Review

Methods to produce marker-free transgenic plants

Behrooz Darbani[1], Amin Eimanifar[2], C. Neal Stewart, Jr.[3] and William N. Camargo[4]

[1]Agriculture Biotechnology Research Institute for Northwest & West of Iran, Tabriz, Iran
[2]Iranian Artemia Reference Center, Urmia, Iran
[3]Department of Plant Sciences, University of Tennessee, Knoxville, TN, USA
[4]Fisheries and Illinois Aquaculture Center, Southern Illinois University at Carbondale, Carbondale, IL, USA

Selectable marker genes (SMGs) have been extraordinarily useful in enabling plant transformation because of the low efficiency of transgene integration. The most used SMGs encode proteins resistant to antibiotics or herbicides and use negative selection, *i.e.*, by killing nontransgenic tissue. However, there are perceived risks in wide-scale deployment of SMG-transgenic plants, and therefore research has recently been performed to develop marker-free systems. In this review, transformation using markers not based on antibiotic or herbicide resistance genes, as well as different systems of marker gene deletion, are discussed.

Received 13 September 2006
Revised 16 October 2006
Accepted 30 October 2006

Keywords: Biosafety · Marker genes · Negative selection · Selectable markers · Site-specific recombination · Transposons

1 Introduction

To produce transgenic plants, selection systems are used that lead to the selective growth of transformed cells. Genes encoding for resistance to specific antibiotics or herbicides have been found to be particularly effective for selection and provide a means for rapidly identifying transformed cells, tissues, and regenerated shoots. Antibiotics and herbicides kill cells by a variety of mechanisms and resistance genes have been widely used in transgenic plant production. For instance, kanamycin and related aminoglycoside antibiotics kill cells by inhibition of protein translation [1, 2] and the bacterially derived *npt*II gene, encoding neomycin phosphotransferase, inactivates these antibiotics by phosphorylation [3]. The herbicide phosphinothricin (PPT), an analog of glutamine, is toxic to plants by irreversibly inhibiting glutamine synthetase, a key enzyme for ammonium assimilation and the regulation of nitrogen assimilation in plants [4, 5]. The *bar* gene, cloned from the bacterium *Streptomyces hygroscopicus*, encodes phosphinothricin acetyltransferase (PAT), which converts PPT into the nontoxic acetylated form [4]. The application of selection pressure in regeneration media, transformed cells with selectable marker genes (SMGs) can survive, while non-transformed cells are killed. Screening approaches for the identification of transgenic lines among many more non-transgenic lines are time consuming, and have not been widely used in the absence of SMGs. In addition, in the absence of SMGs, the size of the regenerated plant events is reduced almost tenfold [6, 7].

However, because SMGs are integrated into the plant genome, there are concerns about widespread occurrence of transgenes in novel ecosystems (*e.g.*, antibiotic resistance in crops and their agroecosystems). Horizontal gene transfer from plants to environmental or medically related bacteria, or from plant products consumed as food to intestinal microorganisms or human cells, are generally considered to be not likely, but the inherent risks have not been totally addressed, and therefore there remain both regulatory and public concerns in many places in the world. Transfer of plant DNA into microbial or mammalian cells under normal conditions of dietary exposure [8] would require all of the following events to occur: (i) removal of the relevant gene(s) from the plant genome, probably as linear fragments; (ii) protection of the gene(s) from nuclease degradation in the plant as well as animal gastrointestinal tract; (iii) uptake of the gene(s) with dietary DNA; (iv) transformation of bacteria or competent

Correspondence: Professor C. Neal Stewart Jr., Department of Plant Sciences, University of Tennessee, 252 Ellington Plant Sciences, Knoxville, TN 37919, USA
E-mail: nealstewart@utk.edu

Abbreviation: SMG, selectable marker gene

mammalian cells; (v) insertion of the gene(s) into the host DNA by rare repair or recombination events into a transcribable unit; and finally, (vi) continuous stabilization of the inserted gene, e.g., there would be low-to-no-cost to the host harboring the new gene in the absence of selection pressure. Numerous experiments have evaluated the possible transfer of plant DNA into microbes and mammalian cells. There are reports that bacteriophage and plasmid DNA, when fed to mice at very high levels, can later be detected in their cells [9], but no data exist to demonstrate that plant DNA can be transferred into and be stably maintained or expressed in mammalian cells [8]. There are some experimental data indicating the transfer of plant DNA into bacteria under laboratory conditions, but only if homologous recombination is facilitated [10, 11]. However, there is no evidence that the transgenic markers presently in use pose a health risk to humans or domestic animals. Nevertheless, some researchers and regulators have concluded that, although the transformation risk of plant-transmitted antibiotic resistance genes to pathogenic bacteria is quite small, the use of markers conferring resistance to clinically relevant antibiotics should be phased out as suitable alternative technologies become available in plant biotechnology [8, 12, 13].

In addition to the risk of horizontal gene transfer, there is also a "vertical cross-species" transfer risk that could potentially create enhanced weediness problems [14]. Production of marker-free transgenic crops eliminates risk of horizontal gene transfer and could mitigate vertical gene transfer. We discuss two strategies to achieve these goal: one approach uses markers not based on antibiotic or herbicide resistance genes, and the second is to excise or segregate marker genes from the host genome after regeneration of transgenic plants [15], which includes co-transformation (*i.e.*, separate transformation of marker and transgene), site-specific recombinase-mediated marker deletion (*e.g.*, Cre/*loxP*, FLP/*FRT* and R/*RS* site-specific recombination systems), transposon-based expelling systems (*e.g.*, Ac transposon), intrachromosomal recombination based excision, and transformation by marker genes not based on herbicide or antibiotic selection.

2 Co-transformation

Co-transformation is a method for production of marker-free transformants based on *Agrobacterium*- or biolistics-mediated transformation in which a SMG and gene of interest are on separate constructs. Three approaches are used for co-transformation: (i-a) introduction of two T-DNAs, in separate *Agrobacterium* strains or (i-b) biolistics introduction of two plasmids in the same tissue; (ii) introduction of two T-DNAs carried by different replicons within the same *Agrobacterium* strain; and (iii) introduction of two T-DNAs located on the same replicon within an *Agrobacterium* [16, 17]. In all of these variants, SMGs can subsequently be removed from the plant genome during segregation and recombination that occurs during sexual reproduction by selecting on the transgene of interest and not the SMG in progeny. Therefore, co-transformation methods cannot be used for vegetatively propagated plants [18]. These procedures not only require fertile plants, but are also very time consuming [19]. In sexually reproducing plants, selection schemes are required to select the stable integration of two T-DNAs, which can be segregated in subsequent progeny [20]. However, tight linkage between co-integrated DNAs limits the efficiency of co-transformation. Indeed, integration of an SMG and the transgene of interest on separate loci are required [21]. Moreover, it is not applicable to transgenic trees with long generation times [22] that require the use of transient selection [23]. The outcome of one co-transformation study indicated that co-transfer of T-DNAs present in the same "super-binary" plant transformation vector in one strain was considerably more efficient than transfer from two different strains [24]. However, the overall advantages of these methods remain unclear [16, 25, 26].

2.1 Using plant DNA

Recent studies have shown that plants have T-DNA border-like sequences in rice and Arabidopsis [27, 28] and these might be used in transformation. Because this so-called plant DNA (P-DNA) lacks any open reading frames and contains a high A/T content, it is likely the footprint of ancient *Agrobacterium*-mediated natural transformation events via horizontal gene transfer [29]. It has been demonstrated that plant-derived P-DNA fragments can be used to replace the universally employed *Agrobacterium* T-DNA for transformation [23]. In addition, co-transformation of the inserted desired transgene into P-DNA and SMG-containing T-DNA is capable of producing marker-free and backbone-free transgenic plants [23]. Moreover, insertion of mutated *Agrobacterium virD2* gene into T-DNA served to impair the integration of transferred T-DNAs [30] and thus allowing a higher frequency for marker-free P-DNA population compared with T-DNA containing an SMG. Apparently, the mutated VirD2 protein has no effect on P-DNA integration. Inserting the bacterial *ipt* cytokinin expression cassette into the backbone of P-DNA vector enabled an increase in the frequency of backbone-free transgenic plant in the recovered population [23].

2.2 Negative selection

An alternative and potentially more efficient strategy is based on the incorporation of a negative selection step. The use of a negative SMG next to a positive SMG in the same construct is a powerful method to create marker gene-free transgenic plants. In this method, transformed offspring are selected for the absence of negative SMG

under the selection pressure of a negative marker gene and the presence of the desired transgene. This negative selection method allows researchers to decrease their search for selectable marker-free transgenic plants without having to resort to copious molecular analyses, *i.e.*, thousands of PCRs [31]. Finally, the combination of using a mixture of mechanisms, transient selection, sequential transformation, negative marker genes, P-DNA and a mutated *virD2* gene together should be capable of producing high frequency marker-free transgenic plants by co-transformation methods. Recently, a novel marker gene has been characterized, *dao1*, encoding D-amino acid oxidase that it can be used as for either positive or negative marker, depending on the substrate [32]. Therefore, it is possible to apply the negative selection after a positive selection using one marker gene, *dao1*, via changing D-alanine or D-serine to D-isoleucine or D-valine for the substrates.

3 Site-specific recombination-mediated marker deletion

Recombination is a universal phenomenon that can occur at any place along two homologous DNA molecules. In temperate bacteriophages, there is a second type of recombination called site-specific recombination, which takes place only between defined excision sites in the phage and in the bacterial chromosome. Positions of the site-specific recombination in the bacterial and phage DNA are called the bacterial and phage attachment sites, respectively. Each attachment site consists of three segments. The central segment has the conserved nucleotide sequence that sites the recombination event. A phage protein, an integrase, catalyzes the site-specific recombination events, which lead to physical exchange of DNA. Excision requires the phage enzyme integrase plus an additional phage protein called excisionase [33]. There are three well-described site-specific recombination systems that might be useful for the production of marker-free transgenic plants: Cre/*loxP* system from bacteriophage P1, where the Cre enzyme recognizes its specific target sites [34, 35], FLP/*FRT* recombination system from *Saccharomyces cerevisiae*, where the FLP recombinase acts on the *FRT* sites [36, 37] and R/*RS* recombination system from *Zygosaccharomyces rouxii*, where R and *RS* are the recombinase and recombination site, respectively [38]. Recognition sites for recombinases consist of palindromes, which are flanked with 7–12-bp core sequences [39]. Cleavage of the sites occurs at the borders between the recombinase binding elements and the core sequence (Fig. 1). In these systems, elimination of SMG would require recombinase expression in transgenic plants. The recombinase gene cassette can be introduced into transformed plants that contain the SMG between two recognition sites. Alternatively, a transgenic plant of interest can be crossed with a plant that expresses a recombinase gene. After segregation, marker-free transgenic progeny plants can be identified. To eliminate the breeding step, a co-transformation based on transient expression of the site-specific recombination system in combination with a conditional lethal dominant gene, *coda* was proposed [40–42]. Furthermore, the characterization and use of inducible promoters, CLX vector system, and GST-MAT vector system (multi-auto-transformation) including oncogenes for cell proliferation and regeneration of transgenic plants (see Fig. 2), to express of recombinase genes would be useful. After applying the induction agent, the recombinase would be expressed with induced excision of SMGs and all sequences between the two recombination sites [43, 44]. Also, tissue-specific promoters for producing marker-free transformants could be useful for fine-tuning the excision patterns. Recently, plant Cre virus vectors (TMV-Cre and PVX-Cre) for transient expression of *cre* recombinase has been developed as an alternative method for the production of marker-free transgenic *N. benthamiana* plants. In this method, transgenic plants containing lox sites and the bar SMG are inoculated with PVX-Cre and TMV-Cre recombinant viruses. PVX-Cre and TMV-Cre systemically infect leaves and allow regeneration without selection pressure. This strategy can be applied to plant species that depend on organogenesis or somatic embryogenesis for regeneration, particularly, soybean, potato and a number of woody plant species [45]. Also, a tightly controlled microspore-specific promoter and a site-specific recombination system was recently employed in an efficient marker gene removal in tobacco pollen [46].

Another strategy was proposed employing two site-specific recombination systems: one for integrating the DNA in a recombination site into the host genome at the designated genomic target site, and a second for removing sequences that are not needed after DNA transfer. This strategy is based on the tandem use of the Cre/*lox*, FLP/*FRT* and R/*RS* inducible systems (Fig. 3). In this method it is feasible to achieve site-specific integrations at an efficient rate with predictable transgene expression.

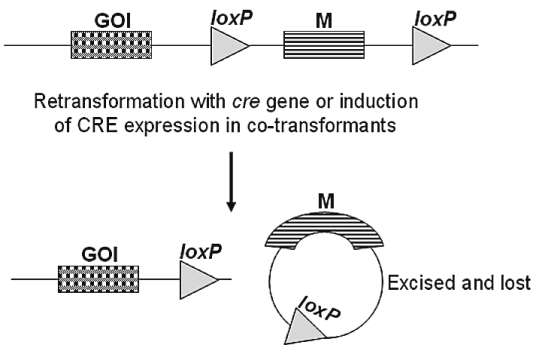


Figure 1. Cre/*loxP* recombination system (M: marker gene, and GOI: gene of interest). See [34].

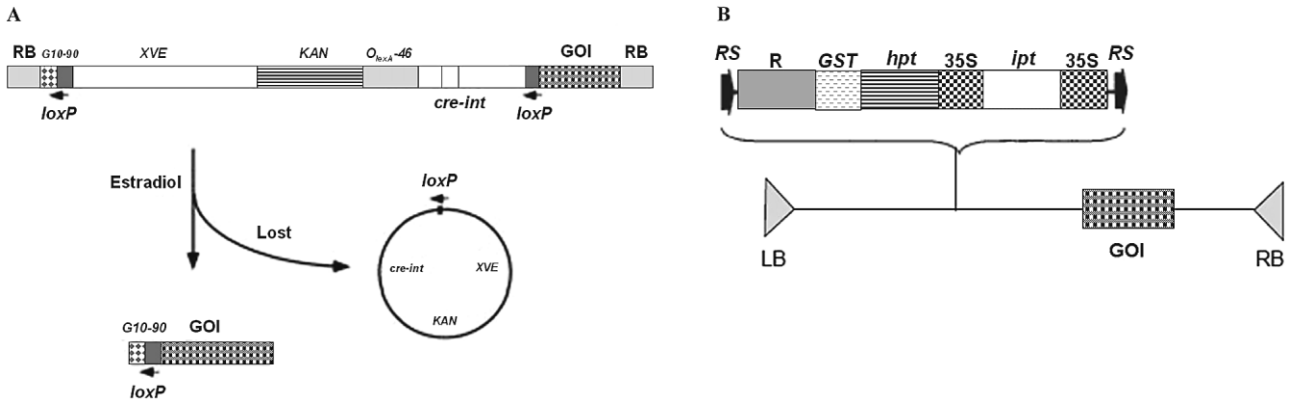


Figure 2. (A) Structure of the CLX vector system. See [44]. C*re-int* includes eight copies of the *LexA* operator sequence fused to the -46 CaMV*35S* promoter. Sequence of Cre is interrupted by an intron. Excision is produced via the ,-estradiol-induced site-specific DNA recombination. (B) MAT Vector System. See [43]. Recombinase genes (*R*) promoting with *GST* promoter and *hpt* marker gene flanked by two directly oriented RS sites. XVE *G10-90* and GOI are hybrid transactivator constitutive promoter and gene of interest, respectively.

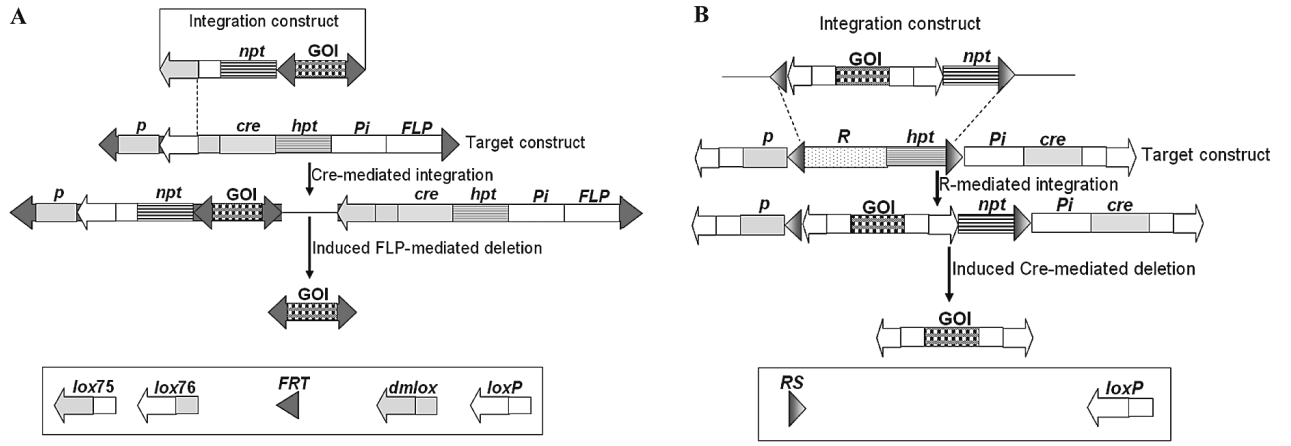


Figure 3. 'Combined step' strategy based on two site-specific recombination systems to remove excess DNA after site-specific integration. See [47]. (A) Use of Cre/*lox* and inducible FLP/*FRT* system to integrate a circular DNA and deleting of excess DNA from the integration locus, respectively. Introduction of circular DNA containing a lox75 (left arm mutant) site into cells containing a lox76 (right arm mutant) locus, site-specific integration of the gene of interest (GOI), and formation of a double mutant lox (dmlox) site, which stabilizes the integration locus. (B) Use of R/*RS* system to integrate a linear T-DNA, delivered by *Agrobacterium*, into target RS sites, followed by use of the inducible Cre/*loxP* system to remove excess DNA from the integration locus. *hpt*, hygromycin phosphotransferase gene; *npt*, neomycin phosphotransferase; P, promoter; Pi, inducible promoter (key in the box).

In this system single copy events at the designated target site ranged from 40% to 60% [47].

4 Transposon-based marker methods

In the 1940s, Barbara McClintock made an astonishing discovery. She detected two factors of DNA transposition in maize: a Ds (disassociation) element that was located at a chromosome break site and an unlinked genetic factor (Ac) that was required to activate the breakage of chromosome 9. McClintock concluded that such an unstable phenotype resulted from the movement or transposition of Ds [48]. These came to be known as transposons. Transposons are DNA sequences between hundreds to thousands of bases long. They code at least one protein, which enables them to replicate. The most widely studied transposon is the P element from the fruitfly (*Drosophila melanogaster*) [49]. Transposable elements can also be used to produce marker-free transgenic plants (Fig. 4). Use of transposable elements for marker gene removal involves several steps: (i) insertion of the marker gene onto a transposon, a segment of DNA that "hops" around in the plant's genome; (ii) co-transformation with gene of interest; and (iii) segregation of the marker gene.

A MAT vector system containing the *ipt* gene and *Ac* element has been designed so that when tobacco leaf segments were transformed and selected, subsequent excision of the modified *Ac* produced marker-free transgenic tobacco plants without sexual crosses or seed production

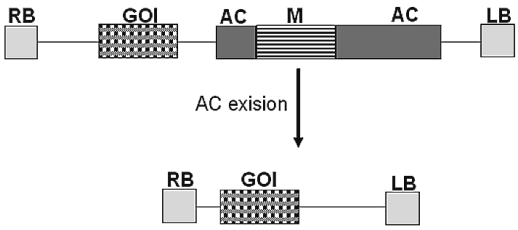


Figure 4. *Ac* transposon-based expelling of nuclear marker genes (M: marker gene, and GOI: gene of interest). See [50].

[50]. However, there are several drawbacks of using a transposable element system for marker gene removal: (i) different species have variable rates of transposition efficiency; (ii) this method requires labor and time costs for crossing transgenic plants and the selection of the progeny [19, 51]; (iii) there is low efficiency of marker-free transgenic plant generation, because of the tendency of transposable elements to reinsert elsewhere in the genome; (iv) imprecise excision; (v) generation of mutations because of insertion and excision cycles; and (vi) genomic instability of transgenic plants because the continuous presence of heterologous transposons [18] decreases efficiency.

5 Intrachromosomal recombination system

A variant of site-specific recombination systems described above employs an intrachromosomal recombination system. As above, recombination sites are engineered into the plant, but no recombinase is expressed. The attachment site from phage origin is denoted *POP'* (*P* for phage) or *attP*, and the attachment site from bacterial origin is denoted *BOB'* (*B* for bacteria) or *attB* [33]. Intrachromosomal recombination in plants [52] is obtained by insertion of SMG between two direct repeats of *attP* that facilitates spontaneous excision. Base composition of the *attP* site sequence is A + T rich, which is conjectured to play a recombination-stimulating role [53]. Possibly, the formation of a recombination hot spot is caused via the induction of double-strand breaks (DSBs) [54], but may also reduce of the stability of transgene sequences later on. Thirty-three percent efficiency of marker gene by I*Sce*I expression, a site-specific homing endonuclease encoded by a mitochondrial intron of *Saccharomyces cerevisiae*, demonstrated that induced DSB-mediated recombination by highly specific endonucleases could be a feasible alternative to site-specific recombinases for marker elimination [55, 56]. In addition, the inclusion of a transformation booster sequence (TBS) from *Petunia hybrida* inserted into the construct adjacent to the attachment sequences increased the frequency of intrachromosomal recombination and illegitimate recombination events in *Petunia*, *Nicotiana* and maize [57]. The potential advantages are: (i) expression of a heterologous recombinase and sexual reproduction are not necessary; (ii) there is a one step selection procedure for transgenic calli (lengthy propagation two-step time as above might increase the risk of somaclonal variation); (iii) it utilizes a natural nuclear recombination systems present in plants; (iv) the frequency of intrachromosomal recombination between two homologous sequences in plants might be increased by stimulation of repair systems; and (v) the efficiency of homologous recombination is directly correlated with the size of the homologous regions [18, 51, 52].

6 Removal of chloroplast marker genes

Mitochondria and chloroplasts have independent genomes in plants that have been the target (especially chloroplasts) of genetic transformation. Biosafety might be facilitated by maternal inheritance, which is the case in most plant species, in which transgenes in plastids would not be disseminated via pollen [58]. Chloroplast transformation vectors are designed with homologous flanking sequences on either side of the transgene. In addition, chloroplast engineering overcomes the challenges of gene silencing, position effects, and multi-step engineering of multiple genes, which are current limitations of nuclear transformation [58–60]. Homologous recombination (the use of identical sequences for example in promoters and terminators between genes) ([61], Fig. 5) and site-specific recombination (for example Cre/*lox* recombination-based systems) or transient expression of recombinase [62] are all potentially suitable for producing marker-free engineered chloroplast of plants. Re-transformation using the same marker gene has been recently demonstrated, and provides first rigorous proof that despite the high copy number of chloroplast genes, all copies of a marker gene can be removed by homologous recombination [63]. Recently, a construct co-integration system followed by a homologous recombination event between single homologous regions in vector and plastome was developed (Fig. 6). After recombination, co-integrates are inherently unstable because of direct repeats. Therefore, subsequent loop-out recombination events create either the stable integration of a transgene of in-

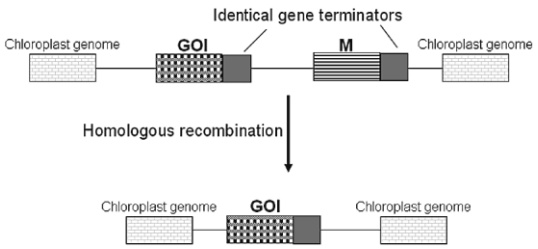


Figure 5. Homologous recombination based removal chloroplast marker gene (M: for marker gene and GOI: gene of interest). See [61].

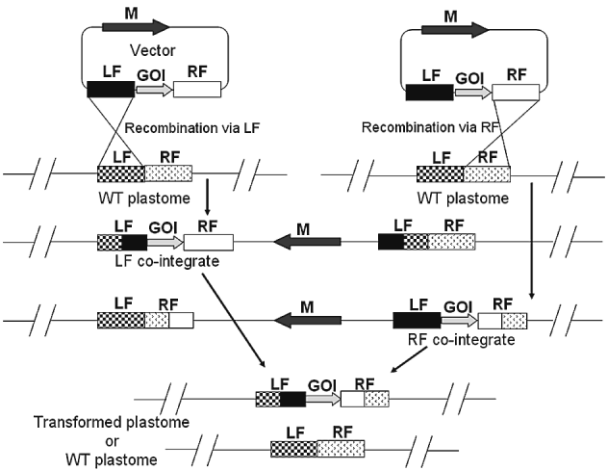


Figure 6. Co-integrated based marker excision method for generation of marker-free plastid transformants (M: marker gene, LF: left and RF: right homologous fragments, GOI: gene of interest, and WT: wild type). See [64].

terest or loss of the integrated vector, which then yields a wild-type plastome [64].

7 Markers not based on antibiotic or herbicide resistance

Recently, researchers have described substitute marker genes of non-bacterial origin that could have inherently increased biosafety. Of most interest are marker genes from plants themselves [65]. One potential alternative method to produce transformants without any antibiotic/herbicide marker gene is so-called positive selection systems. Recently, an *Escherichia coli*-derived phosphomannose isomerase (PMI) was used to convert mannose-6-phosphate to fructose-6-phosphate for positive selectable marker in plant transformation. Only transformed cells are capable of utilizing mannose as a carbon source [66]. PMI has been used as a selectable marker for transformation of many plant species, such as sugar beet [67], maize [68, 69], wheat [70], rice [71], pearl millet [72], and canola [66]. However, this system may not be as effective in plant species that contain endogenous PMI. As a possible solution, the xylose isomerase (*xylA*) gene of *Streptomyces rubiginosus* can be used 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 *xylA* transformed cells with are capable of growing on xylose. The xylose isomerase selection system was tested in potato, leading to a 10-fold transformation frequency compared with kanamycin selection. The levels of enzyme activity in transgenic plants selected on xylose were 5–25-fold higher than the enzyme activity in control plants [73].

Most recently, a notable replacement of the bacterial kanamycin-resistant *nptII* gene is an *Arabidopsis thaliana* ATP binding cassette (ABC) transporter (*Atwbc19*) gene. When overexpressed in transgenic tobacco, it yielded roughly equivalent degrees of kanamycin resistance in plants; however, because of cellular targeting to the tonoplast, it is not expected to confer kanamycin resistance in bacteria if horizontal gene flow were to occur [74]. Other plant-based markers are plant counterparts of aspartate kinase (AK), and dihydrodipicolinate synthase (DHPS) genes for lysine inhibition [73].

8 Conclusions and perspectives

There are many compelling reasons to produce transgenic plants with as little foreign DNA as possible. Many companies that produce transgenic crops are taking this minimalist approach now in their research and marketing strategies, resulting in fewer regulatory and consumer-based concerns for their products. Concurrently with this implementing this corporate strategy, there has been a recent explosion of technologies that, when further developed, will allow the removal of DNA in plants almost as easily as it is inserted today. In fact, we might expect technological development to move at a similar pace to that of transformation technology in the 1980s and 1990s. In a 10-year period, techniques were developed so that nearly all row crops could be transformed with both biolistics and *Agrobacterium*. SMGs are the most obvious candidate sequences to be deleted from transgenic plants since they largely serve no purpose after selection of transformants, thus their removal will be beneficial to end-users. There is no doubt that transgene removal will eventually be routine as some of the techniques reviewed here are perfected.

9 References

[1] Herrera-Estrella, L., De Block, M., Messens, E., Hernalsteens, J. P. *et al.*, Chimeric genes as dominant selectable markers in plant cells. *EMBO J.* 1983, *2*, 987–995.

[2] Bevan, M. W., Flavell, R. B., Chilton, M. D., A chimeric antibiotic resistance gene as a selectable marker for plant cell transformation. *Nature* 1983, *304*, 184–187.

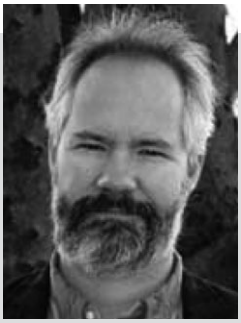
[3] Goodwin, J., Pastori, G., Davey, M., Jones, H., Transgenic plants: methods and protocols. *Methods Mol. Biol.* 2004, *286*, 191–202.

[4] De Block, M., Botterman, J., Vandewiele, M., Dockx, J. *et al.*, Engineering of herbicide resistance in plants by expression of a detoxifying enzyme. *EMBO J.* 1987, *6*, 2513–2518.

[5] Miflin, B. J., Lea, P. J., Amino acid metabolism. *Plant Physiol. Rev.* 1977, *28*, 299–329.

[6] De Vetten, N., Wolters, A.M., Raemakers, K., Vander Meer, I. *et al.*, A transformation method for obtaining marker-free plants of a cross-pollinating and vegetatively propagated crop. *Nat. Biotechnol.* 2003, *21*, 439-442.

Behrooz Darbani has a degree in plant breeding and holds a MSc in plant biotechnology from the Tabriz University of Iran. He joined the genomics laboratory of the Agriculture Biotechnology Research Institute for Northwest & West Iran as a researcher in 2005. Presently, his research interests focuse on recombinant protein production in prokaryotes as well as on molecular markers and genetic engineering of plants.



Neal Stewart obtained his PhD in Plant Physiology from the Virginia Polytechnic Institute and State University in 1993 (VA; USA). From 1993 to 1995 he was Postdoctoral Associate in Plant Genetics (Department of Crop and Soil Sciences, The University of Georgia, GA; USA) and Assistant & Associate Professor of Biology, University of North Carolina (Greensboro, NC, USA) in 1995–2002. He joined the University of Tennessee (TN, USA) in June 2002 assuming the Racheff Chair of Excellence in Plant Molecular Genetics. His laboratory is housed in the new state of the art Plant Biotechnology Building. Research spans the biosafety of transgenic plants, phytosensor research and development, to genomics. The lab has recently begun a potentially large effort in bioenergy research using switchgrass biotechnology.

[7] Birch, R. G., Plant transformation. Problems and strategies for practical application. *Plant Physiol. Rev. Plant Mol. Biol.* 1997, *48*, 297–326.

[8] Food and Agriculture Organization/World Health Organization, *Safety aspects of genetically modified foods of plant origin*. *Report of a Joint FAO/WHO Consultation on Foods Derived from Biotechnology*. World Health Organization, Geneva 2000.

[9] Schubbert, R., Hohlweg, U., Renz, D., Doerfler, W., On the fate of orally ingested foreign DNA in mice: chromosomal association and placental transmission to the fetus. *Mol. Gen. Genet.* 1998, *259*, 569–576.

[10] Nielsen, K. M., Bones, A. M., Smalla, K., Van Elsas, J. D., Horizontal gene transfer from transgenic plants to terrestrial bacteria–a rare event? *FEMS Microbiol. Rev.* 1998, *22*, 79–103.

[11] Kay, E., Vogel, T. M., Bertolla, F., Nalin, R. *et al.*, *In situ* transfer of antibiotic resistance genes from transgenic (transplastomic) tobacco plants to bacteria. *Appl. Environ. Microbiol.* 2002, *68*, 3345–3351.

[12] National Research Council, *Genetically modified pest-protected plants: science and regulation*, National Academy Press, Washington, D.C. 2000.

[13] Royal Society, *Genetically modified plants for food use*. The Royal Society, London 1998.

[14] Dale, P. J., Clarke, B., Fontes, M. G., Potential for the environmental impact of transgenic crops. *Nat. Biotechnol.* 2002, *20*, 567–574.

[15] Jianru, Z., Niu, Q., Ikeda, Y., Chua, N., Marker-free transformation: increasing transformation frequency by the use of regeneration-promoting genes. *Curr. Opin. Biotechnol.* 2002, *13*, 173–180.

[16] Gelvin, S. B., *Agrobacterium*-mediated plant transformation: the biology behind the "Gene-Jockeying" tool. *Microbiol. Mol. Biol. Rev*. 2003, *67*, 16–37.

[17] Afolabi, A. S., Worland, B., Snape, J., Vain, P., Multiple T-DNA co-cultivation as a method of producing marker-free (clean gene) transgenic rice (*Oryza sativa* L.) plant. *African J. Biotechnol.* 2005, *4*, 531–540.

[18] Scutt, C. P., Zubko E., Meyer, P., Techniques for the removal of marker genes from transgenic plants. *Biochimie* 2002, *84*, 1119–1126.

[19] Ebinuma, H., Sugita, K., Matsunaga, E., Endo, S. *et al.*, Systems for the removal of a selection marker and their combination with a positive marker. *Plant Cell Rep.* 2001, *20*, 383–392.

[20] Komari, T., Hiei, Y., Saito, Y., Murai, N. *et al.*, Vectors carrying two separate T-DNAs for co-transformation of higher plants mediated by *Agrobacterium tumefaciens* and segregation of transformants free from selection markers. *Plant* J. 1996, *10*, 165–174.

[21] Konig, A., A framework for designing transgenic crops – science, safety and citizen's concerns. *Nat. Biotechnol*. 2003, *21*, 1274–1279.

[22] Holger, P., Marker-free transgenic plants. *Plant Cell Tissue Org. Cult*. 2003, *74*, 123–134.

[23] Rommens, C. M., Humara, J. M., Ye, J., Yan, H. *et al.*, Crop improvement through modification of the plant's own genome. *Plant Physiol*. 2004, *135*, 421–431.

[24] Depicker, A., Herman, L., Jacobs, A., Schell, J. *et al.*, Frequencies of simultaneous transformation with different T-DNAs and their relevance to the *Agrobacterium*/plant cell interaction. *Mol. Gen. Genet.* 1985, *201*, 477–484.

[25] Xing, A., Zhang, Z., Sato, S., Staswick, P. *et al.*, The use of the two T-DNA binary system to derive marker-free transgenic soybeans. *In Vitro Cell. Dev. Biol- Plant*. 2000, *36*, 456–463.

[26] Komari, T., Hiei, Y., Saito, Y., Murai, N. *et al.*, Vectors carrying two separate T-DNAs for co-transformation of higher plants mediated by *Agrobacterium tumefaciens* and segregation of transformants free from selection markers. *Plant* J. 1996, *10*, 165–174.

[27] Buell, C. R., Yuan, Q., Ouyang, S., Liu, J. *et al.*, *Oryza sativa* chromosome 3 BAC OSJNBa0093I13 genomic sequence. Genbank accession number AC097279. 2003.

[28] Jordan, N., Bangert, S., Wiedelmann, R., Voss, H. *et al.*, *Arabidopsis thaliana* DNA chromosome 3, BAC clone T18B22. Genbank accession number AL138652. 2005.

[29] Depicker, A., Mironov, V., Terras, F., Broekaert, W. *et al.*, Optimized T-DNAs and vectors therefore. World Patent Application No. 01/44482. 2001.

[30] Shurvinton, C.E., Hodges, L., Ream, W., A nuclear localization signal and the C-terminal sequence in the *Agrobacterium tumefaciens* VirD2 endonuclease are important for tumor formation. *Proc. Natl. Acad. Sci. USA* 1992, *89*, 11837–11841.

[31] Jeongmoo, P., Young, L., Bong, K., Won, C., Co-transformation using a negative selectable marker gene for the production of selectable marker gene-free transgenic plants. *Theor. Appl. Genet*. 2004, *109*, 1562–1567.

[32] Erikson., O., Hertzberg, M., Nasholm, T., A conditional marker gene allowing both positive and negative selection in plants. *Nat. Biotechnol.* 2004, *22*, 455–458.

[33] Hartl, D. L., Jones, E. W., *Genetics: principles and analysis*, 4th edn., Jones and Bartlett, London 1998, pp. 330–359.

[34] Sternberg, N., Hamilton, D., Bacteriophage P1 site-specific recombination between loxP sites. *J. Mol. Biol.* 1981, *150*, 467–486.

[35] Hoess, R. H., Abremski, K., The Cre/*lox* recombination system. *Nucleic Acids Mol. Biol*. 1990, *4*, 99–109.

[36] Cox, M. M., DNA inversion in the 2 µm plasmid of *Saccharomyces cerevisiae*, in: Berg, D. E., Howe, M. M. (Eds.), *Mobile DNA*, American Society for Microbiology, Washington D.C. 1989, 661–670.

[37] Huang, L. C., Wood, E. A., Cox, M. M., A bacterial model system for chromosomal targeting. *Nucleic Acids Res.* 1991, *19*, 443–448.

[38] Matsuzaki, H., Nakajima, R., Nishiyama, J., Araki, H. *et al*., Chromosome engineering in *Saccharomyces cerevisiae* by using site-specific recombination system of a yeast plasmid. *J. Bacteriol.* 1990, *172*, 610–618.

[39] Kurin, H., Ola, S., Lars, S., Non-palindromic *aatI* sites of integrons are capable of site-specific recombination with one another and with secondary targets. *Mol. Microbiol*. 1997, *26*, 441–453.

[40] Dale, E. C., Ow, D.W., Intra- and intermolecular site-specific recombination in plant cells mediated by bacteriophage P1 recombinase. *Gene*. 1990, *91*, 79–85.

[41] Gleave, A. P., Mitra, D. S., Mudge, S. R., Morris, B. A., Selectable marker-free transgenic plants without sexual crossing: transient expression of cre recombinase and use of a conditional lethal dominant gene. *Plant Mol. Biol*. 1999, *40*, 223–235.

[42] Perera, R. J., Linard, C. G., Signer, E. R., Cytosine deaminase as a negative selective marker for Arabidopsis. *Plant Mol. Biol.* 1993, *23*, 793–799.

[43] Sugita, K., Kasahara, T., Matsunaga, E., Ebinuma, H., A transformation vector for the production of marker-free transgenic plants containing a single copy transgene at high frequency. *Plant* J. 2000, *22*, 461–469.

[44] Zuo, J. R., Niu, Q. W., Moller, S. G., Chua, N. H., Chemical-regulated, site-specific DNA excision in transgenic plants. *Nat. Biotechnol.* 2001, *19*, 157–161.

[45] Kopertekh, L., Schiemann, J., Marker gene elimination mediated by transient expression of bacteriophage P1 Cre recombinase in plants. *Status Seminar „Sicherheitsforschung und Monitoring*". Berlin 2004.

[46] Mlynarova, L., Conner, A.J., Nap. J.P., Directed microspore-specific recombination of transgenic alleles to prevent pollen-mediated transmission of transgenes. *Plant Biotechnol. J.* 2006, *4*, 445–452.

[47] Srivastava, V., Ow, D.W., Marker-free site-specific gene integration in plants. *Trends Biotechnol*. 2004, *22*, 627–629.

[48] Griffiths, A. J. F., Miller, J. H., Suzuki, D. T., Lewontin, R. C, Gelbart, W. M. (Eds.), *An Introduction to Genetic Analysis*, 8th edn., Freeman, San Francisco 2005, pp. 424–450.

[49] Pierre-Henri, G., Jean-Pierre, H., Jacques, A. (Eds.), *GENE AVATARS: The neo-darwininan theory of evolution*, Kluwer Academic, New York 2002, pp. 174–175.

[50] Ebinuma, H., Sugita, K., Matsunaga, E., Yamakado, M., Selection of marker-free transgenic plants using the isopentenyl transferase gene. *Proc. Natl. Acad. Sci. USA* 1997, *94*, 2117–2121.

[51] Ow, D. W., The right chemistry for marker gene removal? *Nat. Biotechnol.* 2001, *19*, 115–116.

[52] Zubko, E., Scutt, C., Meyer, P., Intrachromosomal recombination between attP regions as a tool to remove selectable marker genes from tobacco transgenes. *Nat. Biotechnol.* 2000, *18*, 442–445.

[53] Muller, A. E., Kamisugi, Y., Gruneberg, R., Niedenhof, I. *et al*., Palindromic sequences and A+T-rich DNA elements promote illegitimate recombination in *Nicotiana tabacum*. *J. Mol. Biol*. 1999, *291*, 29–46.

[54] Puchta, H., Removing selectable marker genes: taking the shortcut. *Trends Plant Sci*. 2000, *5*, 273–274.

[55] Siebert, R., Puchta, H., Efficient repair of genomic double- strand breaks via homologous recombination between directly repeated sequences in the plant genome. *Plant Cell*. 2002, *14*, 1121–1131.

[56] Perez, C., Guyot, V., Cabaniols, J. P., Gouble, A. *et al*., Factors affecting double-strand break-induced homologous recombination in mammalian cells. *BioTechniques*. 2005, *39*, 109–115.

[57] Galliano, H., Muller, A. E., Lucht, J. M., Meyer, P., The transformation booster sequence from *Petunia hybrida* is a retrotransposon derivative that binds to the nuclear scaffold. *Mol. Gen. Genet*. 1995, *247*, 614–622.

[58] Daniell, H., Ruiz, O., Dhingra, A., Transgenic Plants: Methods and Protocols. *Methods Mol. Biol*. 2004, *286,* 111–138.

[59] Daniell, H., Dhingra, A., Multigene engineering: dawn of an exciting new era in biotechnology. *Curr. Opin. Biotechnol.* 2002, *13*, 136–141.

[60] Daniell, H., Khan, M. S., Alison, L., Milestones in chloroplast genetic engineering: an environmentally friendly era in biotechnology. *Trends Plant Sci.* 2002, *7*, 84–91.

[61] Iamtham, S., Day, A., Removal of antibiotic resistance genes from transgenic tobacco plastids. *Nat. Biotechnol*. 2000, *18*, 1172–1176.

[62] Kerry, A. L., Massimo, H. B., Pal, M., Plastid marker-gene excision by transiently expressed CRE recombinase. *Plant* J. *2006,* 45, 447.

[63] Kode, V., Mudd, E.A., Iamtham, S., Day, A., Isolation of precise plastid deletion mutants by homology-based excision: a resource for site-directed mutagenesis, multi-gene changes and high-throughput plastid transformation. *Plant J*. 2006, *46*, 901–909.

[64] Klaus, S. M. J., Huang, F. C., Golds, T. J., Koop, H. U., Generation of marker-free plastid transformants using a transiently cointegrated selection gene. *Nat. Biotechnol*. 2004, *22,* 225–229.

[65] Daniell, H., Muthukumar, B., Lee, S. B., Marker free transgenic plants: engineering the chloroplast genome without the use of antibiotic selection. *Curr. Genet*. 2001, *39*, 109–116.

[66] Sonntag, K., Wang, Y., Wallbraun, M., A transformation method for obtaining marker-free plants based on phosphomannose isomerase. *Acta Univ. Latviensis Biol.* 2004, *676*, 223–226.

[67] Joersbo, M., Donaldson, I., Kreiberg, J., Petersen, S. G. *et al*., Analysis of mannose selection used for transformation of sugar beet. *Mol. Breed*. 1998, *4*, 111–117.

[68] Negrotto, D., Jolly, M., Beer, S., Wenck, A. R. *et al*., The use of phosphomannose-isomerase as a selectable marker to recover transgenic maize plants (*Zea mays* L.) via *Agrobacterium* transformation. *Plant Cell Rep.* 2000, *19*, 798–803.

[69] Wright, M., Dawson, J., Dunder, E., Suttie, J. *et al*., Efficient biolistic transformation of maize (*Zea mays* L.) using the phosphomannose isomerase gene, *pmi*, as the selectable marker. *Plant Cell Rep*. 2001, *20*, 429–436.

[70] Reed, J., Privalle, L., Powell, M. L., Meghji, M. *et al*., Phosphomannose isomerase: an efficient selectable marker for plant transformation. *In Vitro Cell Dev. Biol. Plant*. 2001, *37*, 127–132.

[71] Lucca, P., Ye, X., Potrykus, I., Effective selection and regeneration of transgenic rice plants with mannose as selective agent. *Mol. Breed*. 2001, *7*, 43–49.

[72] Okennedey, M. M., Burger, J. T., Botha, F. C., Pearl millet transformation system using the positive selectable marker gene phosphomannose isomerase. *Plant Cell Rep*. 2004, *22*, 684–690.

[73] Jaiwal, P. K., Sahoo, L., Singh, N. D., Singh, R. P., Strategies to deal with the concern about marker genes in transgenic plants: Some environmentally friendly approaches. *Curr. Sci*. 2002, *83*. 128–136.

[74] Mentewab, A., Stewart, C. N. Jr., Overexpression of an *Arabidopsis thaliana* ABC transporter confers kanamycin resistance to transgenic plants. *Nat. Biotechnol*. 2005, *23*, 1177–1180.