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**The ability to efficiently introduce foreign genes into plants is key to the success of the emerging plant biotechnology industry. Genetic transformation of crop plants is becoming increasingly routine both in terms of the number of plant species which can be transformed and the frequency of transformation, resulting in a number of transgenic products which are ready or close to market introduction<sup>1</sup>. The imminent commercialization of transgenic plants has generated debate about the desirability of the transgenic products containing selectable marker genes, or in fact any other ancillary DNA sequences not directly contributing to the final product<sup>2-6</sup>. In this review, we discuss these issues and examine transformation systems recently developed to selectively eliminate particular transgene sequences from the final transgenic plant.**

**A** number of systems have been developed for the introduction of foreign DNA into plant cells (reviewed in refs. 7, 8). Because, with all these methods, only a relatively small percentage of cells become stably transformed, genes that confer a selectable advantage to transformed cells are almost always included in the transformation system. Table 1 is a summary of selectable marker genes currently in use, their source, and the agents used to apply the selection.

Risk assessments of selectable marker genes have to date focused largely on the most widely used selectable marker gene, neomycin phosphotransferase (*nptII*), in large part because of its presence in Calgene's FLAVR SAVR tomatoes<sup>3,29-32</sup>. The overall conclusions from these studies are clear: There are no scientific reasons on grounds of human, animal, or environmental safety to deny or restrict the use of the kanamycin resistance gene. The process does, however, serve to highlight the areas of concern that need to be addressed before contemplating commercialization of plants containing this and other selectable marker genes or sequences. Potential areas of concern regarding safety to both the environment and the consumer will be discussed below. We will first however discuss a practical problem regarding the presence of a selectable marker gene in a transgenic cultivar, namely that the presence of a functional selectable marker gene in a particular line will preclude the use of the same selection system in subsequent transformations of derivatives of that line.

Superior crop cultivars generally contain several distinct traits which make them of commercial value, for example multiple disease resistance genes. If transgene technology proves to be commercially successful, it seems likely that future cultivars will contain multiple independent transgenes which have been sequentially added into the cultivar as they become available. While hybridization will be used in many cases to pyramid the transgenes into one cultivar, there will also be cases where

repeated transformations into the same cultivar will be more effective. Since the presence of a particular marker gene in a transgenic plant precludes the use of that marker in subsequent transformations, it will be necessary to use different selectable marker systems for each transformation. While this may not currently seem to be a limitation for species for which multiple selection systems are available, in the long term there will almost certainly be more transgenes one wishes to introduce than suitable selectable markers available. This is already the case for plants recalcitrant to transformation and for which selectable markers are at a premium. Hence, the removal of a selectable marker gene may be desirable if for no other reason than that the same (and most likely optimal) selection procedure can be used in subsequent transformations<sup>4,33,34</sup>.

A second pragmatic issue which needs to be considered relates to the stability of homologous transgenes following multiple rounds of transformation. In a number of reported cases it has been noted that expression of a primary transgene is altered or lost following a secondary transformation event<sup>35</sup>. While the genetic mechanisms responsible for the observed instabilities have not yet been fully elucidated, they have been correlated with the presence of homologous sequences on the primary and secondary transgene DNA (reviewed in ref. 36). Therefore, it may be desirable to eliminate all ancillary sequences, including selectable markers, which are present on different transgene constructions in order to minimize regions of homology which may contribute to instability.

In the health and safety arena, one of the major apprehensions with the commercialization of transgenic products has been the concern that selectable marker genes or their products might be toxic or allergenic when consumed. Additionally, when selectable markers for antibiotics which have clinical or veterinary applications are used, the concern has been raised that the marker gene could be transferred into microorganisms and increase the number of resistant pathogenic microorganisms

in the human or animal gut. This would in turn compromise both clinical and veterinary applications of the antibiotic. In the area of environmental safety, the following concerns have been raised: (1) a marker encoding either antibiotic or herbicide resistance may change the transgenic plant into a weedy pest; (2) horizontal transmission of the marker into wild relatives may transform them into weedy pests, and (3) spread of the selectable marker to other organisms may upset the balance of the ecosystem<sup>5,6,31</sup>.

It is the responsibility of the appropriate companies to conduct the necessary risk assessment experiments, in accordance with governmental regulations, to insure the safety of new transgenic products. Such risk assessment of the *nptII* gene and its protein product has been carried out with the conclusion now widely accepted in the scientific community that there are no human or animal health risks associated with the use of this particular marker gene in transgenic crops<sup>29-32</sup>. Potential problems associated with the development of new weedy species would be realized only if there were sufficient levels of the selectable agent such that transgenic organisms would be given a selective advantage over others without the gene. This is highly unlikely for *nptII* and indeed, assessments have indicated that, at least in the Netherlands, such risks to the environment are negligible<sup>31</sup>.

It is however not possible to conclude that every marker gene

will be equally as safe<sup>6</sup>. For example, Gressel<sup>5</sup> makes the point that it may be unwise to grow transgenic oats containing a glufosinate resistance marker gene in regions where glufosinate is used to combat related wild oat weed pests. Other unacceptable scenarios are also easily imagined. Clearly risk assessments will need to be on a case by case basis with respect to the selectable marker gene, the target crop and the environment in which it is to be grown.

In addition to the scientific risk assessments, it is extremely important for those who want to commercialize transgenic products to be sensitive to risks perceived by the consumer. Bryant and Leather<sup>2</sup> argue that while there may be a tendency for scientists to dismiss public perception of risks as irrational, the public nevertheless has the right to avoid a product perceived as presenting an unacceptable risk. Consumer acceptance of the product in the marketplace is the bottom line regardless of the assurances described in risk assessment reports. Companies interested in commercializing transgenic products, particularly foods, should carefully assess the market value of offering selectable marker free products.

It has been suggested that it may be desirable to keep selectable markers in transgenic plants as an aid to breeders and regulatory agencies in the monitoring of transgenes in the environment<sup>3</sup>. However, there are limitations to using marker genes for this purpose<sup>37</sup>. First, screening based on gene activity would

**TABLE 1. Selectable marker genes for plant transformation.**

Marker gene	Gene product	Source	Selection	Reference
<i>nptII</i>	neomycin phosphotransferase	Tn5	kanamycin, G418, paromomycin, neomycin	9,10,11
Ble	bleomycin resistance	Tn5 and <i>Streptoalloteichus hindustanus</i>	bleomycin phleomycin	12,13
<i>dhfr</i>	dihydrofolate reductase	plasmid R67	methotrexate	11
<i>cat</i>	chloramphenicol acetyl transferase	phage p1Cm	chloramphenicol	14
<i>aphIV</i>	hygromycin phosphotransferase	<i>E. coli</i>	hygromycin B	15,16
SPT	streptomycin phosphotransferase	Tn5	streptomycin	17
<i>aacC3</i> , <i>aacC4</i>	gentamycin-3-N-acetyltransferase	<i>Serratia marcescens</i> ; <i>Klebsiella pneumoniae</i>	gentamycin	18
<i>bar</i>	phosphinothricin acetyl transferase	<i>Streptomyces hygroscopicus</i>	phosphinothricin, bialaphos	19,20
EPSP	5-enolpyruvylshikimate-3-phosphate synthase	<i>Petunia hybrida</i>	glyphosate	21
<i>bxn</i>	bromoxynil specific nitrilase	<i>Klebsiella ozaenae</i>	bromoxynil	22
<i>psbA</i>	Q <sub>B</sub> protein	<i>Amaranthus hybridus</i>	atrazine	23
<i>tfdA</i>	2,4-D monooxygenase	<i>Alcaligenes eutrophus</i>	2,4 dichlorophenoxyacetic acid	24
DHPS	dihydrodipicolinate synthase	<i>E. coli</i>	S-aminoethyl L-cysteine	25
AK	aspartate kinase	<i>E. coli</i>	high concentrations of lysine and threonine	25
<i>sul</i>	dihydropteroate synthase	plasmid R46	sulfonamide	26
<i>csrl-1</i>	acetolactate synthase	<i>Arabidopsis thaliana</i>	sulfonylurea herbicides	27
<i>tdc</i>	tryptophan decarboxylase	<i>Catharanthus roseus</i>	4-methyl tryptophan	28

be inconclusive because genes can be silenced by methylation or other suppression mechanisms<sup>36</sup>. Second, a screen based on identifying a selectable marker would not be specific for particular gene constructions since all plants bearing the same marker gene would be scored as positive. In order to monitor specific transgenes, it will be necessary to screen for specific gene constructions by PCR or Southern blot analysis. In the long term such screening would become very complicated, particularly assuming that a wide range of different selectable markers and an even wider range of different gene constructions should be in use.

### Transformation Systems Allowing Marker Gene Elimination

**Co-transformation.** Co-transformation of two separate DNAs, one incorporating a gene of interest and the other the selectable marker gene, may provide a simple system for the elimination of selectable marker genes, providing two criteria are fulfilled: (1) the efficiency of co-transformation needs to be reasonably high and, (2) the co-transformed DNAs must integrate at genomic locations sufficiently unlinked to allow effective recovery of recombination events.

Some early reports had indicated that co-transformation efficiencies using *Agrobacterium* were equal to the product of the efficiency of two independent transformation events<sup>38</sup>. However, more recent work has shown that significantly higher rates of co-transformation are achieved with both *Agrobacterium* mediated and direct transformation methods<sup>39,40</sup>. DeBlock and colleagues co-transformed *Brassica napus* with two *Agrobacterium* strains each carrying a T-DNA bearing a different selectable marker. Following selection for one of the markers, it was found that about 60–80% of these plants had also received the second T-DNAs, about 78% of the time at linked sites<sup>39</sup>. In another example, McKnight and coworkers<sup>40</sup> co-transformed tobacco with two *Agrobacterium* strains, one containing a T-DNA with an *nptII* gene and the second containing a T-DNA with a nopaline synthase gene. Of eleven plants selected and scored positive for NPTII, three were also nopaline synthase positive. NPTII and nopaline synthase activities segregated independently in progeny of all three co-transformed plants, indicating that co-integration was to unlinked sites in each plant. The differences observed between these two examples may be due to a number of factors such as the transformation vector, the *Agrobacterium* strain, the transformation procedure, and the species being transformed. Nevertheless, the data indicate that under some conditions, integration of different T-DNAs can occur to unlinked sites at reasonably high frequencies. Thus under some circumstances, co-transformation may be a suitable method for producing transgenic plants which do not contain a selectable marker gene.

**Site specific recombination systems.** Site specific recombination can be used to eliminate selectable markers as first demonstrated using the *Saccharomyces cerevisiae* 2  $\mu$ m circle site specific recombination system. Cregg and Madden<sup>41</sup> cloned the *S. cerevisiae* ARG4 gene between the asymmetric inverted repeat sequences (FRTs) which are the substrates for the site specific recombinase FLP. The ARG4-FRT construction was transformed into an *arg4* mutant of the yeast *Pichia pastoris* and transformants selected by Arg<sup>+</sup> prototrophy. A plasmid expressing FLP was then introduced in a second transformation and a recombination event was identified by selecting for the loss of Arg prototrophy. This experiment demonstrated how the same selection system could be used for multiple rounds of transformation.

The FLP site specific recombination system was soon demonstrated to be effective in transgenic *Drosophila*<sup>42</sup>, mammalian cells<sup>43</sup> and maize and rice protoplasts<sup>44</sup>. Since then, at least three

additional systems have been used to mediate site specific recombination in plant cells; the bacteriophage P1 Cre/*lox* system<sup>45,46</sup>, the pSR1 system of *Zygosaccharomyces rouxii*<sup>47</sup>, and the Gin recombinase system of phage Mu<sup>48</sup>. All of these are simple two component systems requiring a single peptide enzyme which acts *in trans* to catalyze recombination between two short, specific DNA sequences.

Of these four recombination systems, that derived from the phage P1 is most advanced for plant studies. In this system, the Cre enzyme catalyzes recombination between two 34 bp *loxP* sequences resulting in excision of internal sequences if the *lox* repeats are in direct orientation. The Cre gene can be introduced into the *lox* containing plant by either transformation or sexual crossings<sup>45,49</sup>. When the Cre gene is introduced by transformation, a marker gene cloned between two *lox* sites is eliminated from about 95% of the secondary transformants. The sexual crosses also lead to the marker being lost in a significant percentage of plants, though in this case the F1 individuals are largely chimeric. The rate of recovering F2 progeny without the *lox* flanked marker is variable between F1 individuals. This likely reflects the degree of chimerism in the F1 which itself is a function of the Cre insertion since different Cre transformants give rise to different levels of chimerism<sup>49</sup>.

If the *lox* sequences are inverted with respect to each other, Cre will catalyze the inversion of internal sequences. It has been proposed that generating inversions might be useful for converting functional genes to their antisense derivatives<sup>45</sup>. Alternatively, recombination between *lox* sites located on non-homologous chromosomes, perhaps mobilized to different positions by transposable elements, could generate reciprocal translocations. Finally, site specific recombination could be used to target the insertion of an incoming plasmid to particular chromosomal locations containing *lox* sites. Such a system would not substitute for targeted gene transfer, since a *lox* site needs to preexist in the desired chromosomal location. It might however be useful to compare different transgenes without complications of position effects<sup>45</sup>. In all cases, the desired events would need to be stabilized by removal of the Cre function.

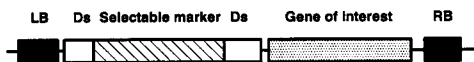
**Intra-genomic relocation of transgenes via transposable elements.** Many maize transposable elements maintain their transposition competence when transformed into other plant species<sup>50,51</sup>. Transposition of at least two of the best characterized maize element families, the *Ac/Ds* and the *Spm/dSpm* family, is by a conservative, cut-paste mechanism resulting in excision of the elements from one locus prior to reinsertion into a second (see ref. 52 for review). In general, transposition occurs to linked and unlinked sites in roughly equal proportions in both maize and transformed dicots<sup>53,54</sup>. We estimated that in tomato, about 10% of the elements that excise do not reinsert, or reinsert into a sister chromatid which is subsequently lost by somatic segregation<sup>55</sup>. The autonomous *Ac* element has two functions essential for transposition which can be physically separated, a transposase gene and inverted repeat termini. *Ds* elements lack the transposase function and are stable in the absence of *Ac*; they can however be trans-activated by introducing *Ac* transposase coding sequences *in trans*. Importantly, sequences cloned between the inverted repeats of a *Ds* element are also mobilized to new genomic locations in the presence of a transposase gene<sup>56,57</sup>. These observations have led to the development of a novel series of transformation vectors which incorporate transposable elements to eliminate selectable marker genes and other ancillary sequences<sup>34</sup>.

The T-DNA region of two classes of these vectors are shown in Figure 1. In the first type of vector, the gene of interest is inserted between the *Ds* inverted repeats (Fig. 1a) while in the second the selectable marker gene is flanked by the *Ds* repeats. In the presence of an active transposase, which can be intro-

### a) Type 1



### b) Type 2



**FIGURE 1.** Diagram of two types of transposable element based transformation vectors. A type 1 vector containing the gene of interest flanked by *Ds* sequences is shown in (a), a type 2 vector containing the selectable marker flanked by *Ds* is in (b). In figure (a) the transposase gene is shown as an integral component of the vector. As discussed in the text this feature is an option with either type of vector. In both figures the right and left T-DNA borders are shown as RB and LB respectively.

duced into the plant as an additional component of the T-DNA (as illustrated in Fig. 1a), or by a secondary transformation, or by crossing to a plant containing transposase, the *Ds* element will transpose to a new genomic location. In about 90% of the events, the *Ds* element, either carrying the gene of interest or the selectable marker gene, will reinsert; about one half of these insertions will be genetically unlinked to the primary site. Recombination between the original and new insertion site will result in progeny plants which segregate for the presence and absence of the chimeric *Ds* element and the presence and absence of the residual integrated T-DNAs (including in some cases the transposase gene). In this way, plants can be recovered which contain a stable transgene either internal to the *Ds* sequences (type 1) or linked to the T-DNA (type 2), and from which the selectable marker has been eliminated.

An advantage of the type 1 system in which the gene of interest is located within the *Ds* element is that by relocating the transgene, different levels of expression, both quantitative and qualitative, can be achieved<sup>34</sup>. This change in expression pattern as a function of the genomic location at which the element inserts is generally termed a position effect. Transposon mediated intra-genomic relocalization of the transgene provides a useful alternative to carrying out multiple independent transformations in order to achieve optimal transgene expression; particularly useful for species difficult to transform. An advantage of the type 2 vector system is that it can be constructed such that minimal amounts of vector sequences are retained. For example, if the construction contains only the 25 bp T-DNA repeat sequences essential for transformation, when the selectable marker is removed via transposition of the *Ds* only the gene of interest flanked by sequence residues of the direct repeats will remain in the plant. A second advantage is that the selectable marker will be lost in some percentage of somatic tissue by failure of the *Ds* element to re-integrate. This makes this strategy applicable for marker removal from vegetatively propagated crop species such as grapes, chrysanthemums, potatoes, strawberries, etc.

**Tissue specific expression of selectable marker genes.** In principle it should be possible to regulate transcription of the selectable marker gene by using a promoter which is preferentially expressed, either temporally or spatially, at the site of transformation. This would allow selection of transformants without expression of the marker in mature plants. The wound

inducible promoter AoPR1, isolated from *Asparagus officinalis*, shows promise as such a system<sup>58</sup>. An *nptII* gene driven by this promoter is sufficiently expressed at the wound sites of tobacco leaf disks to allow efficient selection of transformants. Mature leaf tissue showed however very little expression of NPTII and in some transformants it was virtually undetectable.

**Targeted gene replacement.** Ideally a transformation system would result in the chromosomal replacement of an endogenous gene with the corresponding transgene. Homologous recombination occurs frequently in some eukaryotes and transformation systems exploiting this mechanism have been successfully employed in fungal and animal systems<sup>59,60</sup>. Unfortunately, gene replacement in plants is disappointingly inefficient<sup>61-63</sup>. There are two general approaches to optimizing gene replacement systems; enhance the frequency of homologous events or reduce the frequency of non-homologous events<sup>64</sup>. Recent advances in our understanding of the biochemistry of recombination in plants may suggest alternative approaches to these problems<sup>65,66</sup>.

## Conclusions

In summary, the removal of selectable marker genes may be a reasonable procedure for the long term development of transgenic cultivars if one envisages that a number of transgenes will eventually be incorporated. Also, it may prove to be a sound marketing strategy. In terms of human, animal and environmental safety, different selectable marker genes will need to be independently assessed, as has been done for NPTII, to insure their safe use in commercial crops.

Novel transformation procedures are emerging which allow the generation of transgenic plants containing only the gene of interest without selectable marker sequences or other ancillary sequences. The choice of which transformation system to use is best made on a case by case basis for each particular need, gene and species. In co-transformation the frequency of recovering the desired events is a function of the transformation frequency as well as the degree of linkage between insertion sites. As these parameters are most likely dictated by the choice of vector, strains and transformation conditions, they need to be independently evaluated for each case. Site-specific recombination and transposition based methods are amenable to both *Agrobacterium* and direct transformation procedures providing the enzyme systems are active in the target plant. A unique advantage to the transposon systems is the ability to exploit genomic position effects by moving the gene of interest to alternative genome sites. The use of tightly regulated promoters are in principle applicable to all species irrespective of the transformation method. In contrast to the other systems however, use of tissue specific promoters does not allow re-transformation using the same selectable marker system and may be prone to leakiness or unexpected expression. Finally, while targeted gene replacement would be the best transformation system, it is unlikely to be widely useful without further enhancements. While the rationale for removing certain sequences from transgenic plants will continue to be debated, the ease with which some of the sequence removal strategies can be incorporated into ongoing transformation programs makes them important options to consider.

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