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Cre-Lox: A Tool for Removal of Marker Genes to Make GM Foods Safe

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Abstract

The green revolution has significantly helped in increasing food production. So far, various breeding methods have been exploited, including recombination DNA technology provides another approach for increasing food production. By means of this technology, the losses in food production incurred by various biotic and abiotic stresses can be effectively controlled. In most transgenic studies, scientists have used antibiotic resistant genes as markers for easy selection of transformants but there are risks involved in the use of GM foods. To make such foods safer and environment friendly, we have discussed a novel strategy, i.e. Cre-lox, which involves site specific recombination. By means of Cre-lox, the marker genes can be specifically removed once the selection of transformants is over. In addition, this strategy can be used to module the hybrid chromosomes, avoid gene silencing, and incorporate a single copy of a transgene for its higher expression.

Key words: Cre-lox, site specific recombination, marker genes, gene silencing

For centuries various strategies have been exploited to keep the pace of food production with the growing population, but in modern times we have well understood the mechanisms of inheritance and gene manipulation and their outcomes. It is predicted that by exploiting the recombinant technology for crop improvement, food production can be increased to levels sufficient to feed the whole population. In this context, the development of GM crops / foods is not only important, but essential. Though lot of progress has been made in this regard, there is a threat of unacceptance among common people. This is especially so in European countries where people do not accept these foods because of health and environmental concerns. The presence of antibiotic resistance genes makes GM foods unsafe as these genes may get transferred into the living systems with continuous consumption. We have mentioned a few such reports which emphasize the adverse effects of GM foods. Taking into consideration health and environmental issues, there is an urgent need to utilize / develop some strategies which can help in making such foods safer.

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The primary concern about the possible risks to health caused by GM foods can be very well understood from some of the scientific reports. In report by Pusztai, it was found that by feeding GM potatoes to rats there was a deleterious effect observed on their growth rate and immunity (Ewan and Pusztai 1999). On the contrary, the Organization for Economic Corporation and Development (OECD) Edinburgh conference on "GM food safety: Facts, Uncertainties and Assessments" stated in February 2000: "Many consumers eat GM foods. No significant adverse effects have yet been detected on human health." The other group of scientists tested the effect of transgenic sweet peppers and tomatoes on rats, and by feeding GM soya to mice and rats no adverse effect was observed (Teshima et. al. 2000). Another concern is the environmental issue, which includes the transfer of genes to related species, production of super weeds, the creation of new diseases or disease susceptibility, an increase in allergic reactions and the disruption of existing ecosystems (http//www.Nuffield.org/bioethics/publication/modifiedcrops/index.html).

In most transformation experiments, marker genes like the

neomycin phosphotransferase II (*nptII*) gene, which confers resistance to kanamycin and neomycin, have been employed and judged by several regulatory bodies such as the US Food and Drug Administration (FDA). The FDA has accepted that the use of *nptII* does not fulfill the safety criteria. As with *nptII*, it has been argued that antibiotic resistance is already widespread in bacteria, so a rare gene transfer from a GM food source is unlikely to be of practical consequence. By contrast, even though the level of resistance to antibiotics is high, the situation might be so serious that no increase in antibiotic levels, however marginal, would be acceptable, especially when the gene retains a bacterial promoter (Pechere 1997; 1998). In an another study, it has been reported that though the DNA might get degraded after consumption, small amounts remain intact and these levels might be enough to generate a low frequency of bacterial transformants (Mercer et al. 1999). Chambers et al. (2002) looked for the fate of the ampicillin resistance gene in chicken after feeding GM *E. coli* and GM maize both containing the ampicillin resistance gene. They found that the maize gene was present in the mouth of chickens but not further down in the gastrointestinal tract, where the bacterial gene was protected by its host and remained detectable through the gastrointestinal tract. These studies have investigated DNA transfer from GM plant material to microorganisms and tend to confirm the view that such an event would be extremely rare, but not impossible (Gasson 2000). These concerns are worrying the public about the acceptance of GM foods but it is curious that the public accepts the products of conventional plant breeding without the detailed evaluation of unintended effects, ignoring the fact that conventional breeding can involve treatments such as mutagenesis and induced polyploidy through colchicines, which are more likely to cause unintended changes in gene expression (Dale 1999).

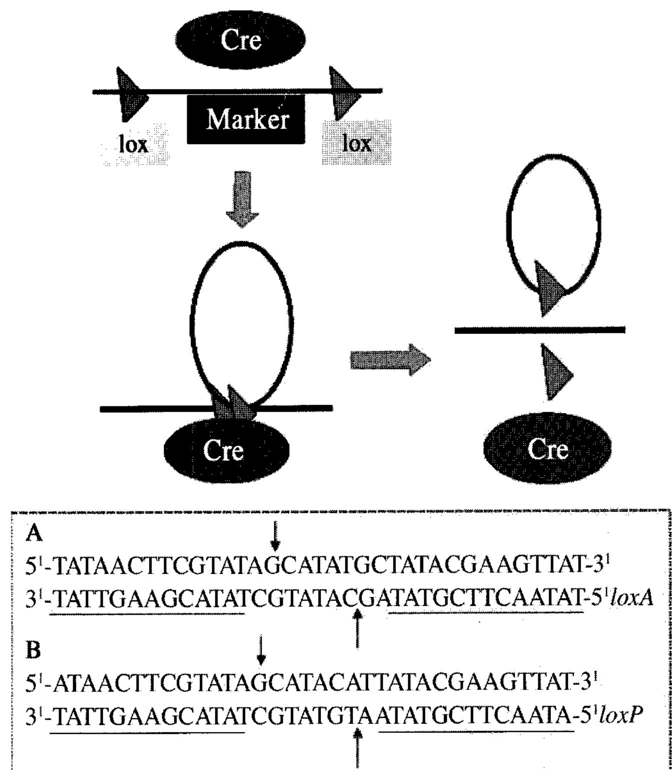

Fig. 1. Schematic representation of the strategy for removal of antibiotic resistant genes (marker) from transgenic plants using Cre-*Lox* recombination method. Red color triangles indicate the *lox* sites and the line between them indicates the antibiotic resistant gene (marker). The Cre enzyme leads to the recombination between lox sites removing the marker gene along with one *lox* site. The gene of interest lies besides the *lox* sites. The boxed region shows the molecular structure of A) *Lox* A site B) *Lox* P site. The13 bp inver- ted repeats in *lox* A and *lox* P sites are underlined, arrows in the figure indicate the site of cleavage of phosphate groups. Central 8 bp are involved in strand exchange.

Why selection of transformed cells is required

The low transformation efficiency for many crops necessitates the use of selectable marker genes to identify transgenic plants. These dominant genes confer resistance to an antibiotic or herbicide that kills non-transformed cells. The most widely-used antibiotic resistance marker for the selection of the transformed plant cells is the *nptII* gene, which confers resistance to the antibiotics neomycin and kanamycin. This gene is present in most GM plants submitted for marketing in Europe. For example, it has been used to develop the delayed ripening tomato, herbicide tolerance, and insect-protected corn and cotton varieties. Thus, single cells with an integrated transgene within a bulk of non-transformed cells can be easily identified. During recent years, concerns have been raised, mainly by environmentalists and consumer organizations, that the presence of such genes within the environment or food supply might cause an unpredictable hazard to the ecosystem or to human health. Herbicide resistance genes might get transferred by outcrossing into weeds (Dale et al. 2002). The presence of antibiotic resistance genes might theoretically lead to the spread of these resistances via intestinal bacteria in human populations. Their absence will make GM foods acceptable by common people and help in speeding up the commercial release of new products (Daniell 2002; Kuiper et al. 2001; Smyth et al. 2002). Co-integration of multiple marker genes with large number of transgenes used for developing multiple pest resistance will increase safety concerns as well as lead to gene silencing in the transformed crop. So there is a need to develop the strategies considering the safety issues and the different ways to get rid of marker genes.

Strategies for removal of marker genes from GM crops:

a) Selection of transformants using molecular methods

Totally avoiding the selectable marker genes and selecting transformants directly by molecular methods is only possible if transformation efficiency can be increased. In spite of having enough automated systems, such a project is still highly demanding. A first report published indicates that this technique will be set up in the near future (Aziz and Machray 2003).

b) Use of screenable markers

The use of such markers does not kill the non-transformed cells, rather the transformed cells experience a metabolic or developmental advantage. Some examples include: the bacterial β-glucuronidase (Joersbo and Okkels 1996), xylose isomerase (Haldrup 1998), and phosphomannose isomerase genes (Negrotto et al. 2000). With the development of these new markers,

concerns about the spread of herbicide or antibiotic resistance into the environment become irrelevant. However, such markers may not be favorable in the long run.

c) Co transformation of two transgenes
By transforming two transgenes (desirable gene and the marker) on two T-DNAs within same binary vector (Depicker et al. 1985) or two binary vectors within same *Agrobacterium* (Daley et al. 1998), a high number of transformants can be obtained carrying the desired gene and marker gene as unlinked copies. The non-linked transgene loci have to be separated by crossing. Therefore, this method not only requires fertile plants but is also very time consuming. It is also not useful for the development of transgenic trees which have long generation times.

d) Excision of selectable marker genes by transposition
In this method, the transgene or the selectable marker is linked to the transposable sequence in such a way that the two genes can be separated after transformation and selection. The marker gene can be linked to the transposable element which is lost after transformation (Gorbunova and Levy 2000). In the case of transgenic tobacco, the marker was successfully removed after linking it to the transposable element Ac (Ebinuma et al. 1997a; Ebinuma et al. 1997b). However, in this approach the segregation of the transgene and the marker is needed and the transgenes tend to jump into the linked positions. This approach is very time consuming.

e) Excision of selectable marker genes using homologous recombination
In this method, the marker gene gets removed due to the induction of double strand breaks (DSBs) at the respective loci (Puchta 2000). DSBs lead to the excision of marker genes by both homologous recombination as well as non-homologous end joining (NHEJ) (Gorbunova and Levy 1999). Here, the marker gene is placed between two homologous sequences. The expression of bacterial proteins such as Rec A and Ruv C have been found to enhance homologous recombination in somatic plant cells (Shalev et al. 1999).

f) Elimination of selectable markers using site specific recombination
More than a decade ago, it was demonstrated for the first time, that marker genes can be removed from the genome of a transgenic plant. It was a big achievement in the field of plant biotechnology. The marker gene (kanamycin) was placed between two lox sites and the marker was removed by the expression of Cre recombinase (Dale and Ow 1991). This technique exploits the means of site specific recombination leading to the removal of marker genes. The various site specific recombination means, which can be used for the removal of selectable markers include: 1) Cre- *lox*, 2) FLP/*ftr* of *Saccharomyces cerevisiae* (Gidoni et al. 2001), and 3) R-*RS* system of *Zygosaccharomyces rouxii* (Ebinuma and Komamine 2001). The Cre, FLP, and R are the members of the integrase family. All three of these recombinase do not require any modification or host specific factors to function in plants.

Cre-*lox*

Cre is a bacteriophage recombinase enzyme which catalyses the site specific recombination between two 34 base pair *loxP* sites and can be employed for the removal of the marker genes. The site specific DNA recombination has been very well understood in viral integration and excision at *attp* and *attb* sites where the recombinase enzymes recognize the specific DNA sequences and catalyse reciprocal exchange of DNA strands between these sites. In the case of Cre-*lox* mediated recombination, four recombinase enzymes and two lox P sites form a synapse structure in which the DNA resembles the model of four-way Holliday junction intermediates. Cre is an enzyme of the integrase family which cleaves their DNA substrates by a series of staggered cuts, during which the recombinase becomes covalently linked to the DNA by a catalytic tyrosine residue. Recombination takes place by exchanging one set of strands to yield a Holliday structure intermediate followed by the exchange of another set of strands to resolve the intermediate into recombinant products. The Cre recombinase with a molecular weight of 38 K from bacteriophage P1 does not require any accessory factor in contrast to bacteriophage λ, which require integrase to facilitate the recombination. The Cre recombinase mediates site-specific recombination between two *loxP* sites; such site-specific recombination can be performed *in vitro* with a variety of DNA substrates. Guo et al. (1997) have determined the three dimensional structure at 2.4A^0 resolution of a covalent intermediate in the site specific recombination reaction between Cre recombinase and the symmetrized *loxP* sites.

Structure of the Cre-*loxA* complex

The structure of the Cre-*loxA* complex reveals that two Cre molecules are bound to a single *loxA* site. By replacing the asymmetric 8-base-pair spacer sequence between inverted repeats in *loxP* with the symmetric spacer, the modified substrate is what we call *loxA* (see Fig1). Each Cre molecule contacts the outermost 15 base pairs of one *loxA* half-site, which includes the 13-base-pair inverted-repeat sequence and the first two base pairs of the central strand-exchange region. One of the two Cre molecules bound to the *loxA* site cleaves the DNA substrate to form a covalent 39-phosphotyrosine linkage. Two anti-parallel Cre-*loxA* complexes then associate to form a dyad-symmetric recombination synapse. A similar protein-protein interface is formed between Cre molecules bound to the same *loxA* site and between Cre molecules bound to different *loxA* sites in the synapse, generating pseudo-C4 symmetry in the synaptic assembly.

Since the two Cre molecules are bound to the *loxA* site, each Cre molecule contacts a *loxA* half-site and one of the two molecules form a covalent 39-phosphotyrosine linkage with the DNA. The amino- and carboxy-terminal domains of Cre form a clamp around the half-site, making extensive contacts with both the major and minor grooves. A total of 39 contacts to the *loxA* site involving arginine, lysine, and histidine side chains are formed by the two Cre subunits. Although the two Cre-DNA interfaces are similar, a clear asymmetry exists that may result in part from conformational changes associated with cleavage of the DNA substrate by only one of the two Cre subunits.

Structure of Cre recombinase

Cre folds into two distinct domains that are separated by a short linker. The amino-terminal domain (residues 20-129) contains five α-helical segments connected by short loops. The larger carboxy-terminal domain of Cre recombinase includes 132-341 amino acids and is primarily helical. The C terminal domain of Cre recombinase is structurally similar to the lambda integrase and HP1 integrase catalytic domains (Hickman et al. 1997; Kwon et al. 1997). Unlike the amino-terminal domain, the C-terminal domain differs significantly between the two *loxA* bound Cre molecules, with the largest structural deviations coming from the C-terminal segments leading to helix N.

Active sites and the phospho-Tyr linkage

The Cre active site contains the conserved catalytic triad residues Arg 173, His 289, and Arg 292 and the conserved nucleophilic Tyr 324 and Trp 315. The amino acids in a single active site are all derived from the same subunit, indicating that Cre recombinase does not form a shared active site like Flp recombinase (Chen et al. 1992). There are two distinct active sites in the Cre-*loxA* complex, one for each Cre subunit bound to a *loxA* half-site. As the synapse is formed by the association of two Cre-*loxA* complexes, the synapse tetramer contains a total of four active sites, corresponding to the four phosphates in the site-specific recombination reaction. One active site in the Cre-*loxA* complex (two per synapse) contains a 39-phosphotyrosine DNA-Cre linkage resulting from cleavage of the DNA substrate. This covalently-attached phosphate is activated for the strand-exchange step of the reaction, which requires nucleophilic attack of the phosphotyrosine by the 59-hydroxyl released upon cleavage of the partner substrate.

Applications and achievements of Cre-lox system

Gene transfer without the incorporation of antibiotic resistance markers in the host genome would ease public concerns over the field release of transgenic organisms expressing such traits. Moreover, it would avoid the need for different selectable markers in subsequent rounds of gene transfer into the same host. Site specific integration and deletion can be joined into a combined step method of introducing DNA into plants. This combined step strategy makes use of two site-specific recombination systems: one for integrating the DNA and a second for removing sequences that are no longer needed after DNA transfer (Srivastava and Ow 2004). In a study by Dale and Ow (1991), they described a general method of gene transfer that does not leave behind a selectable marker in the host genome. They introduced a luciferase gene into the tobacco genome by using the hygromycin phosphotransferase gene (*hpt*) as a linked selectable marker. Flanked by recombination sites from the bacteriophage P1 Cre/*lox* recombination system, the *hpt* gene was subsequently excised from the plant genome by the Cre recombinase. The Cre-catalyzed excision event in the plant genome was precise and conservative, i.e. without loss or alteration of nucleotides in the recombinant site. After removal of the Cre-encoding locus by genetic segregation, plants were obtained that had incorporated only the desired transgene. The application of the bacteriophage P1Cre-*lox* site specific recombination system has also been utilized for the easy identification of chromosome deletions and inversions in plants. The rearrangement involves three steps: a) the introduction of two *lox* sites into the plant genome with one *lox* site within a modified *Ds* transposone, b) Ac transposes-mediated transposition of the *Ds-lox* element to a new locus on the same chromosome, and c) Cre-mediated site specific recombination between the two *lox* sites that cover the chromosomal segment. In tobacco, one deletion and three inversion events were observed on application of Cre-*lox* mediated recombination (Medberry et al. 1995). Employing this strategy, we can well understand the deleterious effects caused by various mutations. Cre-*lox* mediated marker gene excision has successfully worked in case of transgenic maize. Two strategies for the marker removal were followed. In one, plants expressing Cre recombinase were crossed to the plants bearing transgene construct including the marker gene flanked by two *lox* sites. A complete somatic and germline marker gene excision was seen in F1 plants. In the other strategy (auto excision method), the Cre gene under the control of heat shock inducer promoter is excised along with the *nptII* marker gene. A transient heat shock treatment of the primary transgenic callus is sufficient for inducing Cre and excising the Cre and *nptII* genes (Zhang et al. 2003).

Xiang-Lei et al. (2003) utilized the copper inducible Cre-*loxP* recombination DNA excision system from development of transgenic tobacco plants. Cu inducible system was used for the control of the expression of Cre recombinase. The GUS reporter gene flanked by two direct lox sites was excised from the transgenic tobacco genome. Quantitative fluorometric GUS assay, Northern blot, and PCR analysis showed a high efficient, Cu dependent, and Cre-*loxP* mediated DNA recombination in all the tested transgenic lines. The Cu inducible foreign gene excision might be of great potential in the genetic control of transgenic crops. The Cre-*lox* system was used to create hybrid chromosomes between *Arabidopsis thaliana* and *Nicotiana tabacum*. The protoplasts of two plants were fused to allow site-specific recombination to join a promoter from tobacco to a hygromycin resistance coding region from Arabidopsis. The screening was done for hygromycin resistance calli. Molecular analysis of this hybrid indicated that a small portion of the north arm of the Arabidopsis chromosome V is present in the tobacco genome. The feasibility of site-specific recombination between genomes of different species offers new possibilities for engineering hybrid chromosomes that may be maintained in cell culture (Koshinsky et al. 2000). As such, we can examine the effect of chromosomal segment rearrangements on their gene expression levels.

Cre-mediated recombination can also be used for consistent transgene expression. The higher levels of expression variability found in transgenic rice have been minimized by utilizing this strategy. The lox sites were first integrated into the genome and then the transgene was delivered using biolostic method of transformation for its integration at previously lox integrated sites. Eighty percent of the lines were observed possessing the transgene as single copy (SC) (Srivastava et al. 2004). Integration of a gene at a single site results in the desired and appropriate expression level; when compared, the multiple copy integration can lead to

gene silencing. Thus, the described recombination method can be effectively utilized for avoiding gene silencing induced by multiple copy integration.

Conclusion and future perspectives

The present need of is to develop transgenic crops for increasing food production to meet the demands of the growing population. But there are always health and environmental concerns, which needto be taken into consideration in the development of GM foods. Here we have discussed a strategy called Cre-lox which can be well utilized for making these foods safer for human consumption. Cre-*lox* involves a site specific recombination for the removal of antibiotic resistant genes. As such, the potential of this technology can be well understood and henceforth, can be exploited in the development of safe transgenic crops. The genetically modified foods obtained from transgenic crops lacking the antibiotic resistance genes will not only satisfy the consumers in regards to health and environmental aspects but will also increasingly promote molecular farming on a larger scale.

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