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Review Article

APPROACHES FOR BIOLOGICAL IMPROVEMENT OF ECO-FRIENDLY CROPS

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DOI: 10.7897/2321-6328.01325

Article Received on: 12/07/13

Accepted on: 16/09/13

Abstract

Marker gene is a DNA sequence whose phenotype can be easily recognized and identified. It can be used to flag another gene. A selective marker encourages the growth in the presence of a selective agent, which kills or prevents the growth of a cell. Antibiotics resistance gene is used to tag target gene or gene carrying a trait of interest. Antibiotics are used in crops during transfer of gene of interest in crops improvement programmes. Antibiotics and antibiotics resistance gene has been used for a long time in plant biotechnology. Not even one Gene transfer method has so much efficiency so that screening allows the selection of transformed cells having marker gene together with target gene which inactivates the antibiotic and protect the cells from antibiotic while non transformed cells do not have marker gene so they are killed in presence of antibiotics during screening.

Keywords: Cre/lox crossing, FLP/FRT, R/RS System, GUS gene, Homologous recombination system.

INTRODUCTION

Marker gene is a DNA sequence whose phenotype can be easily recognized and identified. It can be used to flag another gene. A selective marker encourages the growth in the presence of a selective agent which kills or prevents the growth of a cell. Genetic diversity enables for long term sustainability and agricultural self-reliance and also has been known to increase or decrease in response to domestication. Extensive recombination and selection result in successful interspecific introgression of genes. Transgenic plants may contain several DNA elements, most notably the selectable marker genes, that are not needed after the selection of the transgenic line. Using site-specific recombination system, such as Cre/lox, targeted deletion of these so-called unneeded DNA elements can be achieved[1]. Bayley and his co-worker used the Cre-lox site-specific recombination system of bacteriophage P1 to excise a firefly luciferase (luc) gene. The excision event was due to site-specific DNA recombination between two lox sequences flanking the luc gene and was catalyzed by the Cre recombinase introduced by cross-fertilization[2]. Genetic diversity resulting from interspecific introgression can be evaluated with morphological characteristics, seed protein, isozymes and DNA markers. A large number of polymorphic markers are required to measure genetic relationship and genetic diversity in a reliable manner. Molecular genetic markers have developed into powerful tools to analyze genetic relationship and genetic diversity. Molecular technologies have provided numerous methods which associate the molecular sites on DNA of the plant genome. Antibiotics and antibiotics resistance gene has been used for a long time in plant biotechnology. Antibiotics resistance gene is used to tag target gene or gene carrying a trait of interest. Not even one gene transfer method has so much efficiency so that screening allows the selection of transformed cells having marker gene together with target gene which inactivate the antibiotic and protect the cells from antibiotic while non transformed cells do not have marker gene so they are killed in presence of antibiotics during screening. Different methods have been developed to remove selectable marker genes and production of marker free crops like site specific recombination, transient expression. A selectable marker gene is incorporated in the plastid genome to evenly change the thousands of genome copies in a tobacco cell. However when transformation is achieved the marker gene becomes undesirable[3]. There exist a large number of marker genes for plants but only a few marker genes are used for most plant research and crop development. For research, a variety of selection systems are necessary as no single selectable marker gene was found to be sufficient for all conditions[4]. Some researchers worked on systems where marker genes are eliminated efficiently soon after transformation. Alternatively, transgenic plants are produced by the use of marker genes that do not depend on antibiotic or herbicide resistance but instead promote regeneration after transformation[5]. The Cre–loxP system consists of two components: (a) two loxP sites each consisting of 34 bp-inverted repeats cloned in direct orientation flanking a DNA sequence and (b) the cre gene encoding a 38- kDa recombinase protein that specifically binds to the loxP sites and excises the intervening sequence along with one of the loxP sites[6]. Park and his co-worker suggested site-specific recombination-based systems such as the phage-derived cre/loxP system, the yeast derived R/RS system, and maize Ac/Ds system. However, at present their applications are limited to a few model plants, such as Arabidopsis[7]. Sreekala and his co-worker reported the production of marker-free transgenic rice by using a chemically regulated, Cre/loxP-mediated site-specific DNA recombination in a single transformation[8]. Hoff and his co-worker constructed and

tested a Cre-loxP recombination-mediated vector system termed pCrox for use in transgenic plants. This single vector system avoids the need for genetic crosses required by other dual recombinase vector systems. The pCrox system may prove particularly useful in instances where transgene over-expression, or under-expression by antisense, would otherwise affect embryo, seed or seedling viability[9]. Luo and his co-worker developed a highly efficient 'genetically modified (GM)-gene-deletor' system to remove all functional transgenes from pollen, seed or both. The 'GM-gene-deletor' reported here may be used to produce 'non-transgenic' pollen and/or seed from transgenic plants and to provide a bioconfinement tool for transgenic crops and perennials, with special applicability towards vegetatively propagated plants and trees[10].

Lutz and co-worker reported plastid marker-gene excision with a transiently expressed CRE, site-specific recombinase. This is a novel protocol that enables rapid removal of marker genes from the approximately 10000 plastid genome copies without transformation of the plant nucleus[11].

Resistance

Bacteria are present everywhere (ubiquitous). It produces chemicals to prevent growth of others and to reduce competition. Microorganisms produce chemicals at very high rate that neutralize the antibiotic before it can do harm and become resistance to antibiotics. Use of marker gene creates problems such as- (1) presence of enzymes coded by these resistance markers does not pose as much threaten as much as transfer of resistance gene form food to gut microorganisms and in environment (2) multiple drug resistance against *Staphylococcus*, *Mycobacterium* etc. (3) negative effect on proliferation and different ion of plant cells in presence of selective agents.

Guidance in Industries

The criteria for using an antibiotic resistance marker gene should be - (1) an assessment of potential toxicity (2) whether protein has potential to cause allergic reactions (3) protein encoded by resistance gene, would compromise therapeutic efficiency of orally administered antibiotics. FDA acknowledges that efficiency of transfer of resistance genes from plant to microbe is remote in comparison to microbes. But FDA believes that use of marker genes in crops by developers should be evaluated on the basis of- (1) whether antibiotic is an important medication (2) whether it is frequently used (3) whether it is orally administered (4) whether it is unique or not (5) whether there would be selection pressure for transformation process to take place (6) level of resistance against antibiotic among bacterial populations. If in any condition product of marker gene could compromise use of relevant antibiotic, marker gene product should not be present in finished food. FDA also lays emphasis on that if there are few antibiotics to treat certain clinical conditions, marker gene for these antibiotics should be avoided.

Removal of Marker Genes in Crops and Methods

Site specific recombination system

Site specific recombination systems are common in bacteria and in lower eukaryotes. A site-specific excision recombination strategy is used for elimination of undesirable transgenic from crops. They have been categorized on the basis of structure in active site. Tyrosine family (Cre, FLP, R, Lambda integrates) use catalytic tyrosine hydroxyl group for nucleophilic attack on phosphodiester bond of targeted DNA and conserved serine residue of serine family (Hin, Gin recombinase).

Cre/lox crossing

The Cre-lox site-specific recombination system has been widely studied for marker removal. In fact, the first marker free commercial transgenic plant was developed using Cre-lox technology[14]. The cre/lox recombination system is very important tool in genetics, simple and no accessory factors are required. This system allows the induction of gene at desired time and in desired cell. This system can be used for removal of extra gene in genome in plant for targeted insertion.

Cre/lox system has three strategies:

1. Re transformation,
2. Crossing
3. Inducible auto excision.

In this approach, plant having cre gene is crossed with plant having transgene construct in which selective marker is flanked by two 34 bp lox p sites in same orientation. Sometime there is incomplete removal of marker gene in F1 generation. It is also a cumbersome method. It cannot be used for all types of vegetative plants. Cre/lox system has two components: cre recombinase and two 34 bp specific lox p DNA sequence. Cre recombinase recognize the two 34 bp sequence and cleave at particular sites within recognition sites in same orientation and remove the DNA segment present in between these two lox p sites. In this method, a binary plasmid vector is constructed in which there is cre gene under heat shock promoter (Gmhsp17.5-E promoter), a marker gene nptII under control of CaMV 35S promoter were placed between two lox p sites. When heat induction is given, cre gene is transcribed and results in excision of marker as well as cre gene itself. A developmentally regulated Cre-lox site-specific recombination system is for excision of a bar marker gene by using seed-specific napin promoter. Auto excision can be used efficiently only for flowering plant not for vegetative propagated plants. Auto excision eliminate marker gene for T1 generation and next generation free of marker gene. This strategy uses functional promoter like floral promoter. This system was used with cre lox recombination in rice first time. In this recombination we use promoter that can be chemically induced and conditionally induced like heat shock, wound etc. Cre lox P has been used successfully in wheat, a foreign DNA fragment flanked by recombination sites (lox511) in the opposite orientation and the loxP sites (in the same orientation) flanking the bar gene were crossed to cre-expressing transgenic parental plants[12]. Transient expression of Cre was sufficient to self-excise the cre gene and to recombine lox sites (either the original loxP sites or its spacer variants such as lox511 and lox2272) integrated into ectopic loci in tobacco[13]. This was called a "forced" self excision because the activation of a marker gene was required to identify auto excision events.

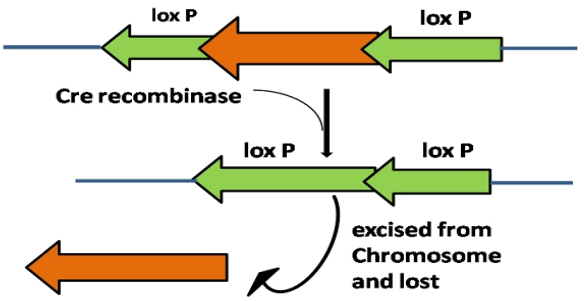

Figure 1: Cre Function Model

FLP/FRT

FLP is originally 599 bp but trimmed up to 30 bp (constructed from 2 micron plasmid). It is easy to work with. It does not require accomplice proteins and is used in many crop improvement programs. Retransformation method show highest recombinant activity.

In addition, a potato virus X or tobacco virus can be used to deliver Cre gene to transgenic tobacco plant having lox p sites. There is removal of cre gene in recovered transgenic products[15,16], the product and substrate are same (the FRT sites) and also not effected by state of DNA like linear, coiled present on same DNA etc. recombination is reversible. Covalent bond are formed between enzyme and substrate so require stoichiometric than catalytic amount of enzymatic protein. First maize cells were used to see functionality in plants. There is not yet much information available on transgenic rice plants expressing FLP as cross pollination is difficult in rice. Radhakrishnan and his co-worker introduced the gene for FLP recombinase under the control of the maize ubiquitin-1 promoter, into the rice genome to develop an FLP-FRT recombination system- (derived from 2 m plasmid of *Saccharomyces cerevisiae*) based marker gene removal application for rice[17].

R/RS System

In these systems, different inducible system can be used according to convenience like heat inducible, wound inducible etc. Then a new approach came 'multi auto transformation' in which firstly ipt (isopentyl transferase) was used and Ac transposon mediated excision method was used. But persistence over expression in some somatic embryogenesis is not preferred and R/RS system was used to remove marker gene. The auto excision system was applied in strawberries[18]. The result were promising, a hybrid negative or positive coded A gene was fused to npt II and this hybrid was put between two RS sites and rat glucocorticoid receptor was integrated with R to make protein coded by R inactive unless activated. Many efforts were done like codon usage according to plant for the R gene. R/RS system is found in *Zygosachromyces rouxii*.

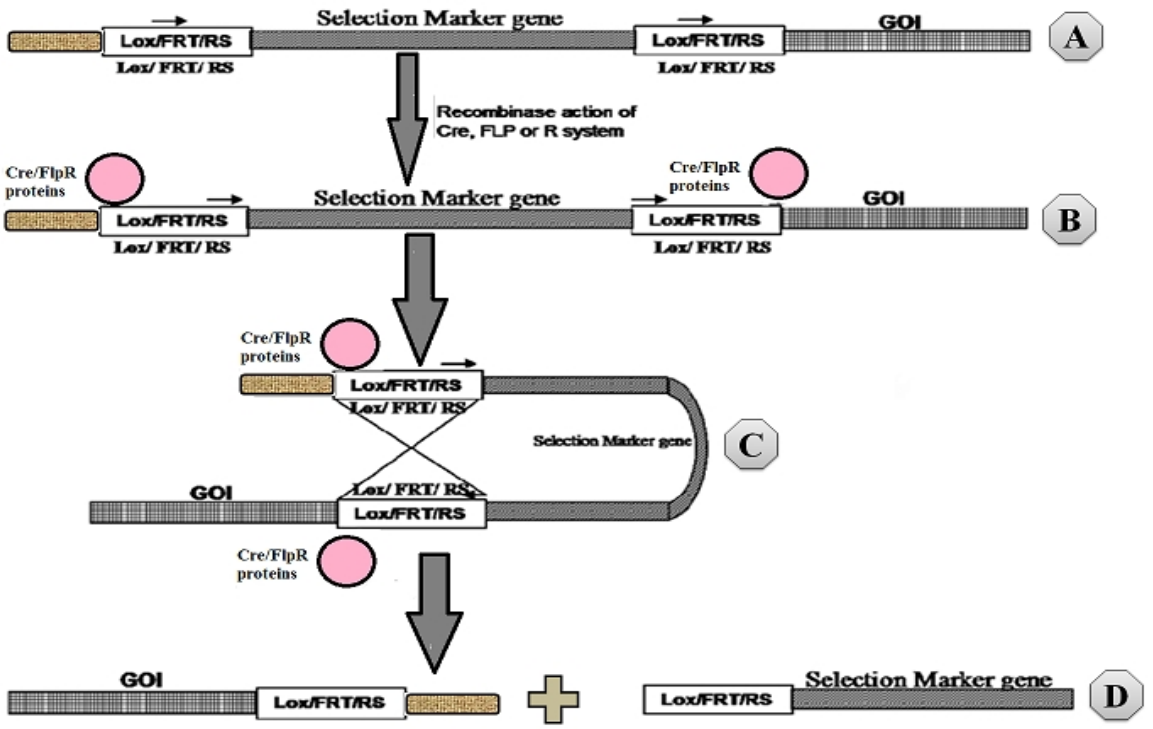

Figure 2: Action mechanism of microbial recombinase systems, Cre-lox, Flp/Frt and RS/R

The gene construct contains the Lox/Flp/R recombinase sequences flanked by the selection marker gene while the gene of interest lies outside the recombinase sequences. (A) When the transgenic containing the Cre/FRT/RS is crossed with the other transgenic contains the Cre, FLP or R recombinase gene sequences (B), the recombination process starts (C) and the marker genes are removed (D) from chromosome. Remaining Cre recombinase sequence in chromosome is separated out during the progression of the generation. SMG (Selection marker gene), GOI (Gene of interest). Symbol cycle in the figure represent the recombinase (Cre, FLP or R) proteins.

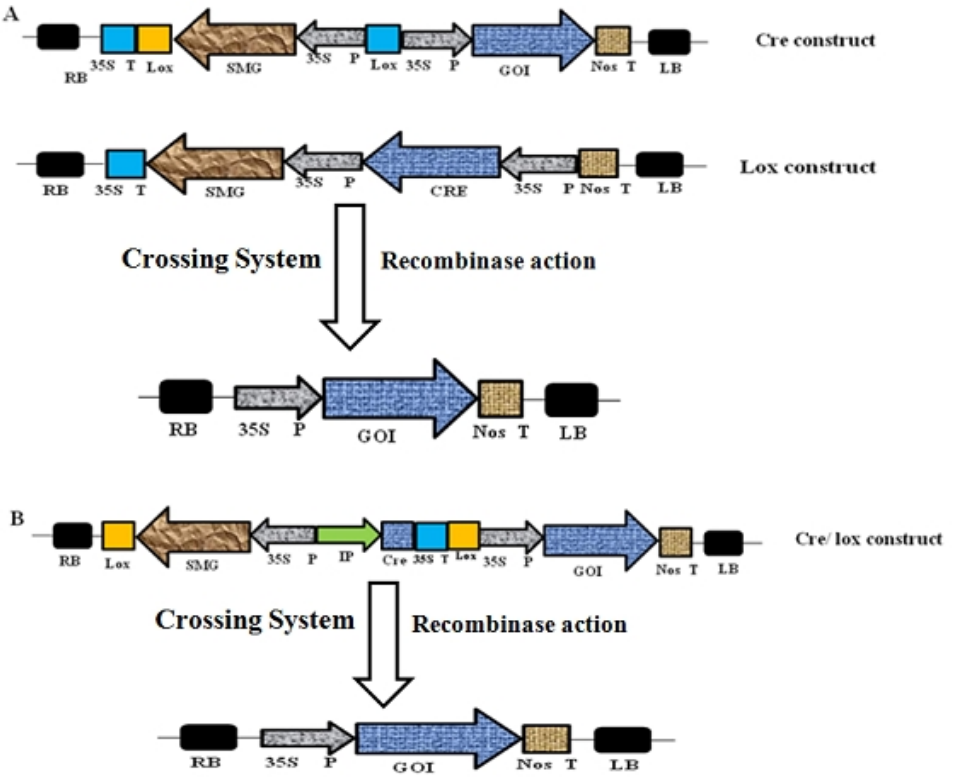

Figure 3: Cre-lox site-specific recombination system for removal of selection marker gene from transgenic plants

A shows, The lox gene construct and the Cre gene construct to be crossed with lox transgenic; B shows, The inducible system where the Cre gene is under the control of inducible promoter (IP).

Integrases

Cre and FRT does not require co factors and lead to a reversible reaction while lambda require accessory factors and can catalyze the reverse reaction, but a specific set of co-factors result into irreversible reaction**.** ΦC31 requires no cofactor for integration[19] and precise integration has been demonstrated in the fission yeast *Schizosaccharo mycespombe*.

Iso pentyl transferase gene for marker free plants

In this a chimeric ipt gene is inserted in the transposable element Ac which is used for selectable marker for transformation. Ipt gene is found on Ti plasmid of *A. tumefaciens*. Ipt gene code for isopentenyl transferase that is responsible for condensation of isopentenyl pyrophosphate and AMP and result in to AMP isopentenyl. Ipt gene product is also helpful in differentiation and proliferation in transformed cells. But this ipt gene result into loss of apical dominance and lack of root due to over production of cytokinin. Ac transposable element in maize can move to new location. It is used to remove ipt gene in transgenic cells of ESF shoots after transformation. When Ac excise, 10 percent does not re-integrate, lost or sometime integrated in sister chromatid. So when Ac element was used along with it then this Ac element resulted in removal of itself and Ipt gene and production of marker free plant in hormone free medium. This process is called as multi auto transformation (MAT) as there is no limit for number of genes to integrate into plant genome as there is low number of particle available marker gene. Rol or Ipt genes are used that is inserted within Ac or R/RS system. It does not require sexual cross for marker gene or recombines and can be used for vegetatively propagated plants. The only limitation is that prolonged expression can cause unwanted harmful effects. But this problem can be solved by using recent version of MAT system with glutathione S transferase promoter from maize for delay in excision. The one step MST system have been used successfully for marker gene free tobacco[20], apricot[21] and rice[22] etc.

Gene Deletor System

In this system, lox P and FRT recognition sites were fused to generate a hybrid site called as LF. Cre/lox is found in bacteriophage P1and FRT sites are formed from 2 micro plasmid *S. cerevesiae*. Cre gene or flp gene lead to 100 percent removal of marker gene in tobacco plant. In this binary expression vector is constructed and recombinase gene is under control of cold inducible promoter that is inactive in plant tissue at normal temperature but active at low temperature. This system is very useful in producing transgenic plants, as heat inducible promoter activate in presence of heat but lead to change in fluidity of membrane.

phiC phage specific recombinase system

This system is used for removal of selectable marker gene from tobacco plasmid. A recombinase enzyme called integrase is used, that recognize bacterial (attB) and bacteriophage P1 (attP). pCK 2 vector is constructed having a streptomycin gene (aad A) flanked by attB and attP sites. This vector is used to transform the plastid. After that, integrase gene is introduced into cell and causes excision of

site and marker gene. This system is better than CRE/lox P system used for plasmid. The vector also carries the bar gene that show resistance against phosphinothricin in absence of aadA gene. Nowadays, plastids are also used for foreign gene expression in some species but routinely in tobacco.

Development of Marker Free Plants

Nick de and his co-worker described the transformation of potato by *A. tumefaciens* without any selectable marker. This was an efficient method. But the problem was due to presence of vector backbone in transformed behaved like selectable marker gene[23].

Positive selection

Some genes are available if they are used as selectable marker, they do not cause harm to transformed plant. These genes code for enzymes that metabolize substances that enhance growth of plants in comparison to normal plant like GUS, manA, xyl A gene.

GUS gene

This gene is found in *E. coli* which acts as a reporter gene and codes for beta-glucuronidase enzymes. It is used extensively in modified plant but not much in crop improvement. In this system of positive selection, a glucuronide derivative of benzyladenine (benzyladenine N-3-glucuronide) is used as selection agent. This enzyme breakdown the glucuronide supplied in media into benzyladenine that is a cytokinin and favour growth of transformed plant.

ManA gene

Phosphomannose isomerase (PMI) converts mannose-6-phosphate into fructose-6-phosphate, which favors growth of the transformed cell. This gene helps the transformed plant cells to utilize mannose as a carbon source to grow and differentiate on media containing mannose as a selection agent. Though most of the plants are sensitive to mannose yet there are some plants insensitive like carrot, tobacco, sweet potato and legumes that are highly insensitive to mannose.

The xylA, DOGR1 and AtTPS1 genes

The xylA gene isolated from *Thermoanaero bacterium thermosulfuro genes* or *Streptomyces rubiginosus*[24] code for xylose isomerase that convert D xylose into D xylulose. Transformed cell can grow in sole xylose medium while non-transformed starves. It is also known as glucose isomerase.

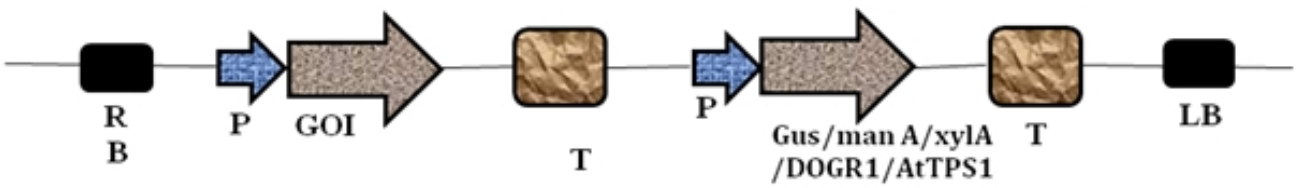

Figure 4: T-DNA of plant transformation vector harboring positive section marker genes for producing marker-free transgenic plants

GUS, β-glucuronidase; pmi, phosphomannoisomerase; xylA, Xylose isomerase A; DOGR1, 2- deoxyglucose-6-phosphate phosphatase; AtTPS1, trehalose-6- phophate synthase.

Negative Selection System

In this negative selectable marker gene is placed next to positive marker gene in same construct. Negative marker gene free plants can be selected by using negative selection. D-amino acid oxidase can be used as positive or negative marker gene by changing substrates D-alanine or D-serine to D-isoleucine or D-valine.

Co transformation of two transgenes

In this two vectors are constructed that integrate the genes of interest at different target sites in the genome. One containing target gene while other contains marker genes that are carried by *A. tumefaciens;* that is most important tool in plant biotechnology for plant transformation. The three methods for these two vectors for transformations are- (1) two different *A. tumefaciens* containing two vectors (2) One *A. tumefaciens* containing two vectors (3) two T DNAs in single binary vector. The marker gene is removed from genome of transgenic plants during segregation and recombination during sexual reproduction by selecting the transformed plants carrying the target gene. But they require fertile plant and is time consuming and require improvements and not advisable for trees with long generation times.

Homologous Recombination System

This system was used for first time in the unicellular green alga *Chlamydomonas reinhardtii*[25] and in higher plants[26]. Homologous recombination takes place between identical gene promoters and terminators flanking target gene and marker gene. Marker gene removal produced 25 percent of marker free transgenic lines[26]. Dufourmantel and his co-worker used various homology recombination technology for production of marker free soya bean and tomato with strong herbicide tolerance. A plastid transformation vector aad A gene was used to interrupt herbicide resistance gene[27], after removal of marker gene transplastomic plant having efficient herbicide gene, 4-hydroxyphenylpyruvate dioxygenase (HPPD)

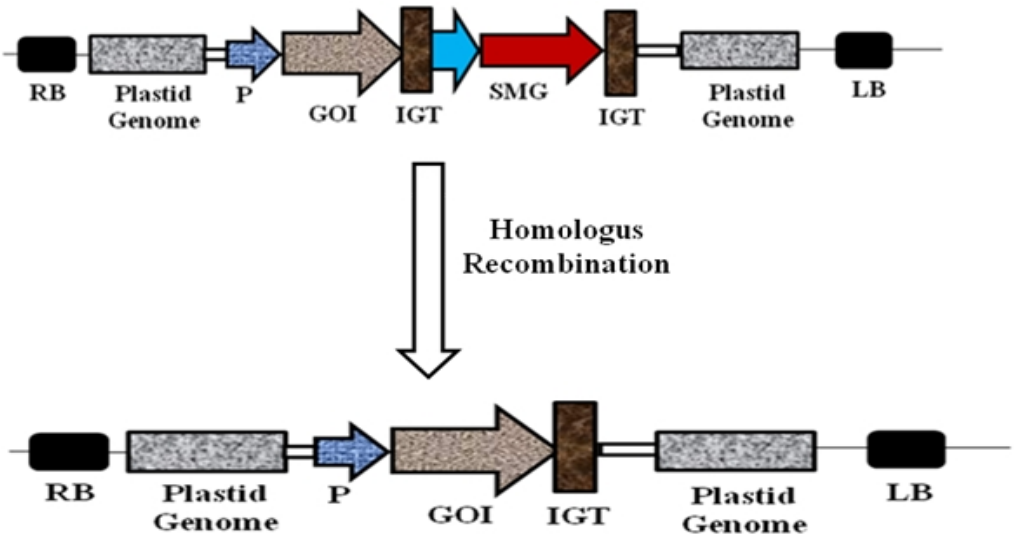

Figure 5: Mechanism of marker gene removal mechanism from chloroplast by homologous recombination

Transient co-integration of the marker gene

In this marker gene and target gene are placed in tDNA by homologous recombination step via targeting sequence. Marker gene is placed outside the targeting region.

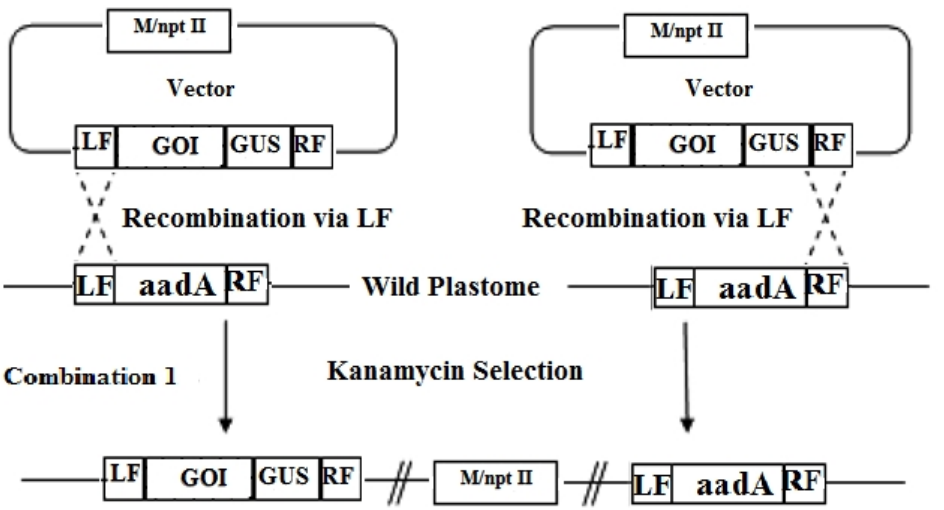

Figure 6: Transient co-integration based marker removal from chloroplast genome of transplastomic plants

M/nptII: Marker, neomycin phosphotransferase-II, LF and RF: Left and right homologous fragments, GOI: Gene of interest, GUS: Reporter gene encoding β-glucuronidase, aadA: Gene for Spectinomycin resistance.

Temporary expression of marker gene

The expression of selectable marker gene takes place under specific conditions only because promoter used for marker gene gets activated in only specific condition (inducible promoter). This system was successfully used in tobacco first time for suppression of npt II wound inducible promoter AoPRi[28]. This system allowed selection of transformed cells and less or negligible expression of npt II after selection.

Advantages

- No need of introduction of foreign gene and no need to produce second generation to eliminate cre gene.
- It can be used for vegetative propagated plants like potato.
- It is easy and time saving[29].

Disadvantages

No control over growth of selected transformed plant.

CONCLUSION

Various approaches and methods have been described here but appropriate choice depends on ease of use, crop, and method of transformation, generation time and intellectual

property rights. These all approaches help in producing selective marker free plants that are valuable and health oriented products. The construction of molecular map will open the door for many applications of DNA markers in plant breeding. Though the number of economically important genetic loci that have been tagged via linkage to molecular markers is currently limited. Work towards this end can now accelerate rapidly. To take advantage of the potential molecular biology techniques, a great deal of time and effort must be devoted to mapping the genetic loci responsible for the tremendous array of characters that breeders are concerned about in population or varietal improvement programs. Much of these efforts involve analysis of specially designed crosses to determine where the genes of interest lie in relation to other mapped phenotypic or molecular markers. As the current cotton genome map evolves toward saturation, new technologies will give rise to new types of genetic molecular marker possibilities for locating and cloning genes of interest. The opportunity to effectively integrate molecular analysis of genetic variation in plant improvement program becomes increasingly apparent.

ACKNOWLEDGMENTS

Mr. Siddharth Priyadarshi is thankful to UGC, New Delhi, India for awarding the Fellowship RGNF 2012-13 to him. The authors acknowledge the Director CSIR-CFTRI and Head PPSFT Department CSIR-CFTRI Mysore, India for the facilities provided and keen interest in the work.

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Cite this article as:

Priyadarshi Siddharth, Shukla Anubha and Borse Babasaheb Bhaskarrao. Approaches for biological improvement of eco-friendly crops. J Biol Sci Opin 2013;1(3):243-249 http://dx.doi.org/10.7897/2321–6328.01325