ORIGINAL RESEARCH PAPER

Excision of selectable marker gene from transgenic tobacco using the GM-gene-deletor system regulated by a heat-inducible promoter

Keming Luo · Min Sun · Wei Deng · Shan Xu

Abstract To excise a selectable marker gene from transgenic plants, a new binary expression vector based on the 'genetically modified (GM)-gene-deletor' system was constructed. In this vector, the gene coding for FLP site-specific recombinase under the control of a heat shock-inducible promoter HSP18.2 from *Arabidopsis thaliana* and isopentenyltransferase gene (*ipt*), as a selectable marker gene under the control of the cauliflower mosaic virus 35S (CaMV 35S) promoter, were flanked by two loxP/FRT fusion sequences as recombination sites in direct orientation. Histochemical staining for GUS activity showed that, upon induction by heat shock, all exogenous DNA, including the selectable marker gene *ipt*, β-glucuronidase (*gusA*) gene and the FLP recombinase gene, between two loxP/FRT sites was eliminated efficiently from primary transgenic tobacco plants. Molecular analysis further confirmed that excision of the marker gene (*ipt*) was heritable and stable. Our approach provides a reliable strategy for auto-excising a selectable marker gene from calli, shoots or other tissues of transgenic plants after transformation and producing marker-free transgenic plants.

Introduction

In plant transformation systems, selectable marker genes, which confer resistance to selective chemical agents such as antibiotics or herbicides, are often required to select transformants from non-transformed plant cells and tissues during the process of production of transgenic plants (Yoder and Goldsbrough 1994). However, most transformed cells do not regenerate easily because these selective agents are not only toxic to non-transformed cells but also to transformed cells. An alternative is provided by so-called positive selection systems such as the β-glucuronidase (*gusA*) gene from *Escherichia coli* (Joersbo and Okkels 1996), the xylose isomerase gene from *Thermoanaerobacterium thermosulfurogenes* (Haldrup et al. 1998) and oncogenes from *Agrobacterium tumefaciens* or *A. rhizogenes* (Christy et al. 1997).

The isopentenyltransferase gene (*ipt*) from *A. tumefaciens* is effective as a positive selectable marker gene for plant transformation (Smigocki and Owens 1988; Ebinuma et al. 1997; Sugita et al. 1999, 2001).

K. Luo (✉) · M. Sun · S. Xu
Key Laboratory of Eco-environments of Three Gorges
Reservoir Region, Ministry of Education, School of Life
Sciences, Southwest University, 1, Tiansheng Road,
Beibei, Chongqing 400715, China
e-mail: luokeming@hotmail.com

W. Deng
Genetic Engineering Research Center, College
of Bioengineering, Chongqing University,
Chongqing 40044, China

One major drawback using it as a selectable marker is that transgenic plants exhibit an extreme shooty phenotype (ESP) due to continuous expression of the marker gene (Ebinuma et al. 1997; Sugita et al. 1999, 2001). To date, several approaches, such as cotransformation, transposition and site-specific recombination (Goldsbrough et al. 1993; Komari et al. 1996; Ebinuma et al. 1997; Sugita et al. 1999, 2001; Hohn et al. 2001; Hare and Chua 2002; Srivastava and Ow 2004), have been successfully explored to excise selectable marker genes from transgenic plants. However, excision efficiencies of these systems are generally low in higher plants (Russell et al. 1992; Lyznik et al. 1996; Luo et al. 2000; Hare and Chua 2002; Ow 2002). More recently, a novel technology, GM-gene-deletor, based on Cre/loxP and FLP/FRT site-specific recombination systems derived from bacteriophage P1 and yeast was developed for addressing this pitfall (Luo et al. 2007). Using this system, all functional foreign genes were automatically eliminated with a high efficiency from pollen and seeds of transgenic tobacco.

In the present paper, we describe an auto-excision strategy based on the GM-gene-deletor system for removal of selectable marker genes from transgenic plants. Utilizing this system, all extraneous DNA flanked by two loxP/FRT sites in direct orientation was eliminated efficiently from transgenic tobacco plants after the heat shock treatment, when the FLP gene was expressed under the control of a heat-shock promoter Hsp18.2 from *Arabidopsis thaliana* (Takahashi and Komeda 1989).

Materials and methods

Vector 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 by Sambrook et al. (2001). The pLF_HSP-FLP-*ipt* vector was constructed as follows. A *Hin*dIII fragment containing the chimeric 35S-*ipt*-nos gene was excised from pBluescript II KS (Y. Li, unpublished work), then blunt-ended and ligated into the *Sma*I site of the vector pLF (Luo et al. 2007), which carried the 35S-GUS:NPTII-nos fusion gene (Fabijanski et al. 2001) flanked by two loxP/FRT

sites in direct orientation, to generate pLF_*ipt*. Subsequently the Hsp18.2-FLP-nos fusion gene was digested with *Kpn*I plus *Sal*I from pBluescript II KS (Luo et al. 2005), inserted into the same restriction sites of the pLF_*ipt* vector, and produced the vector pLF_HSP-FLP-*ipt* (Fig. 1b).

The plasmid obtained above was introduced into *A. tumefaciens* LBA4404 using a freeze-thaw method (Höfgen and Willmtzer 1988). As controls, the binary vectors pBI121 (Clontech) and pBIG (Luo et al. 2006) carrying a chimeric *ipt* gene driven by the CaMV 35S promoter but without the GM-gene-deletor system were also introduced into *A. tumefaciens* (Fig. 1a). Transformants were selected on YEB (Vervliet et al. 1975) solid medium supplemented with
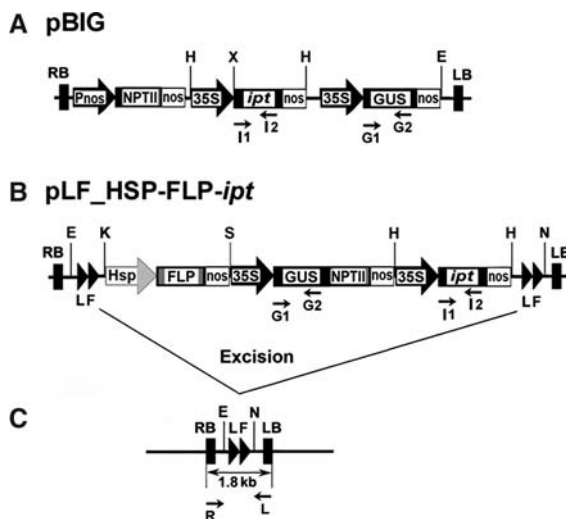

Fig. 1 Schematic representation of T-DNAs used for plant transformation. (**a**) The T-DNA region of vector pBIG containing the isopentenyl transferase gene (*ipt*) under the control of the CaMV 35S promoter. (**b**) The T-DNA region of vector pLF_HSP-FLP-*ipt*. The FLP gene and the GUS-NPTII fusion gene are driven by the heat shock promoter HSP18.2 and the CaMV 35S promoter, respectively. (**c**) The expected T-DNA fragment following excision of the GM-gene-deletor system. RB, right border; LB, left border; Pnos, promoter of nopaline synthase gene; 35S, CaMV 35S promoter; HSP, promoter of heat shock gene HSP18.2; nos, polyadenylation sequence of nopaline synthase gene; GUS, glucronidase gene; *ipt*, isopentenyl transferase gene; NPTII, neomycin phosphotransferase gene; FLP, recombinase gene of yeast site-specific recombination FLP/FRT system; LF, loxP and FRT fusion recognition sites. Restriction sites are indicated for: E, *Eco*RI; H, *Hin*dIII; K, *Kpn*I; N, *Nhe*I; X, *Xho*I; S, *Sal*I. Small arrows represent PCR primers to amplify the *ipt*, GUS, and post-excision fragments

50 mg kanamycin/l plus 100 mg rifampicin/l, and further confirmed by restriction enzyme analysis.

Plant transformation

Nicotiana tabacum cv. Xanthi was grown in a greenhouse under an 18 h light/6 h dark photoperiod at 25°C. *Agrobacterium tumefaciens*-mediated transformation of *N. tabacum* was performed according to Li et al. (1992) with the following changes: the infected leaf discs were transferred to hormone- and kanamycin-free MS medium with 150 mg rifampicin/l to eliminate *Agrobacterium* after co-cultivation for 3 days. The explants were cultivated at 25°C in a 16:8 h (light:dark) photoperiod and transplanted to fresh MS medium every 2 weeks. Adventitious shoots regenerated from infected leaf discs were excised and transferred to fresh kanamycin- and hormone-free MS medium supplemented with 150 mg rifampicin/l, and finally cultured at 25°C under 3,000 lux.

Heat-shock treatment

Regenerated shoots with *ipt*-shooty phenotype were cultured on solid hormone-free MS medium containing 150 mg rifampicin/l approximately 2 months after infection with the construct pLF_HSP-FLP-*ipt*. The Hsp18.2 heat shock promoter was used to control expression of the FLP gene in the heat induction experiments. As described by Liu et al. (2005), heat induction experiments were performed with two consecutive heat treatments such that plants were incubated at 37°C for 8 h, allowed to recover for 48 h at 25°C, and then incubated at 37°C for 16 h. These plants were analyzed 7 days after the second treatment.

Histochemical staining and fluorometric assay of GUS activity

Histochemical assays for GUS activity of transgenic plants were performed as described by Jefferson (1987) to confirm the presence of transgenes in the tobacco genome. Leaf segments of transgenic plants were vacuum-infiltrated for 10 min in 1 mM X-Gluc in 50 mM sodium phosphate buffer, pH 7.0, 2 mM DTT and 20% (v/v) methanol, then incubated at 37°C for 24–28 h. To remove chlorophylls and other pigments, leaf sections were soaked in 75% (v/v) ethanol at 65°C for at least 1 h.

Fluorometric assays of GUS activity were used to measure as described by Jefferson et al. (1987). The substrate for GUS activity measurements in vitro was used MUG (4-methylumbelliferyl-β-D-glucuronide). Plant samples were lyzed in extraction buffer [50 mM phosphate buffer, pH 7.0, 10 mM EDTA, 0.1% (v/v) Triton X-100, 0.1% (v/v) sodium lauryl sarcosine, and 10 mM β-mercaptoethanol] by freezing with liquid N_2 and grinding by mortar and pestle with silicon. Aliquots of the extracts (100 μl) were added to 1 ml of assay buffer (extraction buffer containing 1 mM MUG), pre-warmed and incubated at 37°C. After 0, 5 and 20 min, 100 μl samples were removed and placed in 1.9 ml stop-buffer (200 μM sodium carbonate). Fluorometric measurements of GUS activity were carried out using a Multi-Detection Microplate Reader (Bio-TEK Synergy HT). Total protein content in the extracts was determined by the Bradford method (1976). Relative GUS activities were calculated and expressed as picomole MUG per minute per milligram protein with data from three technical replicates per sample. Five independent lines were tested per treatment.

DNA preparation and analysis

Total genomic DNA was isolated from regenerated plants by CTAB method as previously described (Doyle and Doyle 1990). The presence of transgene inserts in the genome of transgenic plants was determined by PCR. The primers used to amplify the GUS gene were G1/G2 (5′-GTGGAATTGAT CAGCGTTGG-3′ and 5′-CCGACAGCAGCAGTTT CATC-3′). The predicated size of the amplified GUS segment is 985 bp. Another pair of primers I1/I2 (5′-GCGTCTAATTTTCGGTCCAAC-3′ and 5′-CGAATG GTGGGCCTTCAAATC-3′) was used to amplify a 704-bp fragment of the *ipt* gene. PCR reactions were performed under standard conditions with 5 min pre-denaturation at 95°C, and then 1 min denaturation, 1 min annealing and 1 min extension at 94, 60 and 72°C, respectively, for 35 cycles.

Genomic DNA from transgenic plants after treatment with heat shock was used to establish the post-excision signal. Two oligos R and L, 5′-GAACG TGGCGAGAAAGGAAGG-3′ and 5′-ACTGACAG

AAGGGCAACGTTG-3′, specific to the T-DNA sequences outside the pLF_HSP-FLP-*ipt* sequences were used as primers for PCR reactions. PCR reactions were carried out for 40 cycles at 94°C for 1 min, and 60°C for 1 min for and then 72°C for 8 min. DNA fragments were amplified by Ex Taq polymerase (TaKaRa, Dalian, China). PCR products for sequencing analysis were separated and 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, CA, USA). Sequencing from both the sense and antisense orientations was performed for confirmation.

Results and discussion

Construction of pLF_HSP-FLP-*ipt* vector

Previous studies have demonstrated that the *ipt* gene is efficient as a dominant and visual selectable marker for *Agrobacterium*-mediated plant transformation because transgenic explants with abnormal shooty morphology can be generated on a hormone-free medium (Smigocki and Owens 1988; Ebinuma et al. 1997; Sugita et al. 1999). To eliminate the marker gene *ipt* from transgenic plants after transformation, an auto-excision vector pLF_HSP-FLP-*ipt* based on the GM-gene-deletor system was constructed as shown in Fig. 1b. Two loxP/FRT hybrid sequences as the recognition sites flanked all transgenes, including FLP recombinase gene, GUS:NPTII fusion gene as a model gene of interest and *ipt* as a selectable marker. The heat-shock HSP18.2 promoter, which showed no detectable expression in *Arabidopsis* under normal conditions except for weak expression in secondary root hairs and could be induced strongly in the presence of heat stress (Takahashi et al. 1989, 1992), was fused to FLP recombinase to control removal of *ipt* gene. Theoretically, heat shock-induced expression of FLP gene leads to elimination of all sequences between two loxP/FRT sites (Fig. 1c). The morphological changes caused by the presence or absence of *ipt* allows visual selection of *ipt*-shooty or marker-free plants, respectively. Therefore, it is expected that excision of *ipt* gene by the 'heat shock (HS)-GM-gene-deletor' system would be feasible for producing marker-free transgenic plants.

Production of marker-free transgenic plants

In a previous study, we demonstrated that the GM-gene-deletor system, which was controlled by pollen- and seed-specific gene promoters, could auto-excise all functional transgenes from transgenic pollen and seeds with high efficiency (Luo et al. 2007). Here we evaluated the GM-gene-deletor system controlled by a heat-shock gene promoter HSP18.2 for removal of a selectable marker gene *ipt* in transgenic tobacco plants. Leaf discs of *N. tabaccum* were infected with *A. tumefaciens* harboring pBIG and pLF_HSP-FLP-*ipt* respectively, and cultivated on solid hormone-free MS medium. A large number of adventitious buds were differentiated from leaf discs approximately 4 weeks after infection (Fig. 2a). In contrast, no buds were formed on hormone-free MS medium when the binary vector pBI121 (Chen et al. 2003), which did not contain *ipt* gene, was introduced into leaf discs (data not shown). These adventitious buds were removed from the leaf discs and transferred to fresh hormone-free MS medium. After culture below 25°C for a further 3 weeks, some of these buds developed into putative transgenic explants which exhibited *ipt*-shooty phenotype (Fig. 2b). Similar to a previous report (Sugita et al. 2001), an equal regeneration frequency of *ipt*-shooty explants was obtained when transformed with the vectors pBIG and pLF-HSP-FLP-*ipt*, respectively (Table 1). In the GUS histochemical assay, approximately 60% of the *ipt*-shooty plants transformed with these two vectors were GUS-positive (Table 1). These data suggested that *ipt* was highly effective as a selectable marker gene for transformation and excision of the GM-gene-deletor system was controlled strictly by the HSP18.2 promoter.

To obtain marker-free transgenic plants directly from adventitious shoots, 20 *ipt*-shooty explants regenerated from leaf discs infected with pLF_HSP-FLP-*ipt* were selected randomly and transplanted to fresh hormone-free MS medium. After heat shock treatment, 12 out of these *ipt*-shooty explants developed into phenotypically normal shoots within 1 month of cultivation (Fig. 2c). However, no phenotypic changes were observed in the *ipt*-shooty shoots carrying the vector pBIG, which had the chimeric 35S-*ipt*-nos gene but lacked the GM-gene-deletor system, even in the presence of heat shock (data not shown). Subsequently, both *ipt*-shooty and
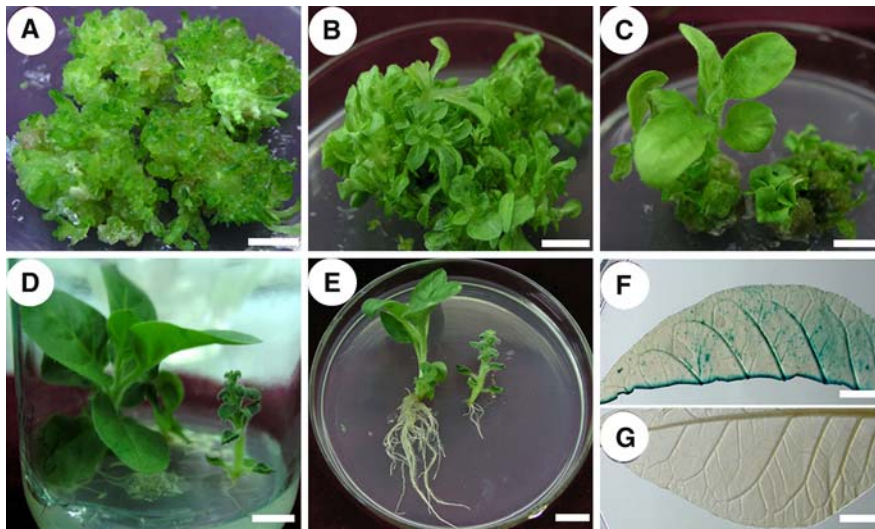
Fig. 2 Production of transgenic shoots from tobacco leaf discs after transformation. (**a**) Regeneration of adventitious buds on hormone-free MS medium. (**b**) *ipt*-shooty, approximately 3 weeks after transplantation of adventitious buds. (**c**) Normal phenotypic shoots (putative marker-free transgenic shoots) appeared from these *ipt*-shooty within 1 month of cultivation after heat shock treatment. (**d**) *ipt*-shooty and phenotypically normal shoots rooted. (**e**) Comparison of *ipt*-shooty and phenotypically normal shoots after an additional 2 weeks of rooting. (**f**) Histochemical staining of GUS activity of transgenic leaves before induction of heat shock. (**g**) Histochemical staining of GUS activity of transgenic leaves after induction of heat shock. Scale bars: (**a**) 0.4 cm, (**b**) 0.6 cm, (**c**) 0.8 cm, (**d**) 1.0 cm, (**e**) 1.0 cm, (**f**) 1.0 cm, (**g**) 1.0 cm

Table 1 Efficiencies of adventitious bud formation and putative transgenic shoot regeneration from tobacco leaf explants infected with *Agrobacterium tumefaciens* LAB4404 carrying the binary vectors pBI121, pBIG and pLF_HSP-FLP-*ipt*

Vector	No. of leaf discs	No. of adventitious buds	No. of *ipt*-shooty explants (%)	No. of GUS-positive explants (%)
pB121	14	0	0 (0)	0 (0)
pBIG	22	89	72 (81)	45 (63)
PLF_HSP-FLP-*ipt*	20	82	65 (79)	39 (60)

Adventitious buds were regenerated on solid hormone-free MS medium and then transferred to the same medium

phenotypically normal shoots were transplanted to fresh hormone-free MS medium and rooted (Fig. 2d). After an additional 2 weeks, the former showed loss of apical dominance and inhibition of root formation while the later grew normally (Fig. 2e). These results suggested that the GM-gene-deletor system driven by the HSP18.2 promoter was highly efficient to remove the selectable marker gene, resulting in generation of putative marker-free transgenic tobacco plants from an *ipt*-shooty.

Verification of marker-free shoots by GUS assay

The phenotypic alterations of putative transgenic plants have indicated that excision of the marker gene

ipt could occur upon induction of heat shock. We further confirmed excision events in transgenic shoots through analysis of GUS expression. Histochemical GUS staining revealed GUS activity in leaves of putative transgenic plants hosting plasmid pLF_HSP-FLP-*ipt* (Fig. 2f), but this activity disappeared 7 days after heat-shock induction (Fig. 2g). Furthermore, a quantitative fluorometric GUS assay was carried out with leaves from uninduced and induced primary lines with *ipt*-shooty phenotype. As a control, transgenic plants carrying pBI121 were also produced on MS medium containing 100 mg kanamycin/l and plant hormones (0.1 mg NAA/l and 1 mg BA/l). The results showed that heat shock treatments did not lead to reduced GUS expression in the pBI121 and pBIG

plants (Fig. 3a, b). Strong GUS activities comparable with those in the pBI121 plants were observed in the uninduced transgenic *ipt*-shooty plants harboring pLF_HSP-FLP-*ipt* vector, but GUS activity in four out of five lines tested was reduced drastically 5 days after treatment with heat shock (Fig. 3c, lines 1–4). These results indicated that heat shock treatment resulted in excision of the foreign genes, including GUS:NPTII, FLP and *ipt*, flanked by loxP/FRT sites.

Molecular analysis of heat shock-induced excision in transgenic plants

To investigate the heat shock-induced DNA excision events at the molecular level, GUS-positive primary transgenic shoots were subjected to polymerase chain reaction (PCR) analysis. Two pairs of the gene-specific primers G1/G2 and I1/I2 were designed to confirm the integration of the GUS and *ipt* genes, respectively (Fig. 1b). Consequently, both the predicted 1.0-kb GUS and 0.7-kb *ipt* fragments were detected in all five uninduced *ipt*-shooty plants (Fig. 4a, b). No PCR products were obtained when genomic DNA isolated from untransformed plants was used as DNA templates. These results indicated that the transgenes were incorporated into the tobacco genome.

Through heat-shock treatment, all extraneous DNA sequence between two direct loxP/FRT sites should be excised by expression of FLP gene, resulting in production of a 1.8-kb DNA fragment, as shown in Fig. 1c. As a result, FLP, *ipt* and GUS:PTII genes will be not detected. To verify the excision events, another pair of primers R/L was designed to amplify the post-excision sequence. After heat-shock induction, both the primers R/L and G1/G2 were used simultaneously to determine the presence of the transgenes. PCR results revealed that a 1.8-kb post-excision DNA fragment was detected in all five transgenic plants tested, but the 1.0-kb GUS fragment disappeared in four transgenic lines (Fig. 4c, lanes 1–4). Interestingly, in the fifth transgenic line (Fig. 4c, lane 5), both the GUS and post-excision fragments were detected, indicating that excision of the transgenes in this line was incomplete. The result was consistent with the previous data from the quantitative fluorometric GUS assay of transgenic plants, in which higher GUS expression was observed in this partly cured line after heat-shock induction, compared to other four
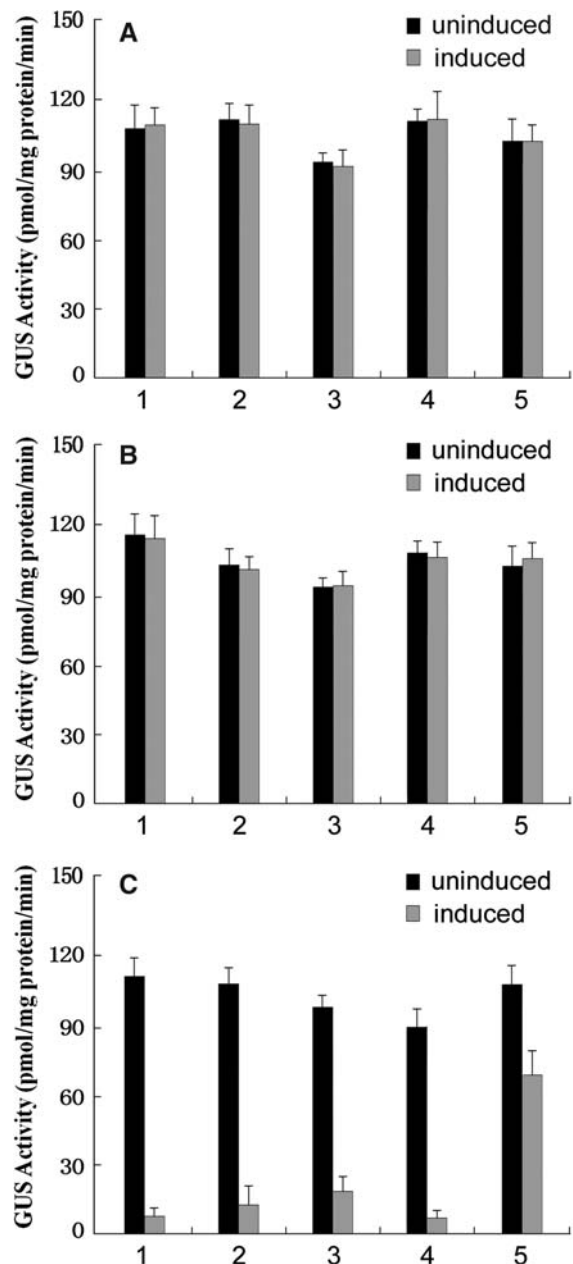

Fig. 3 Quantitative fluorometric GUS assay of heat shock-induced transgenic plants and controls. Shoots with true leaves from induced and uninduced transgenic shoots were assayed for GUS activity, which is expressed in quantity (pmol) of 4-methylumbelliferyl-β-D-glucuronide (MUG) per minute per milligram protein. (**a**) GUS activities of five pBI121 transgenic lines. (**b**) GUS activities of five pBIG transgenic lines. (**c**) GUS activities of five pLF_HSP-FLP-*ipt* transgenic lines. *Error bars* indicate SE values ($n = 3$)

completely cured transgenic lines (Fig. 3c). Similar results on variation in excision efficiency have been reported previously (Odell et al. 1990; Bayley et al.
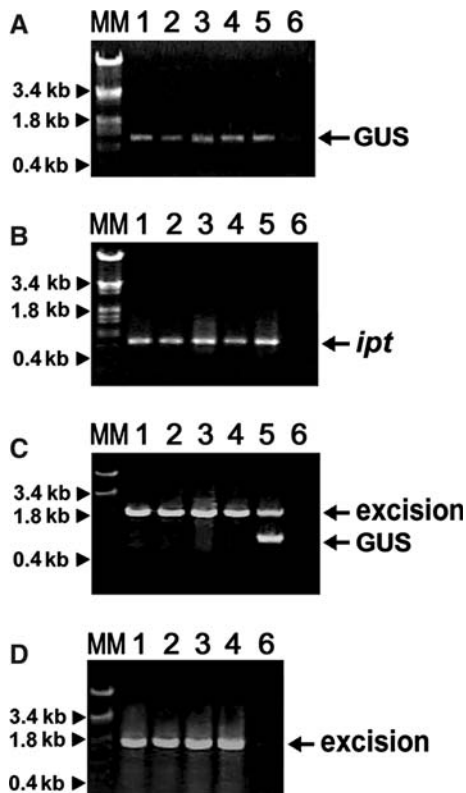
Fig. 4 PCR analysis of putative marker-free transgenic tobacco plants. PCR reactions were performed as described in "Materials and methods" using primers G1/G2, I1/I2, and R/L which were represented in Fig. 1c. (**a**) PCR amplification of the GUS fragment. (**b**) PCR amplification of the *ipt* fragment. (**c**) PCR amplification of the post-excision fragment from transgenic plants after heat shock treatment. (**d**) PCR amplification of the post-excision fragment from T1 progeny of transgenic plants. Lanes 1–5: PCR products from transgenic plants. Lane 6: PCR products from untransformed plants. Lane MM: DNA molecular size marker

1992; Russell et al. 1992; Hoa et al. 2002; Luo et al. 2007). Presumably variations in excision efficiency were caused by chromosomal position of the transgenic loci in and/or the difference in recombinase activity in plants (Gilbertson 2003). In addition, the 1.8-kb signal was not found in non-transformed plants. Taken together, these results suggested that marker-free transgenic tobacco plants were obtained from GUS-positive *ipt*-shooty lines through the HS-GM-gene-deletor system.

Heritability analysis of marker-free plants

Finally, we also determined heritability of marker-free transgenic tobacco plants. Four independent

transgenic lines mentioned above, in which the *ipt* gene was excised successfully after heat shock treatment, were chosen for heritability analysis. All T1 progeny of these transgenic lines appeared phenotypically normal. Histochemical assay for GUS expression revealed no GUS activity in the leaves, stems, flowers or roots of the transgenic progeny plants (data not shown). PCR amplification with primers I1/I2 showed that a 1.8-kb post-excision DNA fragment was obtained in all the T1 progeny plants (Fig. 4d), suggesting that the excision of the marker gene was inherited stably. Hence, our results indicate that the heat inducible GM-gene-deletor system might be a useful tool in transgenic manipulation to generate marker-free transgenic plants.

References

Bayley CC, Morgan M, Dale EC, Ow DW (1992) Exchange of gene activity in transgenic plants catalyzed by the Cre-lox site-specific recombination system. Plant Mol Biol 18:353–361

Bradford MM (1976) A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248–254

Chen PY, Wang CK, Song SC, To KY (2003) Complete sequence of the binary vector pBI121 and its application in cloning T-DNA insertion from transgenic plants. Mol Breed 11:287–293

Christy MC, Sinclair BK, Braun RH (1997) Regeneration of transgenic vegetable brassicas (*Brassica oleracea* and *B. campestrei*) via Ri-mediated transformation. Plant Cell Rep 16:587–593

Doyle JJ, Doyle JL (1990) Isolation of plant DNA from fresh tissue. Focus 12:13–15

Ebinuma H, Sugita K, Matsunaga E, Yamakado M (1997) Selection of marker-free transgenic plants using the isopentenyl transferase gene as a selectable marker. Proc Natl Acad Sci USA 94:2117–2121

Fabijanski SF, Robert L, Schernthaner J, Wu T (2001) Methods and constructs for *Agrobacterium*-mediated plant transformation. Patent WO 0159086 and GenBank Accession No. M26402

Gilbertson L (2003) Cre-lox recombination: Cre-active tools for plant biotechnology. Trends Biotechnol 21:550–555

Goldsbrough AP, Lastrella CN, Yoder JI (1993) Transposition mediated re-positioning and subsequent elimination of

marker genes from transgenic tomato. Biotechnology 11:1286–1292

Haldrup A, Petersen SG, Okkels FT (1998) The xylose isomerase gene from *Thermoanaerobacterium thermosulfurogenes* allows effective selection of transgenic plant cells using D-xylose as the selection agent. Plant Mol Biol 37:287–296

Hare PD, Chua N-H (2002) Excision of selectable marker genes from transgenic plants. Nat Biotechnol 20:575–580

Hoa TTC, Bong BB, Huq E, Hodges TK (2002) Cre/*lox* site-specific recombination controls the excision of a transgene from the rice genome. Theor Appl Genet 104:518–525

Höfgen R, Willmtzer L (1988) Storage of competent cells for *Agrobacterium* transformation. Nucleic Acids Res 16:9877

Hohn B, Levy AA, Puchta H (2001) Elimination of selection markers from transgenic plants. Curr Opin Biotechnol 12:139–143

Jefferson RA (1987) Assaying chimeric genes in plants: the gus gene fusion system. Plant Mol Biol Rep 5:387–405

Jefferson RA, Kavanagh TA, Bevan MW (1987) GUS fusions: *β*-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. EMBO J 6:3901–3907

Joersbo M, Okkels FT (1996) A novel principle for selection of transgenic plant cells: positive selection. Plant Cell Rep 16:219–221

Komari T, Hiei Y, Saito Y, Murai N, 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

Li Y, Hagen G, Guilfoyle TJ (1992) Altered morphology in transgenic tobacco plants that overproduce cytokinins in specific tissues and organs. Dev Biol 153:386–395

Liu HK, Yang C, Wei ZM (2005) Heat shock-regulated site-specific excision of extraneous DNA in transgenic plants. Plant Sci 168:997–1003

Luo H, Lyznik LA, Gidoni D, Hodges TK (2000) FLP-mediated recombination for use in hybrid plant production. Plant J 23:423–430

Luo KM, Zhao DG, Li Y, Pei Y (2005) The application of a novel recombination system for removing foreign genes from transgenic plants. Chin High Technol Lett 15:61–66

Luo KM, Zheng XL, Chen YQ, Zhao DG, McAvoy R, Pei Y, Li Y (2006) The maize Knotted1 gene as a positive selectable marker gene is effective for *Agrobacterium*-mediated transformation in tobacco. Plant Cell Rep 25:403–409

Luo KM, Duan H, Zhao DG, Zheng XL, Deng W, Chen YQ, Stewart CN, McAvoy R, Jiang XN, Wu YH, He AG, Pei Y,

Li Y (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 5:263–374

Lyznik LA, Rao KV, Hodges TK (1996) FLP-mediated recombination of FRT sites in the maize genome. Nucleic Acids Res 24:3784–3789

Odell J, Caimi P, Sauer B, Russell S (1990) Site-directed recombination in the genome of transgenic tobacco. Mol Gen Genet 223:369–378

Ow DW (2002) Recombinase-directed plant transformation for the post-genomic era. Plant Mol Biol 48:183–200

Russell SH, Hoopes JL, Odell JT (1992) Directed excision of a transgene from the plant genome. Mol Gen Genet 234:49–59

Sambrook J, Fritsch EF, Manistis T (2001) Molecular cloning: a laboratory manual, 3rd edn. Cold Spring Harbor Laboratory Press, New York

Smigocki AC, Owens LD (1988) Cytokinin gene fused with a strong promoter enhances shoot organogenesis and zeatinlevels in transformed plant cells. Proc Natl Acad Sci USA 85:5131–5135

Srivastava V, Ow DW (2004) Marker-free site-specific gene integration in plants. Trends Biotechnol 22:627–629

Sugita K, Matsunaga E, Ebinuma H (1999) Effective selection system for generating marker-free transgenic plants independent of sexual crossing. Plant Cell Rep 18:941–947

Sugita K, Kasahara T, Matsunaga E, Ebinuma H (2001) A transformation vector for the production of marker-free transgenic plants containing a single copy transgene at high frequency. Plant J 22:461–469

Takahashi T, Komeda Y (1989) Characterization of two genesencoding small heat-shock proteins in *Arabidopsis thaliana*. Mol Gen Genet 219:365–372

Takahashi T, Natio S, Komeda Y (1992) The *Arabidopsis* HSP18.2 promoter/GUS gene fusion in transgenic *Arabidopsis* plants: a powerful tool for the isolation of regulatory mutants of the heat-shock response. Plant J 2:751–761

Vervliet G, Holsters M, Teuchy H, Van Montagu M, Schell J (1975) Characterization of different plaque-forming and defective temperate phages in *Agrobacterium* strains. J Gen Virol 26:33–48

Yoder JI, Goldsbrough AP (1994) Transformation systems for generating marker-free transgenic plants. Biotechnology 12:263–267