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ANTIBIOTIC RESISTANCE MARKER-FREE TRANSGENIC PLANTS

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Summary

A major goal of plant biotechnology is to improve existing cultivars and to develop new and elite cultivars. Different approaches for elimination of selectable marker genes have been developed over the last several years, and further improvements are now underway. These techniques are in the process of being applied to many useful crop species. It seems that concerns about uncontrolled spread of the transgene(s) in ecosystem will become irrelevant in the near future. Some of the new approaches remain speculative but in the time to come, it is expected that many new useful genes will be discovered, in this context, the focus would shift from frequency of transformation to the development of tailor-made transformation system.

Introduction

The global food supply comes primarily from agriculture, but the major problem in this context is the growing population, which needs an increasingly greater amount of food supply. The agricultural land is continuously declining, while increasing the land area for cultivation of crops without having a serious impact on the environment and natural resources is an unlikely possibility. Undoubtedly, modern agricultural practices have increased production of food, but this has promoted the large-scale use of pesticides and fertilizers that are expensive and potentially hazardous to human health and the ecosystem. A major challenge faced by human today is to increase world food production without damaging the ecosystem (Anonymous, 2000). An input use efficient agricultural system would involve the use of genetically divergent crop cultivars for proper crop rotation, leading to yield enhancement and higher economic returns. Sustainable agriculture would require efficient utilization of water resources, crop rotation and diversification, and inbuilt resistance to pests and pathogens. The recombinant DNA technology coupled with important developments in other areas could offer a sustainable strategy for increasing food production, improving the efficiency of production and reducing the adverse effects on agriculture (Pental, 2003).

Apart from many successful medical applications, one of the most prominent developments in recombinant DNA technology has been the transgenic varieties. Transgenic plants are created by transfer and expression of a specific gene virtually from any source into the desired plant species belonging to any taxon (Lycette, 1990). This has allowed a rapid, yet logical progression of the use of transgenic plants into crop improvement programmes.

Transgenic plants

Transgenic plants usually normal in appearance and character differ from the normal plant with respect to function and influence of the inserted gene. Genetic engineering is a technique, which enables us to insert and express desirable genes into plants as well as its qualitative and quantitative characteristics (Ratner, 1989). These genes are obtained from different sources, the new gene incorporated into host plant genome are called *transgene* and plants are called *transgenic,* which are expected to provide beneficial, new, stable, and inherited traits (Subramanian, 2003).

The selection of transformed cells is not only difficult but it is impossible if transgene is inserted without marker or reporter gene. Marker gene or reporter gene produces a specific phenotype, which permits either an easy selection or quick identification of the cell in which it is present (Braun, 2001). Marker genes are of two types selectable and scoreable. A *selectable marker* governs a feature, which enables only such cells that possess it to survive under the selective conditions e.g. antibiotic resistance gene. The *scoreable markers* produce distinct phenotypes, which allow identification of cells possessing marker gene from those, which are devoid of such genes *e.g., gus* gene (Singh, 2005). These marker genes allow multiple transformations of selected lines and optimization of the selection process for different species. In addition, scoreable markers are valuable aids for demonstrating the targeting of transformation to particular cell types, as well as analyzing gene expression and heritability

of foreign DNA inserts (Ratner, 1989). The identification and selection of cells into which, a new gene has been introduced is an important aspect of genetic engineering. Antibiotic resistance genes have the ability to selectively inactivate certain antibiotic and consequently protect cells against these antibiotics. An antibiotic resistance gene can be used to tag a gene carrying a trait or characteristics of interest Braun, 2001).

Presence of selectable marker gene (e.g., antibiotic resistance and herbicide tolerance gene) has now become matter for public concern, because if it goes into the human and animal food chains, it may cause allergic reaction or other health effects. There are reports that ampicillin resistance gene, which is commonly present in transgenic crop, could be passed from food and kill the useful micro-flora present in the gut of human and animals (Diamond, 2001). There are risks that transgenic pollens may contaminate our conventional crop varieties and it could lead to the development of super-weeds which is danger for wild life and biodiversity (Poppy et al. 2002).

Marker gene

Antibiotics inhibit cell growth by blocking some of its essential metabolic processes. Bacterial strains producing a specific antibiotic have to carry resistance to escape the inactivating effects of the corresponding antibiotic, and thereby prevent their own self-destruction. In the evolutionary race between microbes, the production of new antibiotic is usually countered through the development of resistance mechanism both by the producing and the target organism. As a result, there is, in nature, a wide range of antibiotic producing and corresponding antibiotic resistance genes. For development of their own resistance mechanism, the targeted bacteria will, in general, acquire antibiotic resistance genes, which are already present in the bacterial pool surrounding them (Braun, 2001).

There are the following two types of antibiotic resistance marker genes used in the development of transgenic plants.

1. Genes driven by bacterial promoters are used during the initial stages of the assembly of the pieces of DNA intended for transfer into the plant cells. The purpose of these genes is to select for the amplification of the pieces of constructed DNA in the receiving bacteria. The gene providing resistance to ampicillin belongs to this category.
2. Genes, which allow the selection of plant cells, which have taken up piece of DNA carrying the trait or characteristic of the interest. The insertion of a gene into a plant cell by transformation is a very inefficient process, since only a few thousand cells of the millions subjected to transformation would take up the desired gene. The transfer of an antibiotic resistance marker gene together with the gene of interest allows these very few cells to be selected as only those cells that have taken up both the genes will survive and multiply in the presence of corresponding antibiotic in the growth medium. Genetically engineered plants are then regenerated from the selected cells, and the marker is no longer needed (Braun, 2001).

The main problem in transgenic development is to identify the tissues which are transformed from the bulk of non-transformed cells. Marker genes produce a phenotype that allows easy identification/selection of the cells in which they are present. The marker gene is integrated along with the gene of interest into the vector so that cells that include the selective genes survive under the effects of selection agents (Kin et al., 2002). The marker genes are of following two types:

Scorable marker

These markers produce a distinct phenotype, which allows an easy identification of the cells expressing the inserted gene from those that do not. Scorable markers are introduced into genetically engineered plants and provide valuable tools for identifying and tracking genetic modification in plants. It does not facilitate survival of transformed cells under particular conditions. They are also important where the genetically modified plants can not be regenerated from single cells and direct selection is not feasible or effective. In addition, they are useful in quantifying both transformation efficiency and gene expression in transformant (Flavell et al., 1992).

Most frequently used scoreable markers are â-glucuronidase (*gus*), luciferase (*lux*), Octopine synthase (*oes*) and nopaline synthase (*nos*). Genetically modified plant tissues expressing the *gus (uidA)* gene turn blue

when incubated with the substrate 5-bromo-4-chloro-3-indoyl-l-glucmonidase (Jafferson et al., 1987). A major limitation of this approach is that this reporter gene also expressed in *Agrobacterium* despite the use of promoters. This problem has been overcome by inserting introns within the *uidA* gene, which is processed in plants but not in *Agrobacterium* (Veluthambi et al., 2003). Green fluorescent protein (*gfp*) from jelly-fish finds immense applications in transgenic research. Unlike *gus*, the expression of *gfp* can be visualized in living cells. Green fluorescent protein gene (*gfp*) when expressed in prokaryotic or eukaryotic system produces a fluorescent product. Since exogenous substrate and cofactors are not required for the fluorescence (it occurs under UV/ blue light in the presence of oxygen), *gfp* expression can be used to monitor gene expression and protein localization in living organism thus can be used in selection of transgenic plants (Chalfie et al., 1994). Another reporter gene, luciferase *(lux),* requires an externally added substrate for its detection (Sheen et al., 1995).

Selectable marker

Presence of a suitable marker is necessary to facilitate the selection of few transformed cells and the detection of transgenic plants during their development. These genes have no further desirable function in the genetically engineered organism, as no useful product is derived from them. These selectable marker genes produce a phenotype that enables only the plants that contain and express this gene to survive the selection pressure imposed on them. These genes confer resistance to various compounds toxic to plant cells. Plants regenerated from the Surviving cells, therefore, would contain the selectable marker joined to the gene of interest.

Two main classes of selectable markers have been used in genetic modification of plants, the first group includes such selectable markers that confer resistance to antibiotics such as, kanamycin, chloramphenicol (Herrera-Estrella et al., 1983), hygromycin B (Waldron et al., 1985), bleomycin (Hille et al.. 1986), streptomycin (Jones et al., 1987), gentamycin (Hayford et al., 1988) and phleomycin (Pere et al. 1987). The other group consists of those genes whose products confer tolerance to herbicides such as phosphinothricin (DeBlock et al., 1987), 2,4-D (Strebel et al., 1988) etc. Most commonly used selectable marker is a gene (*nptII*) derived from Transposon 5 *(Tn* 5) of *E. coli* K12; this genes encodes aminoglycoside-3-phosphotransfrase II [APH(3')II]. This enzymes is also commonly known as neomycin phosphotransferase II (NPTII), it inactivates kanamycin, G418 and neomycin by phoshorylation of the antibiotics (Flavell et al 1992).

Antibiotic resistance genes: The original and most widely used selectable marker is a bacterial gene for neomycin phoshotransferase *(npt II),* an enzyme which inactivates a number of related antibiotics, including kanamycin. After introduction of constructs containing the *nptII* gene into plant cells, kanamycin is applied to kill non-transformed cells. Transformed cells express the *nptII* gene, and are protected from the antibiotic. A number of other systems employing antibiotic resistance as a selectable marker employed in the production of genetically engineered plants. These includes *aad* gene for streptomycin and spectinomycin resistance (Khan et al., 1999), the *nptII* gene conferring resistance to Hygromycin (Akiyoshi et al., 1984), and the ampicillin resistance conferring *bla* gene.

Bleomycin, a DNA damaging glycopeptide, resistance gene is located on Transposon 5 *(Tn5)* of *E. coli*. It has been cloned in plant expression vector and introduced into *Nicotiana plumabaginifolia* using *Agrobacterium tumefaciens*. Expression of this determined in plant cells makes them resistant to and allows selection of transformed cells (Hille et al., 1986).

Herbicide resistance genes: While antibiotic resistance genes continue to be used as a selection agent for the generation of a wide range of transgenic plants, resistance to specific herbicides also provide an effective means for plant selection. In many cases, herbicide resistance genes have provided a more effective selection system for plant transformation (Chandler, 1995). Genes conferring resistance to a number of herbicide groups including the triazines, sulphonylureas, bromoxynil, glyphosate and phosphinothricin are readily available. Of these, the *bar* gene isolated from *Streptpmyces hygroscopicus* (Thompson et al., 1987) has been widely used as an effective selectable marker for resistance to the herbicide phoshinothricin and has been proved useful in cereals and grasses (Vasil et al., 1993). Herbicide resistance marker genes may provide considerable advantages over antibiotic resistance marker genes in cases where either higher level the antibiotic may

interfere with plant regeneration process or where plant tissue may exhibit a high level of intrinsic resistance (Huppatz et al., 2000).

Concerns with the use of reporter genes

The antibiotic resistance genes used for the selection of transformed cells are expressed in every cell of the resulting transgenic plants. It has been argued that such genes and their protein products could cause problems to human health and the environment.

Gene-flow from transgenic crops: Gene-flow between cultivars, between crops and their wild relatives is a very common phenomenon that always takes place in nature. Most of the crops are compatible with their wild relatives therefore natural hybrids have been reported, typically without ecological consequences. Mating system, mode of pollination, mode of seed dispersal and the habitat of the crop are critical factors that influence the gene-flow (Hancock et al., 1996), which are very difficult to evaluate and quantify (Messeguer, 2003). Possibility of transgene-flow from engineered crops to other cultivars/wild/weedy relatives is one of the major concerns in relation to the ecological risks associated with the commercial release of transgenic plants. It is important to quantify this gene-flow and to develop strategies for minimize or control, taking into account the possible effects of the newly introduced genes. Natural and engineered traits are likely to have similar patterns of gene dispersal. As a consequence, problems associated with cross-pollination with the wild relatives in transgenic crops will be the same as those encountered in traditional breeding programs (Messeguer, 2003).

Loss of biodiversity is another dangerous consequence of crop-to-weed gene-flow. The crops most likely to increase the risk of extinction by gene-flow are those that are planted in a new location, into the vicinity of their wild relatives, thereby, increasing the hybridization rate due to their proximity (Ellstrand, 2001). In recent years concerns have been raised mainly by environmentalists and consumer organizations that the presence of transgene in the environment or the food supply might pose an unpredictable hazard to the ecosystem or to human health. For example, herbicide resistance genes might be transferred by out-crossing into making their control difficult (Dale et al., 2002).

The presence of resistance genes against antibiotics in food products might be theoretically led to the spread of these resistance genes to intestinal bacteria in human, although there is no evidence supporting this proposition. The absence of resistance gene in transgenic plants could also lower the cost for developing and marketing of genetically modified products and might speed up commercial release of new products (Kupier et al., 2001; Daniell , 2002; Smyth et al., 2002).

Environmental issues : With the transgenic technology another speculated fear is the spread of transgenes. It is thought that related species, which have crossability with the transgenic crop, may invade the crop as weeds, and after receiving the transgene, become superweeds. Another possibility is accumulation of transgene product (e.g., Cry protein) in the environment. An inconclusive report has been put forth showing the susceptibility of non-target organism to Cry proteins (*e.g.*, Monarch butterfly larvae). In recent years the concept of genetic trait control technology (many call it "terminator technology") has emerged, where a chemical (inducer) is used to permit the expression of transgene. If this kind of technology comes to routine use it will enhance the use of chemicals in agriculture. Finally, there are many other concerns like effect of transgene on rhizosphere and phyllosphere micro-flora. These concerns demand that the transgenic materials must be put to rigorous test for their influence on environmental factors.

Agricultural implications : Since much work is being done on insect resistant transgenic plants, it is predicted that a number of target organism will develop resistance over a period of time. To overcome this situation, researchers are now using '*refugia strategy*' where non-transgenic crop is also put in the transgenic field, which serves to maintain Cry susceptible insect population. In USA it is mandatory to plant 25% field with non-transgenic crop. Another situation is that because of transgenic crop monoculture will come in to practice leading to genetic erosion. The pests can also start searching new host because of toxicity of the host transgenic plants.

Toxicity and allergenicity : In the health and safety arena, one of the major apprehensions with the commercialization of transgenic food products has been the concern that selectable marker genes or their products might be toxic or allergenic when consumed. Additionally, when selectable markers confer

resistance to such antibiotics, which have clinical or veterinary applications, the concerns has been raised that the marker gene could be transferred into microorganisms and increase the number of resistant pathogenic microorganisms in the human or animal gut. This would, in turn compromise both clinical and veterinary applications of the antibiotics (Yoder and Goldsbrough 1994). However, it should not be concluded that every antibiotic resistance marker gene will be equally safe and unsafe.

Strategies for developing antibiotic-resistance marker-free transgenic plants

The potential of genetically engineered crops to transfer foreign genes through pollen to related plant species has been cited as an agricultural and environmental concern. Laboratory biosafety and regulatory considerations would require the adoption of gene containment approaches for future generations of genetically engineered crops as studies related to environmental impact of novel genes on indigenous crops and weeds are long-term in nature. Plant regeneration in culture conditions is an indispensable and integral part of transgenic technology. In recent past many techniques have been developed which obviate the need of tissue culture process. In addition, different constructs using an array of genes have been developed where either the antibiotic resistance marker gene is excised or they are not used. Some of these strategies are described below.

a. *In planta* transformation : The small size of *Arabidopsis* plant led to the development of *in planta* transformation by floral dips. This method obviates plant tissue culture and eliminates somaclonal variation. Infiltration of flowering plants was superior to infiltration of young seedlings. Another version of this technique, which is *in planta* embryo transformation, involves infection of embryonic axis with *Agrobaeterium,* so that it subsequently grows directly into a transformed plant. This infection is directed towards the plumule, cotyledonary node and surrounding regions of the young seedlings where one cotyledon is broken off to provide a wound site. Seedlings grow into mature plants, and the T_0 and T_1 generation plants are analysed to check the presence of transgene. This method is not only tissue culture independent but also is genotype independent and permits screening of a large number of transformants in a short span of time.

b. Co-transformation : In this method the selectable marker gene is separated from the gene of interest at the time of transformation. Co-transformation involve two separate DNA assembly, one having gene of interest and another having the marker gene. For successful application of this technique, two criteria must be fulfilled, (i) efficiency of co-transformation must be very high and (ii) the co-transformed DNA must integrate at sufficiently unlinked locations of the genome to allow recovery of the recombinants (Yoder and Goldsbrough, 1994). The desired gene and marker gene can be put on two T-DNA within same binary vector (Depicker et al., 1985, Komari et al., 1996, Lu et al., 2001) or on two different binary vectors (in same *Agrobacterium* or two *Agrobacterium*).

Co-transformation of *Brassica napus* was done with two *Agrobacferium* strains, each carrying a T-DNA bearing a different selectable marker; on selection for one of the marker, it was found that about 60-80% of these plants has also received the second T-DNA, about 78% of the times at linked sites (DeBlock et a1., 1991). In tobacco use of two *Agrobacterium* strains one containing a T-DNA with an *npt II* gene and the second containing a T-DNA with a nopaline synthase gene yielded similar results (McKnight et al., 1987). The co-transformation frequency may be as high as 100% in populations of the model plant species tobacco *(Nicotiana tabacum)*. Over all, 40-50% of the lines demonstrated the capacity for separation of co-transformed T-DNA regions through segregation.

In a co-transformation study a single *Agrobacterium* strain bearing a single binary plasmid contained either two or three T-DNA regions each with a selectable marker by using the binary vector with these three T-DNA regions, the frequency of co-integration of the third independent T-DNA in a population of transformants was higher than expected.

Presently, two different methods of *Agrobacterium* mediated co-transformation are being used. In first method, a single *Agrobacterium* strain carrying two separate binary vectors, one carries the marker gene while another carrying target gene is used. In the alternate method, two different *Agrobacterium* strains each carrying separate binary vectors are employed. The results indicate that the first method is highly efficient for co-transformation (Aztakanandam et al., 2001). Co-transformation frequencies observed are much higher than expected for independent events.

An inherent limitation of this strategy is that the non-linked transgene loci have to be separated by crossing. Therefore, the procedure not only requires fertile plants, but also is time consuming. In addition, the technique can not be used for tree species with long generation times.

c. Site-specific recombination : It is now possible to manipulate newly introduced DNA after genetic transformation procedures; this process is more predictable and reliable (Lyznik et al., 2003). The ability of microbial site-specific recombinases to cleave DNA at specific sites and ligate it to the cleaved DNA at a second target sequence has enabled their wide-spread use in manipulating DNA in higher eukaryotes. The apparatus used by prokaryotes and lower eukaryotes to perform site-specific recombination between-specific target sites is well suited to excising selectable markers from plants. Most of the site-specific recombination systems shown to be functional in plants; they are members of the integrase family and consist of recombinase (Cre, Flp and R; Table 1) and its corresponding recognition sites for recombination *(loxP, FRI,* and *RS* respectively).

Site-specific recombination is one of the strategies for generating marker-free transgenic plants. The well characterized site-specific recombination systems are the cre/lox system of bacteriophage P1 (Odell et al., 1990; Stuurman et al., 1998; Gleave et al., 1999). The excision of kanamycin resistance (*nptII*) gene placed between two *lox* sites has been reported in tobacco by expression of *cre* recombinase (Dale et al., 1991), the R-RS system from pSR1 plasmid of *Zygosacchromyces rouxii* (Onouchi et al., 1995), Gin recombinase of the phage Mu (Maeser and Kahmann 1991; Odell and Russel, 1994), and FLP/FRT recombination system of 2μ plasmid of yeast. The FLP recombinase activity has been reported in *Arabidopsis* (Kilby et al. 1995), tobacco (Bar et al., 1996, Davies et at., 1999), and in rice and maize (Lyznik et al., 1996).

All (FLP/FRT, cre/lox, R/RS, Gin) recombination systems are simple, two component system, each requiring a single peptide enzyme, which acts *in trans* to catalyze recombination between two short, specific DNA sequences. Of these four recombination systems, which are derived from the phage P1 is the most advanced for plant studies. In this system, the cre enzyme catalyzes recombination between two 34 bp *loxP* sequences resulting in excision of internal sequences. The *cre* gene can be introduced into lox containing plant by either transformation or crossing (Dale and Ow, 1990; Russell et al., 1992). If the *lox* sequences are inverted with respect to each other, cre will catalyze the inversion of the internal sequences. It has been proposed that generating inversions might be useful for converting functional genes to their antisense derivatives (Dale and Ow, 1990). Alternatively recombination between *lox* sites located on non-homologous chromosomes, perhaps mobilized to different positions by transposable elements, could generate reciprocal translations.

Unlike most recombinases, cre, flp, and R do not require modification or host-specific factors to function in plants. The most common feature of all these systems is that after the first round of transformation, transgenic plants are produced that contain the respective recombinase and the sequence to be eliminated between two directly oriented recognition sites. After expression of single chain recombinase, the recombination reaction is initiated resulting in transgenic plants devoid of the selectable marker (Puchta, 2003). Crystal structures of the complexes formed between several site-specific recombinases within their DNA targets have revealed conservation within their mode of action (Van Duyno, 2001). The cre recombinase of bacteriophage P1 converts dimeric phage P1 plasmids into their monomeric constituents through recombination between two directly repeated loxP sites in the genome of *E. coli*. The yeast FLP and R recombinases enable efficient replication of plasmids bearing the FR, T and RS sites. Inversion of a segment of the plasmid that is flanked by two opposite oriented recombination sites promotes replication by switching the relative orientations of the replication forks.

Different types of genome manipulation could be achieved by controlling the expression of respective recombinase and specific allocation of the recombination sites within a transgene construct. In addition, the cre/lox system can also be used to avoid transgene silencing. Instead of complex integration, a single transgene copy insertion can be achieved by flanking the transgene of interest with inverted recombination sites; the selectable marker and recombinase gene with direct repeats ensure simultaneous elimination of the selectable marker (Ow, 2001).

One-step transformation strategy has also been designed taking advantage of an effective, estrogen-regulated cre induction system. Upon induced DNA excision, both the selectable marker gene and the *ere* recombinase gene (self-excision) were removed (Zuo et al., 2001). In this way, the techniques does not require crossing of two transgenic parental plants or retransformation.

d. Transposition : Since their discovery and introduction into heterologous host, maize transposable elements served as versatile genetic tools for the isolation of genes and for generating novel mutations. Many of the transposable elements, when transformed into plant species maintain their transposition competence (Yoder et al., 1988). A conservative cut-paste mechanism resulting in excision of the elements from one prior locus to reinsertion into a second is the most interesting feature of the two best characterized maize element families, the Ac/Ds and Spm/dspm family (Fedoroff et al., 1991). It is reported that in maize and transformed dicots, transposition usually occurs to both linked and unlinked sites in roughly equal proportions (Jones et al., 1990). In order to obtain marker- free transgenic plants, a transposable sequence is used to connect either the transgene or the selectable marker within in such a way that the two entities can be separated from each other in a controlled reaction after transformation and selection (Puchta, 2003).

Two types of strategies are generally employed. In the first strategy, the marker gene is placed on a mobile element which is lost after transposition (Gorbunova et al., 2000). Using this strategy, by inserting the selectable *ipt* gene into the transposable element *Ac*, marker-free transgenic tobacco plants have been generated at low frequencies (Ebinuma et al. 1997a,b). In second strategy, the desired gene is excised away from the original transgene locus by transposon-induced dissociation. The feasibility of these approaches has been successfully demonstrated in tomato (Goldsbrough et al., 1993; Yoder et al., 1994), where ~10% of the excised elements do not reinsert into a sister chromatid and subsequently lost by somatic segregation (Belzile et al., 1989). The autonomous *Ac* element has two well-defined regions essential for transposition; a transposase encoding gene and the inverted repeat termini. The *Ds* elements lack transposase function and are stable in the absence of *Ac*. They can however be transactivated by introducing *Ac* transposase encoding sequences *in trans*. Sequences cloned between the inverted repeats of a *Ds* element are also mobilized to new genomic locations in presence of a transposase gene (Lissner et al., 1989; Masterson et al., 1989).

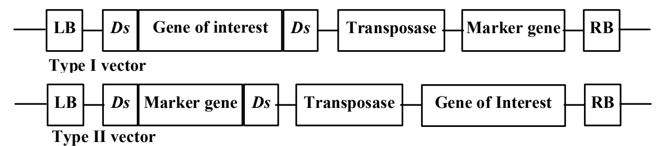


Figure 1. Two types of transposition-based vector systems.

These processes have led to the development of some novel transformation vectors, which incorporate transposable elements to eliminate selectable marker genes and other ancillary sequences (Goldbrough et al., 1993). The T-DNA regions of the two classes of vectors arc shown in Figure 1; in the first type of vector, the gene of interest is inserted between the *Ds* inverted repeats, but in the second type of vector the selectable marker is flanked by the *Ds* repeats. An advantage of the type I vector system, in which the gene of interest is located within the *Ds* element, is that by relocating the transgene, different levels of expression (both quantitative and qua1itative) can be achieved (Goldsbrough et al., 1993). Similarly, the advantage of the type II vector system is that it can be constructed such that minimal amount of vector sequences are retained. For example, if the construct contain only the T-DNA repeat sequence essential for transformation, then after the transposition selectable marker is removed due to the *Ds* sequences and only the gene of interest flanked by sequence resides of the direct T-DNA repeats will remain in the plant genome. In addition, the selectable marker will be lost in some of somatic tissues by failure of the *Ds* element to reintegrate.

e. Intra-chromosomal recombination between *attP* regions : Deletion of sequences positioned between direct repeats in the genome via homologous recombination has been reported to occur at low frequencies in somatic cells (Puchta et al., 1995). Therefore, for a long time homologous recombination was not considered to be a feasible technique for the removal of marker genes. A less complicated approach utilizing intra-chromosomal homologous recombination (ICR) between two homologous sequences has been developed to induce DNA

deletions. Although, ICR can be enhanced by stimulation of DNA repair systems, their frequencies are too low for an efficient application this system to produce deletions of transgene regions. In tobacco, Puchta et al. (1995) reported less than 10 ICR events among all cells of a six week tobacco plant. Such low ICR frequencies might be increased by the use of recombination substrates that provide a more efficient target for the recombination machinery.

In this approach a vector containing *npt II* gene flanked by two 352 bp attachment P *(attP)* region of bacteriophage ë is used and somatic tissues with deletion events are identified. Since *attP* system does not require the expression of helper proteins to induce deletion events, or a genetic segregation step to remove recombinase genes, it is proved to a useful tool to remove undesirable transgene regions, especially in vegetative propagated species (Zubko et al., 2000). It addition, the system is quicker than procedures involving re-transformation or cross-pollination, and also avoided potential problems associated with the expression of a site-specific recombinase.

f. Tissue-specific expression of marker gene : It is possible to regulate transcription of the marker gene by using a promoter, which is deferentially expressed temporarily/spatially at the site of transformation. This would allow the selection of transformants without expression of the marker gene in mature plants (Yoder et al. 1994). A number of promoters are known, which control expression of genes in the specific cells or stage of the plants. Inducible or tissue-specific promoters would be particularly useful for a better control of the expression of pest resistance genes. Rice lipo transfer protein (*lpt*) gene, hydroxypraline-rich glycoprotein (*hrgp*) and maize protease inhibitor (*mpi*) genes from maize are some of the examples of such wound inducible or pathogen responsive promoters (Cordero et al. 1997). In addition, vascular tissue-specific promoters from maize streak virus (MSV) and *Arabidopsis thaliana*, fruit and seed-specific promoters from wheat and *A. tha!iana* have also been identified and cloned. Green tissue-specific promoters (*PEPcP, CIY*) have shown preferential tissue-specific expression in rice and significantly produced expression of the gene in grains (Datta et al. 1998). For specific and desired gene expression, in future promoters would be designed on the basis of known cis-regulatory sequences, which mediate certain gene expression profiles.

g. GST -MAT vector : *Agrobaclerium*-mediated transformation is highly dependent upon competence of the target plant tissues. A system, which results in cell proliferation/differentiation only of transformed cells, is highly desirable for plant transformation. A GST-MAT vector system (MATIMH) consists of *ipt* gene coupled with *iaaM/H* genes as the selectable marker and the *CST-II* promoter for site-specific recombination (Ebinuma and Komamaine 2001; Endo et al., 2002).

In case of an *ipt* type MAT vector, the *ipt* gene of *A. tumefaciens* Po22 is used for regenerating transgenic plants (Sugita et al. 1999); *ipt* gene codes for the isopentinyl transferase that catalyses cytokinin synthesis and causes proliferation of the transgenic cells and differentiation of adventitious shoots (Smigocki et al. 1988). In addition, a site specific recombination (R/RS) system is also combined to remove the *ipt* gene from the transgenic cells after transformation (Sugita et a!., 1999). In many cases, it is difficult to regenerate transgenic plants *in vivo* using only the *ipt* gene. Therefore, a MAT vector is composed of *ipt* gene and *iaaM/H* genes are used to increase their regeneration ability *in vivo* for non-tissue culture based transformation. The *ioaM* and *iaoH* genes encode indole acetamide hydrolase and tryptophan monooxygenase respectively, that catalyses auxin synthesis (Sitbon et a!., 1992). Thus the combination of *iaaNf/H* gene with *ipt* gene efficiently produces marker-free transgenic plants.

h. Conditional lethal dominant gene for marker elimination : In this system, a conditional lethal dominant gene is used, which converts a normal non-toxic compound into a toxic compound. *E. coli* cytosine deaminase (*cod A*; Perera et al., 1993: Stoughgaard, 1993), gene product converts 5-fluororocytosine to 5 fluorouracil, which irreversibly inhibits the thymidylate synthase activity and consequently deprives the cells of dTTP for DNA synthesis.

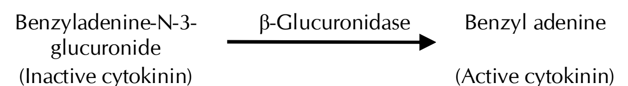
i. Marker-free transplastomic plants using *uidA* reporter gene : Clean gene transformation technologies allow the introduction of desirable genes into crops without antibiotic marker genes or vector sequences (Yoder et al., 1994). Transplastomic plants in tobacco, *Arabidopsis* and potato have been developed using *aadA* gene, which encodes aminoglycoside 3′-adenyl transferase and confers resistance to the antibiotics spectinomycin and streptomycin.

A procedure has been developed by Lamtham and Day (2000) in which plastid DNA recombination and cytoplasmic sorting was used to remove *uidA* gene flanked by *bar* and *uidA* genes. The *bar* gene confers tolerance to the herbicide glufosinate. Excision of *aadA* and *uidA* genes, mediated by two 174 *bp* direct repeats generated *aadA*-free to transplastomic plants containing only the *bar* gene. Removal of *aadA* and *bar* genes by three 418 *bp* direct repeats allowed isolation of marker- free T_2 plants containing a plastid genome having *uidA* reporter gene (Lamtham and Day, 2000).

j. Positive selection system : In genetic transformation generally antibiotic or herbicide resistance genes are used for isolate transformants. In most of the plant systems Hygromycin (Armstrong et al., 1990: Ishida et al., 1996), kanamycin (Schroeder et al., 1991; Kikkert, 1993), bleomycin (Hille et al., 1986), bromoxynil (Stalker et al., 1988), chloramphenicol (Fraley et al., 1983), 2,4-D (Strebel et al., 1989), methotrexate (Golovkin et al., 1993), bialaphose (Gorden-kamm et al., 1990), chlorsulphan (Fromm et al., 1990), and phosphinothricin (Koziel et al., 1993) have been used as selection agents to eliminate the non-transformed tissues or cells. The corresponding resistance genes are in many cases not relevant to the desired transgenic trait and they may be undesirable after selection has been accomplished. As stated elsewhere, there are growing concerns on using antibiotic/herbicide resistance genes in a selection system, Therefore, new marker systems are needed to minimize the use of antibiotic and herbicide selectable marker genes and to address some of the growing concerns in the area of bio-safety associated with the use of antibiotic/herbicide resistance genes as selectable markers. A selection system that does not require antibiotic or herbicide resistance gene would avoid such risks and concerns.

Positive selection system is based on the selection agent, which arrests the growth and development of non-transgenic cells, while, it favors the growth of transgenic cells. In contrast, to an antibiotic/herbicide resistance gene-based selection systems, in which transgenic cells acquire the ability to survive on a selection medium, while the non-transgenic cells are killed, the positive selection system favors growth and development of the transgenic cells, while non-transgenic cells are starved, but not killed. Therefore, this selection strategy is called positive selection (Bojsen et al., 1994; Joerbso et al., 1996). Presently four types *viz*., Benzyladenine-N-3-glucuronide, Xylose, Mannose and tryptophan are mostly being used as positive selection agents.

i. Benzyladenine-N-3-glucuronide-based selection: Benzyladenine-N-3-g1ucuronide is a glucuronide derivative of cytokinin benzyl adenine, which is an inactive form of cytokinin. Upon hydrolysis by the action of GUS enzyme it gets converted into active cytokinin. Therefore, in this method of selection, inactive glucuronide derivative is used in the culture medium in place of active cytokinin as a selection agent. Cells which have acquired the *gus* gene by transformation are able to convert the cytokinin glucuronide to active cytokinin, while untransformed cells are unable to do so and there growth and development is arrested (Joerbso et al., 1996). This methos has successfully been demonstrated in tobacco.

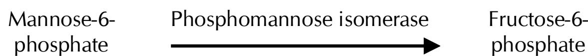


Because of metabolite advantage, the transgenic cells are able to regenerate into plants on the selection medium containing Benzyladenine-N-3-glucuronide, while the untransformed cells are not able to regenerate. In addition, *gus* gene serves the dual purpose of selectable as well as a scoreable marker.

ii. Xylose-based selection system : In most of the experiments, sucrose is added in plant tissue culture medium as a carbon and energy source. Some times sucrose is replaced by other sugars, such as fructose or glucose, but not by xylose, mannose etc., as xylose is toxic to the cells. An enzyme xylose isomarase (D-xylose keto-isomerase) catalyzes the reversible isomerization between D-xylose and D-xylulose, which functions in the xylose metabolic pathway in microorganisms. A gene for xylose isomerase (*xylA)* from *Thermoanaerobacterium thermosulferogenes* has been isolated, cloned and expressed in the potato. Tobacco, and tomato and transgenic plants (Haldrup et al., 1998). When plant cells were transformed with *xylA* gene were placed on selection medium containing xylose, only the transformed cells carrying the gene and its product were able to convert xylose into xylulose, while, untransformed cells are unable to do. Threrefore, transgenic cells could utilize xylose

as a carbon source, while the untransformed cells are starved in the absence of enzyme. Haldrup et al. (1998) also used sucrose in addition to xylose in the slection medium to reduce the toxic effects.

iii. Mannose-based selection system : Many of the plants *e.g.*, maize, can not metabolize mannose as they do not contain a gene to convert mannose to fructose (Wang et al., 2000). Similarly, if mannose is used as carbon source in medium, it strongly inhibits root growth, respiration (Morgan and Street, 1959) and germination (Pego et al.. 1999). An enzyme PMI (Phoshomannose isomerase) has been identified, which catalyzes the reversible inter-conversion of mannose 6-phosphate and fructose 6-phoshate. PMI is common in nature and is found across kingdoms including human, however it is not present in plants, except soybeans (Lee and Matheson, 1984). The *pmi* gene has been isolated from *E. coli* (Miles and Guest, 1984) and has been successfully transformed into sugar beet (Joersbo et al., 1998), maize (Wang et al., 2000; Wright et al., 2001), cassava (Zhang et at, 2000) and in pepper (Kin et al., 2002).



When plant cells transformed with *pmi* gene are placed on selection medium containing mannose as a carbon source, only transformed cells carrying the product of *pmi* gene were able to convert mannose into fructose while untransformed cells are unable to do so (Wang et al., 2000).

iv. Tryptophan-based selection system: An enzyme tryptophan decarbosylase (TDC) from *Catharanthus roseus,* has been identified, which catalyzes L-tryptophan to tryptamine (Noe et al., 1984). TDC is able to utilize tryptophan and its derivatives like 4-methyl tryptophan (4-mT), 4-f1uoro tryptophan and 5- f1uoro tryptophan (Berlin et al., 1987; Sasse and Buckholz, 1983). These compounds are toxic *to* plant cells lacking TDC, they are converted into non-toxic tryptamine derivatives by TDC. In *Peganum harmala* cell suspension, 4-mT has been successfully used *to* select the cells with TDC activity (Berlin et al., 1987). In this way, cDNA clone of the *Catharanthus roseus tdc* gene could be used as a selectable marker gene in transformation of those plant species that have no detectable endogenous TDC activity.

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