MARKER FREE TRANSGENICS

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Abstract : The encouraging future of biotech industry relies on the ability to efficiently introduce foreign genes into plants. Transformation studies have reached newer heights as along with the increase in number of transgenic plants; the frequency of transformation is also increasing which leads to more number of transgenic products either ready or close to market introduction. This imminent commercialization of transgenic plants have opened a debate regarding the desirability of the transgenic products containing genes which do not directly contribute to the final product and has raised questions regarding the use of selectable marker genes. This review focuses on these issues and study recently developed transformation systems to selectively eliminate particular marker sequence from the final transgenic plant.

Introduction

Genetic engineering of plants modifies the plant genome by integration of genetic material (either single or multiple genes) into the recipient plant. This technology has an enormous potential to precisely improve crops of interest. In this process, selection systems are employed 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 of transformed plants. Selectable marker genes (SMGs) along with reporter genes are crucial in plant genetic engineering for the development of transgenic crops. These are mostly always present in engineered DNA plasmids molecules used for genetic transformation of plant tissue (Lee and Gelvin, 2008). Summary of selectable markers along with their source and agents applied for their selection are presented in table 1.

A comprehensible debate has been acquainted along with the development and commercialization of transgenic crops. Commercialization of products obtained by genetically modified (GM) plants is largely criticized by concerns regarding possible risks related to the introduction of SMGs because it is integrated into the plant genome increasing the widespread occurrence of transgenes in novel ecosystems. (Darbani *et al*., 2007) it also affects medically related bacteria or dietary plant products to intestinal microorganisms or human cells by the horizontal gene transfer from transgenic plants which remains as primary concern to the consumers of transgenic plants.

There are apprehensions about normal plant growth and development being altered by continuous expression of these markers. (Ebinuma et al. 1997) Introducing multiple genes (gene stacking) by using different SMGs may lead to un-anticipated gene silencing due to duplication of promoters and polyA signals.

Along with the former risks, there is also a "vertical cross-species" transfer risk that could potentially create enhanced promotion of growth of weeds which can be eliminated using marker free transgenic plants. (Dale *et al*., 2002) It is necessary to use different selectable marker systems for each transformation as the presence of particular marker genes may preclude the use of that marker in subsequent transformations. This system would not be advantageous if one desires to introduce higher transgenes than suitable selectable marker available. Hence it is desirable to remove a selectable marker gene unless expression is under stringent control. (Goldsbrough *et al*., 1993)

Stability of homologous transgenes in multiple transformation events is another pragmatic issue which needs focus. In many cases it is reported that the expression of primary transgene is lost or modified following multiple transgenic events. (Matzke *et al*., 1989) Though the reasons are still elusive, the loss of expression can be correlated with the sequence homology. Therefore removal of all the ancillary sequences, which include selectable markers are desirable in pretext to minimize the regions of homology which may be responsible for instability. (Jorgensen, 1991)

Marker-free transgenic crop production can eliminate the risk of horizontal gene transfer and could mitigate vertical gene transfer. To achieve the goal of obtaining marker free transgenic plants, we have to adopt two strategies: the first approach is to avoid using markers based on antibiotic or herbicide resistance genes and the second approach is to excise or segregate marker genes from the host genome after regeneration of transgenic plants. For the excision of marker genes, techniques like co-transformation, site-specific recombinase-mediated marker deletion, transposon-based expelling systems, intrachromosomal recombination based excision and transformation by marker genes not based on herbicide or antibiotic selection can be employed which would be briefly discussed in the current review.

Transformation systems allowing marker gene elimination

Co- transformation

Simultaneous transformation of two different DNAs, out of which one incorporates a gene of interest and the other the selectable marker gene can provides a simple system for the elimination of SMGs. In this system, selectable marker genes and target genes are loaded into separate T-DNAs, which are expected to segregate independently in a Mendelian fashion. (Ramana Rao *et al*., 2011) SMG can be eliminated from the plant genome during segregation and recombination. This occurs during sexual reproduction

by selection of transgene of interest and not the SMG in progeny. This approach can be fulfilled under two conditions: (1) If the efficiency of co transformation is high and (2) If the co transformed DNAs integrate at location of genomic regions where unlinked genes prevail and allow effective recovery of recombination events. Co-transformation system can be conducted by using the following three methods: (1) Different Agro bacterium strains carrying two different strains. (De Neve *et al*., 1997) and biolistic introduction of two plasmids in the same tissue (Kumar *et al*., 2010); (2) same *Agrobacterium* cell carrying two different vectors (Sripriya *et al*., 2008); and (3) a single binary vector can develop two T-DNAs. (2 T-DNA system) (Miller *et al*., 2002)

There are several advantages of co-transformation though over all advantages are still elusive. This method is highly adaptable to conventional, unmodified *Agrobacterium*-mediated gene transfer methods, it is easy to handle the binary vector as the two T-DNA are separated and, hence, target gene TDNA can be handled independently of selectable marker gene T-DNA. This method relies greatly on the co-transformation efficiency and the independent integration of T-DNA into the plant genome. The co-transformation efficiency is generally, in the range of 30–50%, which is acceptable for practical applications (McCormac *et al*., 2001). Some reports indicate that the co-transformation efficiency using *Agrobacterium* was similar to the efficiency of two different transformation events. (Depicker *et al*., 1985) Recent studies show that co-transformation achieved significantly higher efficiency in *Agrobacterium* mediated and direct transformation methods. (De Block, M. and Debrouwer, D. 1991)

In spite of all these advantages, there are several inevitable limitations. This method is highly time consuming and compatible only for fertile plants. The tight linkage between co-integrated DNAs restricts the efficiency of co-transformation. If both SMG and transgenes integrate in the same loci then it is not feasible for co-transformation. Several papers also comment this method to be useful only for flowering plants.

Site specific recombination systems

Recombination occurs between two homologous DNA molecules. Site specific recombination takes place between defined excision sites in the phage and in the bacterial chromosome in bacteriophage. This exchange occurs between segments possessing only a limited degree of sequence homology. This method was first used in *Saccharomyces cerevisiae* 2µm circle site specific recombination system. Basically three site-specific recombination systems are well known mentioned as under.

Cre/lox site-specific recombination system: There are two components present in this system (a) two loxP sites each consisting of 34 bp inverted repeats cloned in direct orientation flanking a DNA sequence and (b) a 38 kDa recombinase protein encoded by two loxP sites. These sites excise the intervening sequence along with one of the loxP sites. The Cre/loxP have been tested in several plants including *Nicotiana*, (Dale and Wo, 1991) *Zea mays* (Zhang et al. 2003) and *Oryza sativa*. (Sreekala *et al*. 2005)

Cre/lox system has an advantage of being specific for its 34 bp recognition sequence. However limitations to this system are that it is difficult to insert and to excise genes with precision in the plant genome without a site-specific recombination system. Excision of marker gene from transgenic plants requires re-transformation and out-crossing approaches which are laborious and time consuming. (Dale and Wo, 1991). To overcome these obstacles, different approaches like the use of some chemical inducers (Zhang *et al*., 2006) and heat shock (Cuellar *et al*., 2006) have been developed.

FLP/FRT recombination system: This system is recombination system from the 2 µm plasmid *Saccharomyces cerevisiae*, where the FLP recombinase acts on the *FRT* sites and R/*RS* recombination system from *Zygosaccharomyces rouxii*, where R and *RS* are the recombinase and recombination site, respectively. (Huang *et al*., 1991) The FLP enzyme efficiently catalyses recombination between two directly repeated FLP recombination target (frt) sites and eliminates the sequence between them. Cleavage of the sites occurs at the borders between the recombinase binding elements and the core sequence The controlled expression of the FLP recombinase and specific allocation of the frt sites within transgenic constructs allows the system to be applied to eliminate the marker genes after selection (Cho, 2009). In these systems, recombinase expression in transgenic plants is required to eliminate SMG hence to fulfil this objective; the recombinase gene cassette could be introduced into transformed plants that contain the SMG between two recognition sites.

Recent reports show that this method is efficient to obtain marker free transgenic plants by this method. (Cuellar *et al*., 2006; Woo *et al*., 2009; Li *et al*., 2010; Nandy and Srivastava, 2011)

R/RS recombination system: Site-specific recombination mediated by recombinase of the R/RS system is mainly employed during subculturing to produce morphologically normal marker-free transgenic plants. However, this system offers low frequency of marker-free transgenic plants, and also reinsert elsewhere in the genome shortly after their excision. Hence this system can be employed to cells with transposition errors.

Another proposed strategy employed two site specific recombination systems: one for integration of DNA in a recombination site at the designated genomic target site into the host genome and a second for removal of sequences that are not needed after DNA transfer. This strategy used the Cre/*lox*, FLP/*FRT* and R/*RS* inducible systems. It is feasible to achieve site-specific integrations at an efficient rate with predictable transgene expression by this method as in this system a single copy event at the designated target site ranged from 40% to 60%. (Srivastava and Ow, 2004)

Transposon-based marker methods

Transposons are DNA sequences ranging from hundreds to thousands bases. They code at least one protein, which aids in their replication. The P element from the fruitfly (*Drosophila melanogaster*) is the most widely studied transposon; such elements can also be used to produce marker-free transgenic plants. There are several uses of transposable elements, including as a marker gene.

Marker gene removal involves several steps: (i) insertion of the marker gene onto a transposon; (ii) co-transformation of segment with gene of interest; and (iii) segregation of the marker gene.

Ebinuma *et al*., (1997) demonstrated this strategy by designing a MAT vector system containing the *ipt* gene and *Ac* element. When tobacco leaf segments were transformed and selected followed by subsequent excision of the modified *Ac*, marker-free transgenic tobacco plants were produced which required neither sexual crosses nor seed production.

However, transposon-based marker method for marker gene removal have several drawbacks: (i) transposition efficiency is different in different species; (ii) this method is time consuming for crossing transgenic plants and the selection of the progeny requires labor; (iii) there is low efficiency of marker-free transgenic plant generation, as the tendency of transposable elements to reinsert elsewhere in the genome is high; (iv) excision is imprecise; (v) generation of mutations regenerated is high because of insertion and excision cycles; and (vi) transgenic plants are genomic unstable because of the continuous presence of heterologous transposons decreasing efficiency.

Intrachromosomal recombination system

Intrachromosomal recombination system is a variant of site-specific recombination systems described above. Intrachromosomal recombination in plants is accomplished by the insertion of SMG between two direct repeats of *attP* (site sequence is A + T rich) that facilitates spontaneous excision as the base composition is conjectured to play a recombination-stimulating role. (Muller *et al*., 1999) It is predicted that formation of a recombination hot spot is caused via the induction of double-strand breaks (DSBs) (Puchta, 2000) but it may also decrease the stability of transgene sequences afterwards.

It was demonstrated that induced DSB-mediated recombination by highly specific endonucleases could be a feasible alternative to site-specific recombinases for marker elimination as the efficiency of marker gene by I*Sce*I expression was thirty-three percent (Perez *et al*., 2005)

The potential advantages of this strategy are: (i) there is no necessity for the expression of a heterologous recombinase and sexual reproduction; (ii) this strategy avoids lengthy procedure as it is a one step selection procedure for transgenic calli (lengthy propagation increases the risk of somaclonal variation); (iii) it utilizes a natural nuclear recombination system which is present in plants; and (iv) it may increase the frequency of intrachromosomal recombination between two homologous sequences in plants by stimulation of repair systems.

Markers not based on antibiotic or herbicide resistance

Recently, substitute marker genes of non-bacterial origin are described by researchers. Such markers can inherently increased biosafety. Since marker genes of interest are mainly obtained from plants themselves (Daniell *et al*., 2001). One of the potential alternative method to produce transformants without any antibiotic/ herbicide marker gene is called positive selection systems. Some marker genes for positive selection provide the identification and selection of genetically modified cells without injury or death of the non-transformed cell population (negative selection). Recently, *Escherichia coli*-derived phosphomannose isomerase (PMI) was utilized to convert mannose- 6-phosphate to fructose-6-phosphate as a positive selectable marker for plant transformation. This allowed only transformed cells being capable of utilizing mannose as a carbon source. (Sonntag *et al*., 2004) Though there are several reports showing success of this strategy, (Joersbo *et al*., 1998; Negrotto *et al*., 2000; Wright *et al*., 2001) this system may not be as effective in plant species that contain endogenous PMI.

The solution for this limitation is the use of the xylose isomerase (*xylA*) gene of *Streptomyces rubiginosus* as the selectable marker and xylose as the selective agent. The enzyme obtained from *S. rubiginosus* catalyses the isomerization of D-xylose to D-xylulose which makes the transformed cells capable of growing on xylose. The xylose isomerase selection system was studied in potato, which lead to 5–25-fold higher expression level of enzyme activity in transgenic plants selected on xylose (Jaiwal *et al*., 2002)

A notable replacement of the bacterial kanamycin-resistant *nptII* gene is an *Arabidopsis thaliana* ATP binding cassette (ABC) transporter (*Atwbc19*) gene which was studied recently. Transgenic tobacco developed using above reporter gene yielded roughly equivalent degrees of kanamycin resistance in plants; due to the cellular targeting to the tonoplast, if horizontal gene flow were to occur it is not expected to confer kanamycin resistance in bacteria. (Mentewab *et al*., 2005) Other plant-based markers that can be used for transformation are plant counterparts of aspartate kinase (AK), and dihydrodipicolinate synthase (DHPS) genes for lysine inhibition. (Jaiwal *et al*., 2002)

Conclusion

In summary, the excision of selectable marker genes may be a reasonable procedure for long term development of transgenic plants. It also ensures a safe use in commercial crop development. It may also prove to be an appropriate marketing strategy as there are many compelling reasons to produce transgenic plants with as little foreign DNA as possible. Many companies that produce transgenic crops are employing this approach now in their research and marketing strategies, resulting in fewer consumer based concerns for their products. Implementing this corporate strategy, there has been a recent rise of technologies that, when further developed, will allow the removal of DNA in plants almost as easily as it is inserted today. The choice of transformation strategy can be made on basis for each particular need, gene and even species. While the rationale for removing specific sequences from transgenic plants is still under debate, the ease at which sequence removal strategies take shape into ongoing transformation programs makes them important options to consider.

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