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Removal of the selectable marker gene from transgenic tobacco plants by expression of Cre recombinase from a Tobacco Mosaic Virus vector through agroinfection

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Abstract

Selection markers are often indispensable during the process of plant transformation, but dispensable once transgenic plants have been established. The Cre/*lox* site-specific recombination system has been employed to eliminate selectable marker genes from transgenic plants. Here we describe the use of a movement function-improved Tobacco Mosaic Virus (TMV) vector, m30B, to express Cre recombinase for elimination of the selectable marker gene *nptII* from transgenic tobacco plants. The transgenic tobacco plants were produced by *Agrobacterium*-mediated transformation with a specially designed binary vector pGNG which contained in its T-DNA region a sequence complex of 35S promoter-*lox*-the *gfp* coding sequence-*rbcS* terminator-*Nos* promoter-*nptII*-*Nos* terminator-*lox*-the *gus* coding region-*Nos* terminator. The expression of the recombinant viral vector m30B:Cre in plant cells was achieved by placing the viral vector under the control of the 35S promoter and through agroinoculation. After co-cultivating the pGNG-leaf discs with agro35S-m30B:Cre followed by shoot regeneration without any selection, plants devoid of the lox-flanked sequences including *nptII* were obtained with an efficiency of about 34% as revealed by histochemical GUS assay of the regenerants. Three of 11 GUS expressing regenerants, derived from two independent transgenic lines containing single copy of the pGNG T-DNA, proved to be free of the *lox*-flanked sequences by Southern blot analysis. Excision of the *lox*-flanked sequences in the three plants could be attributed to transient expression of Cre from the viral vector at the early stage of co-cultivation, since the *cre* sequence could not be detected in the viral RNA molecules accumulated in the plants, nor in their genomic DNA. The parental marker-free genotype was inherited in their selfed progeny, and all of the progeny were virus-free, apparently because TMV is not seed-transmissible. Therefore, expression of Cre from a TMV-based vector could be used to eliminate selectable marker genes from transgenic tobacco plants without sexual crossing and segregation, and this strategy could be extended to other TMV-infected plant species and applicable to other compatible virus–host plant systems.

Abbreviations: 35S promoter – the Cauliflower Mosaic Virus 35S promoter; CTAB – cetyltrimethylammonium bromide; GFP – green fluorescent protein; GUS – β-glucuronidase; *Nos P* – the nopaline synthase gene promoter; *Nos T* – the nopaline synthase gene terminator; *nptII* – the coding sequence of neomycin phosphotransferase II; *rbcS T* – the ribulose bisphosphate carboxylase small subunit gene (E9) terminator

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Introduction

In recent years, more and more plant species have been genetically modified to improve their agricultural practices and nutritional value. In order to single out transgenic plants conveniently, selectable markers conferring antibiotic or herbicide resistance are generally used in the process of plant transformation (Yoder & Goldsbrough, 1994; Hare & Chua, 2002). However, the presence of selectable genes is no longer required for growing transgenic plants in fields and even causes environmental problems and food safety concerns. In addition, it is necessary to remove the selectable marker gene from transgenic plants when repeated transformation for introducing several transgenes into the same plant is to be performed, especially for that only a very limited number of selectable markers have proved to be safe through risk assessment. It is therefore sensible to eliminate selectable marker genes from transgenic plants.

To date, several methods, such as co-transformation, transposition, site-specific recombination, have been successfully employed for removal of selectable marker genes from transgenic plants (for reviews, see e.g., Hohn et al., 2001; Hare & Chua 2002). Among several site-specific recombination systems, the bacteriophage P1 Cre/*lox* system (Hoess & Abremski, 1985) is the most widely used one. This system consists of two components, the 38 kDa Cre recombinase and its 34-bp *lox* target sites. When the two *lox* sites are directly orientated, the Cre recombinase catalyses excision of the DNA sequence between them (Craig, 1988; Dale & Ow, 1990; Odell et al., 1990). Cre-mediated excision of the selectable marker gene from transgenic plants was first shown by Dale and Ow (1991) followed by several other reports (Russell et al., 1992; Corneille et al., 2001; Hajdukiemicz et al., 2001). All these procedures employed retransformation or sexual crosses to introduce the *cre* gene, commonly accompanied by another selectable marker gene, into the treated plants, followed by genetic segregation to obtain transgenic plants devoid of the secondary selectable gene and the *cre* gene. To circumvent the time-consuming segregation process and avoid the phenotypic aberrations caused by stable expression of Cre in plants (Que et al., 1998; Coppoolse et al., 2003), strategies for transient expression of the Cre recombinase in plant cells, either by co-cultivation with recombinant *Agrobacterium tumefaciens* (Gleave et al., 1999) or by using an inducible self-excising Cre expression vector (Zuo et al., 2001), were developed.

Plant virus expression systems offer an alternative approach for transient Cre expression in plants, which could be at high levels and occur in a large proportion of plant cells. It was recently shown that a Potato Virus X (PVX)-based vector expressed functional Cre leading to the excision of a *lox*-flanked GFP expression cassette in the *Nicotiana benthamiana* genome (Kopertekh et al., 2004a). Furthermore, the PVX-Cre vector has been successfully used to eliminate a selectable marker gene from transgenic *N. benthamiana* plants (Kopertekh et al., 2004b). In this report, we describe the use of another well-developed plant virus vector, TMV 30B (Shivprasad et al., 1999), to transiently express Cre recombinase for removal of a selectable marker gene from transgenic plants. By using a movement protein (MP) gene-modified 30B, named m30B, Cre-mediated excision of the selectable gene worked well in *N. tabacum*. Moreover, our results indicate that the m30B:Cre-infected tobacco cells retained the potential to develop into fertile plants and the marker-free genotype was transmitted to their progeny which showed to be devoid of virus.

Materials and methods

Generation of pGNG-transformed tobacco plants

The pGNG binary vector was derived from pBI121 (Jefferson et al., 1987) and constructed as described before (Jia et al., 2004), in which the GFP gene was linked to the expression cassette *Nos* P-*nptII*-*Nos* T and the two units were cloned between two directly orientated *lox* sites (Figure 1(a)). Upon Cre-mediated recombination, the GUS gene would be brought next to the 35S promoter (Figure 1(b)). *A. tumefaciens* LBA4404 carrying the binary vector pGNG was used to transform *N. tabacum* cv. SR1 by the leaf disc transformation procedure (Horsch et al., 1985). The regenerated shoots with kanamycin resistance were analyzed by Southern blotting to verify that the pGNG T-DNA was integrated into plant chromosomes. Combining with the segregation ratio of kanamycin resistance in the progeny of self-pollinated tobacco transformants, two independent transgenic tobacco lines both with a single transgene locus, G1 and G4, were chosen

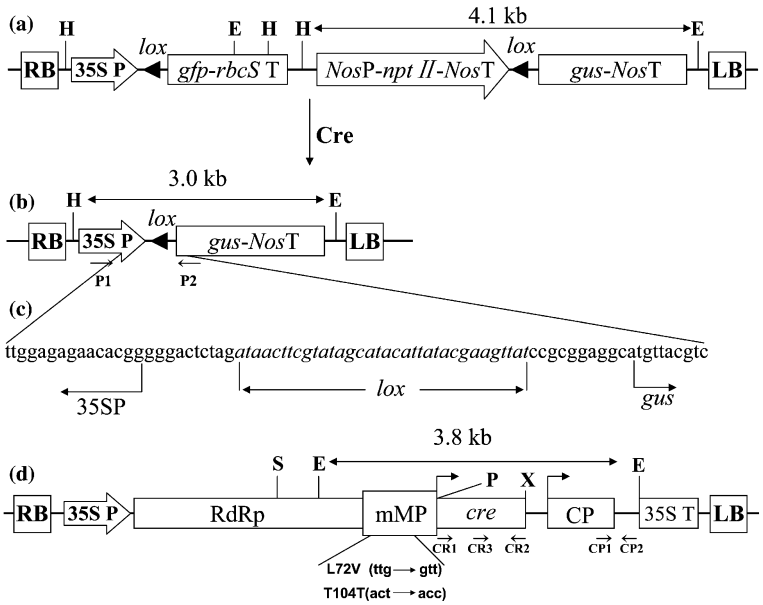

Figure 1. Excision of the selectable marker gene from pGNG-transformed tobacco plants by agroinoculation with agro35S-m30B:Cre. (a) The T-DNA region of pGNG. The *nptII* expression cassette (*Nos* P-*nptII*-*Nos* T) and an upstream-linked *gfp* gene (*gfp-rbcS* T) are flanked by two directly orientated *lox* sites. The 35S promoter (35S P) driving *gfp* expression and a promoter-less *gus* gene (*gus-Nos* T) are located in front of and behind the *lox*-encompassing sequences, respectively. Restriction sites of *Hin*dIII (H) and *Eco*RI (E) used in Southern blot analysis are indicated and the fragment (4.1 kb) that hybridizes to the *gus* probe is shown. (b) Cre-mediated excision of the *lox*-flanked sequence in regenerated plants after co-cultivation with agro35S-m30B:Cre. In the recombined transgenic DNA, *gus-Nos* T is under the control of 35S P, leading to GUS expression. The 3.0-kb *Hin*dIII (H) – *Eco*RI (E) fragment hybridizing to the *gus* probe found in Southern blot analysis is indicated. (c) DNA sequence of the recombined loci in selection marker-free regenerants and their GUS-expressing T1 progeny. The 455-bp PCR fragments harbouring the recombined loci were cloned and sequenced. Shown here is a region containing a single 34-bp *lox* site connected with in the upstream the 35S P sequence and in the downstream the *gus* coding sequence. Nucleotides between 35S P and *lox* and between *lox* and *gus* are derived from cloning procedures in construction of pGNG. (d) Schematic diagram of the T-DNA of p35S-m30B:Cre. Open boxes in the virus vector indicate the following genes: RdRp, RNA-dependent RNA polymerase; mMP, modified movement protein (three nucleotide substitutions are indicated); *cre*, Cre recombinase; CP, TMGMV CP. The bent arrows represent subgenomic RNA promoters of TMV CP and TMGMV CP, respectively. The 3.8-kb *Eco*RI (E) fragment that would hybridize to the *cre* probe but was not detected in Southern blot analysis of marker-free plants is indicated. S, X and P: restriction sites of *Sac*I, *Xho*I and *Pac*I used for generation of p35S-m30B or insertion of the *cre* gene. P1, P2, CR1, CR2, CR3, CP1 and CP2: primers used for the PCR or RT-PCR experiments.

(Jia et al., 2004) and their GFP-expressing T1 progeny were used in the selectable marker-elimination experiments.

Construction of TMV vectors expressing GFP or Cre recombinase

The 30B vector was kindly provided by W.O. Dawson (University of Florida, USA). Based on a derivative of pCAMBIA1300 with the *Sal*I-*Xho*I fragment (containing the 35S promoter-hygromycin resistance gene) deleted, a plant binary vector p35S-30B in which the expression of the 30B cDNA is driven by the 35S promoter, was constructed as described earlier (Jia et al., 2003). In order to improve the movement ability of the 30B vector carrying a foreign gene in tobacco plants, three nucleotide mutations in the MP gene, i.e., ttg → gtt (L72V) and act → acc (T104T) which exhibited the best performance in enhancement of cell-to-cell movement of 30B-GFP in *N. tabacum* cv. MD609 as revealed in a DNA shuffling study (Toth et al., 2002), were chosen and introduced simultaneously into p35S-30B. For this purpose, two synthetic oligonucleotides, MP1 (5′-aagcttattgatagtggatacgtc tgtttagccggtgtttcgtcacg-3′) and MP2 (5′-agccgacga-ggccaccct-3′) harbouring the intended mutations (boxed), were used separately as one PCR primer, and oligonucleotide MP3 (5′-acccctcgaggtttaaacac cgg-3′, corresponding to the 3′ sequence of the multiple cloning sites of 30B) as the other primer to amplify the respective *mp* regions using 30B as a

template. The two PCR products were separately cloned in pGEM-T vector (Promega) and the expected mutations were confirmed by DNA sequencing. The MP2/MP3 amplified fragment was cut with *Bgl*I and *Xho*I (their recognition sites are underlined in the primer sequences) and ligated into the *Xho*I-digested and *Bgl*I-partially digested recombinant pGEM-T clone harbouring the MP1/MP3 amplified sequence to replace the same but the MP T104 codon-unmodified sequence. The resultant clone thus contained a 0.7-kb *mp* region with three designed mutations bounded by a *Hin*dIII site (underlined in the MP1 sequence) and an *Xho*I site. The *Hin*dIII site and the adjacent *Sac*I site in the polylinker of pGEM-T were used to receive a 2.9-kb *Sac*I/*Hin*dIII fragment spanning from nucleotide (nt) 2144–5080 of the TMV genome. The combined 3.6-kb *Sac*I/*Xho*I fragment containing the three nucleotide substitutions in *mp* was put back into p35S-30B pre-digested with single-cutting enzymes *Sac*I and *Xho*I, resulting in p35S-m30B.

The *gfp* coding sequence was amplified from pBIN-*mgfp5-ER* (kindly provided by J. Haseloff, University of Cambridge, UK) using a pair of primers GFP1 (5′-attaattaatgagtaaaggagaagaacttttc-3′, the introduced *Pac*I site is underlined) and GFP2 (5′-actcgagttatttgtatagttcatccatgcc-3′, the added *Xho*I site is underlined), with the following cycling conditions: 94°C for 3 min; 35 cycles at 94°C for 45 s, 56°C for 1 min, 72°C for 1 min 30 s; followed by a final incubation at 72°C for 10 min. The PCR product was cloned as a *Pac*I-*Xho*I fragment into the identical cloning sites of p35S-30B and p35S-m30B to yield p35S-30B:GFP and p35S-m30B:GFP, respectively. In order to obtain p35S-m30B:Cre, the *cre* coding region was amplified from pMM23 (generously provided by D.W. Ow, Plant Gene Expression Center, USA) with primers CR1 (5′-attaattaatgtccaatttactgaccgtacac-3′) and CR2 (5′-actcgagctaatcgccatcttccagcag-3′). The PCR procedure was the same as for amplification of *gfp*. The PCR product was digested with *Pac*I and *Xho*I and inserted into p35S-m30B to form p35S-m30B:Cre (Figure 1(d)). *A. tumefaciens* strain EHA105 was transformed with p35S-30B:GFP, p35S-m30B:GFP or p35S-m30B:Cre by electroporation and the resulting transformed clones were designated as agro35S-30B:GFP, agro35S-m30B:GFP and agro35S-m30B:Cre, respectively.

Agroinoculation with TMV vectors

Agroinoculation was carried out essentially as described previously (Turpen et al., 1993; Jia et al., 2003). The transformed agrobacterial clones were grown overnight at 28°C in LB medium containing kanamycin (25 mg/l) and rifampicin (15 mg/l). The bacterial cells were harvested by centrifugation, resuspended in MMA solution (10 mM $MgCl_2$, 10 mM MES pH 5.6, 100 μM acetosyringone), and adjusted to a final OD_{600} of 1.0. After standing at room temperature for 3 h without shaking, agro35S-30B:GFP or agro35S-m30B:GFP was pressure-infiltrated into leaves of wild-type *N. tabacum* cv. SR1 plants using a 2-ml syringe without needle. Similarly, agro35S-m30B:Cre was used to inoculate the pGNG-transformed tobacco plants.

Detection of GFP fluorescence and histochemical staining of GUS

Under illumination of a 100 W hand-held long-wave ultraviolet lamp (UV Products, model B 100AP), the GFP fluorescence in intact plants was directly visualized or photographed using a digital camera (Nikon Coolpix 995).

Plant tissues assayed for GUS activity were incubated in a solution of 2 mM 5-bromo-4-choro-3-indolyl-β-D-glucuronic acid (X-Gluc) in 50 mM sodium phosphate (pH 7.0) for 8 h at 37°C. Photosynthetic tissues were cleared of chlorophyll by bleaching in 70% ethanol at 50°C. The samples were observed or photographed with Nikon Coolpix 995.

Regeneration of marker-free shoots

The GFP-positive T1 progeny of pGNG-transformed tobacco plants for co-cultivation with *Agrobacterium* were grown on Murashige and Skoog (MS) medium (Sigma) at 25 °C under 16 h light/8 h darkness cycle. An overnight agro35S-m30B:Cre culture was diluted 12-fold with half strength MS liquid medium and the leaf discs were mixed with the diluted culture for 15 min. The explants were blotted dry on sterile filter paper and then placed onto MS medium without kanamycin. The plates were sealed with parafilm and kept in darkness at 25°C for 2 days. After co-cultivation, the leaf discs were transferred to MS medium containing

0.1 mg/l IAA, 1.0 mg/l 6-BA and 500 mg/l carbenicillin. The regenerated plantlets were excised and rooted on half strength MS medium with 0.5 mg/l IBA and 500 mg/l carbenicillin.

Southern blot analysis

Plant genomic DNA was extracted from tobacco plants by the CTAB method (Dellaporta et al., 1983). About 30 μg of total DNA digested with *Hin*dIII and *Eco*RI or *Eco*RI alone was electrophoresed in 0.8% agarose gel, denatured and transferred by capillarity to Hybond N+ membrane (Amersham) as described (Sambrook et al., 1989). A *Bam*HI-*Sac*I fragment from pBI121 containing the GUS gene or the *cre* coding sequence PCR-amplified by CR1 and CR2 was used as a template for probe preparation. Probes were labelled with ^{32}P-labeled dCTP using Random Primed DNA Labeling Kit (Roche). Prehybridization, hybridization and washes were done at 65°C, and the membrane was analyzed by autoradiography.

Reverse transcription-polymerase chain reaction

Total plant RNA was isolated from regenerated plants by Trizol Reagent (GibcoBRL) according to the manufacturer's instructions. First-strand cDNA primed with oligonucleotide CP2 (see below for detailed information) or CR2 was synthesized by using AMV Reverse Transcriptase (Promega) following the supplier's protocols. A fraction of the first-strand cDNA was used as a template for PCR. For detecting the Tobacco Mild Green Mosaic Virus (TMGMV) coat protein (CP) gene transcript derived from the TMGMV CP subgenomic RNA promoter embedded in the m30B vector (Figure 1(d)), primers CP1 (5′-tgaactggttcgtggaactggc-3′, identical to nt 393–414 of the TMGMV CP gene) and primer CP2 (5′-ggccgctacccgcggtta-3′, complementary to the 3′ end sequence of the TMGMV 3′-non-translated region), which are separated by 403 nucleotides (Shivprasad et al., 1999), were used. Primers CR3 (5′-agatatctcacgtactgacggtgg-3′, corresponding to nt 549–572 of the *cre* coding sequence) and CR2 which would yield a PCR product of 484 bp, were used for detection of the *cre* gene transcript that would be generated from the upstream TMV CP subgenomic promoter upon replication of the viral sequence. VentR® DNA Polymerase (BioLabs) was used for PCR amplifications with the following cycling conditions: 94°C for 3 min; 35 cycles at 94°C for 45 s, 54°C for 1 min, 72°C for 1 min; followed by a final incubation at 72°C for 10 min. The RT-PCR products were analyzed by 1.0% agarose gel electrophoresis.

Polymerase chain reaction and DNA sequencing

Genomic DNA was extracted from the leaves of transgenic tobacco plants derived from co-cultivation with agro35S-m30B:Cre using the CTAB method (Dellaporta et al., 1983). Oligonucleotides P1 corresponding to the sequence of −365 to −348 (5′-ctcagaagaccaaagggc-3′) of the 35S promoter and P2 (5′-cacgggttggggtttctacagg-3′) complementary to nt 10–31 of the *gus* coding sequence were used as a pair of primers to amplify the recombined construction of pGNG. The cycling conditions were the same as described above in the RT-PCR experiments except that the annealing temperature was 52°C. The PCR products were resolved by electrophoresis in a 1.0% agarose gel. The 455-bp DNA fragments were purified from the gel and cloned into pGEM-T vector for sequencing the recombined loci by using T7 primer.

Results

Improvement of the movement ability of the virus vector

The 30B vector carrying the GFP gene (30B-GFP) can systemically infect *N. benthamiana* plants (Shivprasad et al., 1999). However, it does not move efficiently into upper leaves of tobacco plants, where the loss of the inserted gene was frequently observed (Rabindran & Dawson, 2001). Modification of the MP gene resulted in improvement in both cell-to-cell and systemic movement of 30B-GFP in tobacco plants (Toth et al., 2002). Based on this information, m30B which incorporates three critical nucleotide substitutions in *mp* was constructed and tested for efficient and stable expression of GFP in tobacco plants via agroinoculation.

Under long-wave UV illumination, green fluorescent spots could be visually detected in locally

inoculated leaves of *N. tabacum* cv. SR1 infiltrated with either agro35S-30B:GFP or agro35S-m30B:GFP at 5 days post-inoculation (dpi). However, diffuse green fluorescence could only be readily visualized in systemic leaves of infected plants inoculated with agro35S-m30B:GFP at approximately 8–10 dpi. By 21 dpi, m30B:GFP had systemically infected 2–3 upper leaves (Figure 2a), whereas 30B:GFP was still constrained in inoculated leaves (Figure 2b). These results indicated the introduced modifications to the 30B MP gene were effective and the m30B vector is preferable to expressing Cre in tobacco plants.

Removal of selectable marker gene nptII *from pGNG-transformed tobacco plants mediated by virus-expressed Cre recombinase*

Excision of the *lox*-flanked *nptII* expression cassette together with the upstream GFP gene in the genome of pGNG-transformed tobacco plants by agroinfection of m30B:Cre was first shown by GUS assay. As preliminary experiments, intact leaves of the transgenic plants were inoculated with agro35S-m30B:Cre, and diffuse blue colour developed around the infiltrated sites after histochemical staining with X-Gluc at 21 dpi (data not shown), indicating that m30B expressed sufficient amount of functional Cre recombinase to catalyse the deletion of the *lox*-flanked sequence in tobacco cells. Histochemical GUS staining was then performed on regenerated shoots produced by co-cultivation of leaf discs of the GFP-positive T1 progeny of two independent pGNG-transgenic plant lines (G1 and G4) with agro35S-m30B:Cre. Among 32 regenerated shoots (22 derived from G1 progeny and 10 from G4 progeny), 11 regenerants displayed GUS expression in leaf cross-sections. However, in 8 of the 11 shoots, GUS activity was restricted to some regions of leaf tissues. These shoots were regarded as genetic chimeras as Cre-mediated recombination might occur only in some cells, and therefore not used in further experiments. The other three regenerants, two derived from G1 (G1R1, G1R3) and one from G4 (G4R4), which exhibited a uniform blue colour in all leaf tissues as well as in the petioles and roots (data not shown), appeared to arise from single selectable marker-free cells (Table 1).

To provide molecular evidence for the Cre-mediated recombination in the three plants, total genomic DNA was extracted and assayed by Southern blotting. *Hin*dIII/*Eco*RI-digested DNA was probed with the *gus* coding sequence. The original pGNG T-DNA would give rise to a signal at the size of 4.1 kb. The loss of the *lox*-flanked 1.1-kb region would produce a band of 3 kb (Figure 1(a, b)). As expected, a 3-kb hybridizing fragment was observed in the digested DNA of G1R1, G1R3 and G4R4 (Figure 3, lanes 2, 3 and 6), whereas the parental transgenic lines yielded a 4.1-kb signal (Figure 3, lanes 1 and 5).

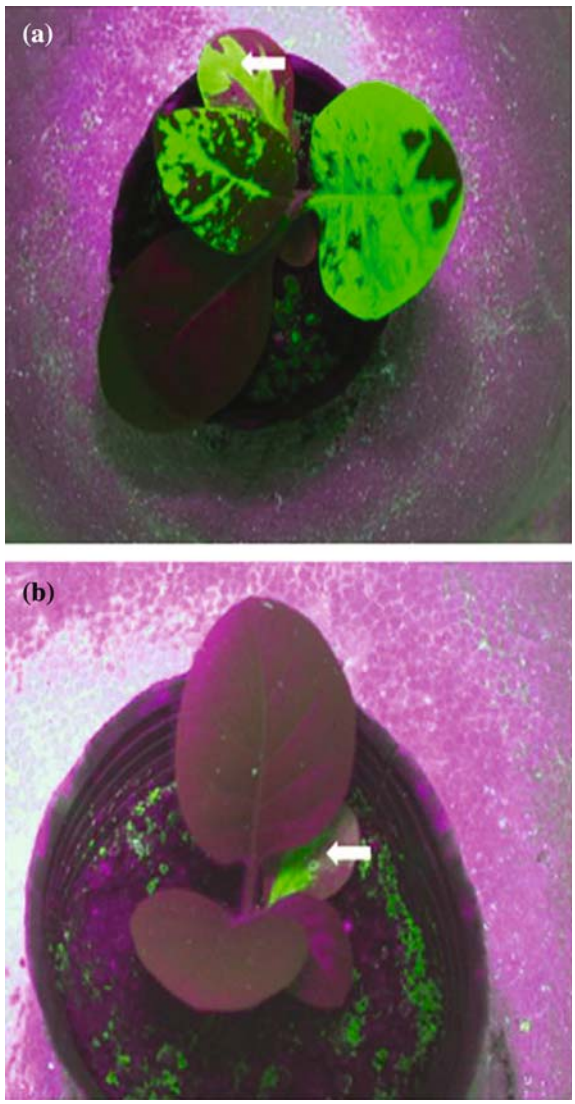

Figure 2. Movement of the virus vector and expression of GFP in systemic leaves of *N. tabacum* cv. SR1 at 21 dpi inoculated with agro35S-m30B:GFP (a) and agro35S-30B:GFP (b). Arrows indicate the agroinoculated leaves.

Table 1. Histochemical GUS assay on regenerated shoots

pGNG-plant line	No. of regenerated shoots		
	Assayed	GUS activity in some tissues	GUS activity in all tissues
G1	22	7	2
G4	10	4	1

Transient expression of Cre recombinase in marker-free plants

To test whether the T-DNA of p35S-m30B:Cre is integrated into the genome of the three marker-free plants, the *Eco*RI-digested plant DNA was analyzed by Southern blotting using the ^{32}P-labeled *cre* sequence as a probe. A 3.8-kb signal would be expected if the T-DNA is inserted into the plant genome (Figure 1(d)). As shown in Figure 4, there was no hybridizing band detected in the genomic DNA of G1R1, G1R3 or G4R4. The results revealed that the *cre* gene in the T-DNA of p35S-m30B:Cre did not integrate into the chromosomes of the three plants and suggest that the excision of the *lox*-flanked region of pGNG T-DNA was caused by transient expression of Cre recombinase from the unintegrated T-DNA of p35S-m30B:Cre.

Moreover, our experimental data revealed that the *cre* gene sequence was only transiently present in the viral molecules generated in the regenerated plants. Total RNA was extracted from G1R1, G1R3 and G4R4, and RT-PCR was performed using *cre*-specific primers CR2 and CR3 to determine whether the *cre* gene sequence is present in the viral genome and the subgenomic RNA. No signal corresponding to 484 bp that would be produced from viral RNA molecules retaining the *cre* sequence was detected (Figure 5, lanes 1–3). By contrast, when primers CP1 and CP2 were used in RT-PCR, a 443-bp band derived from the TMGMV CP gene and its 3′ non-translated region was amplified from all three RNA samples (Figure 5, lanes 7–9). The results indicate that the virus vector expressed from the introduced p35S-m30B:Cre replicated in the regenerated plants and the inserted *cre* sequence was not stably retained in the virus vector.

Together, our results suggest that the excision of the *lox*-flanked sequence occurred in G1R1, G1R3 and G4R4 plants could be attributed to transient expression of Cre recombinase at early stage of co-cultivation with agro35S-m30B:Cre.

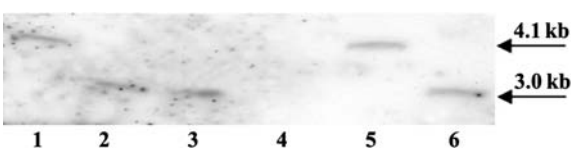

Figure 3. Southern blot analysis of marker-free tobacco transformants. Plant DNAs were digested with *Hin*dIII and *Eco*RI, resolved in 0.8% agarose gel, blotted onto the Hybond N+ membrane and hybridized with a ^{32}P-labeled *gus* probe. Parental pGNG-transgenic lines G1 (lane 1) and G4 (lane 5) yielded a band of 4.1 kb, whereas their marker-free regenerants G1R1 (lane 2), G1R3 (lane 3) and G4R4 (lane 6) gave a band of 3.0 kb. Lane 4, wild-type *N. tabacum* cv. SR1 as a negative control.

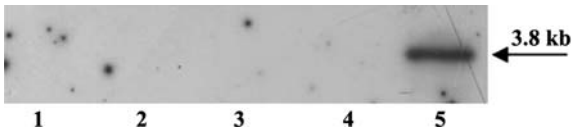

Figure 4. T-DNA of p35S-m30B:Cre is not integrated into the genome of the marker-free plants. Plant DNAs digested with *Eco*RI were subjected to Southern blot analysis essentially as described in the legend of Figure 3 except using *cre* gene as a probe. Lanes 1–3, DNA from G1R1, G1R3 and G4R4, respectively. Lane 4, DNA of wild-type *N. tabacum* cv. SR1. Lane 5, *Eco*RI-digested p35S-m30B:Cre DNA as a positive control which produced a 3.8-kb signal.

Inheritance of the marker-free trait in progeny

The selectable marker-free plants G1R1, G1R3 and G4R4 were self-pollinated. Plant tissues from

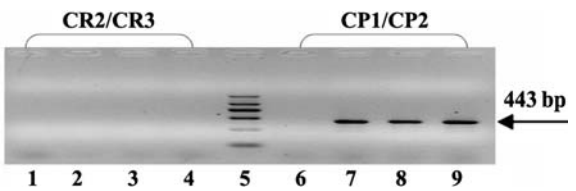

Figure 5. RT-PCR analysis. RT-PCR was carried out with total RNA extracted from regenerated marker-free shoots (G1R1, G1R3 and G4R4) and wild-type *N. tabacum* cv. SR1. RT-PCR primers used for detection of the *cre* gene transcript (CR2/CR3) and the TMGMV CP gene transcript (CP1/CP2) are depicted on top of the picture. Lanes 1 and 7, RT-PCR products from G4R4; lanes 2 and 8, RT-PCR products from G1R3; lanes3 and 9, RT-PCR products from G1R1; lanes 4 and 6, RT-PCR products from wild-type *N. tabacum* cv. SR1 plant; lane 5, DNA ladder (from top to bottom: 1.2, 0.9, 0.7, 0.5, 0.3, 0.1 kb).

their T1 progeny were assayed for GUS expression. The result showed 3:1 GUS positive to negative ratio in their progeny, indicating that the GFP-expressing progeny used in generating G1R1, G1R3 and G4R4 were heterozygous for the transgene locus (data not shown). Two GUS-expressing seedlings from each marker-free plant line were analyzed by PCR amplification to confirm the stable inheritance of Cre-mediated excision. By using one primer located in the 35S promoter and another primer in the GUS gene, a specific 455-bp fragment, indicative of removal of the *lox*-flanked region of pGNG T-DNA, was readily detected in all the T1 progeny as well as in the parental marker-free lines (Figure 6). To show the precise DNA recombination at the *lox* sites, the PCR products were purified from the gel and separately cloned into pGEM-T vector for sequencing. The analyzed sequences of all PCR products were identical and matched to the expected recombined structure (Figure 1c). Furthermore, RT-PCR similar to that in analyzing the TMGMV *cp* sequence in the parental marker-free plants was performed on the T1 seedlings. No amplified product could be observed (data not shown). These results showed that the parental marker-free trait was inherited in their T1 progeny, whereas the virus vector present in the parental lines had not been transmitted to their progeny through plant seeds.

No seeds from self-pollinated G1R1, G1R3 and G4R4 were found to be kanamycin-resistant when T1 seeds were surface-sterilized and germinated on MS medium containing kanamycin (data not shown). In addition, when PCR with *gfp*-specific primers was performed on the T1 seedlings, no amplified product with the expected size was observed (data not shown). The results indicated that the excised *lox*-flanked fragment of pGNG T-DNA was not reintegrated into the plant genome.

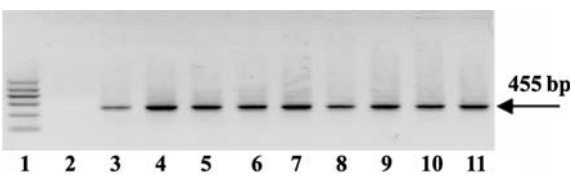

Figure 6. PCR assay showing the inheritance of Cre-mediated excision of the *lox*-flanked sequence in the T1 progeny of G1R1, G1R3 and G4R4. PCR experiments were performed on plant DNAs using primers P1 and P2 that flank the recombined locus. Amplified products all with the expected size (455 bp) were observed in the parental marker-free lines G1R1 (lane 3), G1R3 (lane 6) and G4R4 (lane 9), and their randomly selected GUS-positive T1 progeny (lanes 4 and 5, progeny of G1R1; lanes 7 and 8, progeny of G1R3; lanes 10 and 11, progeny of G4R4). Lane 1, DNA ladder (from top to bottom: 1.2, 0.9, 0.7, 0.5, 0.3, 0.1 kb). Lane 2, wild-type *N. tabacum* cv. SR1 as a negative control.

Discussion

The Cre/*lox* site-specific recombination system of bacteriophage P1 has been widely used to eliminate selection markers from transgenic plants. In earlier studies, *cre* gene was stably transferred into the marker-containing plants by retransformation or sexual crossing. For selection of retransformants or crossing progeny without the *lox*-flanked marker gene, another selectable marker gene needed to be co-introduced with *cre* gene into the plants. Thus at least one step of genetic segregation was required to obtain progeny totally free of selectable marker genes and *cre* gene (Dale & Ow, 1991; Russell et al., 1992). Later on, transient expression of Cre recombinase in tobacco cells from unintegrated T-DNA of co-cultivated agrobacteria was used to directly obtain selectable marker-free plants (Gleave et al., 1999). However, efficiency of this approach was very low (0.25%). By using an ingenious self-excising strategy in which *cre* expression controlled by the β-estradiol-inducible XVE hybrid transactivator led to the loss of a *lox*-flanked region containing the XVE-*cre* transcription unit and an interposed kanamycin-resistant gene, excision of the selectable marker gene from transgenic *Arabidopsis thaliana* plants occurred with an efficiency between 29 and 66% after treatment with the inducer (Zuo et al., 2001).

Plant RNA Viruses-based vectors provide temporary, transient expression system since the foreign genes delivered by viruses into the infected plant cell and amplified by viral replicase do not integrate into the plant genome (Koprowski & Yusibov, 2001). Moreover, rod-shaped Viruses do not have severe constraints on the size of foreign sequences inserted into the viral genome (Hull, 2002). Therefore, these viral vectors should be suitable for transient expression of Cre recombinase to eliminate the *lox*-flanked selectable marker gene from transgenic plants. Indeed, marker-excision in

transgenic *N. benthamiana* plants by a PVX vector expressing functional Cre was recently reported (Kopertekh et al., 2004b). Concurrently, we have carried out a marker-elimination study using another rod-shaped RNA Virus, TMV, as described in this paper. In comparison to the PVX-Cre system, our TMV-Cre method has several distinctive features. First, TMV has a wider host range than PVX (Brunt et al., 1996), which means that the TMV-Cre vector could be applied to more susceptible plant species for marker elimination. Second, by modification of the MP gene of the original TMV vector 30B, a movement function-enhanced vector m30B was created (Figure 2) and employed to transiently express Cre to remove the selectable marker gene from *lox*-target *N. tabacum* cv. SR1 plants. Although the PVX cDNA vector could express an inserted GUS gene in *N. tabacum* cv. Samsun NN (Chapman et al., 1992), PVX-Cre-mediated marker excision was only shown in *N. benthamiana* (Kopertekh et al., 2004b), a wild tobacco species generally used as a model plant in laboratories. Third, agroinoculation was used to deliver m30B:Cre into pGNG-transgenic plants, bypassing the process of purification of the plasmid DNA carrying the virus expression vectors, which was included in preparation of the PVX-Cre inoculum. Finally, the marker-excision efficiency (9.4%) achieved by transient expression of Cre from agro35S-m30B:Cre is much higher than that from the unintegrated T-DNA harbouring a 35S promoter-driven *cre* (Gleave et al., 1999), suggesting a role of the virus vector in expression or spreading of Cre in tobacco cells. However, it should be pointed out that our method relied on agroinoculation to deliver the 35S-m30B:Cre into pGNG-plants and thus has an intrinsic drawback that the T-DNA of p35S-m30B:Cre could be stably integrated into some of the selectable marker-free tobacco plants. In fact, Gleave et al. (1999) found a large fraction (4 out of 6) marker-free plants contained the integrated Cre gene. We also could not rule out the possibility that the T-DNA region of p35S-m30B:Cre is integrated into the genome of the remaining eight GUS-expressing plants (Table 1). In this context, direct introduction of the 35S-m30B:Cre DNA (without the T-DNA borders) into pGNG-plants could be a potential approach to eliminate the drawback of the *Agrobacterium*-mediated TMV-Cre expression. Although manual inoculation with 35S-TMV cDNA clones failed to establish an infection in several cultivars of *N. tabacum* including SR1 (Dagless et al., 1997; our unpublished data), other direct DNA transfer methods are worthy to be tried.

We have shown that the *cre* gene of p35S-m30B:Cre is not integrated into the plant genome of regenerated marker-free plants (Figure 4). Furthermore, the *cre* sequence was no longer present in viral RNA molecules extracted from the rooted regenerated tobacco plants (Figure 5). This result is consistent with the observation that the inserted GFP gene tends to be deleted from the 30B-GFP vector in systemic leaves of infected *N. tabacum* cv. Xanthi plants (Rabindran & Dawson, 2001), but contrary to that observed in PVX-Cre-infected *N. benthamiana* plants where the *cre* sequence was retained in the virus vector and expressed at high levels as detected by Western blot analysis (Kopertakh et al., 2004b). Nevertheless, transient expression of Cre from m30B:Cre derived from the unintegrated T-DNA of p35S-m30B:Cre at early stage of co-cultivation should provide sufficient recombinase activity for inducing excision of the *lox*-flanked sequence in pGNG-plants.

Although all of regenerated marker-free tobacco plants showed mild symptoms caused by the virus vector, they were able to set seeds. As expected, the progeny inherited the marker-free genotype from the parental plants (Figure 6), and proved to be virus-free. Since TMV is not transmitted through seed, it is dispensable to take further measures to clean the virus vector from the regenerated shoots.

In conclusion, the work described here and the previous report (Kopertekh et al., 2004b) together demonstrate that plant virus vectors could be used to express functional Cre recombinase and provide a feasible way to remove the selectable marker genes from compatible host plants.

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