Strategies to deal with the concern about marker genes in transgenic plants: Some environment-friendly approaches

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Selectable marker genes, that confer resistance to antibiotics or herbicides, are generally incorporated along with the gene of interest in a transformation process so as to allow the recognition of the transformed cells from a background of untransformed ones. Subsequent to the generation of transgenic plants, the presence of these marker genes becomes no more of practical utility and thus arguably a matter of public euphoria, speculating the risk they can pose to the environment and health. Since the recurrent transformations to pyramid desirable genes are practically difficult with the same selectable markers used, novel strategies evolved to essentially eliminate these marker genes or to modulate their spatial/temporal expression, and by use of environmentally safe selectable markers and transplastomic plants. The article discusses the state-of-the-art applications and limitations associated with such systems.

THE rapid development of plant genetic engineering has led to the creation of transgenic crops, with genes transferred from organisms across their sexual boundaries, which is otherwise impossible through conventional breeding. Since the first transgenic plant was developed in tobacco in 1984 (ref. 1), transgenics in several economically important plants resistant to herbicides, insects, diseases, and also with superior nutritional and post-harvest quality[2] were developed, some of which are already in use. The dramatic increase in area of cultivation of transgenic crops is evident from a virtually ground zero in 1994 to around 125 million acres of land occupied the world over at the end 2001 (ref. 3). However, the public outcry and vehement protests by green crusaders against the commercialization of transgenics[4], due to possible environment and health hazards, demand thorough assessment on this aspect. One such concern is with genes that code for antibiotic and herbicide resistance. Co-transferred into the host genome as selectable markers, they confer selective advantage to the transformed cell/tissue to grow in the presence of the antibiotic/herbicide. The products of these genes may not be necessarily harmful[5], albeit their presence in transgenic plants increases the chances of their escape through pollen or seed dispersal to the wild and weed relatives of the crops, resulting in genetic pollution[6]. Antibiotic resistance genes may get passed onto pathogenic microorganisms in the gastrointestinal tract or soil, making them resistant to treatment with such antibiotics[6–8]. Similarly, resultant gene products in the transgenic plant may cause unknown health hazards to the consumers. Since a limited number of selectable marker genes are available for plant transformation, the combination of multiple transgenic traits through crosses among different transgenic lines will frequently produce plants that contain multiple copies of the same selectable marker linked to different effector genes. The presence of such multiple homologous sequences in plants enhances the likelihood for homology-dependent gene silencing[9], which could severely limit the reliable long-term use of transgenic crops.

Recently, many new strategies have been developed to eliminate the selectable marker genes from the transgenic plants for the improved safety of both the environment and the consumer. Again, it is possible to re-transform a transgenic plant using the same original marker for multigene transfer, thereby repeatedly stacking the transgene in a stepwise process. The ability to re-use a selectable marker could be particularly helpful for re-transforming species, which are otherwise difficult to transform or regenerate and for which a few markers work well. Besides marker removal systems, several other strategies where removal of marker genes is not needed, e.g. development of environmentally safe selectable markers or transplastomic plants, modulating and spatial/temporal expression of transgenes have been developed to ease the public concerns about health and environment.

Marker genes with potential risks

The use of genes encoding for antibiotic or herbicide resistance has assisted the selection of transformants in the background of untransformed ones. The neomycin phosphotransferase-II (*npt*II) gene that confers resistance to aminoglycoside antibiotics such as kanamycin and its analogues paromomycin and geneticin (G418)[10], and hygromycin phosphotransferase (*hpt*) gene, which con-

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fers resistance to hygromycin[11] have been successfully used as selectable marker in transformation of a variety of crop plants, including dicots and monocots. Similarly the *bar* (bialaphos resistance) and *pat* (phosphinothricin acetyltransferase) genes[12,13] which confer resistance to the herbicide BASTA® and phosphinothricin respectively[14], have also provided an efficient selection of transgenics in a number of crops. It is extremely important to evaluate the potential risks, as perceived by the consumer, associated with the marker genes in the transgenic crops prior to their field release. Such 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. The risk assessment of the *npt*II gene and its protein product has been carried out, with a 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[15]. It is however not possible to conclude that every marker gene will be equally safe[16]. Therefore, necessary risk-assessment experiments, in accordance with government regulations, must be carried out to ensure the safety of products of other marker genes in transgenic crops. Though the spread of marker genes to other organisms is difficult to preclude at this stage, recent evidence of horizontal flow of *bar* gene[17] by pollen dispersal from transgenic to cultivated rice is indeed a great concern, and strongly recommends removal of this gene from transgenic crops prior to their field release. Similar experimental designs using antibiotic resistance genes as tracer marker must be conducted to assess the escape of foreign genes through pollen dispersal from engineered plants to other cultivars of the same species or to weed relatives, before eliminating them from transgenic crops.

Safer marker genes

Green fluorescent protein gene as a visual selectable marker

The green fluorescent protein (*gfp*) gene is an ideal selectable marker and reporter for gene expression analysis and plant transformation[18]. The *gfp* gene, isolated from jellyfish, *Aequorea victoria*, encodes a small, barrel-shaped protein surrounding a fluorescent chromophore, which immediately emits green fluorescent light in the blue to ultraviolet range. Visual detection is possible at any time in living cells without their destruction and without the addition of any cofactor or external substrate. In addition, *gfp* gene product does not adversely affect cell growth, regeneration and fertility of transformed plants. The availability of mutant forms of *gfp* differing in solubilities and emission spectra make it possible to simultaneously monitor multiple transformation events within an individual transformant[19]. GFP has been of great use when the organogenesis or conversion segments of transformation are inefficient under antibiotic and herbicide selection. In the beginning, GFP was used in conjunction with antibiotics and herbicides and has been shown to decrease the number of escapes for a number of tree species[20]. Recently, *gfp* has been used as a sole visual selectable marker in genetic transformation of monocots, i.e. barley[21], oat[22], rice[23] and wheat[24]. GFP selection system seems to be promising for tissue culture/transformation systems that are inefficient, for recalcitrant genotypes, and for plant species for which no system exists.

β-*glucuronidase gene*

In the traditional selection system using antibiotics or herbicides, the transgenic cells convert the selective agent to a detoxified compound that may still exert a negative influence on plant cells. Further, the release of toxic metabolites by dying adjacent cells may also inhibit the growth of transformed cells. In contrast, a new set of markers known as positive selection markers are being developed, which can overcome some of the limitations encountered by the traditional selection system. One such selection system has been established which uses the β-glucuronidase gene from *E. coli* as a selectable gene and a glucuronide derivative of the cytokinins, benzyladenine as selective agent. Benzyladenine N-3 glucuronide is inactive as cytokinins but upon hydrolysis by GUS becomes active cytokinin, stimulating growth and regeneration of transformed cells *in vitro*[25] (Figure 1). The *GUS* gene served as both selectable and screenable markers. The efficiency of the transformation was reported to be about two-fold higher than with kanamycin.

Phosphomannose isomerase (PMI) and xylose isomerase (xylA) genes

Another selection system, also originated from the concept of favouring the transgenic cells while starving rather than killing the non-transgenic cells, is the *man-*

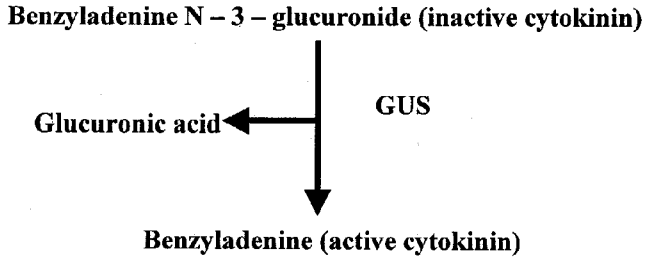

Figure 1. Hydrolysis of inactive cytokinin (benzyladenine N-3-glucuronide) to active cytokinin (benzyladenine) by GUS in cells transformed with a *GUS* gene.

nose selection system. It is based on *E. coli* phosphomannose isomerase (*PMI*) gene as a selectable gene and mannose as selective agent. The *manA* gene encoding PMI was cloned from *E. coli* by Danisco Biotechnology. After uptake by plant cells, mannose is phosphorylated by a hexokinase to mannose-6-phosphate that results in phosphate and ATP starvation, thus depleting energy from critical functions such as cell division and elongation. The mannose-6-phosphate toxicity in plant cells has also recently been shown to cause apoptosis or programmed cell death through induction of an endonuclease responsible for DNA laddering[26]. The non-transgenic PMI negative cells are unable to survive on media containing mannose as the sole carbon source. Plant cells transformed with this gene convert the selective agent to an easily metabolizable compound, fructose-6-phosphate, thus improving the energy status of the transformed cells and avoiding accumulation of derivatized selective agents. Joersbo and colleagues[27] observed that the use of mannose selection in sugar beet resulted in a ten-fold increase in transformation frequency when compared to traditional kanamycin selection. Novartis Agribusiness Biotech. Research Inc., which has licensed the *PMI* gene selection system, has found this marker to be effective in the selection of wheat and maize transgenics, with an astounding high frequency of transformation of 25% and 50% respectively. The *manA* gene has been used successfully in maize[28,29] and cassava[30] transformation. Scientists at Novartis have found that PMI protein is safe and readily digested by mammals. However, this system may not work well in those plants which contain an endogeneous level of phosphomannose isomerase. To overcome this problem, scientists at Danisco have recently devised another new selection system in which xylose isomerase (*xylA*) gene of *Streptomyces rubiginosus* is employed as the selectable marker and xylose as the selective agent. The enzyme from *S. rubiginosus* catalyses the isomerization of D-xylose to D-xylulose. The non-transformed plant cells cannot utilize the D-xylose as a sole carbon source. But cells which are transformed with *xylA* growing on xylose have the ability to convert D-xylose to D-xylulose and utilize it as a C source. The xylose isomerase selection system was tested in potato[31] and the transformation frequency was found to be approximately ten-fold higher than with kanamycin selection. The levels of enzyme activity in transgenic plants selected on xylose were five to 25-fold higher than the enzyme activity in control plants. Transformation using xylose as a selective agent in tobacco and tomato has been successful. Use of *xyl* as a selectable marker in plant transformation is quite safe, as xylose isomerase is used in the food industry on a commercial scale[32–34]. PMI–mannose and *xylA*–xylose appear to be ideal selectable systems for plant transformation as they obviate the need for antibiotic or herbicide, and in addition increase the frequency of recovering transformed plants.

Gene encoding enzymes in the hormone pathway

Genes encoding enzymes of the hormone pathways originating from *Agrobacterium* have been successfully used for the selection of transformed plants, although the presence or activity of the respective gene had to be eliminated or turned down in order to avoid detrimental effects of hormone overdoses on plant development. In 1997, Ebinuma and co-workers[35] developed a multi-auto-transformation (MAT) vector system in which the selectable marker isopentenyl transferase (*ipt*) gene is inserted into the maize transposable element Ac. The *ipt* gene is one of the tumour-inducing genes from *Agrobacterium tumefaciens*, which codes for isopentenyl transferase (IPT) and is involved in cytokinin synthesis in plant cells[36]. Introduction of the *ipt* gene under CaMV 35S in plants results in a marked increase in endogeneous cytokinin and the production of an extreme shooty phenotype, *ipt* shooty, which exhibits the loss of apical dominance and root formation. As the *ipt* selectable gene is combined with Ac in MAT vector system, phenotypically normal transgenic plants can be obtained subsequently by the removal of *ipt* gene by Ac, achieving a goal of marker-free transformation. However, one of the major limitations of using this system is the low frequency of marker-free transgenic plants, as most of the modified transposable elements (containing *ipt* gene) reinsert elsewhere in the genome shortly after their excision and thus only cells with transposition errors would generate phenotypically normal plants. To overcome this problem, a new MAT vector has been created in which the Ac for removing the *ipt* gene is exchanged with a site-specific recombination system *R/rs* isolated from *Zygosaccharomyces rouxii*[37]. The *R/rs* system comprises a *R* recombinase gene and two *rs* recombination site sequences. The *ipt* combined with the (*R*) gene was placed within two directly-oriented recognition sites to remove it from transgenic cells after transformation. The improved MAT vector is used to generate marker-free transgenic plants efficiently. Such a system can be applied to woody plants or vegetatively propagated species to produce marker-free transgenic plants[37] as well as providing the basis for the development of an inducible plant transformation system. Expression of *ipt* gene under dexamethasone-inducible promoter led to the recovery of lettuce and tobacco transformants under inducing conditions[38].

rol genes

The *rol* genes derived from *A. rhizogenes* are responsible for the proliferation of hairy roots, which spontaneously regenerate into transgenic plants with abnormal phenotype such as wrinkled leaves, shortened internodes and reduced apical dominance. Such plants are easily identified, thus necessitating eviction of selectable markers[39].

Dihydrodipicolinate synthase and desensitized aspartate kinase-selectable marker

Perl *et al.*[40] demonstrated that the regulatory enzymes of aspartate family pathway, which leads to the synthesis of the branched-chain amino acids, lysine, threonine, methionine and isoleucine from aspartate, could be used as selectable markers and lysine plus threonine as selective agent. Treatments with millimolar concentration of lysine plus threonine strongly inhibited the growth of a wide range of plant species. This is due to the complete inhibition of aspartate kinase (AK) activity by these amino acids, causing starvation for methionine. AK, the first enzyme of the aspartate family pathway, consists of several isoenzymes which are feedback-inhibited by lysine and threonine. Another regulatory enzyme of this pathway, dihydrodipicolinate synthase (DHPS), is also extremely sensitive to feedback inhibition by lysine, and is the major limiting factor for the synthesis of lysine. Bacterial genes for AK and DHPS enzymes are less sensitive to lysine inhibition than their counterparts in plants, and these genes have been successfully used as selectable markers for genetic transformation of tobacco[41,42], potato[43] and barley[44]. The plants transformed with bacterial desensitized *AK* genes were selected for resistance to the presence of lysine plus threonine in the regeneration medium and those transformed with *DHPS* gene were selected for resistance to the toxic lysine analogue, S-aminoethyl L-cysteine. The transgenic plants showed higher activity of AK and DHPS enzymes, which resulted in overproduction of lysine, threonine and methionine. Thus, in plants where accumulation of these essential amino acids is desired, it is possible to obtain both selection and introduction of agronomically desirable traits simultaneously.

Betaine aldehyde dehydrogenase gene

Recently, Daniell *et al.*[44] engineered chloroplast genome without the use of antibiotic selection. The betaine aldehyde dehydrogenase (*BADH*) gene from spinach was used as the selectable marker. This enzyme is present only in the chloroplast of a few plant species (members of Chenopodiaceae, Poaceae, etc.). Rapid regeneration of chloroplast transgenic plants was obtained under betaine aldehyde (BA). The selection process involves the conversion of the toxic BA by the chloroplast BADH enzyme to a nontoxic glycine betaine, which also serves as an osmoprotectant for enhancing drought and salt stress tolerance in plants[46]. Transgenic plants were morphologically indistinguishable from untransformed plants and the induced trait was stably inherited in the subsequent generations. Chloroplast transformation efficiency was 25-fold higher in BA selection than spectinomycin[45], which is widely used for chloroplast transformation.

Methods for removal of marker genes

Co-transformation

One of the strategies to produce transgenic plants free from selectable marker is that the marker and the gene of interest are placed on two separate T-DNAs in a single plasmid[47,48] or on separate plasmids which are contained in one[49] or more agrostrains[48,50] (Figure 2). The transgene

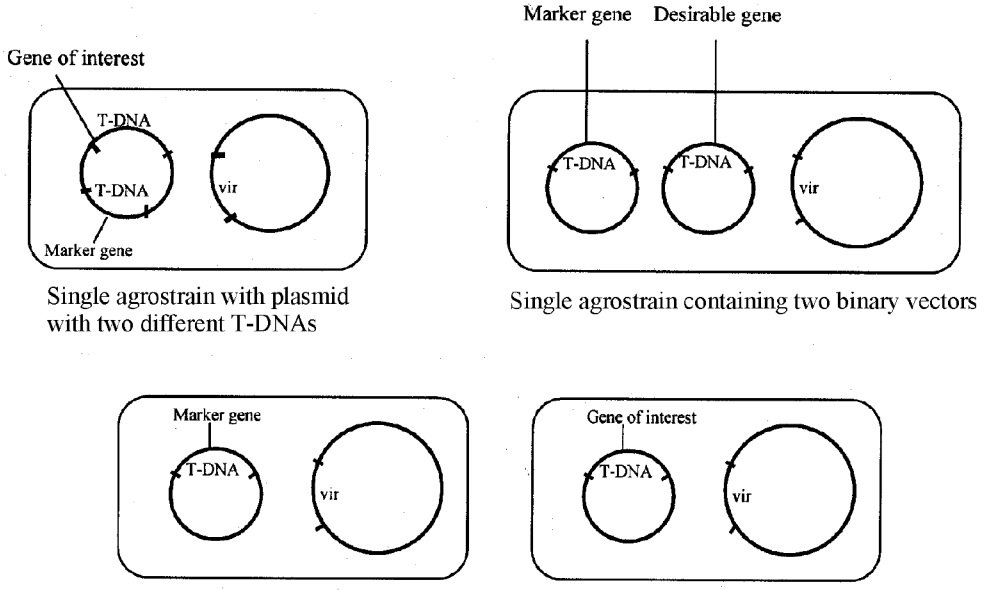

Figure 2. Schematic representation of expression of strains of *A. tumefaciens* and vectors. vir, virulence region.

and marker are thus inserted at the loci, which should recombine at reasonably high frequencies; the gene of interest can be segregated from the selectable marker gene in the next generation. Using this technique, transgenic plants free of a selectable marker gene were developed in rice, *Brassica napus*[50] and *Nicotiana tabacum*[48,51]. Similarly, in direct transformation method of particle bombardment, the transgene and marker gene carried in separate plasmids, upon coating to gold or tungsten particles, can be bombarded to target tissue to produce transgenic plants. Co-transformation frequency more than approximate 50% has been obtained with both *Agrobacterium*-mediated and with a direct gene transfer, but *Agrobacterium*-mediated transformation has been the method of choice for elimination of transgenes[51]. The frequency with which the transgene and marker gene segregate is determined by the location of the transgene and the marker gene in the plant genome, and the type of agrostrain used. Whether one- or two-strain method provides a higher frequency of marker-free plant recovery, is not clear. The use of two plasmids in one octopine-derived agrostrain has been found to give high co-transformation frequency, with high frequency of unlinked integration[48].

Site-specific recombination

Another approach to eliminate the selectable marker gene is to flank the selectable marker gene with direct repeats of recognition sites for a site-specific recombinase, which allows the enzyme to excise the marker gene from the plant genome by enzyme-mediated site-specific recombination (Figure 3 *a*). Among many site-specific recombination systems that have been characterized, bacteriophage P1 Cre/*lox* recombination system is one which consists of the 38-kDa product of the *cre* gene (the Cre recombinase) and the asymmetric 34 base pair (bp) *lox* sites, composed to two 13 bp inverted repeats with 8 bp asymmetric core region that gives directionality to the

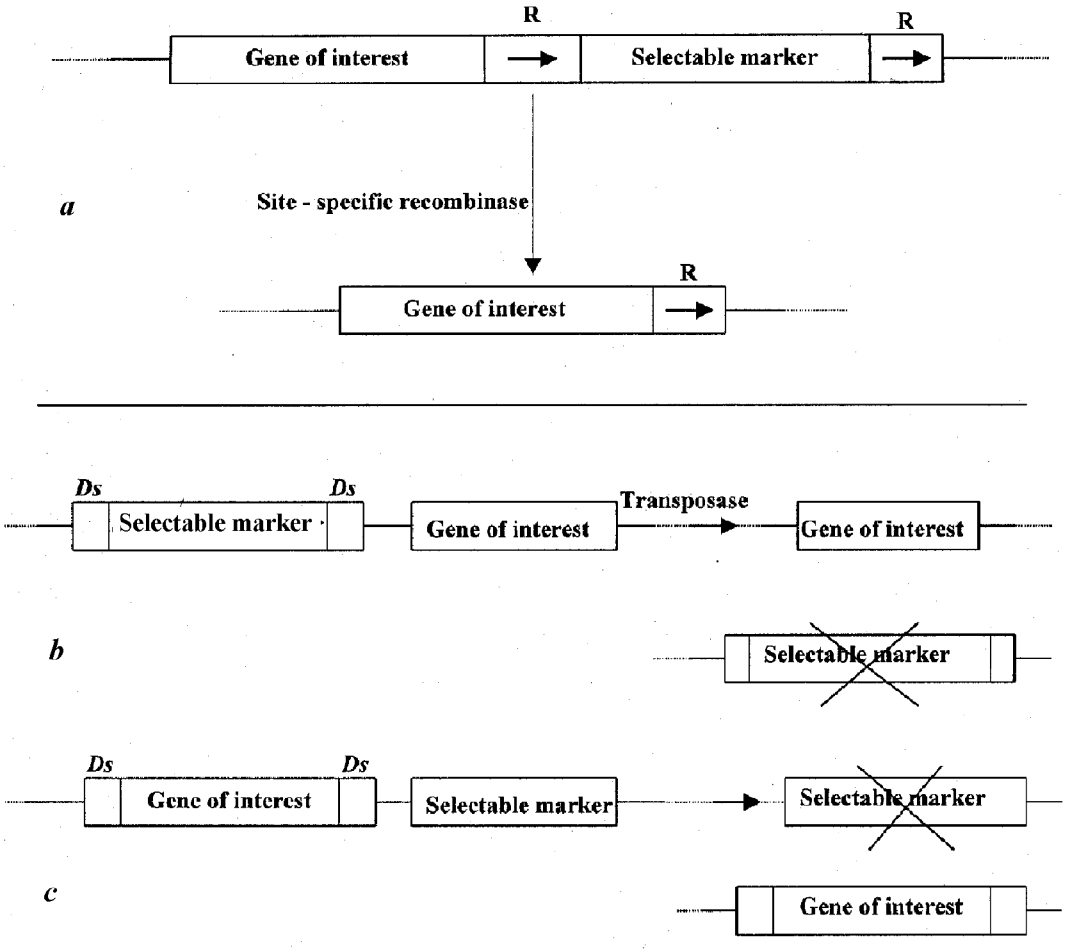

Figure 3. Strategy for elimination of selectable marker gene. ***a***, Selectable marker gene is flanked by direct repeats (R) which are recognized by site-specific recombinase; ***b***, Selectable marker gene is inserted between the *Ds* inverted repeats; ***c***, The gene of interest is flanked by the *Ds* repeats. The activation of transposase allows *Ds* elements to move to a new genomic location. Genomic crosses and/or segregation will dissociate the two transgenes.

site[52]. No additional factors are required for Cre-catalysed recombination between the *lox* sites. The Cre/*lox* system has been used to direct the site-specific integration of incoming plasmids at the *lox* sites previously placed in the genome by direct gene transfer[53] or *Agrobacterium*-mediated transfer[54]. Cre-*lox* system thus offers a method for precise insertion of single copy DNA into genomic targets. Two recombination *lox* sites of the same orientation flank the integrated DNA. Introduction of Cre results in excision of the internal sequence (insert), if the *lox* repeats are in direct orientation. The *cre* gene can be introduced into the *lox*-containing plant by either transformation or sexual crossing. Upon transformation, a marker gene cloned between two *lox* sites is eliminated from about 95% of the secondary transformants. Tobacco[55] and *Arabidopsis*[56] transgenic plants free from a selectable marker gene were recovered using cre/*lox* recombination system. The Cre-catalysed excision events in the plant genome are precise and conservative, i.e. without loss or alteration of nucleotides in the recombinant site. On the other hand, if the *lox* sites are located in opposite direction, Cre will catalyse the inversion of internal sequences. It has been proposed that generating inversion might be useful for converting functional genes to their antisense derivatives. The other single-chain recombinases, which have also been found to be useful for the removal of marker genes are the FLP/*frt* system of 2 μm plasmid of *Saccharomyces cerevisiae*[57,58] and *R/rs* system of pSR1 plasmid of *Zygosaccharomyces rouxii*[59,60]. A common feature of these systems is that the first round of transformation produces transgenic plants with the selection marker between two directly oriented recognition sites for the respective recombinase. After expression of the single-chain recombinase, either by crossing in plants expressing the enzyme, by transient expression via second transformation or by the use of an inducible promotor, the recombinase reaction is initiated resulting in marker-free transgenic plants.

Removal of marker genes by transposases

Transposable elements have been employed for the dissociation of marker and desirable genes in two ways: (i) The marker gene is placed on mobile element, which is lost after transposition[15] (Figure 3 *a*). Marker-free transgenic tobacco and aspen plants have been generated by inserting the selectable *ipt* gene into transposable element Ac[35]. (ii) The transgene by itself is mobile; the activation of transposase allows the relocation of the desired transgene to a new chromosomal position (Figure 3 *b* and *c*). Genetic crosses and/or segregation will dissociate the two transgenes. The feasibility of this approach was demonstrated in tomato[61]. Relocation of the transgene at different genomic positions results in differential levels of expression. In addition, a series of plants with different loci can be obtained from one original transformant, and the one with optimal transgene expression may be useful for species difficult to transform.

Intrachrosomal recombination for marker gene elimination

Recently, Peter Meyer's group[62] developed a novel strategy for the production of transgenics in tobacco devoid of a selectable marker gene after a single round of transformation. This involves DNA deletion based on intrachromosomal homologous recombination (ICR) between two homologous sequences. The selectable marker gene *npt*II and negative selectable marker *tms2* gene were flanked by two 352 bp attachment P (attP) regions of bacteriophage-λ in a binary vector (pattP–ICR). The attP-sites are used by the bacteriophage-λ for integration at the attB-site of the *E. coli* genome, a reaction in which two proteins are involved, those of the phage-encoded integrase (int) and the bacterial integration host factor (IHF). The construct (pattP–ICR) was introduced into tobacco leaves using *A. tumefaciens* and resistant calli were selected with kanamycin. These calli were transferred onto kanamycin-containing shoot regeneration medium. Out of 11 calli, two produced a mixture of green and white shoots, indicating loss of the *npt*II gene. For further studies, white leaves were regenerated on kanamycin-free medium and the regenerated shoots were placed on a medium supplemented with naphthalene acetamide (NAM), which is converted by the *tms2* gene product into the auxin NAA, a phytohormone that blocks root development. Only those shoots in which the *tms2* gene was lost were expected to produce shoots, and DNA analysis confirmed not only the loss of both the *npt*II and *tms2* genes but also that in three out of 23 cases, this event was caused by homologous recombination between the attP-sites. The attP system does not require the expression of helper proteins to introduce deletion events, or a genetic segregation step to remove recombinase genes, thus providing a useful tool to remove undesirable transgene regions, especially in vegetatively propagated species.

Transplastomics to overcome pollen gene transfer

Transplastomics are the transgenic plants with pure population of transformed chloroplasts (integration of transgenes into plastid genome instead of nuclear genome). Escape of transgenes from transplastomic crops poses a negligible risk of pollen-mediated movement of transgenes, as transplastomes are not transmitted by pollen[63]. Chamberlain and Stewart[64] assessed the possibility of pollen-mediated movement of transgenes from transplastomic *Brassica napus* to its wild relative *B. napa*. They

used naturally occurring chloroplast genes as marker for hypothetical transgene escape and confirmed that in contrast to a nuclear-encoded trait, a plastid-encoded trait will not be transmitted from the crop to the weed through pollen. However, the crop plants which exhibit biparental inheritance or those that are propagated vegetatively might be less amenable to this technology. Further introduction of transgenes into the chloroplast genome amplifies the antibiotic resistance genes. Moreover, the compatible protein synthesis machinery between chloroplast and bacteria may enhance the probability of gene transfer from plants to bacteria living in the soil or gastrointestinal tract. Recently, it has been shown that selectable markers can be removed after the generation of the chloroplast transgenic plants. Fischer *et al.*[65], in an investigation on *Chlamydomonas reinhardii*, showed that the homologous recombination between two direct repeats flanking a selectable marker enabled it to be removed under non-selective growth conditions. Jamtham and Day[66] extended this technology to tobacco and demonstrated that the T_0 chloroplast tobacco transgenics were generated by transformation with vector containing two independent sets of direct repeats flanking different genes that were in tandem, *uidA*, *aadA* and *bar*, which code for β-glucuronidase, antibiotic resistance and herbicide resistance respectively. Vegetative propagation using herbicide results in the production of *aadA*-free plants because of the recombination even among flanking direct repeats. Sexual back-cross of the T_1 in the absence of the selection produced marker-free chloroplast transgenic T_2 plants in 24% of the population. The feasibility of this method in other crops is yet to be shown. Recently, transplastonic lines have been developed in *Arabidopsis*[67] and potato[68], but uniparental transgene transmission has not been demonstrated. However, Ruf *et al.*[69] reported generation of fertile transplastomic tomato plants which transmitted the transgenes in a uniparentally maternal fashion, as expected for a plastid-encoded trait.

Modulation of marker gene expression

Antisense technology could be applied to prevent the expression of marker genes. When a marker gene that is cloned in its reverse orientation is introduced into a cell, it encodes antisense RNA that is complementary to the mRNA of the original marker gene and forms an RNA duplex, which renders the mRNA inactive. Catalytic RNA cleaves either itself or other RNA molecules. Examples of catalytic RNA are ribozymes, which can act specifically on target RNA molecules to render them inactive. Catalytic RNA has created new opportunities to repress the expression of selectable marker genes. The expression of a ribozyme gene directed towards the target mRNA of kanamycin selectable marker in plants resulted in a reduction of the kanamycin gene product[70]. Genetically modified plants containing selectable markers could be further modified with genes that encode antibodies against the marker gene protein product[71].

Spatial and temporal expression of selectable marker gene

It is possible to regulate transcription of the selectable marker gene by using a promoter, which is preferentially expressed, either temporarily or spatially at the time/site of transformation. This would allow selection of transformants without expression of the markers in mature plant. An *npt*II gene driven by the wound-inducible promoter AoPRi expressed simultaneously at the wound site of tobacco leaf discs, to allow efficient selection of transformants. Mature leaf tissue showed very little expression of NPTII and in some transformants, it was virtually undetectable[72].

Transfer of desired transgene in the zygote

The single-cell regeneration system, e.g. zygote has a great potential for the generation of marker-free transgenic plants, especially in cereals[73], as the protocols for regeneration of plants from zygote are available in *Oryza sativa*[73], *Triticum aestivum*[74,75], *Zea mays*[76] and *Hordeum vulgare*[77]. Recently, transgenic plants of barley were generated by microinjection of the transgene into the zygote[77] and it has been suggested that the transformation of the individual zygote might allow the identification and regeneration of transgenic plants containing only the gene of interest. The ability to grow a complete plant from microinjected zygote is a valuable approach to avoid the use of selectable markers.

Conclusions and future prospects

Apart from scientific risk assessments associated with selectable marker genes in transgenic crops, sensitive issues related to the risks perceived by the consumers cannot be neglected as consumer acceptance in the market place is the bottom line, regardless of the assurances described in the risk assessment reports. Further, germplasm hierarchy, safety and balance cannot be ignored while commercializing transgenic crops. Where there is ample evidence for marker gene flow as in the *bar* gene, removal of marker gene so as to hasten the commercilization of engineered crops and gene stacking, is a must. Over the past decade, several approaches have been developed either for excising marker genes from transgenic plants or their modulation and spatial/temporal expression. Further, alternative environmentally safe selectable markers or containment of the transgenes through transplastomic plants have given a new dimen-

sion. Though each approach has its own advantages and disadvantages, all of them depend on the frequency and time-frame in which such plants are produced, the ease with which the transgenes are cloned into vectors, the number of genes that can be introduced into plants at one time, and the number of times a system could be used for sequential transformation. The antibiotic/herbicide selectable markers have been removed using several site-specific recombination systems (e.g. *lox*/Cre recombinase, Flp recombination target (FRT)/*flp* recombinase or *Rs*/*R* recombinase) or intrachromosomal recombination between page-attachment region (attP) sites or through a transposable element (A*c*) or a co-transformation system to segregate the gene of interest from the selectable marker gene. These approaches are time-consuming, as sexual crossing or two-step regeneration is required to excise or segregate a marker gene, rather than confined to the plants propagating through sexual crosses. Further, such methods are not convenient for species with long generation times, such as trees. On the contrary, MAT vectors, which do not require either re-transformation or sexual crossing for generation of marker-free transgenic plants, save considerable time and effort. However, the MAT system with *ipt* gene is not suitable for plants regenerating through somatic embryogenesis rather than cytokinin-dependent organogenesis. Although the homologous recombination operates efficiently in plant chloroplasts, it is less predictable and efficient in nuclear DNA. In deleting a nuclear marker, two successive rounds of screening are needed with an overall efficiency of less than one per cent. The transposition method can also be unreliable and excision of transposon from the genome can alter adjacent DNA sequences. Use of alternative selectable markers to antibiotics and herbicides such as β-glucuronidase, green fluorescent protein, isopentenyl, phosphomannose and xylose isomerase genes increases the efficiency and flexibility of plant transformation and does not cause any risk to animal, human or environmental safety. The alternative selectable markers would be valuable for re-transformation of plant species, incapable of sexual reproduction and for which methods that rely on gene segregation for elimination of the selectable marker would not work. 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. The generation of transplastomic plants enhances gene containment because in many crop plants, plastids are inherited from the maternal parent, preventing the pollen spread of transgenes. However, fertile transplastomic plants have so far been developed only in a few plant species. Most of the marker-free transgenics have been developed in model plants, e.g. tobacco or *Arabidopsis*. The feasibility of these approaches has to be demonstrated in other crops. Once the marker gene is eliminated from plants, either a phenotype produced by the gene of interest or detection of the gene itself must be relied upon for identification of transgenic individuals. If a gene does not result in an easily recognizable phenotype, as does a marker gene, the analysis of segregating populations becomes more tedious, expensive and time-consuming. While use of conventional marker genes cannot be ignored at the experimental stage considering the extreme difficulties in recalcitrant crops, use of markers with substantial evidence for potential risks must be discouraged and alternative safer marker genes should be given priority. Further, a case-to-case examination of products of each marker gene, and its consequent flow to neighbouring crops as well as horizontal flow should be carefully examined in accordance with government regulations, before reaching to a conclusion. Some of the selectable marker genes may in future be exchanged for native plant genes, thus altogether eliminating the use of foreign genes as selectable marker.

1. Horsch, R. B., Fry, J. E., Hoffman, N. L., Eichholtz, D., Roger, S. G. and Fraley, R. T., *Science*, 1985, **227**, 1229–1230.
2. Pattanayak, D. and Kumar, P. A., *Proc. Indian Natl. Sci. Acad.*, 2000, **B66**, 265–310.
3. Gupta, P. K., *Curr. Sci.*, 2002, **82**, 4.
4. Sahoo, L., Singh, N. D., Sonia, Sugla, T., Singh, R. P. and Jaiwal, P. K., *Physiol. Mol. Biol. Plants*, 2001, **7**, 1–2.
5. Dale, P., *Biochemist*, October 1999, 10–12.
6. Daniell, H., *Trends Plant Sci.*, 1999, **4**, 467–469.
7. Puchta, H., *ibid*, 2000, **5**, 273–274.
8. Daniell, H. *et al.*, *Curr. Genet.*, 2000, **39**, 109–116.
9. Matzke, M. A. and Matzke, A., *Plant Mol. Biol.*, 1991, **16**, 219–221.
10. Bevan, M., Flavell, R. B. and Chilton, M. D., *Nature*, 1983, **304**, 184–187.
11. van den Elzen, M., Townsend, J., Lee, K. and Bedbrook, J., *Plant Mol. Biol.*, 1985, **5**, 299–302.
12. Thompson, C. J., Rao Movva, N., Tizard, R., Crameri, R., Davies, J. E., Lauwereys, M. and Botterman, J., *EMBO J.*, 1987, **6**, 2519–2523.
13. Strauch, E., Wohlleben, W. and Puhler, A., *Gene*, 1988, **63**, 65–74.
14. Murakami, T., Anzai, H., Satoh, A., Nagaoka, D. and Thompson, C. J., *Mol. Gen. Genet.*, 1986, **205**, 42–50.
15. Yoder, J. I. and Goldsbrough, A. P., *Bio/Technology*, 1994, **12**, 263–267.
16. Dale, P. J., *Plant Physiol.*, 1992, **100**, 13–15.
17. Messeguer, J., Fogher, C., Guiderdoni, E. and Marfa, V., *Theor. Appl. Genet.*, 2001, **103**, 1151–1159.
18. Sonia, Jaiwal, P. K., Ahad, A. and Sahoo, L., *Curr. Sci.*, 1998, **74**, 402–405.
19. Stuber, R. H., Horie, K., Carney, P., Hudson, E. A., Tarasova, N. I., Gaithanaris, G. A. and Pavlakis, G. N., *Biotechniques*, 1998, **24**, 462–471.
20. Tian, L., Seguin, A. and Charest, J. P., *Plant Cell Rep.*, 1997, **16**, 267–271.
21. Ahlandsberg, S., Sathish, P., Sun, C. and Jansson, C., *Physiol. Plant.*, 1999, **107**, 194–200.
22. Kaeppler, H. F., Menon, G. K., Skadsen, R. W., Nuutila, A. M. and Carlson, A. R., *Plant Cell Rep.*, 2000, **19**, 661–666.
23. Vain, P., Worland, B., Kohli, A., Snape, J. W. and Christou, P., *Theor. Appl. Genet.*, 1998, **96**, 164–169.
24. Jordan, M. C., *Plant Cell Rep.*, 2000, **19**, 1069–1075.

25. Joersbo, M. and Okkels, F. T., *ibid*, 1996, **16**, 219–221.
26. Stein, J. C. and Hansen, G., *Plant Physiol.*, 1999, **121**, 71–79.
27. Joersbo, M., Donaldson, I., Kreiberg, J., Petersen, S. G., Brunstedt, J. and Okkels, F. T., *Mol. Breed.*, 1998, **4**, 111–117.
28. Evans, R., Wang, A., Hanten, J., Altendorf, P. and Mettler, I., *In Vitro*, 1996, **32**, 72A, abstr.
29. Wang, A., Evans, R., Altendrof, P., Doyle, M. C. and Rosichan, J. L., *Plant Cell Rep.*, 2000, **19**, 654–660.
30. Zhang, P. and Pounti-Karlaes, J., *ibid*, 2000, **19**, 1041–1045.
31. Haldrup, A., Petersen, S. G. and Okkels, F. T., *ibid*, **18**, 76–81.
32. Report, Food and Drug Administration Fed. Regist. 48, 1983, p. 5715.
33. *Regulatory Aspects of Microbial Food Enzymes*, Association of Microbial Food Enzymes Producers, Brussels, 1992, 4th edn.
34. Bhosale, S. H., Rao, M. B. and Deshpande, V. V., *Microbiol. Rev.*, 1996, **60**, 280–283.
35. Ebinuma, H., Sugita, K., Matsunaga, E. and Yamakado, M., *Proc. Natl. Acad. Sci. USA*, 1997, **94**, 2117–2121.
36. Barry, G. F., Rogers, S. G., Fraley, R. T. and Brand, L., *ibid*, **81**, 4776–4780.
37. Sugita, K., Matsunaga, E. and Ebinuma, H., *Plant Cell Rep.*, 1999, **18**, 941–947.
38. Kunkel, T., Niu, Q.-W., Chan, Y.-S. and Chua, N.-H., *Nature Biotechnol.*, 1999, **17**, 916–919.
39. Edinuma, H., Sugita, K., Matunaga, E., Endo, S. and Kasahara, E., in *Molecular Biology of Woody Plants* (eds Jain, S. M. and Minocha, S. C.), Kluwer, the Netherlands, 2000, pp. 24–46.
40. Perl, A., Galili, S., Shaul, O., Ben-Tzvi, I. and Galili, G., *Bio/Technology*, 1992, **11**, 715–718.
41. Shaul, O. and Galili, G., *Plant Physiol.*, 1992, **100**, 1157–1163.
42. Karchi, H., Shaul, O. and Galili, G., *Plant J.*, 1993, **3**, 721–727.
43. Brinch-Pedersen, H., Galili, G., Knudsen, S. and Holm, P. B., *Plant Mol. Biol.*, 1996, **32**, 611–620.
44. Daniell, H., Wiebe, P. O. and Fernandez-San Milan, A., *Trends Plant Sci.*, 2001, **6**, 237–239.
45. Rathinasabapathi, B. *et al.*, *Planta*, 1994, **193**, 155–162.
46. Depicker, A., Herman, L., Jacobs, S., Schell, J. and Van Montagu, M., *Mol. Gen. Genet.*, 1985, **201**, 477–484.
47. Komari, T., Hiei, Y., Saito, Y., Murai, N. and Kumashiro, T., *Plant J.*, 1996, **10**, 165–174.
48. de Framond, A. J., Back, E. W., Chilton, W. S., Kayes, L. and Chilton, M. D., *Mol. Gen. Genet.*, 1986, **202**, 125–131.
49. De Block, M. and Debrouwer, D., *Theor. Appl. Genet.*, 1991, **82**, 257–263.
50. Daley, M., Knauf, V. C., Summerfelt, K. R. and Turner, J. C., *Plant Cell Rep.*, 1998, **12**, 489–496.
51. Hiei, Y., Komari, T. and Kubo, T., *Plant Mol. Biol.*, 1997, **35**, 205–218.
52. Hoess, R. H. and Abremeski, K., *J. Mol. Biol.*, 1985, **181**, 351–362.
53. Albert, H., Dale, E. C., Lee, E. and Ow, D. W., *Plant J.*, 1995, **7**, 649–659.
54. Vergunst, A. C. and Hooykaas, P. J. J., *Plant Mol. Biol.*, 1998, **7**, 649–659.
55. Dale, E. C. and Ow, D. W., *Proc. Natl. Acad. Sci. USA*, 1991, **23**, 10558–10562.
56. Russell, S. H., Hoopes, J. L. and Odell, J. T., *Mol. Gen. Genet.*, 1992, **234**, 49–59.
57. Kilby, N. J. *et al.*, *Plant J.*, 1995, **8**, 637–652.
58. Lyznik, L. A. *et al.*, *Nucleic Acid Res.*, 1996, **24**, 3784–3789.
59. Onouchi, H. *et al.*, *Mol. Gen. Genet.*, 1995, **247**, 653–660.
60. Sugita, K. *et al.*, *Plant J.*, 2000, **5**, 461–469.
61. Goldsbrough, A. P., Lastrella, C. N. and Yodar, J. I., *Bio/Technology*, 1993, **11**, 1286–1292.
62. Meyer, P., Zubko, E. and Scutt, C., *Nature Botechnol.*, 2000, **18**, 442–445.
63. Carrer, H. and Maliga, P., *Biotechnology*, 1995, 791–794.
64. Chamberlain, D. and Stewart, C. N., *Nature Biotechnol.*, 1999, 330–331.
65. Fischer, N. *et al.*, *Mol. Gen. Genet.*, 1996, **251**, 373–380.
66. Jamthan, S. and Day, A., *Nature Biotechnol.*, 2000, **18**, 1172–1176.
67. Sikdar, S. R., Serino, G., Chaudhuri, S. and Maliga, P., *Plant Cell Rep.*, 1998, **18**, 20–24.
68. Sidorov, V. A., *Plant J.*, 1999, **19**, 209–216.
69. Ruf, S., Hermann, M., Boerger, I., Carrer, H. and Bock, R., *Nature Biotechnol.*, 2001, **19**, 870–875.
70. Steinecke, P., Herget, T. and Schreier, P. H., *EMBO J.*, 1992, **11**, 1525–1530.
71. Hiatt, A., Cafferkey, R. and Bowdish, K. *Nature*, 1989, **342**, 76–78.
72. Ozcan, S., Firek, S. and Draper, J., *Bio/Technology*, 1993, 218–221.
73. Zhang, J., Dong, W. H., Galli, A. and Potrykus, I., *Plant Cell Rep.*, 1999, **19**, 128–132.
74. Kumlehn, J., Schieder, O. and Lorz, H., *ibid*, 1997, **16**, 663–667.
75. Kumlehn, J., Lorz, H. and Kranz, E., *Planta*, 1998, **205**, 327–333.
76. Leduc, N. *et al.*, *Dev. Biol.*, 1996, **177**, 190–203.
77. Holm, P. B., Knudsen, S., Mouritzen, P., Negri, D., Olsen, F. L. and Roue, C., *Plant Cell*, 1994, **6**, 531–543.

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