Transformants were selected in medium lacking isoleucine and the threonine dehydratase enzyme activity was restored. In another example, mutants of *N. tabacum* and *N. plumbaginifolia*, deficient in nitrate reductase, were complemented by transformation with the *nr* gene from *N. tabacum*. Transformants were selected in presence of nitrate and the mutant phenotype was reverted.

Chloroplast transformation

In this system, chloroplasts (or plastids) are transformed with genes for herbicide resistance as selection markers, because most of target proteins for herbicides are compartmentalized within this organelle. Chloroplast transformation could be accomplished by both the biolistic and polyethylene glycol-mediated strategies (Daniell et al., 1998; Hibberd et al., 1998; Kofer et al., 1998; Sidorov et al., 1999). Vectors that specifically address the integration of the herbicide-resistant marker gene in the chloroplasmatic genome should be preferentially used, as the "tobacco vector" or the "universal chloroplast vector". These vectors possess flanking sequences from high conserved chloroplast genes to allow integration by homologous recombination. The advantages that chloroplast expression systems may confer over routinely nuclear expression are (Bogorad, 2000):

- The contention of the transgene flow (especially marker genes) through microspores is reduced. The chloroplast genome possesses maternal inheritance for most crop plants;
- The expression level of the transgene is extremely high, compared with the expression of the same gene integrated in the nuclear genome, due to the large copy number of chloroplast genomes (5,000 to 10,000) per cell;
- The insertion in the genome is driven (site-specific), avoiding position effects and facilitating comparative studies;
- The *quasi* absence of gene silence in the inserted transgenes (Daniell et al., 1998).

In addition, other subcellular organelles, as mitochondria, might also provide more favorable environments than the nuclear-cytoplasmic compartment for certain biochemical reactions or for high, predictable, uniform and stable transgene expression, not subject to gene silencing (Bogorad, 2000).

Antibiotic-resistant marker gene could be also used in this system, facilitating the extension of plastid transformation to non-green plastids such as in embryogenic cells of cereal crops (Kavanagh et al., 1999; Khan and Maliga, 1999).

Strategies to recover marker-free transgenic plants

Antibiotic resistance genes (ARGs) have been introduced into transgenic plant genomes. The ARGs are used under control of prokaryotic promoters to select bacteria aiming the production of vectors for direct plant transformation. In some cases, these prokaryotic ARGs are introduced along with the gene of interest in the vector. In addition, ARGs under the control of eukaryotic promoters are widely used as selection marker genes. Due to biosafety concerns, complex evaluations have been accomplished, aiming to study the potential impacts of ARGs present on transgenic plants for the human health and for the environment. In spite of there be no evidences of deleterious effects on the use of transgenic plants carrying ARGs, its removal is already stated as "good practice of laboratory", by several regulatory committees (US Food, Drug Administration, 1998). So, there is a recommendation that those who are developing genetically modified foods aiming to reach the market, should be encourage to phase out the utilization of ARGs. This recommendation could be also applied to the other selection marker genes, since they are not necessary once the transgenic plant is obtained. Consequently, several strategies to remove the selection marker genes have been developed.

A. MAT (multi-auto-transformation) system

Origin: The *ipt* gene that codes for the enzyme isopentenyl phosphotransferase (EC 2.5.1.27) was isolated from the Ti plasmid of *Agrobacterium tumefaciens*.

Mode of action: The MAT system is firstly based on the visual selection of transgenic plants containing the *ipt* gene (Ebinuma et al., 1997). In the presence of that gene, the transformed plant loses the apical dominance and ability to rooting. The acquired abnormal phenotype was called *extreme shooty phenotype* (ESP) and is easy to be visually detected. In a second step, the unsuitable *ipt* gene is removed of the transgenic plant through the transposition of the *Ac* transposable element from maize, which was transferred along the selection marker gene. In this way, marker-free transgenic plants could be generated with the normal phenotype restored, containing only the gene of interest. In the MAT vectors, the *ipt* gene, under control of the CaMV 35S promoter, is inserted into the transposable element *Ac*.

The selective agent's action: The enzyme IPT catalyzes the condensation of isopentenyl pyrophosphate with AMP to produce isopentenyl AMP, which is a precursor of several cytokinins. Cytokinins stimulate organogenesis in several *in vitro* cultivated plants and are widely used to regenerate plants after the transformation event.

Observation: In the transposition process, only few excised transposable element *Ac* (containing the marker gene) disappear from transgenic cells, because the *Ac* element do not reinsert or because it has reinserted into a sister chromatid that is further lost by somatic segregation. Therefore, the frequency of elimination of the *Ac* element to recover marker-free transgenic plants is low, ranging from 0.1 to 0.5%.

The site-specific recombination system *R/RS* from *Zygosaccharomyces rouxii* can also be used for the elimination of the marker gene (Sugita et al., 1999; 2000). In this system, the removal of *ipt* gene followed by the recovery of marker-free transgenic plants is reported to be improved from 70% over the transposable element *Ac* system.

When the expression of *ipt* gene is controlled by a dexamethasone-inducible system, the co-introduction of multiple genes, in addition to *ipt*, is more efficient and the recovery of marker-free transgenic plants is high (Kunkel et al., 1999).

The MAT system is particularly valuable for plants with long generation cycles such as fruit and forest trees, providing a promising way to shorten breeding time.

B. Co-transformation

In this system, the transformation is achieved utilizing two separate plasmid vectors: one containing the gene of interest and other the selective marker gene. In this way, the selective marker gene can be further eliminated by progeny segregation. Thus, the co-transformation system allows the use of a selective agent during plant regeneration and subsequent recovery of marker-free progeny, which contains only the gene of interest. There are at least two basic requirements to make this system functional: (1) the efficiency of co-transformation should be enough high, and (2) the vectors should be integrated in different loci sufficiently "unlinked" to allow effective recovery of recombination events and/or gene segregation.

De Block and Debrouwer (1991) described that the co-transformation efficiency in oilseed rape with two *Agrobacterium* strains ranged from 60 to 80%. However, 78% of those events were in the same locus (linked sites). Other reports described that, under determined conditions (depending on the transformation vector, transformation methodology, strains of *Agrobacterium*, plant species etc.), the integration of different vectors occur into unlinked sites in a high frequency (Goldsbrough et al., 1993; Yoder and Goldsbrough, 1994; Daley et al., 1998; Tang et al., 1999). Using the biolistic process, the co-transformation of dry bean was 50% for unlinked genes (Aragão et al., 1996).

C. Site-specific recombination system

It is a system of two components, that requires an enzyme that acts *in trans* to catalyze the recombination between two short specific sequences of DNA that flank the selective marker gene to be eliminated. This system was already efficiently demonstrated in yeast. However, in plants, the site-specific recombination rate is very low and the current knowledge of homologous recombination is still limited (Mengiste and Paszkowski, 1999; Vergunst and Hooykaas, 1999). The most common system used to mediate the site-specific recombination in plants is the bacteriophage P1 *Cre/lox* (Yoder and Goldsbrough, 1994; Vergunst and Hooykaas, 1998; Vergunst et al., 1998; Gleave et al., 1999). In this recombination system, the plant should be previously transformed with a selective marker gene cloned between two sequences of the gene *lox*, each with 34 bp, repeats in direct orientation. In a second stage, the *Cre* gene should be introduced in this plant by a second transformation, by sexual crossing or by transient expression. Once the *Cre* gene is expressed, the *Cre* enzyme catalyses the recombination between the *lox* repeat

sequences, eliminating the marker gene in the secondary transformants. This recombination strategy can also be used to target the insertion of new genes in an already transformed plant containing the *lox* sites, or another known sequence, inserted in an "suitable" chromosomal position (Gallego et al., 1999). Site-specific recombination can also be utilized to compare transgenic plant lines, without the effect of the integration site position.

D. Intragenomic relocation of transgenes via transposable elements

In the system, as well as in the MAT system, the selective marker gene should be flanked by the inverted and repeated sequences of the *Ds* element of the maize transposable system *Ac/Ds*. Once obtained the transgenic plant, the *Ds* element and the marker gene will be, when in the presence of the transposase, transferred for a new locus of the plant genome or eliminated. The gene of interest will be left in the first insertion locus (Goldsbrough et al., 1993). The transposase can be introduced in this plant as an additional element in the transformation vector, by a second transformation or by sexual crossing. The advantage of this system is that the selective marker gene will be lost in some somatic tissues due to the failure of the *Ds* element reintegration. This makes the strategy suitable for removal of marker genes in vegetatively propagated plants (Yoder and Goldsbrough, 1994). In a similar way, Zubko et al. (2000) recently described a system to remove selective marker gene by intrachromosomal recombination between the bacteriophage λ attachment (attP) regions.

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