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ORIGINAL PAPER

Auto-excision of selectable marker genes from transgenic tobacco via a stress inducible FLP/FRT site-specific recombination system

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Abstract Antibiotic resistance marker genes are powerful selection tools for use in plant transformation processes. However, once transformation is accomplished, the presence of these resistance genes is no longer necessary and can even be undesirable. We herein describe the successful excision of antibiotic resistance genes from transgenic plants via the use of an oxidative stress-inducible *FLP* gene. *FLP* encodes a recombinase that can eliminate *FLP* and *hpt* selection genes flanked by two *FRT* sites. During a transformation procedure in tobacco, transformants were obtained by selection on hygromycin media. Regenerants of the initial transformants were screened for selective marker excision in hydrogen peroxide supplemented media and both the *FLP* and *hpt* genes were found to have been eliminated. About 13–41% of regenerated shoots on hydrogen peroxide media were marker-free. This auto-excision system, mediated by the oxidative stress-inducible FLP/*FRT* system to eliminate a selectable marker gene can be very readily adopted and used to efficiently generate marker-free transgenic plants.

Keywords Auto-excision · FLP/FRT recombination system · Marker-free · Oxidative stress-inducible · Transgenic tobacco

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Introduction

The use of selectable marker gene systems facilitates the transformation process in plants. But the resistance gene is not generally required for the subsequent expression of the trait genes of interest in the transgenic lines. Moreover, the presence of a selectable marker gene can raise regulatory issues. A number of strategies for the removal of these markers after selection have already been applied such as T-DNA mediated segregation between selectable marker genes and target genes of interest (Depicker et al. 1985; De Block and Debrouwer 1991; Komari et al. 1996; Daley et al. 1998; Xing et al. 2000; McCormac et al. 2001), homologous recombination between direct repeats (Zubko et al. 2000; Puchta 2000) and the use of site-specific recombinases (Sugita et al. 2000; Zuo et al. 2001).

Several site-specific recombination systems from bacteriophages and yeast have been well characterized,

including Cre/*lox* from bacteriophage P1 (Dale and Ow 1990, 1991; Odell et al. 1990; Russell et al. 1992; Zuo et al. 2001; Hoa et al. 2002), FLP/*FRT* from *Saccharomyces cerevisiae* (Lloyd and Davis 1994; Kilby et al. 1995; Luo et al. 2000), R/*RS* from *Zygosaccharomyces rouxii* (Onouchi et al. 1995; Sugita et al. 2000) and Gin/*gix* from the bacteriophage Mu (Maeser and Kahmann 1991). These simple site-specific recombination systems all consist of two basic components, a recombination enzyme and small DNA recognition sites. In addition, they can be grouped according to the placement of the recombinase gene, either on a vector different from the one containing the transgene and the selectable marker, or on the same vector between the recombination sites. In the first strategy, the recombinase gene can be delivered to a transgenic plant by re-transformation and segregated from the trait locus in the next generation following a sexual cross (Odell et al. 1990, Dale and Ow 1991; Lyznik et al. 1996). In the second strategy, often referred to as "auto-excision", expression of the recombinase gene is regulated by chemicals (Sugita et al. 2000; Zuo et al. 2001; Schaart et al. 2004; Sreekala et al. 2005; Zhang et al. 2006, 2003) or environmental factors such as temperature (Kilby et al. 1995; Hoff et al. 2001; Wang et al. 2005; Cuellar et al. 2006) when the marker gene is no longer required. However, there are drawbacks for both of these possible approaches. Re-transformation is quite laborious and time-consuming, whilst the auto-excision requires at least one additional step to activate the recombinase.

In our present study, we describe the construction of a binary expression vector in which an oxidative stress-inducible promoter (peroxidase (POD) promoter) is used to control the FLP/*FRT* recombination system. In a previous report, Kim et al. (2003) showed that this promoter is strongly induced in response to environmental stresses including hydrogen peroxide and wounding, but is not expressed in any tissues in differentiated plants. Using our constructed binary expression vector in transgenic tobacco plants, we report the production of segregated and marker free transformants in the T_1 progeny without chemical induction and also, the successful excision of selectable marker genes via hydrogen peroxide-regulated regeneration.

Materials and methods

Plasmid construction

Molecular manipulation methods, such as plasmid DNA isolation, restriction enzyme analysis, ligation of DNA fragments and transformation of *Escherichia coli*, were performed as described previously by Sambrook et al. (2001). The pHPFB vector was constructed as follows: to generate pBluescript-35S-*FRTm-hpt*, the pBluescript-35S plasmid was first constructed by isolating the 852 bp *Spe*I/*Bam*HI fragment (CaMV 35S promoter) from pBI121 (Clontech, Paro Alto, CA). This fragment was then subcloned into the *Spe*I/*BamH*I sites of pBluescript II KS (Stratagene, La Jolla, CA). A 1,272 bp *Hin*dIII/*Cla*I fragment of *hpt* containing the polyA signal from pCAMBIA1304 (CAMBIA, Canberra, Australia) was next cloned into the pBluescript-35S plasmid via the *Hin*dIII-*Cla*I sites to yield pBluescript-35S-*hpt*. Subsequently, a modified *FRT* (*FRTm*) site (Lyznik et al. 1996) was introduced between the 35S and *hpt* sites. Briefly, the *FRTm* sequences were synthesized with *Bam*HI compatible cohesive-end sequences (5′-GATCATTCCGAAGTTCCTATTCTCTAGAAAGTATAGGAACTTC-3′) and *Hin*dIII compatible cohesive end sequences (5′-AGCTGAAGTTCCTATACTTTCTAGAGAATAGGAACTTCGGAAT-3′) at both ends. The *FRTm* site was then ligated into the *Bam*HI–*Hin*dIII site of pBluescript-35S-*hpt* to generate in pBluescript-35S-*FRTm-hpt*.

To construct the Ppod-*FLP*-T*nos* cassette, the *FLP*-coding region was amplified by PCR from the pOG44 plasmid (Invitrogen, Carlsbad, CA) with the following-primers incorporating the *Bam*HI and *Sac*I sites (forward primer 5′-ATGGATCCCCACCATGCCACAATTTGAT-3′ and reverse primer 5′-ATGAGCTCGCGTGTTTATGCTTAAATGCG-3′). The *Bam*HI/*Sac*I *FLP* fragment of 1,317 bp was cloned upstream of the terminator site in pBI121. The full length POD promoter of 1.8 kb in length was cloned from sweet potato as reported previously by Kim et al. (2003). A 1.2 kb fragment of the POD promoter was amplified by PCR from sweet potato with primers harboring *Cla*I and *Bam*HI site (forward specific primer 5′-CCATGATCAGATCGATAATACCA-3′ and reverse primer 5′-CGGGATCCGGTCAAAGGAAAATGTAAGATTAAG-3′).

The *Cla*I/*Bam*HI POD promoter was cloned into the corresponding sites of the pBI121-*FLP* plasmid. To generate pBluescript-*FRT-bar*-T_{35S}, a 790 bp fragment containing the bar gene and CaMV 35S terminator sequence was amplified by PCR from pCAMBIA 3301 (CAMBIA, Canberra, Australia) with primers containing an *Spe*I and *Xba*I site (forward primer 5′-GGACTAGTCCATGAGCCCA GAACGACGCCCGG-3′ and reverse primer 5′-G GTCTAGAATTCCGCCGAATTAATTCGGGGGA TCTGG-3′). This *bar* fragment was then cloned into the *Spe*I/*Xba*I sites of pBluescript II KS. Another *FRT* sequence was synthesized with *Xma*I compatible cohesive end sequences (5′-CCGGGAAGTTCCTA TTCCGAAGTTCCTATTCTCTAGAAAGTATAGG AACTTC-3′) and *Spe*I compatible cohesive end sequences (5′-CTAGGAAGTTCCTATACTTTCTA AGAATAGGAACTTCGGAATAGGAACTTC-3′) at both ends. This fragment was ligated into the *Xma*I/*Spe*I site of pBluescript-bar-T_{35S} and subsequently excised with *Sma*I and *Sac*I, for subcloning into pBI121. The *Spe*I/*Cla*I fragment containing the 35S-*FRTm-hpt* gene was excised from pBluescript-35S-*FRTm-hpt*, and ligated into the *Spe*I/*Cla*I sites of p35S-*FRTm-hpt*. The Ppod-*FLP*-Tnos cassette was excised from this construct with *Cla*I and *Bam*HI, and then inserted into the same restriction sites of p35S-*FRTm-hpt-FRT-bar* to yield pHPFB. A schematic of pHPFB is shown in Fig. 1.

Transformation of tobacco

The *Agrobacterium tumefaciens* strain LBA4404 harboring pHPFB was used to transform tobacco plants (*Nicotiana tabacum* cv. Xanthi) via the leaf disk method (Horsch et al. 1985). Murashige-Skoog (MS) medium containing 2% (w/v) sucrose, 0.8% (w/v) agar, 1 mg/l 6-benzyladenine (6-BA), 0.1 mg/l naphthaleneacetic acid (NAA), 500 mg/l carbenicillin (Cb) and 10 mg/l hygromycin was used for shoot differentiation. Hygromycin resistant shoots were directly formed on the cut edges of leaf discs. Rooting of hygromycin-resistant plantlets was carried out in selectable MS medium without growth regulators. After acclimation, the plants were transplanted to potting soil and maintained in a greenhouse.

PCR analysis and DNA sequencing of transgenic tobacco

Total genomic DNA was isolated from regenerated plants by the CTAB method as described previously (Murray and Thompson 1980). The presence of transgene inserts was determined by PCR with the primer sets 35F/HR and 35F/BR. The 35F primer corresponds to a region of the CaMV 35S promoter gene, whereas the HR and BR primers recognize the *hpt* and *bar* genes, respectively (Fig. 1).

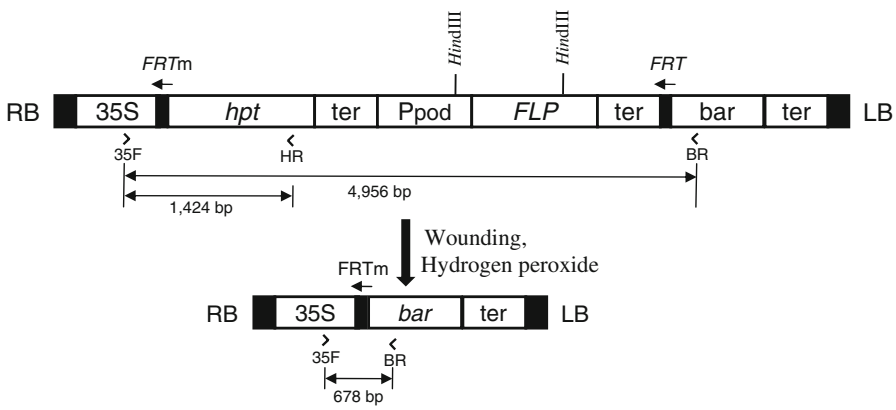

Fig. 1 Schematic diagram of the T-DNA region of pHPFB and an FLP/FRT-mediated DNA recombination event. The regions flanked by *FRTm* and the *FRT* in the *upper diagram* are a modified and full-length *FRT* fragment. 35S, 35S CaMV gene promoter; Ppod, stress-inducible peroxidase gene promoter; *hpt*, hygromycin phosphotransferase gene; *FLP*, FLP: recombinase gene from *Saccharomyces cerevisiae*; *bar*, phosphinothricin acetyltransferase gene; *FRT*, FLP: recognition site; ter, terminator; RB, right border of T-DNA; LB, left border of T-DNA; The cleavage sites for restriction enzyme *Hin*dIII are indicated, as are the recognition sites of the 35F/HR/BR primers used for PCR analysis

The primers used to amplify the P35S-*hpt* gene were the 35F/HR set (5′-GGACCTAACAGAACT CGCCGTAAAGAC-3′ and 5′-CCGTCAACCAAG CTCTGATAGAGTTG-3′). The predicted size of the amplified PCR product is 1,424 bp. The PCR product amplified with the 35F/BR primer set (BR; 5′-GAAGTTGACCGTGCTTGTCTCGATG-3′) would be 678 bp in size if excision had occurred but 5 kb otherwise. PCR reactions were performed using standard cycling conditions of 10 min pre-denaturation at 94°C, and then 30 cycles of 30 s denaturation, 30 s annealing and 1 min extension at 94, 60 and 72°C, for 30 cycles, respectively. The PCR products to be used for sequencing analysis were separated, purified on agarose gels and then eluted. DNA sequences were determined by dye terminator cycle sequencing using an Applied Biosystem 377 DNA sequencer (Perkin-Elmer Corp, Foster City, CA). Sequencing in both directions was performed.

Seed germination assay

T_1 progeny seeds were harvested, surface-sterilized for 15 min in 1.5% sodium hypochloride solution, and rinsed thoroughly (3–4 times) in sterile water. Surface-sterilized seeds were grown on germination medium (MS medium 2% (w/v) sucrose and 0.7% (w/v) agar) containing phosphinothricin (10 mg/l) or hygromycin (30 mg/l). Three weeks later, seedlings grown on the phosphinothricin (ppt) or hygromycin-containing MS media were evaluated for resistance.

RT-PCR analysis of gene expression driven by the POD promoter

To examine *POD* promoter activity in response to H_2O_2, the callus derived from the leaf of transgenic tobacco was sub-cultured on MS medium containing either 0, 1 or 5 mM of H_2O_2 for 1 week. Total RNA from the callus cells was extracted from the powdered tissue using RNeasy Mini Kit and on-column DNase treatment according to the manufacturer's protocol (Qiagen, Valencia, CA). Two μg of total RNA was reverse transcribed using SuperScript^{TM}II Reverse Transcriptase (Invitrogen, Carlsbad, CA) with oligo$(dt)_{20}$. PCR amplifications was carried out for 19–26 cycles, using 100 ng of cDNA and gene-specific primers. The primers used to amplify *FLP*, *bar* and *hpt* are as follows: *FLP* (5′-CGGAACAGC AATCAAGAGAGCCAC-3′ and 5′-CGAGAAACTA GTGCGAAGTAGTGATCAGG-3′), *bar* (5′-ATGG GCCCAGAACGACGCC-3′ and 5′-CATGCCAGTT CCCGTGCTTGAA- 3′) and *hpt* (5′-CTGATCGAAA AGTTCGACAGCGTCT-3′ and 5′-CCGTCAACCA AGCTCTGATAGAGTTG-3′). Ubiquitin rRNA was amplified as an endogenous normalization control. PCR was performed under the following conditions: 10 min at 94°C; followed by 19–26 cycles of 30 s at 94°C, 30 s at 60°C, 1 min at 72°C and a final extension at 72°C for 10 min.

Regeneration of transgenic tobacco

Leaves taken from transgenic tobacco lines were excised squarely in 0.5 × 0.5 cm pieces and placed on MS medium supplemented with 5 mM H_2O_2, 2% sucrose, 0.7% agar, 1 mg/l BAP, 0.1 mg/l NAA and 500 mg/l carbenicillin. To set seeds, rooted plants were transferred to soil.

Southern blotting

Genomic DNA was isolated from the leaf tissues of regenerated plants and 15 μg aliquots was restricted with *Hin*dIII, resolved in 0.8% agarose gel and transferred to a Hybond N^+ nylon membrane (Amersham, Piscataway, NJ). Blots were hybridized with *hpt* and *bar* probes and detected with [α-^{32}P] dCTP (Dupont-New England Nuclear, Boston, MA).

Results

Construction and characteristics of pHPFB

The region between the left and right T-DNA borders in the binary vector pHPFB is depicted schematically in Fig. 1, in which the hygromycin-selectable marker gene (*hpt*) and the recombinase gene *FLP* under the control of oxidative stress-inducible promoter of sweet potato were placed between two opposite *FRT* sites. We used one modified *FRT* site (*FRT*m) and one full-length *FRT* site. Compared with the full-length FRT sequence with three FLP binding sites, the short 39 bp *FRT*m site contains only two symmetry elements for the FLP protein. Importantly, this modification does not substantially affect the function of the FLP recombinase. However, in

transient expression assays, structurally different *FRT* sites provided higher yields of FLP-catalyzed DNA excision products when one full-length and one modified *FRT* site are used as compared with two full-length *FRT* sites (Lyznik et al. 1993, 1996). This system also provides a means to distinguish precise site-specific recombination from spontaneous recombination. In the stress-inducible FLP/*FRT* DNA recombination system, the *hpt* and *FLP* genes will be co-eliminated once the *FLP* gene is induced by stress treatment, resulting in the loss of the hygromycin resistance marker and generation of the phosphinothricin resistant transgenic plants.

Tobacco transformation and identification of transgenes

Tobacco was transformed using the *Agrobacterium* mediated method. Forty lines transformed by pHPFB were then obtained by hygromycin selection. To validate the transgenic plants, 12/40 lines were randomly chosen for molecular analysis. The presence of the introduced gene was assessed by PCR with the gene specific primer sets 35F/HR and 35F/BR, respectively. The results shown in Fig. 2 reveal that a 1,424 bp P35S-*hpt* fragment was detectable in all transgenic plants, but also that 678 bp. The explanation for this is that these transformed plants are chimeric with excised and non-excised T-DNA loci coexisting in the same cells or tissues. Amplification of the full *hpt-FLP* cassette with the 35F/BR primer set did not result in a readily detectable band presumably due to its large size (4,956 bp).

Heritability analysis of T_1 transgenic tobacco plants

Ten of the transgenic lines chosen for PCR analysis were further subjected to heritability analysis. As shown in Table 1, the segregation ratio of the hygromycin gene (*hpt*) was 3:1 in five transgenic plants, and 15:1 in a further four transgenic lines. In addition, the segregation ratio of the *bar* gene in chimeric transgenic plants was also about 3:1 in four lines in which post-excision fragments were detected by PCR. To confirm this independent segregation, T_1 progeny of chimeric transgenic line 1 showing growth retardation were screened on MS media containing hygromycin and the progeny were transferred on MS medium containing phosphinothricin. Among transferred plants, 15% (6 out of 40 seedlings) progeny from line 1 T_0 plants showed ppt-resistance and almost of the T_1 progeny showed ppt resistance had no detectable *hpt* gene in PCR analysis (data not shown). These results may indicate that four T_0 transgenic plants with hpt and ppt resistance might be chimeric transgenic plants comprised of excised and non-excised cells resulting from stress stimuli during the transformation procedure. Nevertheless, a recombined marker excision locus and non-excised gene locus were inherited independently, and as a consequence produced marker free transgenic offspring.

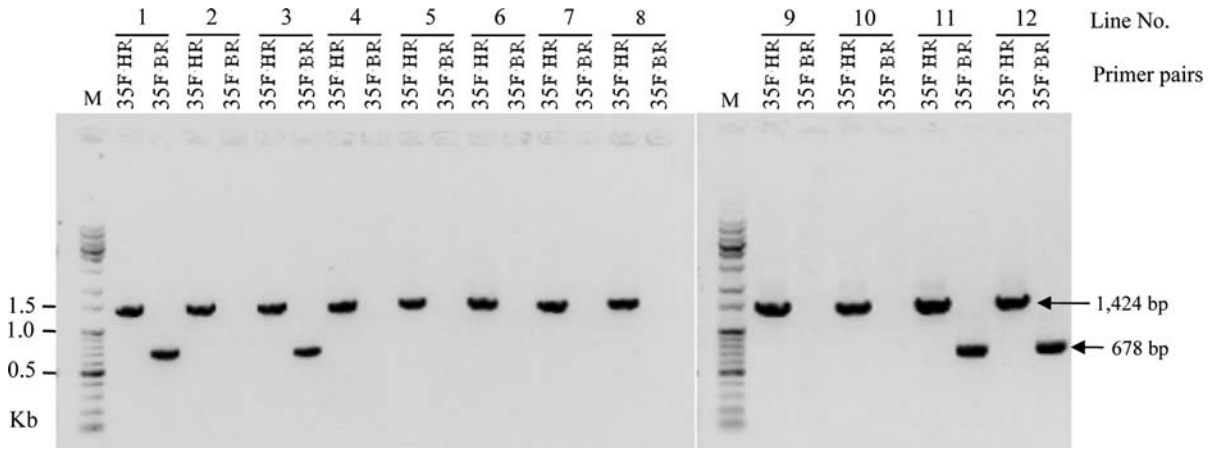

Fig. 2 PCR analysis of genomic DNA prepared from transgenic T_0 plants. *Line numbers* and *primer pairs* used for each PCR reaction are indicated on the *top* of each lane. M, molecular markers

Table 1 Segregation of the *bar* and *hpt* genes in T1 seeds of primary transgenic tobacco plants

Line no.	*bar* (PPT[a] resistance)		Expected ratio	Chi-square	*P* value	*hpt* (hygro-resistance)		Expected ratio	Chi-square	*P* value
	+	−				+	−			
1	59	18	3:1	0.11	0.74	112	37	3:1	0.00	0.96
2	0	89	–	–	–	39	18	3:1	1.32	0.25
3	64	22	3:1	0.02	0.90	94	8	15:1	0.21	0.64
4	0	94	–	–	–	72	26	3:1	0.12	0.73
5	0	47	–	–	–	48	6	15:1	2.19	0.14
6	0	125	–	–	–	117	9	15:1	0.17	0.68
9	0	90	–	–	–	89	29	3:1	0.01	0.92
10	0	128	–	–	–	111	27	3:1	2.17	0.14
11	55	49	3:1	–	NS	108	3	63:1	0.95	0.33
12	78	20	3:1	1.10	0.29	78	3	15:1	0.90	0.34

NS, not statistically significant

[a] PPT, phosphinothricin

Excision analysis in hydrogen peroxide treated leaves of primary transgenic tobacco plants

In a previous study, the strong induction of the peroxidase (POD) promoter in response to several environmental stresses, including hydrogen peroxide, has been reported (Kim et al. 2003). To determine the POD promoter activity levels in tobacco plants in response to different hydrogen peroxide concentrations, we employed RT-PCR to examine the expression of the *FLP* gene in the callus. Leaves from two selected lines (chimeric transgenic plant 3 and transgenic plant 9; Table 1) were placed in MS medium supplemented with various concentration of hydrogen peroxide and regenerated calli were successfully obtained at the 1 and 5 mM levels. Total RNA was extracted from the calli grown under different hydrogen peroxide concentrations to analyze gene expression by RT-PCR. The overall pattern of expression for *FLP* and *bar* following hydrogen peroxide exposure showed a steady upregulation whereas the expression of *hpt* was decreased under these conditions. This demonstrated that the POD promoter was activated by hydrogen peroxide and that FLP/*FRT*-mediated marker gene excision occurred more strongly in the callus following hydrogen peroxide treatment (Fig. 3).

Based on our RT-PCR data, we regenerated some transgenic plants from line 9 in MS medium containing 5 mM hydrogen peroxide and analyzed

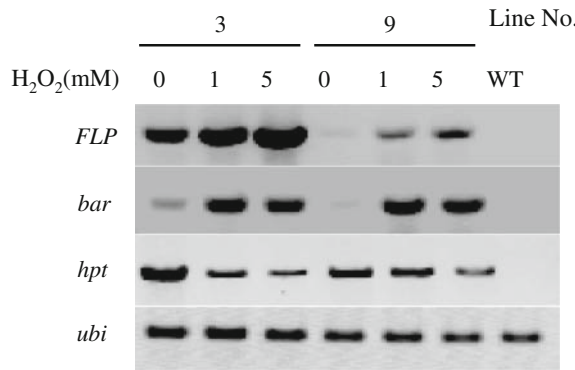

Fig. 3 RT-PCR analysis of *FLP* and *bar* mediated by the peroxidase promoter in calli derived from the leaves of transgenic tobacco plants. Two micrograms of total RNA from hydrogen peroxide treated calli was used for PCR amplification. Tobacco ubiquitin rRNA was used as a normalization. The transgenic line numbers for each callus and concentration of H_2O_2 used for RT-PCR are indicated on the *top* of each lane. WT, wild type tobacco

their excision status by PCR using the 35F/BR and 35F/HR primer sets (Fig. 4). These analyses indicated the presence of the expected 678 bp P35S-*Bar* fragment and the absence of *hpt* gene in regenerated explants. The excision efficiencies of transgenic lines 9 and 10 in hydrogen peroxide treated medium were 13% (8 out of 62 regenerated shoots) and 41% (29 out of 70 regenerated shoots). These differences may correlate with integration position of the transferred gene in the tobacco genome as have been shown to

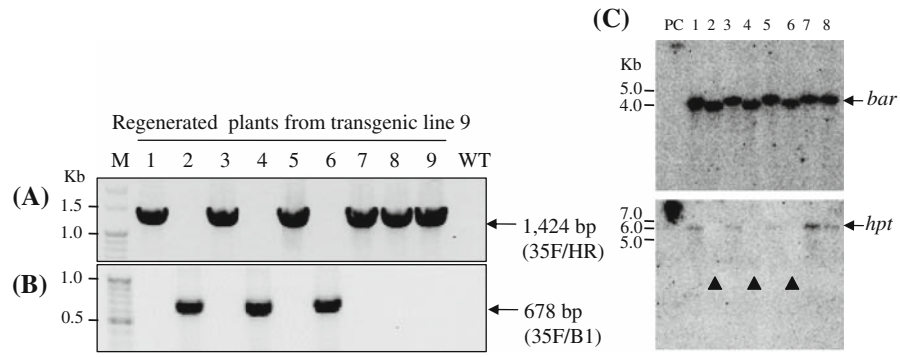

Fig. 4 PCR and southern blot analysis of the elimination of the *hpt* gene in transgenic plants after regeneration with hydrogen peroxide. **A** PCR results with the primer pair 35F/HR. **B** PCR results with the primer pair 35F/BR. M, molecular markers; WT, wild type tobacco. **C** Southern blotting analysis of genomic DNA from regenerated transgenic plants. Genomic DNA was digested with *Hin*dIII, and hybridized with *bar* (*upper*) and *hpt* (*under*) gene probes. PC, pHPFB plasmid; ▲, three regenerated marker-free plants exhibited no signal

integrate both near to and far from transcriptional activating elements, and also into transcriptionally silent regions (Gelvin 2003).

To validated these PCR data, eight plants regenerated from transgenic line 9 were subjected to southern hybridization analysis using *hpt* and *bar* fragments as probes (Fig. 4C). All of these regenerants showed the expected band with the *bar* probe, as the segregation ratio of the *hpt* gene was 3:1, whilst three plants in which marker excision occurred exhibited a smaller band compared with plants that retained *hpt*. In the case of hybridizations with the *hpt* probe, none of the three regenerated marker-free plants exhibited a signal, thus confirming a complete excision event.

To further validate that DNA excision had occurred in transgenic plants, the 678 bp PCR fragments generated from three marker-free plants were sequenced, As shown in Fig. 5, the region between the two *FRT* sites was removed completely and precisely, with only one *FRT*m site left, whilst the flanking regions outside of the two *FRT* sites remained intact.

Hygromycin and phosphinothricin resistance of T_1 marker-free plants

We determined the hygromycin resistance of four marker-free transgenic tobacco lines. The four independent transgenic lines confirmed above, in which the *hpt* gene was excised and the *bar* gene was expressed after H_2O_2 treatment, was chosen for resistance analysis. T_1 tobacco seeds derived from regenerated marker-free tobacco plants that had been selfed were sown on hygromycin and phosphinothricin supplemented medium, respectively. As shown in Fig. 6, all seeds of marker-free regenerated tobacco grew on phosphinothricin supplemented medium but

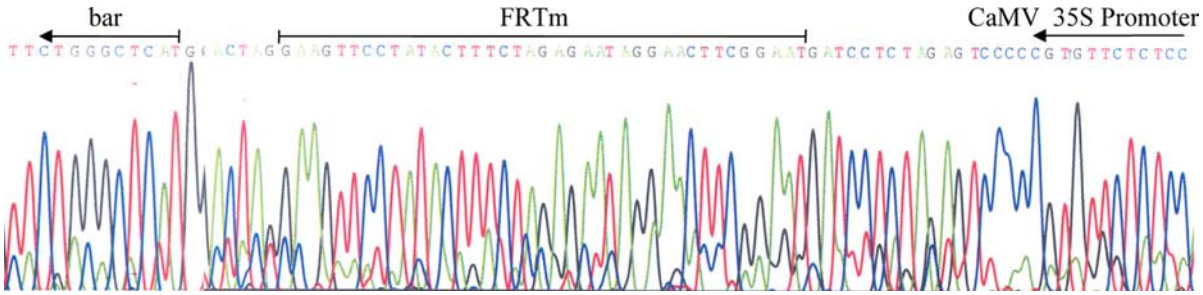

Fig. 5 Sequence analysis to confirm marker gene auto-excision in the genome of transgenic lines. The 678 bp fragments amplified using the primers 35S and BR from four marker-free plants were sequenced. The results indicate that all sequences between the two *FRT* sites were successfully removed

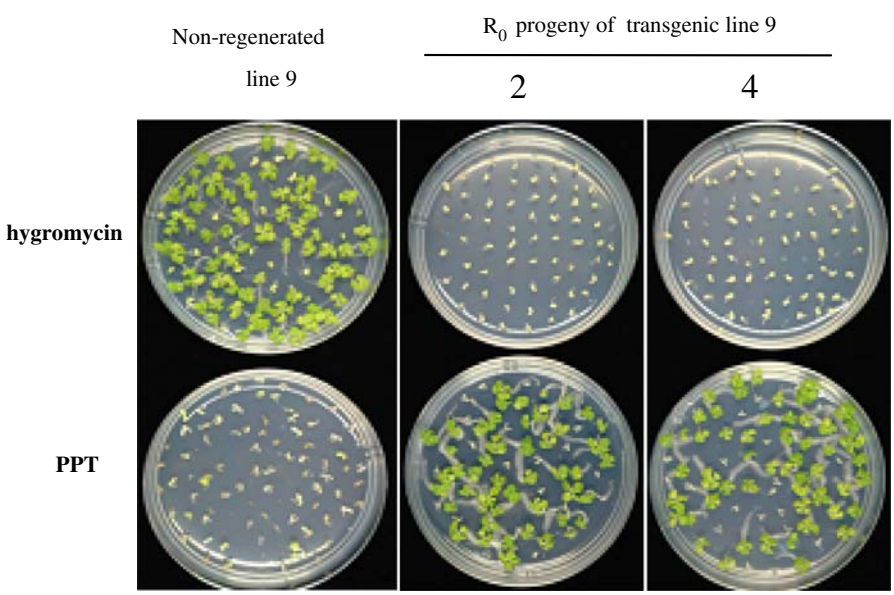

Fig. 6 Hygromycin and phosphinothricin resistance of T_1 progeny derived from regenerated transgenic tobacco plant line 9. Seeds of the primary plants and regenerated marker-free plants derived from this line were sown on hygromycin and phosphinothricin supplemented medium, respectively

not on hygromycin supplemented medium, indicating that the antibiotic gene, *hpt*, was excised successfully by the FLP/*FRT*-mediated marker gene excision system.

Discussion

We successfully obtained marker-free transgenic tobacco plants following hydrogen peroxide treatment of leaves of primary transgenic tobacco plants placed on regeneration media. The auto-excision rate of marker genes by hydrogen peroxide treatment was determined to be 13–41% of regenerated shoots. Marker-free plants were also obtained in the T_1 progeny without chemical induction. This spontaneous excision presumably results from expression of the POD promoter in transgenic cells during the transformation process and subsequent growth and development of T_0 plants. About 15% of marker-free seedlings were obtained amongst the 40 T_1 progenies derived from line 1 T_0 plant. Compared with the approaches used for inducible auto-excision, the XVE system used to eliminate selection markers in *Arabidopsis* showed a 22–66% frequency of marker-excision (Zuo et al. 2001). However the same system was less efficient when used in rice, possibly due to inducer inaccessibility or instability (Sreekala et al. 2005). Using a heat shock inducible recombination system, marker-excision efficiencies in regenerated tobacco exceeded 70% (Wang et al. 2005). However, when *Cre* recombinase was driven by a heat shock promoter, recombinase-mediated marker gene excision occurred in transgenic plants and the bacteria even without heat shock treatment. This may indicate potentially serious problems with maintenance of plasmid and transformation efficiency (Foster et al. 2003; Wu et al. 2001; Wang et al. 2005). In our FLP/*FRT* auto-excision system, the auto-excision rate of marker genes by hydrogen peroxide treatment was determined to be 13–41% of regenerated shoots, showing less efficient. However, this system provides an easier way to eliminate selection markers compare to other systems.

The oxidative stress-inducible POD promoter from sweet potato was used to control the expression of the *FLP* gene in this experiment. Calli formed from the wounded parts of explants in our plant transformation procedure have a high potential to produce various antioxidant compounds to overcome the oxidative stress derived by wounding. Thus, to achieve an easily controlled and also highly efficient marker gene auto-excision system, we constructed a novel FLP/*FRT* auto-excision system in which the *FLP* recombinase gene was placed under the control of oxidative stress-inducible POD promoter. In previous reports, the POD promoter from sweet potato was found not to be expressed in any tissues in differentiated plants, but was strongly expressed and induced by environmental stress conditions including

hydrogen peroxide and wounding in cultured cells of the sweet potato (Kim et al. 1999). In addition, the induction of the POD promoter by environmental stresses has been confirmed in a variety of transgenic plants such as potato, rice, ginseng and tall fescue (Tang et al. 2008; Lian et al. 2004; Kwon et al. 2003; Lee et al. 2007). Hence, one of the advantages of an oxidative stress-inducible auto-excision system is the ability to regulate the recombinase gene using various types of stress, such as wounding and hydrogen peroxide exposure at the callus stage. Another advantage of this system is that it could be a potentially useful tool in various crops including vegetatively propagated plants like potato.

In our present experiments, if 35S-*hpt* was detected in T_0 non-excised transgenic plant, no band for the 35S-*bar* could be amplified by PCR when a forward primer (35F) from 35S promoter sequence and a reverse primer (BR) from the bar coding sequence were used. However, in four T_0 plants out of 12 transgenic tobacco lines that we generated, we could observe both 35S-*hpt* and 35S-*bar* amplification. In addition, these T_0 plants could produce marker-free transgenic segregants with functional recombinant T-DNAs in subsequent generations. This phenomenon had also been previously reported for rice transformations using an XVE system (Sreekala et al. 2005). Problems associated with chimerism were also reported for strawberry transformations using a Cre/*lox* based site-specific recombination system (Schaart et al. 2004). Nevertheless, the control of recombination and excision by inducible promoters directing recombinase expression could provide an alternative solution to the problems of chimerism and inefficient excision (Endo et al. 2002; Ballester et al. 2007).

Generally speaking, there are three strategies used to eliminate marker genes via site-specific recombinase-based systems: re-transformation, cross-pollination and auto-excision. All of these methods have some disadvantages, however. In the case of re-transformation, despite having the highest frequency of marker-excision among the three approaches, a recombinase gene with a new marker gene will be introduced in the second round of transformation, and must be eliminated through segregation and selection in subsequent generations. Likewise, the cross-pollination method requires the selection of transgenic plants that contain only the desired gene without any marker gene in the F_2 population. Although the frequency of marker-excision by auto-excision is lower than that obtained by first two strategies, auto-excision has been widely used with inducible promoters because this is less labor intensive and time consuming. With regard to this efficiency of auto-excision, a GM-gene-deletor based on the Cre/*loxP* and FLP/*FRT* site-specific recombination system has recently been developed (Luo et al. 2007). In this system, the combined use of *loxP* and *FRT* as the recognition sequences for FLP or Cre recombinase was adopted to enhance the efficiencies of these recombinases. The utilization of combined recognition sites in a stress-inducible recombination system might thus improve the efficiency of marker-excision.

In conclusion, selective marker genes can be eliminated efficiently using an FLP/*FRT* recombination system under the control of the oxidative-stress inducible POD promoter. Hence, this oxidative-stress auto-excision strategy provides a novel and useful tool to produce marker-free transgenic plants.

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