EUROPEAN ACADEMIC RESEARCH
Vol. II, Issue 3/ June 2014

Impact Factor: 3.1 (UIF)
DRJI Value: 5.9 (B+)

ISSN 2286-4822
www.euacademic.org

Review

Advances in Production of Marker Free Transgenic Plants: Current Challenges and Future Perspectives

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Abstract:

Plant selectable markers genes are commonly used in genetic transformation experiments to select transgenic plants from non transgenic plants. These selection markers genes affect both the targeted and non targeted living organisms. Various techniques have been developed for excision of lethal selectable marker gene from the genetic engineered plants. Selectable marker genes can be efficiently removed from plants genome by many ways such as by using non toxic chemicals for selection, co-transformation vector system, site specific recombination under the expression of inducible promoters and multi-auto-transformation system. These techniques are very useful to control the environmental hazards associated to these marker genes. In this review we describe the current issues related to various plant selectable marker genes, to highlight various safe techniques to remove these toxic markers genes from transgenic plants and emphasize on the responsible conducts of scientists to develop new marker free transgenic plants by using safe marker free techniques in future.

Key words: Environment, Transgenic Plants, Marker genes.

Introduction:

Selectable marker genes are important for the selection of transgenic plants from non transgenic plants (Lee and gelvin 2008). Various plants selection markers genes such as *hpt* gene (Hygromycin phospotransferase), *npt II* gene (Neomycin phosphotransferase) and herbicides resistant genes such as *bar* gene, *gox* gene etc are commonly used in plant transformation experiments. To date about 50 different marker systems have been introduced in plant transformation experiments (Sundar and Sakthivel 2008; Miki and McHugh 2004). Selectable marker genes such as antibiotics, herbicides and many others toxic substances remain as end products in transgenic plants. The presence of these toxic substances in our food, feed and in environment raise many biosafety issues in many countries (Yeu Yau and Neal 2013). These markers genes provide tolerance to the pathogens against these antibiotics. The excision of selectable markers genes are useful by two ways; to avoid the environmental toxicity associated to these marker genes and to block the expression of these markers genes through transcription gene silencing methods (Hohn *et al.* 2001; Ebinuma *et al.* 2001). By removing these toxic selectable genes are important to develop transgenic plants with low cost and to increase the marketing of these products. Therefore it is important to used marker free technologies to develop new transgenic plants which fulfill the consumer demands (Kupier *et al.* 2001; Daniel 2002; Smyth *et al.* 2002). The marker free genetic engineered plants can be produced by applying following methods.

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Using of Non Toxic Chemicals for Selection of Transgene in Transgenic Plants

The toxicity of selectable markers can be controlled by using non toxic chemicals. These chemicals work like as selectable markers genes but the mechanisms of selection are totally different. These chemicals promote the growth and development of transgenic plants rather than non transgenic plants. Several different types of chemical inducers are used instead of toxic selectable marker genes (Yuan *et al.* 2004; Zhang *et al.* 2006). The enzymes such as glucornidase (Joersbo and Okkels 1996), xylose isomerase (Haldrup *et al.* 1998) and phosphomannose isomerase were efficiently used for selection of transgene in transgenic plants without any toxicity to living organisms (Joersbo *et al.* 1998, Negrotto *et al.* 2000). The dexamethasone inducible promoter driving gene was successfully used for selection of transformants in tobacco and lettuce plants (Kunkel *et al.* 1999). These genes not only increase the selection frequency of transformed plants but also enhance others plant growth metabolic pathways (Ebinuma 1997). *E. coli* phosphomannose isomerase gene (*pmi*) gene is also used as selection in many crops such as sugar beet (Joersbo *et al.* 1998), maize (Negrotto *et al.* 2000), cassava (Zhang *et al.* 2000) and rice (Luka *et al.* 2001) by producing mannose which is less toxic to plants. With the use of these environmental friendly chemicals can significantly decrease the toxicity associated to microbial and herbicide resistant selectable marker genes.

Co- Transformation System for Excision of Marker Genes from Transgenic Plants

Co- transformation method is based on by combining two different T-DNA; one having desired gene while the other T-DNA carrying the marker gene (Fig.1). If both genes

transformed at different loci at T^0 stage follow by their separation at T1 stage lead to removal of lethal marker gene by Mendelian method. In other experiment two different T-DNA having different size in binary plasmid; one larger T-DNA having selectable marker gene while the other smaller T-DNA having gene of interest. The T-DNA of smaller size show more expression than larger size T-DNA. This method serve as a model for developing a binary vector by keeping the selection marker gene on the longer T-DNA while keeping targeting gene on the smaller T-DNA. As a result there will be higher expression of desired gene as compare to marker genes. The co-transformation method was successfully used in tobacco and rice crops plants. The maximum transformation efficiency 47 % was achieved at T^0 stage while the transformation was only 25 % at T1 stage with the excision of marker genes (Komari *et al.* 1996). This method serves as a model for excision of markers genes for other agronomical important crops. The maximum transformation efficiency 86 % was achieved in marker free transgenic rice. The transgenic maize showed 93 % transformation efficiency through this method (Sripriya *et al.* 2011). However this method is totally dependent on fertility of plants for optimum separation of transgenic loci (McKnight *et al.* 1987; De Block and Debrouwer 1991; De Neve *et al.* 1997; Kumar *et al.* 2010). This method is very efficient for flowering plants rather than non flowering plants (De Vetten *et al.* 2003).

Removal of Marker Gene through Cre/lox System

One method to remove the marker gene from transgenic plants is to use the marker gene between the two lox sites follow by their expression under recombinase gene. As a result of this recombination between the marker sequence and the sequence between the two lox sites leads the excision of targeted marker gene. In other method the two lox sites can be expressed in presence of transposase enzyme having jumping ability

followed by site specific recombination. This method is a quick and efficient for removal of the marker gene from the transgenic plants. In other experiment the resulted transgenic plants were regenerated first and then crossed with the wild plants having recombinase gene. The new regenerated plants results from these cross were screened. The resulted progeny showed no marker gene (Dale and Ow 1991). Deb Roy *et al.* (2008) described that heat induced cre/lox system is very efficient to excise *npt II* marker gene from transgenic *Arabidopsis thaliana* plants. Ma *et al.* (2009) reported 41 % transformation efficiency in transgenic tomato through this method. The marker gene which expressed in a particular tissue in plants was eliminated by the expression of recombinase gene under the inducible promoter. This method is very useful to remove marker gene which expressed in particular plants tissue at specific time of plants developmental stage.

Production of Marker Free Transgenic Plants through Transposan and Multi-Auto- Transformation Systems

Transposan mediated method is one of the quick and efficient method for removing marker gene from plant genome. In this method the foreign gene and marker gene are flanked in specialize maize activation/dissociation (Ac/Ds) factors followed by the excision of marker gene by the expression of Ac transposase gene in plant genome (Hua and Rommens 2007). *Npt II* marker free tomato and *hpt II* marker free transgenic rice plants have been successfully achieved through this method (Cotsftis *et al.* 2002). However this technique is less effective in vegetative propagated plants.

In multiple transformation auto system a specialize vector called multi-auto- transformation system (MAT) vector having the Ac element and *IPT* (Isopentyltransferase) gene were expressed under the control of strong constitutive

promoter such as 35sCaMV promoter. After the transformation of desire gene into plants, both the *IPT* gene and marker gene were eliminated from plant genome by expression of Ac transposase gene (Saelim *et al.* 2009). The drawback of this method is the reinsertion of transposable elements into plant nucleus (Ebinuma and Komamini 2001). In order to avoid this problem plant specific inducible promoter were used such as in maize tissue specific ubiquitin promoter was expressed in ovary. The resulted marker free transgenic maize plants showed high transformation efficiency (Yang *et al.* 2009). This method was successfully used for production of marker free transgenic cassava plants (Saelim *et al.* 2009). Khan *et al.* (2010) used this method successfully to produce marker free *petunia hybrid* transgenic plants against *Botrytis cinerea* (gray mold). The IPT system is also vey useful for the production of marker free transgenic potato plants against biotic stresses (Khan *et al.* 2010).

Conclusions:

The major goal of plant biotechnology is to develop transgenic plants for many useful purposes. The presence of selectable marker genes in transgenic plants affects our environment both directly and indirectly by horizontal gene transfer to wild and non targeted organisms. Theses selectable marker provides resistance to microbes against these antibiotics which are also used by human being for its protection against these microbes. Therefore the marker free methodologies are useful to overcome the gene flow from the genetic engineered plants to other living organisms. These selectable marker genes could be used only for early selection of transgenic plants while it should be removed after next generation in order to avoid their toxicity to other living organisms. Different techniques were used efficiently to remove the marker genes from transgenic plants genome such as by using less toxic chemical for selection, co-

transformation mechanisms for marker gene excision, site specific recombination via recombiase gene, transposan mediated and multi-auto- transformation systems. By applying one of these novel techniques we can get safe genetic engineered plants with no or minimum biosafety issues. By using marker free techniques we can develop and commercialize new transgenic plants that will definitely fulfill consumer demands. Now it is the responsibilities of future scientists to use the novel marker free technologies to keep environment clean and safe.

Annex:

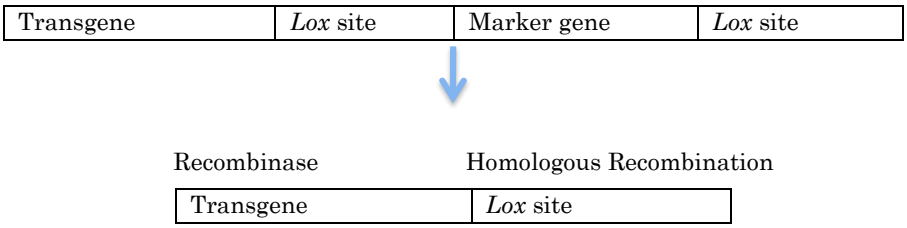

Fig. 1. Strategy to remove the marker gene by Cre-Lox system. The marker gene is targeted by two Lox sites follow by homologous recombination of two identical sequences (modified after Puchta 2000).

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