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Elimination of Transgenic Sequences in Plants by *Cre* Gene Expression

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1. Introduction

The ability to insert foreign DNA into plant cells opened plenty opportunities for the development of new cell lines and improved varieties for agronomic and industrial purposes. Despite the great advances reached there are still some limitations in plant biotechnology based on genetic transformation. In most cases precise engineering of target genomic loci is difficult. Random DNA integration and multi-copy transgene insertions might result in unpredictable expression or gene silencing. Furthermore, commercial application of plant biotechnology products rises numerous regulatory and biosafety concerns about possible spread of the transgenes into the environment or the presence of selectable marker genes. One of the molecular tools that can help to overcome these limitations is site-specific recombination. Several site-specific recombination systems have been shown to be functional in plant cells: the Cre-*lox* system from bactreiophage P1 (Dale and Ow, 1990; Odell et al., 1990, Bayley et al., 1992), the FLP-*FRT* system from *Saccharomyces cerevisiae* (Lyznik et al., 1993; Lloyd and Davis, 1994; Kilby et al., 1995), the R-*RS* system from *Zygosaccharomyces rouxii* (Onouchi et al., 1991), the Gin-*gix* system from bacteriophage Mu (Maeser and Kahmann, 1991), the CinH-RS2 system from *Acetinetobacter* (Moon et al., 2011), the ParA system from a plasmid operon parCBA (Thomson et al., 2009) and the *Streptomyces* phage phiC31 system (Kittiwongwattana et al., 2007, Rubtsova et al., 2008). Currently, Cre-*lox* has become the most commonly employed site-specific recombination system. Although both types of recombination catalyzed by the Cre protein, site-specific integration and excision, found practical application (Ow, 2002; Gilbertson, 2003; Lyznik et al., 2003; Gidoni et al., 2008; Wang et al., 2011), the removal of *lox*-flanked sequences is the most widely used applications of Cre recombinase. The following technologies are based on excisional recombination: (i) regulation of gene expression, (ii) resolution of complex insertion sites to single copy structures, (iii) biological confinement, and (iv) elimination of selectable marker genes. Here we review the progress in the employment of Cre-mediated site-specific excisional recombination for applied plant biology and discuss in detail the advantages, limitations and potential improvements of technologies utilizing the Cre-*lox* system.

2. The Cre-*lox* site-specific recombination system: Structure, biological functions, mode of action

The Cre-*lox* site-specific recombination system from bacteriophage P1 belongs to the tyrosine integrase family whose members use a conserved tyrosine residue as catalytic nucleophile (Grindley et al., 2006). It performs at least two functions in the P1 life cycle: (i) it promotes the circularization of bacteriophage DNA after infection of bacteria (Segev and Cohen, 1981; Hochman et al., 1983), and (ii) it maintains the phage genome as unit-copy plasmid by resolving dimeric plasmids during bacterial division (Austin et al., 1981).

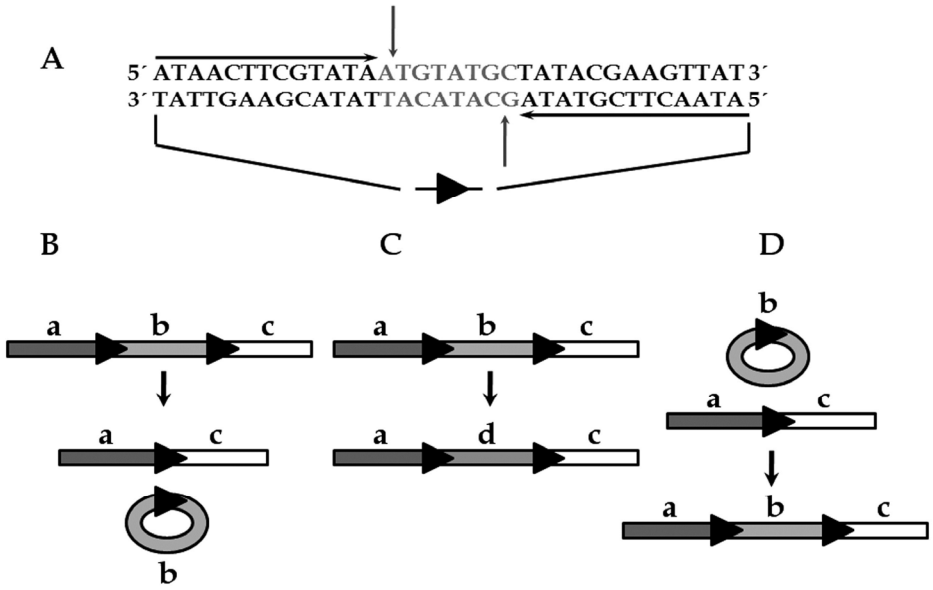

Fig. 1. The Cre-mediated recombination reaction. A: Schematic representation of the *lox* recombination site. The 13 bp inverted repeats are marked by large horizontal arrows. The points of the spacer region at which Cre cleaves the *lox* sites are denoted by small vertical arrows. Cre recombinase mediates inter- and intramolecular recombination leading to deletion (B), inversion (C) or integration (D) events.

The Cre-*lox* system consists of two short DNA recognition sequences known as *lox* (*locus of crossing-over*) and the recombinase protein Cre. Structural studies have revealed that a functional *lox* site is composed of two 13 bp inverted repeats flanking an 8 bp spacer region (Hoess et al., 1982; Hoess and Abremski, 1984) (Figure 1A). The inverted repeats and adjacing 4 bp of the spacer region compose a Cre binding domain. The asymmetry of the 8 bp spacer sequence determines the outcome of the recombination. The second component of the system, the 38 kDa Cre protein, includes two domains: a NH_2-terminal domain and a larger COOH-terminal domain, which contains the active site of the enzyme and major determinants for DNA binding specificity. The Cre recombinase does not require additional proteins or cofactors and performs enzymatic activity under a wide variety of cellular and non-cellular conditions. Crystallographic analysis of Cre-DNA complexes (Guo et al., 1997;

Guo et al., 1999) has revealed the recombination mechanisms. The process of site-specific recombination involves the formation and resolution of a Holliday junction intermediate, during which the DNA is transiently attached to the enzyme through a phosphotyrosine linkage. The reaction can result in integration, inversion or excision, depending on the position and orientation of the recombination sites. Recombination between two *lox* sites in direct orientation on the same DNA molecule results in excision of the *lox*-flanked DNA fragment (Figure 1B). In contrast, recombination between two *lox* sites in inverted repeat leads to inversion of the intervening DNA fragment (Figure 1C). Integration results from recombination between two *lox* sites situated on different DNA molecules (Figure 1D). The recombination reaction is reversible. Since intramolecular excision is kinetically favoured over bi-molecular integration, the excision reaction is essentially irreversible. In contrast, the insertion products are unstable in the presence of Cre recombinase.

3. Cre expression strategies: Efficiency and limitations

According to the presence of the *cre* sequence in the plant genome and the duration of *cre* expression, approaches to combine the *lox* recognition sequences and Cre protein can be grouped into three categories: (i) constitutive, (ii) transient and (iii) temporal expression. In the first group, the recombinase gene is stably integrated into the plant genome and expressed during the whole plant life. There are at least two main possibilities to integrate the *cre* gene into *lox*-containing plants: cross pollination and retransformation. To follow the crossing strategy, *cre* and *lox*-transgenic lines are developed and subsequently crossed (Bayley et al., 1992; Russell et al., 1992; Hoa et al., 2002). Applying the retransformation strategy, the *cre* gene is transformed into *lox*-lines (Odell et al., 1990; Dale and Ow, 1991; Zhang et al., 2003). Constitutive expression provides high recombination efficiencies in both model (Dale and Ow, 1991; Russell et al., 1992) and commercial crops (Hoa et al., 2002; Zhang et al., 2003). However, prolonged *cre* expression has some limitations. It is not optimal for plant species that are propagated by vegetative cuttings, since the crossing/segregation step for the *cre* gene can be problematic. Furthermore, additional time is required to perform a second round of transformation or cross pollination. A further strong argument against constitutive *cre* expression is the possible occurrence of genetic and phenotypic changes caused by the Cre recombinase, which were observed in plastid and nuclear genomes, respectively (Hajdukiewicz et al., 2001; Coppoolse et al., 2003).

Transient expression offers the possibility to reduce/avoid undesired side-effects caused by long-term persistence of the Cre protein. The following approaches have been described in the literature: application of the purified Cre protein and virus- or *Agrobacterium tumefaciens*-mediated *cre* expression.

Addition of Cre protein to induce site-specific recombination was initially demonstrated for animal cells (Baubonis and Sauer, 1993) and extended by Cao and co-workers (2006) to excise *lox*-flanked DNA fragments in plant culture. In theory, direct introduction of the recombinase protein into plant cells could be an elegant solution. In fact, the broad application of this method to commercial crops is highly problematic. Additional time and costs have to be invested to purify an enzymatically active Cre protein and to obtain optimal conditions for cell culture treatment. Reliable regeneration protocols from protoplasts are not available for several crops. Moreover, this regeneration step can introduce additional somaclonal variation.

The Cre function can be provided transiently by *Agrobacterium*-based vectors using T-DNA-independent and T-DNA-dependent expression. T-DNA-independent *Agrobacterium*-mediated *cre* expression is based on fusion of the Cre protein to the NH_2 –terminus of VirE2 and VirF proteins. *Agrobacterium* is able to transfer these fusions into *Arabidopsis* cells resulting in excision events, although detectable efficiency of the process was low (Vergunst et al., 2000). Therefore, this system might be used only for applications where rare recombination rates are essential. T-DNA dependent expression relies on the fact that non-integrated copies of T-DNA may persist in the nucleus for a period of time providing transient expression of genes from T-DNA. The Cre recombinase gene cloned between left and right T-DNA borders can be delivered into plant cells by the agro-inoculation technique and recombine *lox* sites in both nuclear (Gleave et al., 1999; Kopertekh and Schiemann, 2005) and plastid (Lutz et al., 2006) genomes as shown in tobacco. The principle of transient recombinase expression via *A. tumefaciens*-based vectors was proved only in model plant species yet.

Another possibility to deliver Cre protein without *cre* gene insertion into the plant genome is provided by the application of RNA viruses. Two Cre-virus vectors, PVX-Cre (Kopertekh et al., 2004a, 2004b) and TMV-Cre (Jia et al., 2006), have been shown to be functional in *lox*-target *N. benthamiana* and *N. tabacum* plants. In both vectors the *cre* gene was integrated between movement and coat protein genes. Recently, the application of PVX-Cre for marker gene elimination in potato has been demonstrated (Kopertekh et al., 2011). In comparison to the *A. tumefaciens* transient expression system, virus vectors were more efficient in generating recombination events. In general, *Agrobacterium*- and virus-based *cre* expression is mostly suitable for vegetatively propagated species. However, the necessity to develop efficient agroinfiltration methods or infectious Cre-virus vectors, as well as regeneration protocols for plant explants might hamper a broad application of these approaches.

To follow the temporal expression approach, a stably integrated *cre* gene is placed under the control of inducible or tissue specific promoters. To date, a regulated *cre* expression is usually combined with the autoexcision strategy. Self-excision plant transformation vectors contain two recognition sites and the *cre* gene on the same T-DNA molecule. Conditional expression of the *cre* gene results in simultaneous removal of all sequences situated between the *lox* sites. This autoexcision strategy provides several potential advantages. First, all components of the Cre-*lox* system can be incorporated into the plant genome in one transformation step. Second, this strategy could be employed for both generatively and vegetatively propagated species. Several inducible systems responsive to external stimuli have been reported for plants, e.g. heat-shock and β-estradiol regulated. The heat-shock regulated system seems to be the simplest and most familiar for use. Its function has been demonstrated as functional in *Arabidopsis* (Hoff et al., 2001), tobacco (Wang et al., 2005), potato (Cuellar et al., 2006), maize (Zhang et al., 2003), rice (Khattri et al., 2011) and aspen (Fladung and Becker, 2010). In the chemically regulated self-excision system developed by Zuo and associates (2001), the *cre* gene was combined with the XVE system which is induced by β-estradiol. The system was successfully applied to *Arabidopsis* (Zuo et al., 2001), rice (Sreekala et al., 2005) and tomato (Zhang et al., 2006; Zhang et al., 2009). Despite the great advantage of the temporally controlled recombinase expression, heat-shock and chemically regulated promoters require an external signal to be activated and the recombination frequencies are greatly dependent on the penetration of the signal into plant cells, respectively.

A promising alternative to the *cre* regulation described above is the use of developmentally inducible promoters. During the last few years a number of promoters active in different stages of plant development, namely in germline (Verweire et al., 2007; Van Ex et al., 2009), embryo (Li et al., 2007), microspore (Mlynarova et al., 2006; Luo et al., 2007), floral (Bai et al., 2008) and seed (Odell et al., 1994; Moravčíková, et al., 2008; Kopertekh et al., 2010) tissues have been tested to control *cre* expression. High efficiency of such promoters in *Arabidopsis* (Verweire et al., 2007), tobacco (Mlynarova et al., 2006), rice (Bai et al., 2008), soybean (Lie et al., 2007) and oilseed rape (Kopertekh et al., 2009) makes this approach universal for model and agronomically important species. In addition, the employment of germline-specific promoters allows a more efficient transmission of the recombined status to the progeny. The essential feature of conditional Cre systems is a careful regulation with respect to time and tissue. Background Cre activation was observed for heat-shock inducible (Hoff et al., 2001; Wang et al., 2005) and some seed-specific promoters (Odell et al., 1994; Moravčíková, et al., 2008), resulting in reduced efficiency of the systems.

In summary, methodological progress in *cre* gene expression strategies allows to modulate the recombinase activity in a temporal manner. The choice between the Cre expression systems depends mainly on the goals of the experiment, involved plant species, and finally available expertise.

4. Application of Cre-mediated excision in plant biotechnology

The removal of *lox*-flanked DNA fragments by Cre recombinase is broadly used in plant applied research. The applications described in the literature can be grouped into four categories: (i) regulation of gene activity, (ii) simplification of complex transgene structures, (iii) complete excision of a transgene to prevent gene flow, and (iv) marker gene removal.

4.1 Gene regulation

Cre-mediated site-specific recombination offers an effective way to turn on or off gene expression in transgenic plants by removing DNA fragments located between directly repeated recombination sites. What are the potential uses of this technology?

One example is the use of plants as bioreactors to produce recombinant proteins that are toxic to plant cells. Tremblay et al. (2007) designed transgenic *Arabidopsis* plants harbouring a *Turnip Mosaic Virus* (TuMV) amplicon in which a *lox*-flanked translational terminator integrated between the P1 and HCPro coding sequences prevented virus replication. After delivery of Cre recombinase by agroinfiltration, a PVX-Cre vector or a transgenic chemically inducible system, the intervening DNA fragment was eliminated resulting in virus accumulation.

The same strategy was used for conditional recombinase-mediated gene expression in plant cell culture (Joubes et al., 2004). In a plant transformation vector, excision of the *gfp* coding sequence by heat-shock and a dexamethasone inducible Cre recombinase lead to expression of the gene of interest. The system was tested in *N. tabacum* bright yellow-2 (B-2) cells and its efficiency was demonstrated for the *gus* reporter gene and a potent inhibitor of the cell cycle mutant allele of the A-type cyclin-dependent kinase (CDKA).

Another example of recombinase-mediated gene regulation is the restoration of pollen fertility. Transgenic tobacco plants containing a *lox*-flanked stilbene synthase (*sts*) gene under control of a tapetum-specific promoter displayed the male-sterile phenotype (Bayer and Hess, 2005). Pollen fertility was restored after crossing with *cre*-expressing tobacco lines. This method may provide a valuable strategy for the production of hybrid plants.

In contrast to animal systems the few reports describing Cre recombinase-mediated gene regulation in plant systems only demonstrate a proof of principle without practical application yet.

4.2 Generation of single copy transformants by Cre-*lox* recombination

During plant genetic transformation multiple T-DNA copies are often integrated at a single locus. Complex integration sites are commonly associated with intrachromosomal recombination (Srivastava et al., 1996) and transgene silencing (Wang and Waterhouse, 2000; De Buck et al., 2001). Moreover, a single integration pattern may simplify the functional and structural characterization of a transgene. Therefore, single copy transgenic plants are more desirable for commercial practice. Several approaches such as conventional screening amongst a large pool of transformants (De Buck and Depicker, 2004), agrolistics (Hansen and Chilton, 1996), niacinamide application (De Block et al., 1997) or use of Cre-mediated site-specific recombination (Srivastava et al., 1999) have been developed to select/generate single copy lines. The Cre-*lox*-based strategy is based on a transgene flanked by *lox* sites in opposite orientation. In case of tandem insertion of T-DNAs at a single locus, the Cre recombinase resolves multiple units to a single-copy insert.

The proof of concept and successful application of the Cre-*lox*-based strategy was reported for the first time by Srivastava et al. (1999). Four transgenic wheat *lox*-target lines, containing a DNA fragment flanked by recombination sites in inverted repeats, were generated by particle bombardment. The Cre recombinase was provided by crossing with *cre*-expressing plants. Cre-mediated resolution of the complex T-DNA structure was observed in T_2 progeny plants for all four lines investigated. However the authors reported (i) incomplete resolution of complex loci in 20-40% of the T_2 progenies from three lines and (ii) persistence of excised DNA fragment extrachromosomally in one plant.

The strategy described above was modified to generate single-copy maize plants more efficiently. In comparison to the original method, the *cre*-expressing construct was introduced into *lox*-transgenic maize cells transiently by particle bombardment (Srivastava and Ow, 2001). This modification was highly efficient: 85% of regenerated plants contained 1 to 2 copies of the introduced DNA, with 38% harbouring a single copy. In 23% of single copy lines recombination was performed by transient *cre* expression: they harboured only the *lox*-target construct.

The Cre-mediated resolution approach was also functional in *Arabidopsis*. In the *lox*-transformation vector two recombination sites in inverted repeat were cloned inside the T-DNA immediately adjacent to the left and right T-DNA border ends (De Buck et al., 2007). Seven transgenic lines with a complex integration locus were crossed with *cre*-transgenic plants. The progeny of two hybrids demonstrated a single-copy T-DNA status without integration of the released DNA fragment in the plant genome. In some transformants, the Cre-mediated resolution of complex loci increased the transgene expression at least tenfold.

Based on these results an alternative transformation system to generate single copy transformants has been developed and proved in *Arabidopsis* (De Pape et al., 2009). To omit the crossing step between *cre*- and *lox*-plants, a *lox*-target construct was transferred by floral deep transformation into *cre* expressing plants. 55% of primary transformants contained a single copy of the introduced T-DNA. However 73% showed inversion of the DNA fragment between the *lox* sites which can result in variable transgene expression. Further improvement was achieved by introducing only one *lox* site in the transformation vector: 70% of primary transformants harboured a single-copy of T–DNA without inversion.

In summary, the recombinase-based resolution strategy can efficiently resolve complex integration patterns in important agricultural crops, particularly wheat and maize, as well as in the model plant *A. thaliana*. However, the following potential limitations have to be envisaged for this strategy. First, this approach may not be suitable for multiple locus integration events since Cre-mediated resolution can cause chromosomal deletions. Second, incomplete resolution of the complex locus is possible. Finally, released DNA fragment may be present in the plant genome.

4.3 Transgene confinement

One concern related to genetically modified plants is the potential effects resulting from transgene transfer into the environment. To address this issue several biological confinement strategies have been proposed. Current technologies, namely male sterility, chloroplast transformation, cleistogamy and transgene removal from pollen or seeds, offer new possibilities for biological confinement (Daniell, 2002; Keenan and Stemmer, 2002; Moon et al., 2009). In this chapter we will mainly describe biological confinement strategies based on the Cre-*lox* recombination system. Here, all functional transgenes are flanked by two recognition sites in direct orientation. Upon expression of the *cre* gene driven by tightly regulated chemically induced or tissue specific promoters, the transgene sequences are removed leaving only a short recognition sequence in the genome. Since gene flow occurs most frequently via seed or pollen dispersal, transgene removal from seed or pollen by developmentally regulated *cre* recombinase could minimize transgene transfer.

The seed-sterile technology is based on two expression units: *cre*-expression unit and cytotoxic ribosome-inhibitor (RIP) gene expression unit (Daniell, 2002). The *cre* gene is linked with a repressor-operator (Tet) system which allows *cre* expression in the presence of tetracycline. In the second expression unit, a seed-specific late embryonic abundance (LEA) promoter and a *RIP* gene are separated by a *lox*-flanked "spacer sequence". Tetracycline induced *cre* expression results in the removal of the "spacer sequence" and the fusion of LEA promoter and *RIP* gene. The RIP protein destructs the seed tissue resulting in production of non-viable seeds. The following potential problems are linked with this strategy: (i) all three components of the system (Cre, RIP and Tet) should be present together in one plant, (ii) the repressor-operator (Tet) system should display high efficiency in crop plants and the chemical inducer should penetrate the plant tissue uniformly, (iii) the seed-specific LEA promoter can be subjected to silencing causing undesired transgene dispersal.

In the second advanced strategy developed by Mlynarova et al. (2006), a *lox*-embedded cassette includes (i) marker gene, (ii) gene of interest and (iii) *cre* gene driven by the NTM 19 microspore-specific promoter. This design allows autoexcision of all transgenes during

microsporogenesis without application of an additional induction factor. It was highly efficient in tobacco plants: only two out of 16800 seeds (0.024%) contained non-excised transgene sequences. Additionally, the authors did not observe premature activation or absence of activation for the tissue-specific Cre-system under laboratory stress conditions.

The efficiency and reliability of recombinase-mediated confinement methods was further improved by the application of pollen- and seed-specific promoters and hybrid *lox-FRT* recombination sites (Luo et al., 2007). The *lox-FRT* fusion sequences dramatically enhanced the excision frequency: analysis of 25000 progeny seedlings for several transgenic tobacco lines revealed that transgenes in pollen or seeds were excised with 100% efficiency. Despite simplicity and high efficiency of the developmentally regulated Cre-system to prevent gene flow, the need to maintain the hemizygous status may be a great disadvantage for transgenic crops multiplied by seeds.

It should be pointed out that all strategies presented in this section were only tested in model plants such as tobacco and *Arabidopsis*. Therefore, no data are available on the efficiency and stability of these systems in actual crop species under agronomic conditions.

4.4 Cre-mediated excision of marker genes

In most cases, plant transformation is inefficient and transgenic cells and regenerants must be selected from a great number of non-transformed cells via incorporation of selectable marker genes. Once plant transformation is completed, these marker genes can be eliminated. There are several reasons to produce marker-free plants (Hohn et al., 2001; Hare and Chua, 2002; Miki and McHugh, 2004; Goldstein et al., 2005): marker gene removal can prevent the movement of selectable markers within the environment, simplify the regulatory process and allow the reuse of the same marker. Different methods have been identified that enable marker gene removal: co-transformation (Komari et al., 1996), transposon-dependent repositioning (Goldsbrough et al., 1993), as well as homologous (Zubko et al., 2000) and site-specific recombination (Dale and Ow, 1991). Site-specific marker gene removal will be the main topic of this section. The plant material used has been ordered according to species, supposing that this structure of the chapter might help to compare the efficiency of different methods and to choose the optimal approach for the plant to be used. Table 1 provides summarised information about Cre-site-specific marker gene elimination systems and their efficiency in different plant species.

The theoretical concept of Cre-mediated marker gene excision was proved in tobacco about twenty years ago by two research groups (Dale and Ow, 1991; Russel et al., 1992). Marker-free plants were generated by applying the Cre recombinase constitutively either via cross-pollination or a second round of transformation. The authors reported that re-transformation provided much higher recombination efficiency. This principle was also functional in the plastid genome (Corneille et al., 2001). Both methods for constitutive *cre* expression were efficient in tobacco chloroplasts, but *Agrobacterium*-mediated Cre recombinase delivery caused plastid genome rearrangements.

Transient expression vectors - *Agrobacterium*- or virus, - worked efficiently in tobacco. Simple cocultivation of transgenic tobacco leaves harbouring the marker gene with *A. tumefaciens* containing a *cre*-plasmid led to the removal of the flanked region in 0.25% of the regenerants (Gleave et al., 1999). In comparison to cocultivation technique, the

agroinfiltration method greatly increased the recombination efficiency. Regenerants without marker genes were obtained with a frequency of about 34%. In 14% of plants site-specific recombination was performed without stable recombinase integration. Delivery of the Cre protein by agroinfiltration was also adopted to remove marker genes from the plastid genome (Lutz et al., 2006). Another option to perform transient *cre* expression is the use of Cre-virus vectors. The first plant Cre-virus vector was based on PVX and demonstrated high recombination rates (48-82%) in *N. benthamiana* (Kopertekh et al., 2004b). This vector was also suitable to generate marker-free tobacco plants without a regeneration step (Kopertekh et al., 2004a). The second Cre-virus vector described is based on TMV. It was functional in *N. tabacum* plants with an efficiency of about 34% (Jia et al., 2006).

Genotype	Induction factor, expression system/promoter	*cre* expression type	Excision rate	Gene of interest	Reference
Tobacco *N. tabacum*	Cross-pollination, retransformation, 35S promoter	Constitutive	ND	*luc*	Dale and Ow, 1991
Tobacco *N. tabacum*	Cross-pollination, retransformation, 35S promoter	Constitutive	95% (retransformation)	*gusA*	Russell et al., 1992
Tobacco *N. tabacum*	Cross-pollination, 35S promoter	Constitutive	19.2%	*ASAL*	Chakraborti et al., 2008
Tobacco *N. tabacum* (plastid genome)	Cross-pollination, retransformation, 35S promoter	Constitutive	ND	-	Corneille et al., 2001
Tobacco *N. benthamiana*	PVX-Cre expression vector	Transient	48-82%	*gfp*	Kopertekh et al., 2004
Tobacco *N. tabacum*	TMV-Cre expression vector	Transient	34%	*gusA*	Jia et al., 2006
Tobacco *N. tabacum*	*A. tumefaciens*-expression vector	Transient	0.25%	*gusA*	Gleave et al., 1999
Tobacco *N. tabacum* (plastid genome)	*A. tumefaciens*-expression vector	Transient	10%	*bar*	Lutz et al., 2006
Tobacco *N. benthamiana*	*A. tumefaciens*-expression vector	Transient	34%	*gfp*	Kopertekh et al., 2005
Tobacco *N. tabacum*	Heat-shock, HSP17.5E promoter from soybean	Temporal	30-80%	*gusA*	Wang et al., 2005
A. thaliana	Chemical induction, β-estradiol inducible transactivator XVE	Temporal	29-66%	*gfp*	Zuo et al., 2001
A. thaliana	Tissue-specific induction, *AP1* and *SDS* germline specific promoters	Temporal	83-100%	-	Verweire et al., 2007
Rice	Cross-pollination, 35S promoter	Constitutive	58%	*gusA*	Hoa et al., 2002
Rice	Co-cocultivation with a purified Cre-recombinase protein	Transient	26%	*gusA*	Cao et al., 2006

Rice	Heat-shock, HSP17.5E promoter from soybean	Temporal	16%	*gusA*	Khattri et al., 2011
Rice	Chemical induction, β-estradiol inducible transactivator XVE	Temporal	29.1%	*gfp*	Sreekala et al., 2005
Maize	Cross-pollination	Constitutive	ND	*cordapA*	Ow, 2007
Maize	Cross-pollination, 35S promoter Heat-shock, HSP17.5E promoter from soybean	Constitutive Temporal	ND	*gfp*	Zhang et al., 2003
Maize	Cross-pollination, Ubi promoter	Constitutive	ND	*gusA*	Kebrach et al., 2005
Wheat	Cross-pollination, 35S promoter	Constitutive	ND	-	Srivastava et al., 1999
Potato	Heat-shock, hsp70 promoter from *Drosophila melanogaster*	Temporal	4.7%	-	Cuellar et al., 2006
Potato	PVX-Cre expression vector	Transient	20-27%	*gfp*	Kopertekh et al., 2011
Brassica juncea	Cross-pollination, 35S promoter	Constitutive	ND	*gusA*	Arumugam et al., 2007
Brassica napus	Tissue-specific induction, seed-specific napin promoter from *B. napus*	Temporal	13-81%	*vst*I	Kopertekh et al., 2009
Soybean	Tissue-specific induction, *Arabidopsis app1* embryo-specific promoter	Temporal	13%	*gusA, gat*	Li et al., 2007
Tomato	Chemical induction, β-estradiol inducible transactivator XVE	Temporal	15%	*cry*IAc	Zhang et al., 2006
Tomato	Chemical induction, β-estradiol inducible transactivator XVE	Temporal	ND	*atlpk2β*	Zhang et al., 2009

ND, not determined
luc: luciferase gene
gfp: green fluorescent protein gene
atlpk2β: inositol polyphospate 6-/3-kinase gene
gus: beta-glucuronidase gene
*vst*I: stilbene synthase gene from *Vitis vinifera*
bar: phosphinothricin acetyltransferase gene
*cry*IAc: a synthetic *Bacillus thuringiensis* endotoxin gene
ASAL: allium sativum leaf agglutinin gene
gat: glyphosate acetyltransferase gene
cordapA: dihydrodipicolinate synthase gene

Table 1. Cre-based systems for marker gene elimination

Different promoters, including heat-shock and developmentally regulated ones, were tested in autoexcision vectors in tobacco. In the heat-shock inducible system, the Cre recombinase was more effective in somatic tissues in comparison to germline cells: 70-80% of the regenerants derived from heat-treated leaves lost *lox*-flanked DNA fragments, whereas only 30-40% of seeds after heat-shock gave rise to marker-free plants (Wang et al., 2005). A developmentally regulated Cre-*lox* system based on the seed-specific napin promoter was more efficient in *N. benthamiana* plants: genetic and molecular analysis of T1 progeny indicated DNA excision in all transgenic lines tested (Kopertekh et al., 2010).

Both tobacco and *Arabidopsis thaliana* served as model systems to test different gene elimination approaches. An elegant self-excision Cre-system regulated by β-estradiol was applied for the first time in *Arabidopsis* with an efficiency of 29-66% (Zuo et al., 2001). Furthermore, Verweire et al. (2007) reported an almost complete autoexcision driven by germline promoters.

Rice has been intensively studied for Cre-mediated marker gene excision. The efficiency of all three categories of methods, transient, constitutive and temporal expression, has been evaluated. In one of the first studies on the Cre-*lox* system in rice, *lox*- and *cre*-constructs were combined by cross-fertilization of transgenic plants (Hoa et al., 2002). In the Cre-*lox* hybrids from T_2 crosses a high marker gene deletion frequency of 58.3% was observed. Marker gene excision was also accomplished in transgenic rice cells by simple co-cultivation with a purified cell-permeable Cre recombinase protein (Cao et al., 2006). About 26% of regenerants derived from Cre-treated calli were scored as putative recombinants. However, no data are available about germinal inheritance of the recombined "footprint". Thus, it is difficult to assess the efficiency of this of this approach properly. Marker gene excision and inheritance of the excised locus were observed in one transgenic rice line containing a *lox*-target construct and a single copy of the *cre* gene under the control of the HSP17.5E heat-shock inducible promoter (Khattri et al., 2011). An obvious drawback of this co-transformation approach is the necessity to segregate the *cre*-construct after recombination. Sreekala et al. (2005) demonstrated the removal of the flanked fragment from the genome of transgenic rice in a single-step transformation by using the β–estradiol regulation of Cre. In total, 29% of transgenic T_0 plants were marker-free or could segregate marker-free progeny. In the Cre-*lox* system controlled by a floral specific promoter complete auto-excision was observed in three out of eight rice lines with an efficiency of 37.5% (Bai et al., 2008). This approach may be considered as the most promising for the removal of unnecessary sequences in rice since (i) Cre expression is restricted to a special tissue, (ii) recombined lines can be obtained without crossing or additional treatment and (iii) this one-step transformation approach provides high recombination frequencies.

Two strategies - cross-pollination and heat-shock inducible autoexcision - have been shown to be useful to develop transgenic maize plants harbouring only the trait gene. The crossing strategy worked with nearly 100% efficiency in several laboratories (Zahn et al., 2003; Kebrach et al., 2005). Moreover, commercial marker-free maize LYO38 was developed by Monsanto through sexual crossing between *lox*- and *cre*-plants following segregation of the *cre* gene in the next generation. A comparative study by Zhang et al. (2003) also demonstrated that autoexcision induced by heat-shock provided precise, complete and stable marker gene excision.

There is less information available on Cre-mediated marker gene elimination in wheat. Srivastava et al. (1999) combined two potential applications of site-specific recombination in

one plant vector. Transgenic wheat plants harbouring a DNA fragment between mutant *lox*511 sites in opposite orientation and a marker gene between wild type *lox* sites in direct orientation were crossed with a *cre*-transgenic line. Some T_1 plants without the selection marker and a reduced copy number were detected by PCR.

The feasibility of the Cre-*lox* system for the removal of marker genes in *Brassicaceae* was demonstrated in two studies. In the first one, the *lox* sites and *cre* gene under control of a constitutive promoter were combined by cross-pollination to produce marker-free *Brassica juncea* plants (Arumugam et al., 2007). The Cre recombinase displayed low activity in meristematic cells. Thus, an additional regeneration step from leaf explants was necessary to obtain *B. juncea* plants without marker genes. The application of seed-specific napin promoter from *B. napus* to control the *cre* gene seems to be more suitable to perform the germline transmission of the recombination event (Kopertekh et al., 2009). Marker-free *B. napus* plants could be generated with high efficiency (13-81%).

Two techniques, *cre* induction by heat-shock and PVX-Cre-expression have been optimized for vegetatively propagated potato. About 4% of regenerated shoots derived from heat treated internodes and tubes demonstrated the marker-free phenotype (Cuellar et al., 2007). Transient PVX-Cre-based expression resulted in a more efficient excision of the *npt*II gene cloned between recognition sites (Kopertekh et al., 2011). Excision rates of 20-27% were achieved by applying the particle bombardment infection method and the P19 silencing suppressor protein.

In the auto-excision Cre-system developed for soybean transformation, a selectable marker gene was expressed at an early transformation step and then removed by the Cre recombinase driven by *app1* embryo-specific promoter from *A. thaliana* (Li et al., 2007). This excision reaction led to the activation of the glyphosate acetyltransferase (*gat*) gene. It was shown that 13% of events exhibited complete excision of the marker gene.

The application of a chemically-regulated autoexcision Cre-system in tomato was reported by Zhang et al. (2006). β-estradiol treatment resulted in the excision of *cre* and marker genes and subsequently in the fusion of the endotoxin gene *cryIA* with the promoter sequence. 15% of T_1 progeny plants harboured a marker-free phenotype.

Generally, the newly designed Cre-systems have first been tested in tobacco and *Arabidopsis,* and subsequently extended to actual crops. It should be pointed out that the same Cre-systems demonstrate higher recombination efficiencies in model species in comparison to agricultural crops. For example, for the β-estradiol inducible self-excision Cre system in *Arabidopsis* 29-66% efficiency was observed, whereas in rice and tomato only 30% and 15%, respectively. Similarly, heat-shock induction resulted in 30-80% excision rates in tobacco and only 4% in potato. Recently, this tendency was also demonstrated for the transient PVX-Cre vector. In comparison to *N. benthamiana* (48-82%), lower excision rates of 20-27% were shown for potato (Kopertekh et al., 2011).

5. Conclusions

Since the initial work of Dale and Ow (1990) demonstrating the functionality of the Cre-*lox* system in plant cells, a number of technologies based on site-specific recombination have been developed, tested and implemented into transformation protocols. All these technologies rely on two basic genome modifications caused by Cre recombinase:

integration or removal of foreign DNA fragments. In this review we focused on the employment of Cre-mediated elimination of transgene sequences. The literature analysis indicates several current trends in optimizing the recombination strategies and their practical application. First, future employment of the Cre-*lox* system will likely incorporate more precise temporal and spatial control of *cre* expression. To this end a number of conditional and transient excisional Cre-systems have been designed and tested during the last decade (see the paragraph "Cre-expression strategies: efficiency and limitations"). Second, the methodological progress mentioned above allowed extending the recombination technology from model (tobacco and *Arabidopsis* plants) to actual crops, including generatively and vegetatively propagated species, monocots and dicots. Third, the excisional recombination method was combined with trait genes, illustrating the development from laboratory experiments to practical utilization; this tendency can mainly be observed for the marker gene elimination technology. Among commercial traits combined with the Cre-*lox* system are modification of protein composition (Ow, 2007), tolerance to environmental stress (Zhang et al., 2009) as well as herbicide (Lutz et al., 2006; Li et al., 2007) and insect (Chakraborti et al., 2008; Zhang et al., 2006) resistance. The first marker-free commercial maize event LY038, which received the US regulatory approval in 2006, provides higher lysine content (Ow, 2007).

However, the approval process for the commercial utilization of genetically modified plants based on the techniques described above might require additional regulatory costs. The first consideration could be connected to the possible reintegration/persistence of excised DNA fragments. Although it is generally assumed that the elimination product is lost upon cell division there is one report showing the presence of deleted DNA as an extra-chromosomal circle in wheat cells (Srivastava and Ow, 2003). The second consideration is linked with possible unintended effects which might be caused by the Cre-*lox* system. Numerous reports exist that demonstrate high specificity of Cre-mediated recombination. Nevertheless, several articles have described undesirable Cre-mediated changes in mammalian genomes (Schmidt et al., 2000; Thyagarajan et al., 2000; Loonstra et al., 2001; Silver and Livingston, 2001). The impact of Cre activity on the plant genome is not well studied. Two types of effects have been described: phenotypic abnormalities and DNA rearrangements in chloroplasts. In petunia, tomato, tobacco and *Arabidopsis* aberrant phenotypes such as leaf chlorosis, growth retardation and reduced fertility were associated with high levels of *cre* expression (Coppoolse et al., 2003). These phenotypic abnormalities were not connected with chromosomal rearrangements: they always co-segregated with the *cre* transgene. In contrast, non-specific DNA recombination products have been identified in the plastid genome by two research groups (Corneille et al., 2001; Hajdukiewicz et al., 2001). Temporal or developmental regulation of the Cre activity would decrease or eliminate these side-effects and subsequently simplify risk assessment process. Another concern related to the Cre-*lox* application is the presence of a *lox* recognition site in the final product. Theoretically, non-predicted recombination between this *lox* site and pseudo-*lox* sites in the genome can occur in the presence of the Cre protein. In fact, the probability of such an event is extremely low. First, the recombination reaction strongly depends on a sequence similarity between the introduced *lox* and genomic pseudo-*lox* sites. The recombination efficiency is greatly reduced when only a few nucleotides in the *lox* spacer region are different (Hoess et al., 1986). Second, the distance between recombination sites plays an important role: the recombination between *lox* sites located at unlinked chromosomes is less efficient (Qin et al., 1994).

Despite the regulatory issues described above, we expect that site-specific excisional recombination will become a routine method in plant biotechnology and find a broader application for the commercial use of crop plants.

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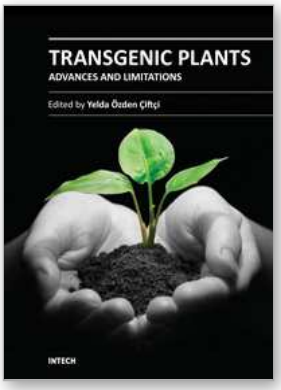

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Development of efficient transformation protocols is becoming a complementary strategy to conventional breeding techniques for the improvement of crops. Thus, Transgenic Plants - Advances and Limitations covers the recent advances carried on improvement of transformation methods together with assessment of the impact of genetically transformed crops on biosafety. Each chapter has been written by one or more experienced researchers in the field and then carefully edited to ensure throughness and consistency.

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