Mol. Cells *33*, 61-69, January 31, 2012
DOI/10.1007/s10059-012-2212-6

Molecules and Cells

Development of a Simple and Efficient System for Excising Selectable Markers in *Arabidopsis* Using a Minimal Promoter::*Cre* Fusion Construct

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The development of rapid and efficient strategies to generate selectable marker-free transgenic plants could help increase the consumer acceptance of genetically modified (GM) plants. To produce marker-free transgenic plants without conditional treatment or the genetic crossing of offspring, we have developed a rapid and convenient DNA excision method mediated by the Cre/*loxP* recombination system under the control of a -46 minimal CaMV 35S promoter. The results of a transient expression assay showed that -46 minimal promoter::*Cre* recombinase (-46::*Cre*) can cause the *loxP*-specific excision of a selectable marker, thereby connecting the 35S promoter and *β-glucuronidase* (*GUS*) reporter gene. Analysis of stable transgenic *Arabidopsis* plants indicated a positive correlation between *loxP*-specific DNA excision and *GUS* expression. PCR and DNA gel-blot analysis further revealed that nine of the 10 tested T_1 transgenic lines carried both excised and non-excised constructs in their genomes. In the subsequent T_2 generation plants, over 30% of the individuals for each line were marker-free plants harboring the excised construct only. These results demonstrate that the -46::*Cre* fusion construct can be efficiently and easily utilized for producing marker-free transgenic plants.

INTRODUCTION

Transferring valuable genes into important crop species has now become routine practice in plant biotechnology. Many crop species have now been genetically modified to increase agricultural yields and nutrient composition. The incorporation of selectable marker genes during transformation is important for the successful generation of transgenic plants. However, once transgenic plants have been produced and characterized, these selectable markers are no longer required and those that confer herbicide or antibiotic resistance have raised public concerns about possible risks related to their expression in genetically modified (GM) plants in commercially use. Hence, the development of simple and efficient techniques for the production of marker-free transgenic plants is an important endeavor for plant biotechnologists (Miki and McHugh, 2004).

Several strategies have been developed to generate marker-free transgenic plants which include a non-selection approach (Li et al., 2009), transposon-mediated repositioning (Ebinuma et al., 1997), the co-transformation of two T-DNA molecules and segregation in subsequent progeny (Depicker et al., 1985; Komari et al., 1996; Mc-Knight et al., 1987; Sengupta et al., 2010), bacteriophage *attP* site-mediated intrachromosomal homologous recombination (Zubko et al., 2000) and site-specific recombination (Qin et al., 1994; 1995). The non-selection approach utilizes either visual marker genes or a PCR methodology in the initial screening for putative transformants but produces numerous chimeric plants (Li et al., 2009). In the transposon-mediated repositioning approach, selectable marker genes flanked by terminal repeat sequences are removed by the Ac transposase (Ebinuma et al., 1997). However, this strategy results in a high percentage of transgene reinsertion. In the co-transformation and segregation method, the selectable marker gene and desired transgene are co-transformed with two separate T-DNAs or plasmids. This strategy is often inefficient however and it can take some time until marker-free transgenic plants are segregated out in the progeny (Daley et al., 1998; De Block and Debrouwer, 1991; Sengupta et al., 2010). In the homologous recombination strategy, large deletions via illegitimate recombination are often observed and the efficiency of recovering selectable marker-free transgenic plants is low (Zubko et al., 2000). Using the site-specific recombinase-mediated excision systems, such as Cre/*loxP* and Flp/*frt*, the recombinases Cre and Flp act on their target sequences, *loxP* and *frt*, respectively, that flank selectable marker genes and excise them from the transgenic plants (Abremski and Hoess, 1984; Miki and McHugh, 2004; Ow, 2002; Wang et al., 2010). Among these strategies, the site-specific recombination technology using Cre/*loxP* has been widely tested to create transgenic plants without retention of selectable marker genes.

In the Cre/*loxP* system, Cre recombinase catalyzes excision of the DNA segment between two *loxP* sites when they are directly orientated (Craig, 1988; Dale and Ow, 1990; Odell et al.,

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Received September 30, 2011; revised November 9, 2011; accepted November 11, 2011; published online November 29, 2011

Keywords: -46 promoter, *Arabidopsis*, Cre, *loxP*, marker-free plant

1990). In earlier Cre/*loxP* systems, the transgenic plants harboring the desired gene and the maker gene flanked by *loxP* sites were crossed with Cre recombinase-expressing lines. Thus, the generation of transgenic plants without the marker or *Cre* gene requires the propagation of multiple generations (Chakraborti et al., 2008; Corneille et al., 2001; Russell et al., 1992). To overcome these shortcomings, an alternative technique was developed in which *Cre* was included on the same DNA segment as the marker gene flanked by *loxP* sites (Hoff et al., 2001; Zuo et al., 2001). In this way, an auto-excision system of marker genes was developed under the control of chemical or heat inducible promoters (Hoff et al., 2001; Sreekala et al., 2005; Wang et al., 2005; Zhang et al., 2003; Zuo et al., 2001). Similar auto-excision systems for marker genes have also been developed using pollen-, seed-, or floral-specific promoters (Bai et al., 2008; Luo et al., 2007). In addition, a transient *Cre* expression system using a virus-based vector has been employed in tobacco (Jia et al., 2006; Kopertekh et al., 2004). These Cre/*loxP*-mediated excision techniques have some notable constraints however, such as the need for chemical or heat treatment to induce *Cre* expression, the requirement for suitable tissue-specific promoters in each target species, and the limited host range of virus-based vectors.

In our present study, we report the development of a novel Cre/*loxP*-based vector system under the control of the -46 minimal CaMV 35S promoter to generate marker-free transgenic plants. This new system provides a simple, rapid and efficient way to remove marker genes without any treatment during the transformation process, thereby more easily producing marker-free transgenic plants for GM crop production.

MATERIALS AND METHODS

Construction of Cre/*loxP* vector

The Cre/*loxP* vector (Fig. 1) was constructed by combining the minimal promoter of the 46-bp upstream sequence of CaMV 35S (termed the -46 promoter), a *Cre* expression insert carrying an intron, *loxP* sites and the backbone of the binary vector pCAMBIA0380 (GenBank Accession no. AF234290; Ouwerkerk et al., 2001). First, the *GUS* gene fragment containing the terminator of the nopaline synthase (*Nos*) gene from the binary vector pBI101.1 (Jefferson et al., 1987) was inserted into the *Hin*dIII and *Eco*RI sites of pCAMBIA0380 (Hajdukiewicz et al., 1994), and the resulting construct was designated pJJ1895. Two fragments, 35S::*Hpt* and Cre/*loxP*, were then amplified with *Hin*dIII/*Xho*I or *Xho*I/*Xba*I sites, respectively, using a PCR-mediated fusion strategy using the primers listed in Table 1 (Sieburth and Meyerowitz, 1997). The 35S::*Hpt* fragment contains the CaMV 35S promoter, a *loxP* site, the *Hpt* gene, a *Nos* terminator, and the -46 promoter. The Cre/*loxP* fragment includes the intron-containing *Cre* recombinase gene, the octopine synthase (*Ocs*) terminator, and a l*oxP* site. The intron-containing *Cre* recombinase gene was constructed by introducing the *Arabidopsis Korrigan1* (*Kor1*) intron into the *Cre* coding sequence according to the method of Zuo et al. (2001) (GenBank Accession no. AF330636). The CaMV 35S promoter, *Hpt*, the intron-containing *Cre* recombinase and -46 promoter regions were amplified from pPZP2Ha3(+) (Fuse et al., 2001), pYLTAC7 (Liu et al., 1999), pHpt-Ds5 (Qu et al., 2009) and pGA2406 (Jeon et al., 2008), respectively. Finally, these two PCR-amplified products, 35S-*Hpt* and Cre/*loxP*, were digested with *Hin*dIII/*Xho*I and *Xho*I/*Xba*I, respectively, and then subsequently cloned into the *Hin*dIII and *Xba*I sites of pJJ1895. More details of the Cre/*loxP* vector construction are available upon request.

Particle bombardment

Tungsten M-10 particles with diameters of 0.7 μm (Bio-Rad) were used for bombardment. The preparation of tungsten particles was performed as described by Hull et al. (1996). The final concentration of tungsten particles was 67 mg ml^{-1} in 50% glycerol. A 45 μl tungsten particle suspension was added to a 1.5 ml tube, and 10 μg of plasmid DNA, 50 μl 2.5 M $CaCl_2$ and 10 μl 0.1 M spermidine were then added with constant vortexing. The mixture was vortexed for a further 3 min and then left on ice for 10 min. The tungsten particles coated with plasmid DNA were centrifuged for 2 s and the supernatant was then removed. The pellet was next washed with 140 μl of 70% ethanol followed by the same volume of 100% ethanol. Finally the tungsten particles were resuspended in 48 μl of 100% ethanol. Six μl of tungsten particles was deposited onto the microprojectiles. Particle bombardment of onion epidermal cells was carried out using a PDS-1000/He Biolistic Particle Delivery System (Bio-Rad) under a vacuum of 28 inches Hg.

Agrobacterium-mediated transformation

To produce transgenic *Arabidopsis* plants, we introduced the Cre/*loxP* vector into *A. tumefaciens* strain GV3101 by electroporation (Mersereau et al., 1990). Transformed colonies were selected on LB medium supplemented with 25 mg L^{-1} kanamycin and confirmed by restriction enzyme analysis. The *A. tumefaciens* GV3101 strain harboring the vector was grown to stationary phase in LB liquid culture with 25 mg L^{-1} kanamycin at 28°C, 250 rpm. *A. thaliana* wild-type Columbia was transformed with the *Agrobacterium* strain using the floral dip method as previously described (Clough and Bent, 1998). Seeds obtained from T_0 plants were germinated on Gamborg B5 medium supplemented with 50 mg L^{-1} hygromycin (Lim et al., 2009). Selected plants were transplanted into soil, self-pollinated and harvested individually up to the T_1 generation. Thereafter, progeny plants germinated without any antibiotic selection were grown in soil at 22°C under a 16-h-light/8-h-dark photoperiod.

PCR analysis

Genomic DNA was isolated from the leaves of transgenic plants using a mini-prep method (Chen and Ronald, 1999). The presence of excised or non-excised forms of the vectors in the transformed plants was detected by PCR amplification using specific primers (Table 1). The reaction conditions were 1 cycle of 5 min at 94°C followed by 35 cycles of 30 s at 94°C, 1 min at 56°C and 1 to 5 min at 72°C depending on the size of the PCR product, and then a final extension step of 7 min at 72°C. The amplified products were loaded onto a 0.7% (w/v) agarose gel.

DNA gel-blot analysis

Total genomic DNA from young leaves of 3 to 4 weeks-old transgenic plants was isolated and used for DNA gel-blot analysis. Thirty μg of genomic DNA was digested with *Eco*RI and *Hin*dIII, electrophoresed in a 1% (w/v) agarose gel and subsequently transferred to a Hybond N+ nylon membrane (Amersham Biosciences). The *GUS* and *Hpt* coding regions were amplified from the Cre/*loxP* plasmid using *GUS* forward and reverse, and *Hpt* forward and reverse primers, respectively (Table 1). The amplified products were labeled with ^{32}P-dCTP using the Amersham™ Rediprime™ random primers DNA labeling system (GE Healthcare), and then used as the probes for DNA gel-blot hybridization. The hybridizations were carried out over 16 h at 65°C in a solution containing 0.5 M sodium phosphate (pH 7.2), 1 mM EDTA, 1% (w/v) BSA, and 7% (w/v) sodium dodecyl sulfate (SDS). After hybridization, the membrane was washed twice using 0.2× SSC (3 M NaCl and 0.3 M so-

Table 1. PCR primers used in this study

Primer	Sequence (5′-3′)
P1	CAAAGATTCAAATAGAGGACCTAA
P2	CAAGCTCTGATAGAGTTGGTCAAG
P3	AGTAAAAACTATCCAGCAACATTT
P4	AAAGAAATCATGGAAGTAAGACTG
P5	TTATGTTTATCGGCACTTTGCAT
P6	AGATGTCGCTATAAACCTATTCAG
GUS forward	CTACACCACGCCGAACACCT
GUS reverse	CAGGCACAGCACATCAAAGA
Hpt forward	TTATGTTTATCGGCACTTTGCAT
Hpt reverse	CAAGCTCTGATAGAGTTGGTCAAG
35S-Hind-F1	CCAAGCTTAGATTAGCCTTTTCAATTTCAG
35S-Lox-R1	AACTTCGTATAATGTATGCTATACGAAGTTATCGTGTTCTCTCCAAATGAAA
Hpt-Lox-F1	ATAACTTCGTATAGCATACATTATACGAAGTTATGAAAAAGCCTGAACTCACCG
Hpt-Mini-R1	AATGAACTTCCTTATATAGAGGAAGGGTCTTGCGTAGTAACATAGATGACACCG
Hpt-Xho-R2	CCCTCGAGCGTGTCCTCTCCAAATGAAATGAACTTCCTTATATAGAGGAAGGGTCTTGCG
Cre-Xho-F1	CCCTCGAGTCGACTCTAGCCTCGACATGTCCAATTTACTGACCGTAC
Cre-Lox-R1	GCTCTAGATAACTTCGTATAATGTATGCTATACGAAGTTATTCCTGCTGAGCCTCGACATGTT

dium citrate) and 0.1% SDS at 65°C for 10 min. After washing, the hybridization signals were recorded with a phosphorimager (Typhoon, Amersham Biosciences).

GUS histochemical assay

Leaves and pollens of transgenic plants were used for GUS staining. Each tissue was infiltrated under a vacuum for 10 min with the reaction buffer containing 0.2 M sodium phosphate (pH 7.0), 10 mM EDTA, 20% methanol, 0.01% Triton X-100, 2% dimethyl sulfoxide (DMSO), 0.1% 5-bromo-4-chloro-3-indole β-glucopyranoside (X-Gluc) as described by Jeon et al. (1999). Pollens were incubated at 37°C for 30 min and other tissues were incubated from 2 h to overnight until sufficient staining was observed. Leaf samples were then transferred into a 70% ethanol solution to remove chlorophyll. GUS staining patterns were visualized under a dissecting microscope SZX12 (Olympus, Japan) and photographed with ImagePro Express (Olympus).

RESULTS

Transient expression assay of a minimal promoter::*Cre* fusion construct

The aim of this study was to develop a novel Cre/*loxP* vector based on the *Cre* gene governed by a 46-bp CaMV 35S minimal promoter containing a TATA box and transcription start site. This -46 promoter alone is unable to drive *Cre* gene expression but can activate the gene in conjunction with neighboring enhancer elements in the plant genome (Springer, 2000). It is noteworthy in this regard that the constitutive expression of the *Cre* recombinase gene causes undesirable phenotypes such as growth retardation, leaf chlorosis patterns and reduced fertility (Coppoolse et al., 2003). Hence, we designed our novel construct so that -46::*Cre* could cause the excision of a *hygromycin phosphotransferase* (*Hpt*) marker gene and the *Cre* construct itself between the *loxP* sites. In the design of this vector, we placed the *Hpt* gene immediately downstream of the CaMV 35S promoter and a *loxP* sequence so that transformed cells could be selected on hygromycin media (Fig. 1), a proven system for the selection of transgenic lines (Qu et al., 2009). To examine the efficacy of our vector, we delivered the Cre/*loxP* fusion construct into onion (*Allium cepa*) epidermal cells by particle bombardment (Hull et al., 1996). Any induction of the *Cre* recombinase gene by the -46 promoter would cause deletion of the region between *loxP* sites containing *Hpt* and lead to the fusion of CaMV 35S promoter to *GUS*, thus enabling a visual analysis of this reporter gene expression. Indeed, in transient assays, we observed a number of GUS staining sectors within the onion epidermal cells (Supplementary Fig. 1), indicating the functionality of the -46::*Cre* fusion system.

Production and selection of transgenic lines harboring the novel Cre/*loxP* vector

From a pool of transgenic *Arabidopsis* plants generated by *Agrobacterium*-mediated transformation, 10 T_1 transgenic *Arabidopsis* plants that grew on hygromycin-containing medium were selected for detailed characterization. All the plants showed no visible phenotype in comparison with wild-type plants. We examined the GUS staining patterns of these lines using young leaves. Nine of the 10 lines exhibited a chimeric GUS staining pattern, indicating the presence of both excision and non-excision forms whereas the remaining line showed no staining, indicating the absence of excision. This result indicated that the -46::*Cre* element could successfully excise the *loxP* regions *in vivo*. The 10 T_1 transgenic plants under analysis were then cultivated until seed setting to evaluate transgene inheritance by the next generation.

Four to nine T_2 progeny plants from the nine T_1 transgenic lines that showed a chimeric GUS staining pattern were germinated on hygromycin-free medium and further grown in soil. We then performed GUS staining to determine the structure of the Cre/*loxP* vector in the genome of the T_2 individual plants.

Table 2. Molecular analysis of 10 selected T_1 lines

Line	Primer					GUS staining[a]	Vector structure[b]
	P1/P2	P3/P4	P1/P4	P5/P6	P5/P2		
4	+	+	+	+	+	+	E/NE
22	-	+	-	+	+	-	NE
41	+	+	+	+	+	+	E/NE
42	+	+	+	+	+	+	E/NE
43	+	+	+	+	+	+	E/NE
44	+	+	+	+	+	+	E/NE
45	+	+	+	+	+	+	E/NE
46	+	+	+	+	+	+	E/NE
47	+	+	+	+	+	+	E/NE
48	+	+	+	+	+	+	E/NE

[a]+, chimeric staining; -, no staining
[b]NE, non-excision form; E/NE, both forms

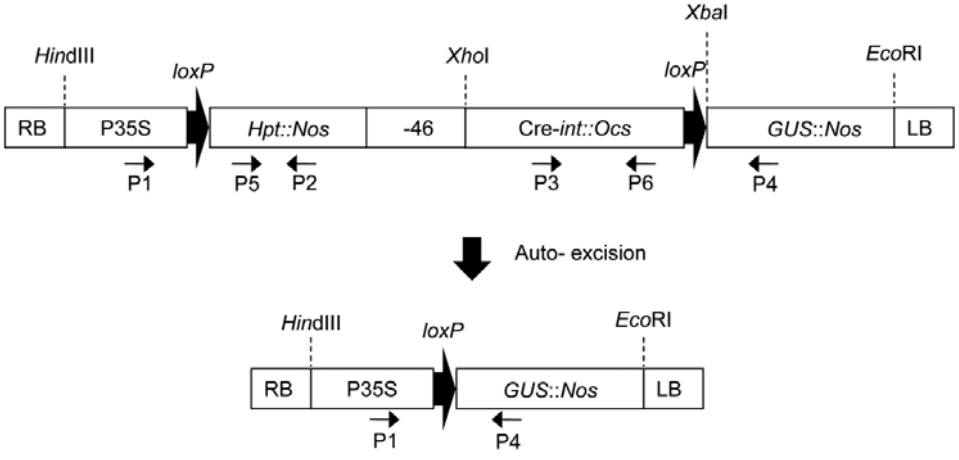

Fig. 1. Schematic representation of the Cre/*loxP* vector and Cre/*loxP* mediated recombination. When *loxP*-specific DNA excision occurs, the selective marker region is eliminated and thereafter a 3 kb band between *Hin*dIII and *Eco*RI is detectable, otherwise the band is 6.5 kb in size. RB, right border; *P35S*, CaMV 35S promoter; *Hpt*, *hygromycin phosphotransferase* gene; *Nos*, nopaline synthase terminator; -46, 46-bp CaMV 35S minimal promoter; *Cre-int*, the bacteriophage P1 *Cre* recombination gene with an intron; *Ocs*, octopine synthase terminator; *GUS*, *β-glucuronidase* complete coding region; LB, left border. P1-P6, primers used in PCR analysis.

Among the 72 T_2 transgenic plants tested, 24 showed a uniform whole tissue staining pattern indicating that they were most likely to be marker-free transgenic plants, i.e. without the *Hpt* resistance gene (Fig. 2C), whilst 29 further lines showed a chimeric GUS staining profile that was characteristic of both excision and non-excision chimeric patterning (Fig. 2B) and 19 were negative for staining (Fig. 2A), likely because they were either wild type segregants or had adopted a non-excision structure in the transgene. This result suggests that the excision form of the vector was successfully inherited into subsequent progeny, thereby generating marker-free transgenic plants with an efficiency of over 30%.

To examine whether the excision form of our construct was present in reproductive tissues, we further performed GUS staining of pollens from the transgenic plants (Figs. 2D, 2E, and 2F). Seven from the uniform leaf staining lines showed whole GUS staining of the pollens, indicating that they are homozygous for the excision form of the -46::*Cre* fusion vector (Fig. 2F), whilst 11 and 23 from the uniform and chimeric leaf staining lines, respectively, exhibited a segregation pattern between GUS stained and non-stained pollens (Fig. 2E), indicating either that they harbor both excision and non-excision forms of the vector or are heterozygous for the excision form. In addition, the remaining transgenic lines (each six from the uniform and chimeric leaf staining lines and all from the non-staining lines) were found to be negative for GUS staining, again indicating that they are either wild type segregants or carry only the non-excision form of the vector in their genomes (Fig. 2D).

Molecular characterization of T_1 and T_2 transgenic lines harboring the -46::*Cre* fusion vector

To examine the structure of our novel Cre/*loxP* vector in the genomes of transformed *Arabidopsis* plants, we performed genomic PCR analysis using multiple primer set combinations (Fig. 1 and Table 1). Amplicon sizes for the non-excision form of the Cre/*loxP* vector using specific primer pairs are as follows: 1389 bp for P1 and P2, 2290 bp for P3 and P4, 4553 bp for P1 and P4, and 2812 bp for P5 and P6. For the excision form of the vector, a distinct 1081 bp fragment should be amplified using the P1 and P4 primers. In addition, the presence of the *Hpt* selection marker can be validated by the P5 and P2 primers which are specific for this gene (Fig. 1). We tested 10 T_1 transgenic lines by PCR using the P1 and P4 primers and found that with the exception of line No. 22, amplified PCR

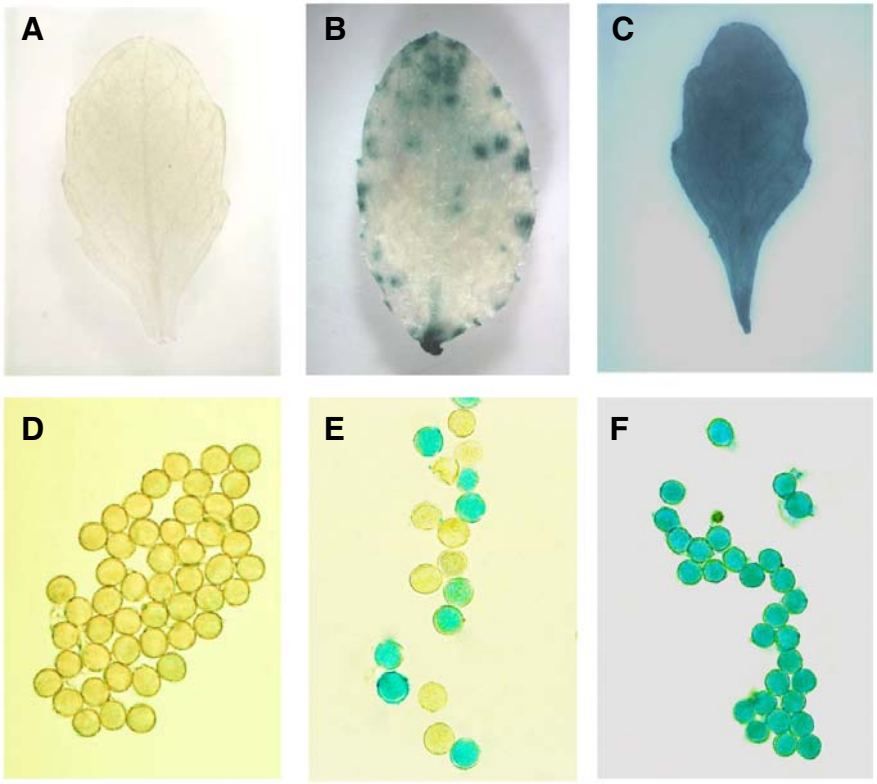

Fig. 2. Histochemical analysis of GUS activity in leaves and mature pollens of transgenic *Arabidopsis* plants. (A-C) Leaf of T_2 transgenic lines 22-2 (A), 46-2 (B), and 46-1 (C). (D-F) Pollens of T_2 transgenic line 22-2 (D), T_2 transgenic line 46-2 (E), and marker-free T_2 transgenic line 46-1 (F). These were selected as representatives for the uniform (46-1) and chimeric leaf GUS staining (46-2), and non-GUS staining (22-2) lines.

products of both 4553 and 1081 bp were obtained in all cases (Table 2 and Supplementary Fig. 2A), indicating that these lines carried both the excision and non-excision forms of the Cre/*loxP* vector. Line No. 22 was subsequently found to be positive for the *Hpt* region, as determined using the P5 and P2 primers (Supplementary Fig. 2B) and for the P3/P4 and P5/P6 amplicons, but no amplified products for the P1/P2 or P1/P4 primers (Table 2). These results likely indicate a modification of the P35S promoter and *loxP* region near to the T-DNA right border (RB), and thus the presence of the non-excision form of the vector only.

To further confirm the excision of the *Hpt* marker gene from our transgenic plants, we performed DNA gel-blot analysis after digestion of genomic DNA with *Eco*RI and *Hin*dIII using *GUS* and *Hpt* gene probes, respectively. If the selective marker gene is excised, a 3032 bp band would be obtained using the *GUS* probe but not the *Hpt* probe. In contrast, when the marker gene region was retained in a non-excision structure, a 6504 bp band would be detected using either the *GUS* or *Hpt* probe. In all of the T_1 transgenic plants tested, hybridization with the *GUS* probe produced both 3032 bp and 6504 bp bands, with the exception of line No. 22 which showed only a single band indicative of the non-excision form of the introduced vector (Fig. 3A). Our experiments with the *Hpt* probe revealed a single band of the non-excision form (Fig. 3B), indicating that nine of the 10 T_1 transgenic plants analyzed carry both the excision and non-excision forms of the Cre/*loxP* vector in their genomes and that line No. 22 contains only the non-excision form.

Using T_2 transgenic lines, we again performed PCR and genomic DNA gel-blot analyses. For this experiment, we selected three representative lines which were No. 22 as a non-excision form, and Nos. 46 and 48 as both form-carrying lines (Table 3). PCR analysis showed that the products were amplified in almost all T_2 lines of No. 22 using the P5/P2, P3/P4 ad P5/P6 primer pairs but not with the P1/P4 or P1/P2 primer pairs (Table 3). The exceptions were T_2 lines 22-1, -3 and -9 which were likely to be wild type segregants.

In contrast to the above results, PCR analysis of the T_2 lines of No. 46 showed that a 1081 bp PCR product was amplified with the P1/P4 primers in lines 46-1, -4, -5, and -9 but that no product was obtained with the P5/P2, P1/P2, P3/P4 or P5/P6 primer pairs (Table 3 and Supplementary Fig. 3). This indicated that these lines were marker-free transgenic plants, which carried only the excision form and did not retain the *Hpt* gene in their genome. All PCR products of the tested primer combinations were successfully amplified in T_2 lines 46-2 and 46-6, indicating that these lines carry both excision and non-excision forms of the Cre/*loxP* vector. In contrast, none of the PCR products were amplified in lines 46-3, -7, -8, confirming that they were wild type segregants (Table 3 and Supplementary Fig. 3). Similar results were observed for T_2 lines of line No. 48 (Table 3).

To further verify these PCR data, we performed genomic DNA gel-blot analysis using *GUS* and *Hpt* gene probes, respectively, in T_2 progeny of line No. 46. The T_2 lines 46-1, -4, -5, and -9 showed a 3032 bp band that hybridized with a *GUS* probe but did not exhibit any band that bound to a *Hpt* probe (Figs. 4A and 4B). This confirmed the findings from the PCR data that these lines were marker-free transgenic plants harboring only the excision form of the transgene and did not retain the *Hpt* gene in their genomes. Consistently, these lines showed a uniform whole tissue GUS staining pattern (Table 3). T_2 lines 46-2 and 46-6 showed both 3032 and 6504 bp bands when hybridized with the *GUS* probe, also validating the observations

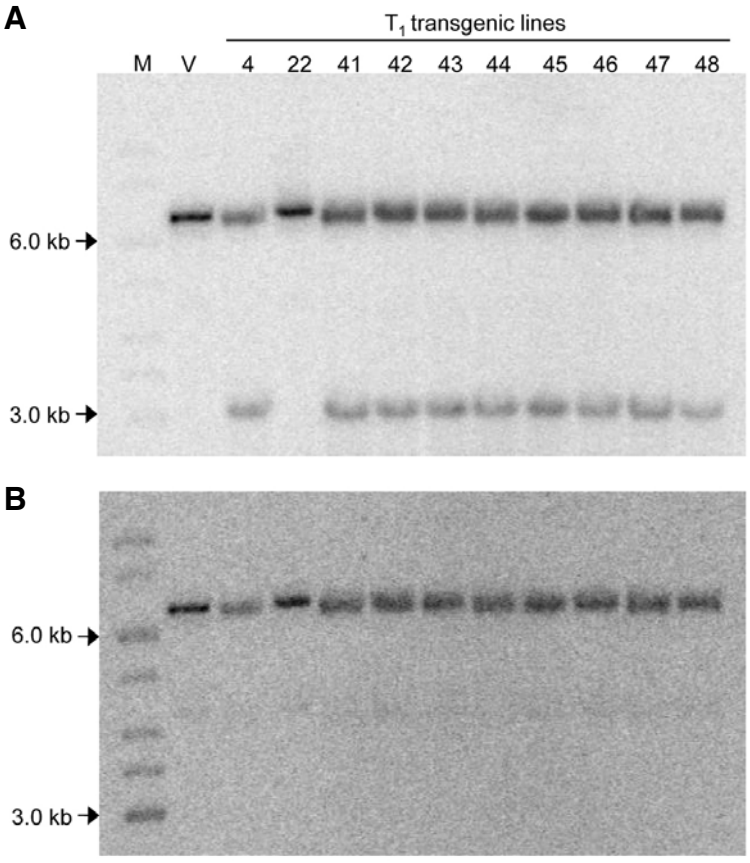

Fig. 3. DNA gel-blot analysis of T_1 transgenic plants. Genomic DNA from the first generation transgenic plants was digested with *Eco*RI and *Hin*dIII and hybridized with *GUS* probe (A) or *Hpt* probe (B). Hybridization of the probes to a 6.5 kb band indicates the non-excision form of Cre/*loxP* vector, whereas a detectable 3.0 kb hybridization fragment indicates that Cre-mediated recombination has occurred. Line M, molecular markers; line V, Cre/*loxP* vector; lines 4, 22, and 41-48 are independent T1 transgenic lines.

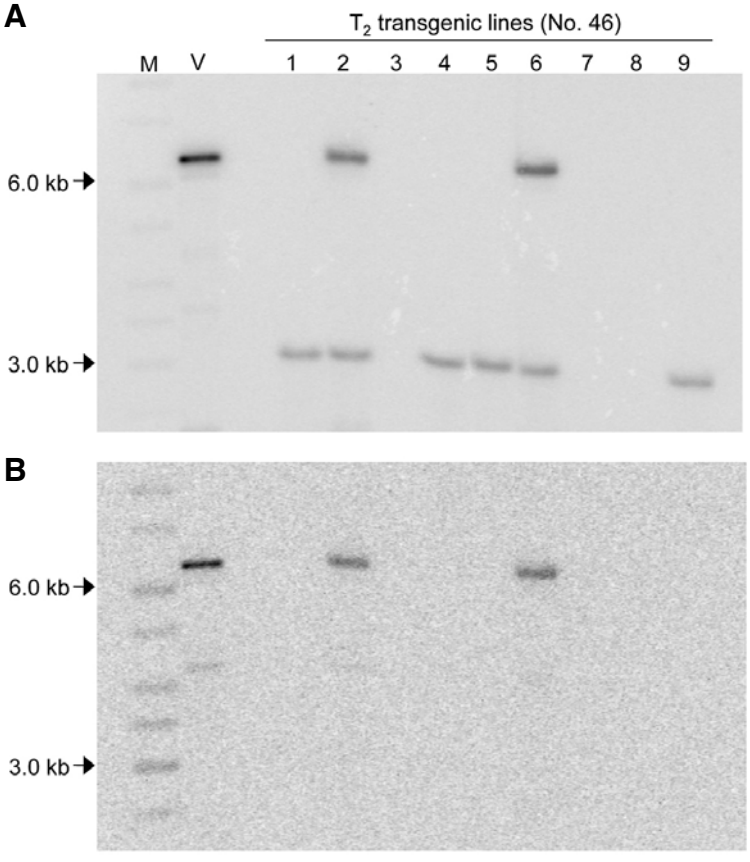

Fig. 4. DNA gel-blot analysis of T_2 transgenic plants. Genomic DNA from the T_2 transgenic plants was digested with *Eco*RI ad *Hin*dIII and hybridized with *GUS* probe (A) and *Hpt* probe (B). Hybridization of the probes to a 6.5 kb band indicates the non-excision form of Cre/*loxP* vector, whereas a detectable 3.0 kb hybridization fragment indicates that Cre-mediated recombination has occurred. Line M, molecular markers; line V, Cre/*loxP* vector; 46-1~46-9 are independent T_2 transgenic lines.

Table 3. Molecular analysis of the T_2 progeny of selected lines

Line	Primers					GUS staining[a]	Vector structure[b]
	P1/P2	P3/P4	P1/P4	P5/P6	P5/P2		
46-1	-	-	+	-	-	+++	E
46-2	+	+	+	+	+	+	E/NE
46-3	-	-	-	-	-	-	W
46-4	-	-	+	-	-	+++	E
46-5	-	-	+	-	-	+++	E
46-6	+	+	+	+	+	+	E/NE
46-7	-	-	-	-	-	-	W
46-8	-	-	-	-	-	-	W
46-9	-	-	+	-	-	+++	E
48-1	-	-	-	-	-	-	W
48-2	-	-	+	-	-	+++	E
48-3	+	+	+	+	+	+	E/NE
48-4	+	+	+	+	+	+	E/NE
48-5	+	+	+	+	+	+	E/NE
48-6	-	-	+	-	-	+++	E
48-7	-	-	+	-	-	+++	E
48-8	+	+	+	+	+	+	E/NE
48-9	+	+	+	+	+	+	E/NE
22-1	-	-	-	-	-	-	W
22-2	-	+	-	+	+	-	NE
22-3	-	-	-	-	-	-	W
22-4	-	+	-	+	+	-	NE
22-5	-	+	-	+	+	-	NE
22-6	-	+	-	+	+	-	NE
22-7	-	+	-	+	+	-	NE
22-8	-	+	-	+	+	-	NE
22-9	-	-	-	-	-	-	W

[a]+++, whole tissue staining; +, chimeric staining; -, no staining
[b]E, excision form; NE, non-excision form; E/NE, both forms; W, wild type segregant

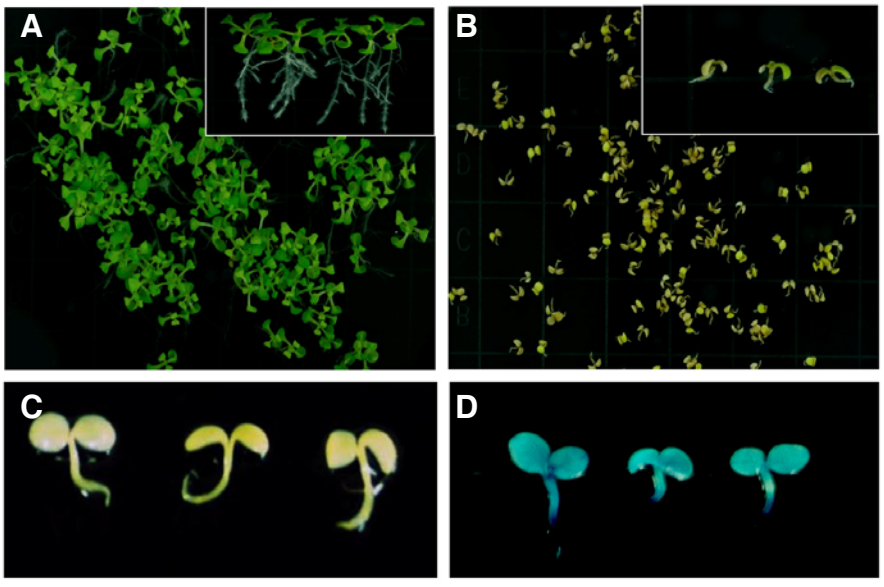

Fig. 5. Confirmation of selectable marker gene excision. (A, B) Growth of the transgenic plants was observed in medium supplemented with 50 mg L^{-1} hygromycin at 10 days after germination. (A) Growth of line 22-2 which harbors the non-excision form of the Cre/*loxP* vector. (B) Growth of line 46-1. (C, D) *GUS* expression patterns of germinated seedlings. (C) GUS negative staining of progeny plants derived from line 22-2. (D) Whole plant GUS staining pattern of marker-free transgenic plants derived from line 46-1.

from the PCR data that these lines carried both the excision and non-excision forms of the Cre/*loxP* vector. In addition, the T_2 lines 46-3 and 46-7 and 46-8, did not hybridize with any probe, indicating that they were wild type segregants (Figs. 4A and 4B).

To demonstrate if the excision form-carrying plants were marker-free transgenic plants, we harvested T_3 seeds of lines 46-1 and 46-4 and germinated them on hygromycin-containing media. As a control, line 22-2 was used as the non-excision form-carrying line. Hygromycin selection tests showed that all of germinated seeds from lines 46-1 and 46-4 did not grow and eventually died (Fig. 5B), whereas progeny plants of line 22-2 grew normally on the hygromycin-containing medium (Fig. 5A). Our PCR analysis using T_3 progeny plants of 46-1 confirmed that the 1081 bp product was amplified with P1/P4 primers in all the plants but not with P5/P2 primers (Supplementary Fig. 4). A GUS staining experiment confirmed that the progeny of lines 46-1 and 46-4 showed positive GUS staining (Fig. 5D) but that the plants derived from line 22-2 were negative for this staining (Fig. 5C), which was consistent with our earlier data that the 46-1 and 46-4 progeny plants were marker-free transgenic plants.

DISCUSSION

Although the use of antibiotic and herbicide resistance genes is important during the process of developing transgenic plants, the existence of such selective marker gene in transgenic plants can be a barrier to further experimental techniques and the eventual commercialization of GM crops. In our present study, we have developed a novel Cre/*loxP* based vector system controlled by a minimal promoter that can excise the selectable marker from the progeny of transgenic plants.

Most (9/10) of the T_1 selected transgenic plants in our analysis were found by PCR and DNA gel-blot analyses to carry excision and non-excision forms of the introduced vector (Table 2, Fig. 3 and Supplementary Fig. 2), indicating that this chimeric excision occurred in nearly all of the transgenic lines. This additionally suggests that a high efficiency of excision can be achieved in plant offspring. In the next generation, we found that over 30% (4/9 of line No. 46 and 3/9 of line No. 48) of the T_2 transgenic plants were indeed marker-free carrying only the excision form of the vector (Table 3, Fig. 4 and Supplementary Fig. 3). This finding demonstrates that this elaborate Cre/*loxP* system based on the -46 minimal promoter::*Cre* fusion works very efficiently in *Arabidopsis*.

An advantage of our present Cre/*loxP* system is that no additional treatment or genetic crossing of transgenic lines is required, which provides simplicity and convenience. In previously developed Cre/*loxP*-based strategies, transient and stable expression of the *Cre* recombinase gene has been accomplished using chemical or heat inducible promoters (Hoff et al., 2001; Sreekala et al., 2005; Zhang et al., 2003; Zuo et al., 2001) or tissue-specific promoters (Bai et al., 2008; Luo et al., 2007). It is known that the -46 minimal promoter alone containing a TATA box and transcription start site is unable to drive gene expression in plants (Lim et al., 2007). Thus, we speculated that any enhancer element neighboring the T-DNA insertions in plant genomes would probably drive *Cre* gene expression. There is the possibility that a chronically leaky expression of *Cre* via the minimal promoter would result in a threshold level of Cre recombinase and thus excision of the *loxP* sites. It is known that constitutive expression of *Cre* affects the growth of transgenic plants (Coppoolse et al., 2003). In this regard, our present system can completely eliminate the -46::*Cre* construct containing the selectable marker gene region between *loxP* sites once *Cre* is expressed, which adds considerably to the usefulness of this vector. Alternatively, the minimal promoter might be activated by the enhancer present in the CaMV 35S promoter of the vector construct that drives the *Hpt* expression. Replacement of the 35S promoter with a different promoter known without any enhancer should help to address the question.

We are currently testing our Cre/*loxP* vector in the model crop species rice (*Oryza sativa* L.). In preliminary experiments, we have observed a large number of GUS staining sectors from transgenic calli (Kim et al., unpublished observations), most likely indicating that -46::*Cre* also functions in rice. Therefore, we believe that this novel Cre/*loxP* vector system is applicable to diverse plant species. To produce this vector more conveniently, we have now developed an additional cloning vector based on the Gateway system (Supplementary Fig. 5).

In conclusion, we have developed a novel Cre/*loxP* vector using a minimal promoter::*Cre* fusion construct to eliminate the selective marker genes used for the genetic transformation of plants. In our experiments, the -46 minimal promoter efficiently induced *loxP* site-specific DNA excision in the T_1 transgenic plants. A high frequency of marker-free transgenic plants was obtained in the T_2 generation. Hence, our present results suggest that this novel Cre/*loxP* vector provides a simple and efficient way to generate marker-free transgenic plants.

Note: Supplementary information is available on the Molecules and Cells website (www.molcells.org).

ACKNOWLEDGMENTS

This work was supported by grants from the Next-Generation BioGreen 21 Program (PJ008114022011 and PJ008156012011), Rural Development Administration of the Korean Ministry of Food, Agriculture, Forestry, and Fisheries, and from the World Class University program (R33-2008-000-10168-0) and the Mid-Career Researcher Program (2010-0026679) from the Korean Ministry of Education, Science and Technology.

REFERENCES

Abremski, K., and Hoess, R. (1984). Bacteriophage P1 site-specific recombination. Purification and properties of the Cre recombinase protein. J. Biol. Chem. *259*, 1509-1514.

Bai, X., Wang, Q., and Chu, C. (2008). Excision of a selective marker in transgenic rice using a novel Cre/loxP system controlled by a floral specific promoter. Transgenic Res. *17*, 1035-1043.

Chakraborti, D., Sarkar, A., Mondal, H.A., Schuermann, D., Hohn, B., Sarmah, B.K., and Das, S. (2008). Cre/lox system to develop selectable marker free transgenic tobacco plants conferring resistance against sap sucking homopteran insect. Plant Cell Rep. *27*, 1623-1633.

Chen, D.H., and Ronald, P.C. (1999). A rapid DNA minipreparation method suitable for AFLP and other PCR applications. Plant Mol. Biol. Rep. *17*, 53-57.

Clough, S.J., and Bent, A.F. (1998). Floral dip: a simplified method for Agrobacterium-mediated transformation of *Arabidopsis thaliana*. Plant J. *16*, 735-743.

Coppoolse, E.R., de Vroomen, M.J., Roelofs, D., Smit, J., van Gennip, F., Hersmus, B.J., Nijkamp, H.J., and van Haaren, M.J. (2003). Cre recombinase expression can result in phenotypic aberrations in plants. Plant Mol. Biol. *51*, 263-279.

Corneille, S., Lutz, K., Svab, Z., and Maliga, P. (2001). Efficient elimination of selectable marker genes from the plastid genome by the CRE-lox site-specific recombination system. Plant J. *27*, 171-178.

Craig, N.L. (1988). The mechanism of conservative site-specific recombination. Annu. Rev. Genet. *22*, 77-105.

Dale, E.C., and Ow, D.W. (1990). Intra- and intermolecular site-specific recombination in plant cells mediated by bacteriophage

P1 recombinase. Gene *91*, 79-85.
Daley, M., Knauf, V.C., Summerfelt, K.R., and Turner, J.C. (1998). Co-transformation with one *Agrobacterium tumefaciens* strain containing two binary plasmids as a method for producing marker-free transgenic plants. Plant Cell Rep. *17*, 489-496.
De Block, M., and Debrouwer, D. (1991). Two T-DNAs co-transformed into *Brassica napus* by a double *Agrobacterium tumefaciens* infection are mainly integrated at the same locus. Theor. Appl. Genet. *82*, 257-263.
Depicker, A., Herman, L., Jacobs, A., Schell, J., and van Montagu, M. (1985). Frequencies of simultaneous transformation with different T-DNAs and their relevance to the Agrobacterium/plant cell interaction. Mol. Gen. Genet. *201*, 477-484.
Ebinuma, H., Sugita, K., Matsunaga, E., and Yamakado, M. (1997). Selection of marker-free transgenic plants using the isopentenyl transferase gene. Proc. Natl. Acad. Sci. USA *94*, 2117-2121.
Fuse, T., Sasaki, T., and Yano, M. (2001). Ti-plasmid vectors useful for functional analysis of rice genes. Plant Biotechnol. *18*, 219-222.
Hajdukiewicz, P., Svab, Z., and Maliga, P. (1994). The small, versatile pPZP family of Agrobacterium binary vectors for plant transformation. Plant Mol. Biol. *25*, 989-994.
Hoff, T., Schnorr, K.M., and Mundy, J. (2001). A recombinase-mediated transcriptional induction system in transgenic plants. Plant Mol. Biol. *45*, 41-49.
Hull, G., Garrido, J.M.G., Parcy, F, Menossi, M., Martinez-Izquierdo, J.A., and Gallois, P. (1996). Use of *lacZ* repoter gene as an internal control for GUS activity in microprojectile bombarded plant tissue. Plant Sci. *120*, 153-160.
Jefferson, R.A. (1987). Assaying chimeric genes in plants: the GUS gene fusion system. Plant Mol. Biol. Rep. *5*, 387-405.
Jeon, J.S., Chung, Y.Y., Lee, S., Yi, G.H., Oh, B.G., and An, G. (1999). Isolation and characterization of an anther-specific gene, RA8, from rice (*Oryza sativa* L.). Plant Mol. Biol. *39*, 35-44.
Jeon, J.S., Lee, S., and An, G. (2008). Intragenic control of expression of a rice MADS box gene OsMADS1. Mol. Cells *26*, 474-480.
Jia, H., Pang, Y., Chen, X., and Fang, R. (2006). Removal of the selectable marker gene from transgenic tobacco plants by expression of Cre recombinase from a tobacco mosaic virus vector through agroinfection. Transgenic Res. *15*, 375-384.
Komari, T., Hiei, Y., Saito, Y., Murai, N., and Kumashiro, T. (1996). Vectors carrying two separate T-DNAs for co-transformation of higher plants mediated by *Agrobacterium tumefaciens* and segregation of transformants free from selection markers. Plant J. *10*, 165-174.
Kopertekh, L., Juttner, G., and Schiemann, J. (2004). PVX-Cre-mediated marker gene elimination from transgenic plants. Plant Mol. Biol. *55*, 491-500.
Li, B.C., Xie, C., and Qiu, H. (2009). Production of selectable marker free transgenic tobacco plants using a non-selection approach: chimerism or escape, transgene inheritance, and efficiency. Plant Cell Rep. *28*, 373-386.
Lim, H.M., Cho, J.I., Lee, S., Cho, M.H., Bhoo, S.H., An, G., Hahn, T.R., and Jeon, J.S. (2007). Identification of a 20-bp regulatory element of the *Arabidopsis pyrophosphate*:fructose-6-phosphate 1-phosphotransferase $\alpha 2$ gene that is essential for expression. Plant Cell Rep. *26*, 683-692.
Lim, H., Cho, M.H., Jeon, J.S., Bhoo, S.H., Kwon, Y.K., and Hahn, T.R. (2009). Altered expression of pyrophosphate: fructose-6-phosphate 1-phosphotransferase affects the growth of transgenic Arabidopsis. Mol. Cells *27*, 641-649.
Liu, Y.G., Shirano, Y., Fukaki, H., Yanai, Y., Tasaka, M., Tabata, S., and Shibata, D. (1999). Complementation of plant mutants with large genomic DNA fragments by a transformation-competent artificial chromosome vector accelerates positional cloning. Proc. Natl. Acad. Sci. USA *96*, 6535-6540.
Luo, K., Duan, H., Zhao, D., Zheng, X., Deng, W., Chen, Y., Stewart, C.N.Jr., McAvoy, R., Jiang, X., Wu, Y., et al. (2007). 'GM-gene-deletor': fused loxP-FRT recognition sequences dramatically improve the efficiency of FLP or CRE recombinase on transgene excision from pollen and seed of tobacco plants. Plant Biotechnol. J. *5*, 263-274.
McKnight, T.D., Lillis, M.T., and Simpson, R.B. (1987). Segregation of genes transferred to one plant cell from two separate Agrobacterium strains. Plant Mol. Biol. *8*, 439-445.
Mersereau, M., Pazour, G.J., and Das, A. (1990). Efficient transformation of *Agrobacterium tumefaciens* by electroporation. Gene *90*, 149-151.
Miki, B., and McHugh, S. (2004). Selectable marker genes in transgenic plants: applications, alternatives and biosafety. J. Biotechnol. *107*, 193-232.
Odell, J., Caimi, P., Sauer, B., and Russell, S. (1990). Site-directed recombination in the genome of transgenic tobacco. Mol. Gen. Genet. *223*, 369-378 .
Ouwerkerk, P.B., de Kam, R.J., Hoge, J.H., and Meijer, A.H. (2001). Glucocorticoid-inducible gene expression in rice. Planta *213*, 370-378.
Ow, D.W. (2002). Recombinase-directed plant transformation for the post-genomic era. Plant Mol. Biol. *48*, 183-200.
Qin, M., Bayley, C., Stockton, T., and Ow, D.W. (1994). Cre recombinase-mediated site-specific recombination between plant chromosomes. Proc. Natl. Acad. Sci. USA *91*, 1706-1710.
Qin, M., Lee, E., Zankel, T., and Ow, D.W. (1995). Site-specific cleavage of chromosomes *in vitro* through Cre-lox recombination. Nucleic Acids Res. *23*, 1923-1927.
Qu, S., Jeon, J.S., Ouwerkerk, P.B., Bellizzi, M., Leach, J., Ronald, P., and Wang, G.L. (2009). Construction and application of efficient Ac-Ds transposon tagging vectors in rice. J. Integr. Plant Biol. *51*, 982-992.
Russell, S.H., Hoopes, J.L., and Odell, J.T. (1992). Directed excision of a transgene from the plant genome. Mol. Gen. Genet. *234*, 49-59.
Sengupta, S., Chakraborti, D., Mondal, H.A., and Das, S. (2010). Selectable antibiotic resistance marker gene-free transgenic rice harbouring the garlic leaf lectin gene exhibits resistance to sap-sucking planthoppers. Plant Cell Rep. *29*, 261-271.
Sieburth, L.E., and Meyerowitz, E.M. (1997). Molecular dissection of the AGAMOUS control region shows that cis elements for spatial regulation are located intragenically. Plant Cell *9*, 355-365.
Springer, P.S. (2000). Gene traps: tools for plant development and genomics. Plant Cell *12*, 1007-1020.
Sreekala, C., Wu, L., Gu, K., Wang, D., Tian, D., and Yin, Z. (2005). Excision of a selectable marker in transgenic rice (*Oryza sativa* L.) using a chemically regulated Cre/loxP system. Plant Cell Rep. *24*, 86-94.
Wang, Y., Chen, B., Hu, Y., Li, J., and Lin, Z. (2005). Inducible excision of selectable marker gene from transgenic plants by the cre/lox site-specific recombination system. Transgenic Res. *14*, 605-614.
Wang, Y., Yau, Y.Y., Perkins-Balding, D., and Thomson, J.G. (2010). Recombinase technology: applications and possibilities. Plant Cell Rep. *30*, 267-285.
Zhang, W., Subbarao, S., Addae, P., Shen, A., Armstrong, C., Peschke, V., and Gilbertson, L. (2003). Cre/*lox*-mediated marker gene excision in transgenic maize (Zea mays L.) plants. Theor. Appl. Genet. *107*, 1157-1168.
Zubko, E., Scutt, C., and Meyer, P. (2000). Intrachromosomal recombination between attP regions as a tool to remove selectable marker genes from tobacco transgenes. Nat. Biotechnol. *18*, 442-445.
Zuo, J., Niu, Q.W., Moller, S.G., and Chua, N.H. (2001). Chemical-regulated, site-specific DNA excision in transgenic plants. Nat. Biotechnol. *19*, 157-161.