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Full Length Research Paper

Selectable gene auto-excision via a cold inducible 'gene deletor' system

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Selectable marker genes are necessary tools for selecting transformants during transgenic plant production. However, once transformation is accomplished, the presence of these selectable marker genes is no longer necessary and can even be undesirable. Here we describe the successful excision of selectable genes from transgenic plants via the use of a cold inducible 'gene deletor' system. During a transformation procedure in tobacco, transgenic plants obtained by selection on kanamycin medium and identified by GUS staining and PCR method. Some shoots regenerated form transgenic tobacco leaves, after cold inducing, were screened for selective marker excision using GUS staining and PCR method, and all the exogenous genes were found to have been eliminated. About 28 - 94% of regenerated plants were marker-free. This excision system, mediated by the cold inducible 'gene-deletor' system to eliminate a selectable marker gene can be very readily adopted and used to efficiently generate marker-free transgenic plants.

Key words: 'Gene deletor', cold inducible, transgenic tobacco, marker free.

INTRODUCTION

Selectable marker gene plays an important role during transgenic plant production (Yoder et al., 1994). But these genes are not generally required for the subsequent expression of the functional genes in the transgenic lines. Moreover, these selectable marker genes have potential problems to human health and the ecosystem (Dale et al., 2002). To resolve these problems, various strategies have been suggested to remove marker genes from the genome of the transgenic plants such as co-transformation through segregation between selectable marker genes and functional genes (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; Bai et al., 2008). Site-specific recombination is the most widely known general approach to eliminate selectable marker genes (Ow, 2002).

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To date, several site-specific recombination systems from bacteriophages and yeast have been well characterized, including Cre/lox from the bacteriophage P1 (Stricklett et al., 1998), Flp/FRT from the 2 μ plasmid of Saccharomyces cerevisiae (Meyer-Leon et al., 1984) or R/RS from Zygosaccharomyce srouxii (Araki et al., 1985). All of these site-specific recombination systems consist of two basic components: a recombination enzyme and short DNA sequences that are recognized by the former. These two components mediated excision between directly orientated recognition sites results in the deletion of the intervening DNA, leaving single recognition site (Gregory et al., 2001; Chen et al., 2003).

Many reports have been published describing the successful removal of herbicide or antibiotic selectable marker genes from various transgenic plants (Luo et al., 2008; Chakraborti et al., 2008; Ballester et al., 2007; Zelasco et al., 2007; Ebinuma et al., 1997; Cao et al., 2006; Li et al., 2007; Wang et al., 2009; Woo et al., 2009; Yang et al., 2009) through site-specific recombination system Cre/loxp, Flp/FRT or R/RS. Luo et al. (2007) developed an efficient site-specific recombination system named gene-deletor. In this system, FRT and loxP

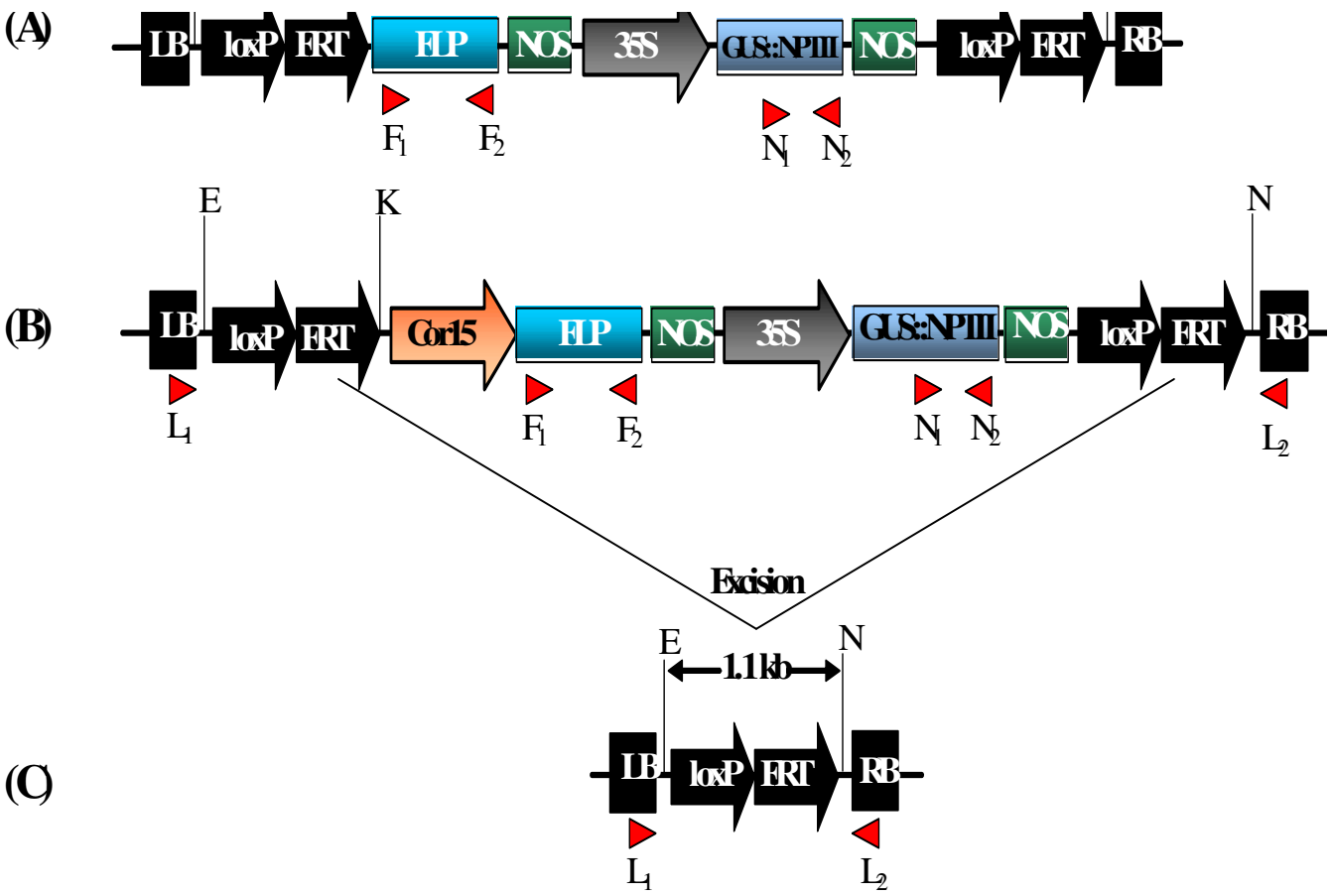

Figure 1. T-DNA region of plasmid and the principles of 'gene-deletor'. (A, B) Gene cassettes used in this research. E, EcoRI; K, KpnI; N, NheI (restriction enzyme sites); Cor15, cold inducible promoter; 35S, 35S CaMV gene promoter; nos, 3'-untranslated sequence from the Agrobacterium NOS gene; LF, fused loxP and FRT (loxP-FRT) recognition sequence; FLP, FLP gene coding sequences from FLP/FRT systems, respectively; LB and RB, left border and right border of T-DNA, respectively. (C) Schematic representation of the FLP-mediated excision of all transgenes from plant. FLP expression should lead to the deletion of all functional transgenes, including FLP from transgenic plant after cold treatment.

recognition sites were fused to generate the LF hybrid recognition sites, expression of either the FLP or Cre gene alone could lead to 100% efficiency in deleting all transgenes from pollen or seed of transgenic tobacco. Subsequently, gene-deletor system regulated by a heat-inducible promoter successfully have been deleted the selectable marker gene from transgenic tobacco (Luo et al., 2007).

In our present study, we described the construction of a binary expression vector in which a cold-inducible promoter (cor15 promoter) is used to control the FLP/LF recombination system. In previous reports, Baker et al. (1994) showed that the cor15 promoter is inactive in most of the tissues and plant organs maintained under temperatures associated with active growth, and that in response to low temperature, it becomes highly activate in the shoots but not in the roots (Baker et al., 1994). Using this constructed binary expression vector, both selectable marker gene and FLP gene can be eliminated from transgenic plants simultaneously through cold treatment.

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 by Sambrook (Sambrook et al., 2001). Ti-plasmid vector of pBin19 (Bevan, 1984) was chosen as the basic vector in this research. The construction carrying "Loxp-FRT" fusion sequences, FLP recombination enzyme gene and GUS::NPTII fusion reporter gene cassette was pLF as described previously (Luo et al., 2007). The Kpn/Xho fragment of a FLP-NOS amplified by PCR was ligated into the Kpn/Sal site of pLF (Luo et al., 2007) to generate 'pLF-FLP' plasmid (Figure 1A), which served as a control, in this cassette, excision was not expected to occur. The Kpn fragment of a Cor15 (0.98 kb) promoter amplified by PCR was ligated into pLF-FLP to generate 'pLF-Cor15-FLP' plasmid (Figure 1B). The plasmid obtained above was introduced into *A. tumefaciens* LBA4404 using a freeze-thaw method (Hofgen and Willmtzer, 1988). Transformants were selected

on YEB (Vervliet et al., 1975) solid medium supplemented with 50 mg kanamycin/l plus 100 mg rifampicin/l, and further confirmed by restriction enzyme analysis.

Plant transformation and regeneration

N. tabacum was grown in a greenhouse at 25℃ and under an 18 h light/6 h dark photoperiod. Expanded leaves were harvested from a wild type tobacco plant of (*Nicotiania tabbacum* cv "Xanthin") and washed with tap water followed by sterilization with 10% bleach plus 0.1% Tween-20 for 15 min. After being immersed in sterile water for 5 times, the leaves were cut into small pieces (approximate 0.5 - 1 cm X 0.5 - 1cm). Leaf discs were incubated with *A. tumefaciens* strain LBA4404 (OD600 = 0.6) for 10 min then put on a sterile filter paper to remove excess bacteria in suspension for 40 - 50 min. The infected leaf discs were transferred onto a co-culture medium without kanamycin and plant hormones and cultured for 3 days in dark at 24℃. The leaf discs were then transferred to selection MS medium with 100 mg/l kanamycin, 100 mg/l timentin (100 mg/l) plus 1.0 mg/l BA and 0.1 mg/l NAA. After one month, the independent shoots were cut and cultured on the 1/2 MS medium plus 1 mg/L IBA for inducing roots. All the media were supplemented with agar (8 g/l), sucrose (20 g/l), and adjusted to pH 5.8.

Histochemical analysis of GUS gene expression

Histochemical staining and fluorometric assays for GUS activity in vegetative organ of transgenic plants were performed as Jefferson's methods (Jefferson et al., 1987). To confirm the presence of transgenes in the tobacco genome, stem segments, leaf segments and roots of transgenic plants were 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℃ for 24 h. To remove chlorophylls and other pigments, the sections were soaked in 75% (v/v) ethanol at 37℃ for at least 6 h. The substrate for GUS activity measurements *in vitro* was used MUG (4-methylumbelliferyl-b-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 b-mercaptoethanol] by freezing with liquid N2 and grinding using mortar and pestle. Aliquots of the extracts (100 ul) were added to 1 ml of assay buffer (extraction buffer containing 1 mM MUG), pre-warmed and incubated at 37℃. After 0, 5 and 20 min, 100 ul samples were removed and placed in 1.9 ml stop-buffer (200 uM 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 per milligram protein with data from three technical replicates per sample. Five independent lines were tested per treatment.

PCR analysis

The incorporation of transgenes into tobacco genome of transgenic plants was determined by the PCR method. Genomic DNA was extracted from each of kanamycin resistance plant lines. The primers used to amplify the FLP gene were F1/F2 (5'-atgccacaatttggtatattatg-3' and 5'-ttatatgcgtctatttatgtagg-3'). The predicated size of the amplified FLP segment is 1272 bp. Another pair of primers N1/N2 (5'-ATGATTGAACAAGATGGATTGCA-3' and 5'-tcagaagaactcgtcaagaaggc-3') was used to amplify a 795-bp fragment of the NPTα gene. PCR reactions were performed under standard conditions with 3 min pre-denaturation at 94℃, and then 1 min denaturation, 1 min annealing and 1 min extension at 94, 55 and 72℃, respectively, for 30 cycles. Genomic DNA from transgenic plants after cold treatment was used to establish the post-excision signal. Two oligos L1 and L2, 5'-gtggtgtaaacaaattgacgc-3' and 5'-ttacccgccaatatatcctgtc-3', specific to the T-DNA sequences outside the pLF-Cor15-FLP sequences were used as primers for PCR reactions. PCR reactions were carried out for 35 cycles at 98℃ for 10 s, and 68℃ for 10 min. DNA fragments were amplified by Ex Taq polymerase (Dalian TaKaRa Biotechnology Co. Ltd, China). PCR products for sequencing analysis were separated and purified on agarose gels and then cloned into pGEM-T-easy Vector (Progema). Sequencing was carried out by Dalian TaKaRa Biotechnology Co. Ltd, China. Sequencing from both the sense and antisense orientations was performed for confirmation.

Cold treatment

To induce expression of FLP gene by cold treatment, whole plant were incubated at 4℃ for under the light (3000 lux) for three days (Khodakovskaya et al., 2005, 2006), allowed to recover for five days at 25℃, and this inducement were done twice. Leaves, stems and roots excised from transgenic tobacco plants were further analyzed. Leaves excised from uninducing transgenic plant were placed on MS medium with 100 mg/l kanamycin, 100 mg/l timentin (100 mg/l) plus 1.0 mg/l BA and 0.1 mg/l NAA. After three weeks, callus or shoots regeneration from leaf disc were incubated at 4℃ for three days, allowed to recover on MS with 100 mg/l timentin (100 mg/l) plus 1.0 mg/l BA and 0.1 mg/l NAA for five days at 25℃, and then incubated at 4℃ for three days. The independent shoots were cut and cultured on the medium 1/2 medium plus 0.5 mg/L IBA and 100 mg/l timentins for inducing roots. The rooting plants were then cultured at 25℃ under a 16 h light/8 h dark photoperiod for further analyzing.

RESULT

Generation of transgenic tobacco plant

The primary putative transgenic shoots were regenerated during the selection. Histochemical GUS assays was examined in vegetative organ of the plants. Gus activities were observed in roots, stems, and leaves (Figure 2 A, B, C). Integration of the NPTII, Flp genes in GUS-positive plants were confirmed by PCR amplification with the specific primers N1/N2 and F1/F2 using recovered DNA as template. 1272 bp Flp fragment and 756 bp NPTII fragment were detected in plants harbored ''pLF-FLP' and 'pLF-cor15-FLP'' (Figure 3 A, B). Consequently, 46 independent transgenic plants were generated using the constructs illustrated in Figure1, which included 22 pLF-FLP transgenic lines (line F1 - line F22) and 24 pLF-cor15-FLP transgenic lines (line C1 - line C 24). Eighteen lines designated C1, C2, C3, C4, C6, C9, C11, C12, C15, C17, C19, and F2, F4, F6, F12, F20, F21, F22 were chosen for further analysis.

Excision efficiency of marker gene by cold treatment of plants regeneration from leaves of primary transgenic tobacco plants

Through cold treatment, the DNA sequence between two

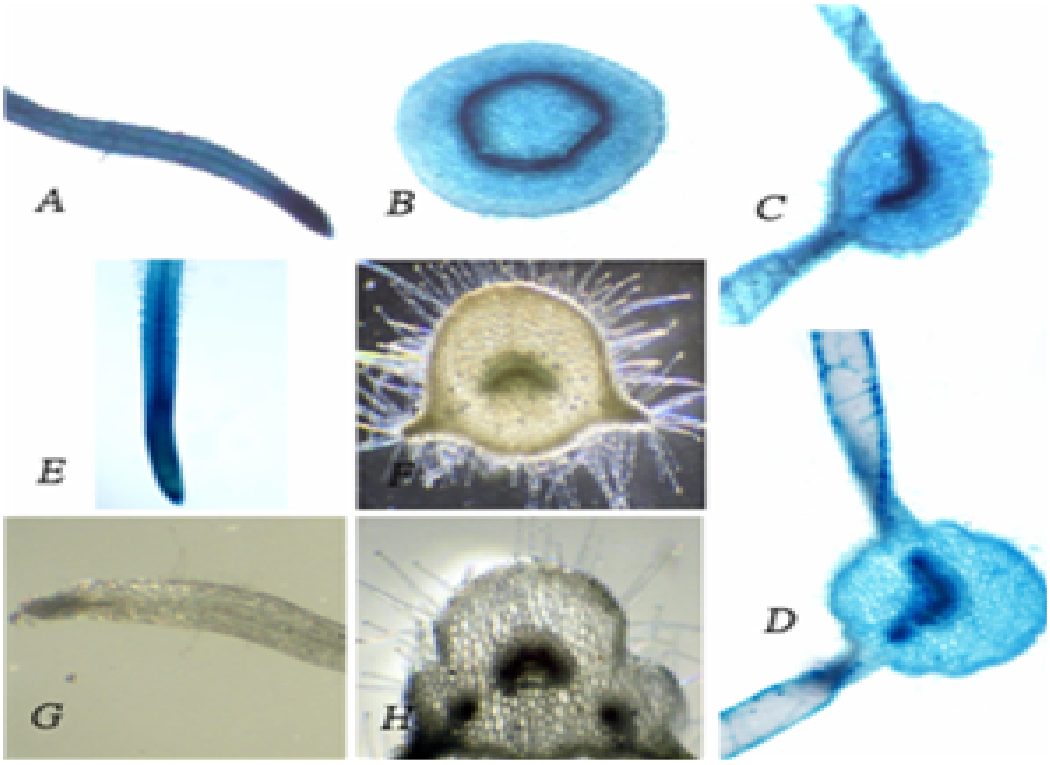

Figure 2. Histochemical staining of GUS activity of transgenic leaves before or after induction of cold treatment. (A) Roots transverse section of pLF-Cor15-FLP transgenic lines C-1. (B) Stem transverse section of pLF-Cor15-FLP transgenic lines C-1. (C) Leaves transverse section of pLF-FLP transgenic lines F-20. (D) Leaves transverse section of pLF-FLP transgenic lines F-20 after cold treatment. (E) Roots of pLF-Cor15-FLP transgenic lines C-1 after cold treatment. (F) Petiole transverse section of pLF-Cor15-FLP transgenic lines C-1 after cold treatment. (G) Roots Gus staining of plants regeneration from leaves of transgenic lines C-1 after cold treatment. (H) Petiole transverse section Gus staining of plants regeneration from leaves of transgenic lines C-1 after cold treatment.

direct LF sites will be excised by the expression of FLP gene. Therefore, after FLP/LF-mediated excision, all of the transgenes including Gus and nptII gene in transgenic plant leaves will be detected. Based on the above principle, to examine the “gene-deletor” mediated excision of the transgenes in plant, some transgenic plants regenerated from cold treated leaves were investigated by Gus staining. As shown in Figure 2 G, H, GUS activity was not observed in leaves and roots of some plants regeneration from transgenic C1 leaves with cold inducement. The statistic results of GUS staining were shown in Table 1. As shown in Table 1, except line C - 9(46.9%) and C - 2 (28.0%), excision efficiencies in all cold-treated lines exceeded 75%. Meanwhile, excision of plant regenerated from leaves transformed with pBLF-FLP was not observed in two untreated lines.

Verification excision of marker gene by GUS fluorometric assay

The GUS gene was driven by the constitutive 35S promoter (Odell et al., 1985), GUS activity served as a visible marker for the presence of transgenes in the cell. Gus expression of transgenic plant leaves have indicated that excision of the marker gene could not occur after induction of cold. We further confirmed excision events in transgenic leaves through analysis of GUS expression. Histochemical GUS staining revealed GUS activity in leaves(Figure 2D) of transgenic plants hosting plasmid pLF-FLP after cold induction, but this activity disappeared in leaves (Figure 2F) and appeared in roots (Figure 2E) of transgenic plants hosting plasmid pLF-Cor15-FLP.

Furthermore, a quantitative fluorometric GUS assay was carried out with leaves from ten independent (C - 3, C - 6, C - 11, C - 12, C - 19, F - 2, F - 4, F - 6, F - 20, F - 22) transgenic plant before cold treatment and after two days of cold treatment. The results showed that GUS expression in the pLF - FLP (Figure 4 A) did not reduce after cold treatments. Strong GUS activities comparable with those in the pLF - FLP plants were observed in the uninduced transgenic plants harboring pLF-cor15-FLP vector, but GUS activity in four out of five lines tested was reduced drastically three days after cold treatment Figure 4 C, lines C - 3, C - 6, C - 11, C - 19). These results indicated that cold treatment resulted in excision

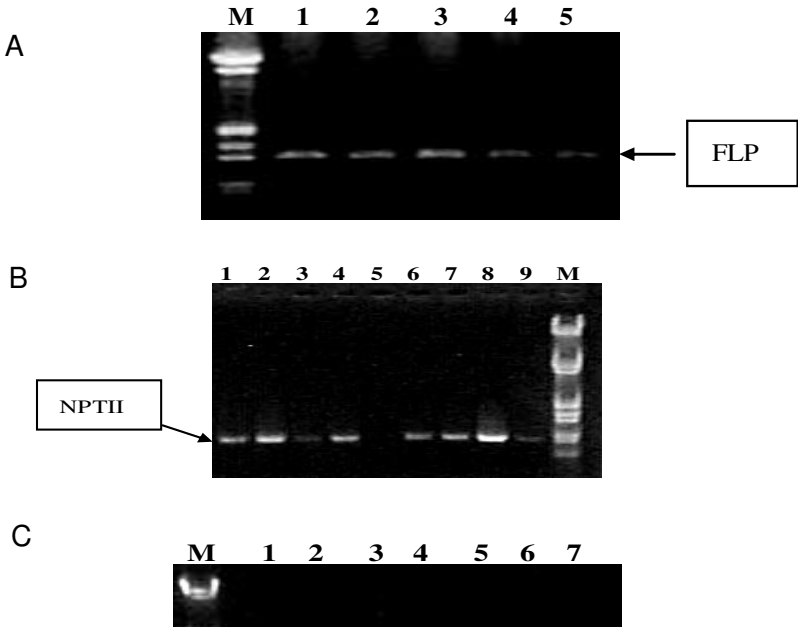

Figure 3. PCR analysis of the transgenes in transgenic plants. (A) PCR reactions were performed as described in Materials and Methods with primer sequences for the FLP gene. Lanes 1 - 5: Representative transgenic Gus positive shoot lines. (B) PCR reactions were performed as described in Materials and Methods with primer sequences for the NPTII gene. Lanes1-4: Representative plant lines transformed with pLF-FLP, Lanes6-9 representative plant lines transformed with pLF-cor15-FLP. Lane 5: Wild-type plant control (Wild-type plant DNA as template). (C) PCR reactions were performed as described in Materials and Methods with primer sequences for the 1.1kb sequence Lanes 1, 2: Representative Gus negative plant lines after cold treatment. Lanes 4 - 7 Representative Gus negative shoots after cold treatment. Lane 3: Wild-type plant control (Wild-type plant DNA as template). Lane M: DNA molecular size marker (Lambda DNA was digested by HindIII and EcoRI).

Table 1. Excision efficiency of marker gene by cold treatment of shoots regeneration from leaves of primary transgenic tobacco plants.

Line no.	Number of leaves	Number of identified plants	Number of marker-free plants	Excision ratio (%)
C-1	15	92	75	81.5
C-2	15	98	46	46.9
C-4	15	106	80	75.5
C-9	15	82	23	28.0
C-15	15	84	79	94.0
C-17	15	78	70	89.7
F-12	15	98	0	0
F-21	15	89	0	0

(of the foreign genes, including GUS:NPTII, FLP and flanked by loxP/FRT sites.

PCR analysis of transgenic tobacco plants after elimination of marker gene

Therefore, after FLP/LF-mediated excision, only about 1.1 kb DNA fragment will be amplified with primers L1/L2, while neither GUS gene nptII gene will be detected. Some transgenic plants regenerated from cold treated leaves with negative GUS staining results were further investigated by PCR with primers L1/L2 to examine the excision of the marker gene. PCR results shown in Figure 3C indicate the presence of the expected 1.1 kb fragment in the marker-free transgenic tobacco plants. To validate the DNA excision in transgenic plants, the PCR products

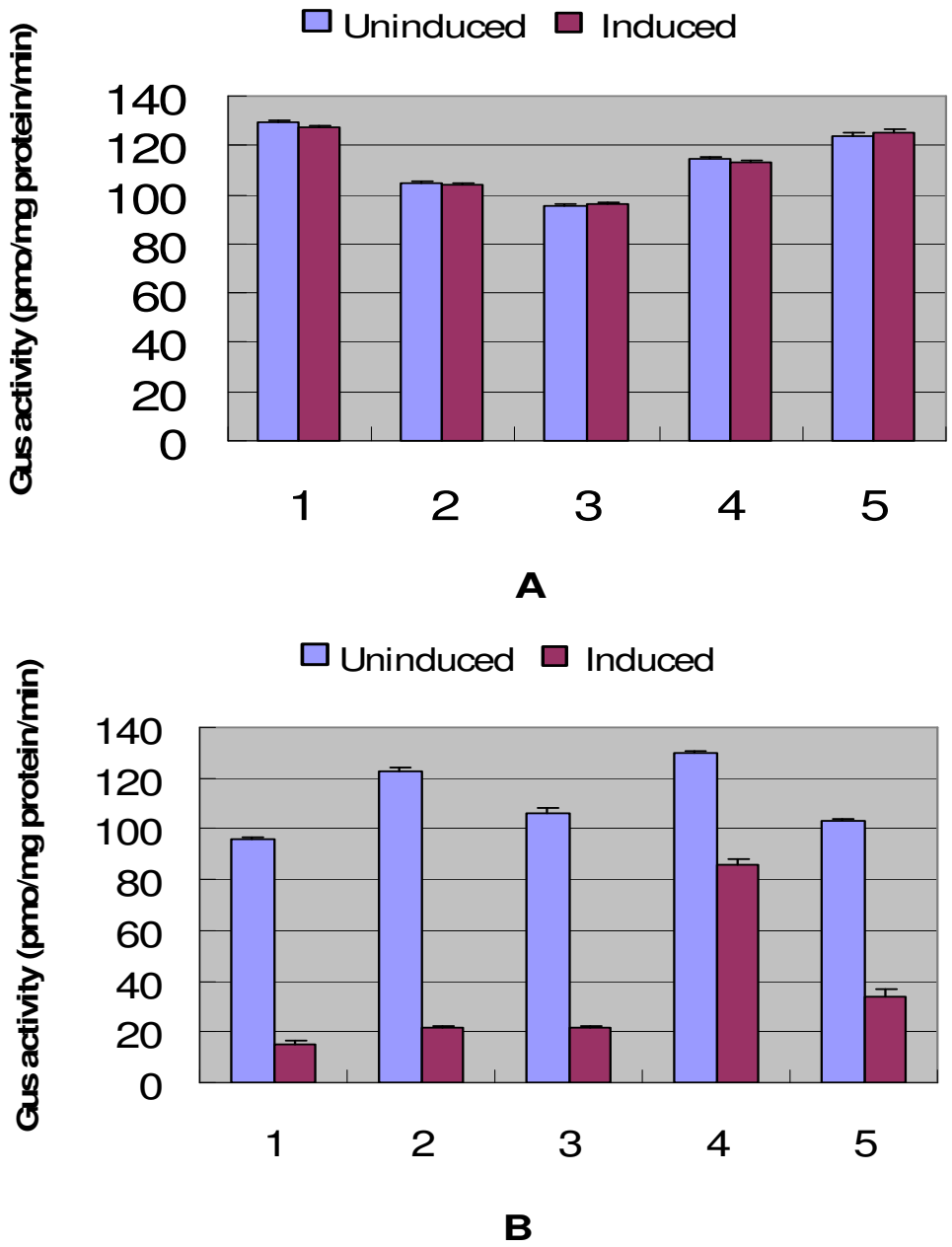

Figure 4. Quantitative fluorometric GUS assay of cold induced transgenic plants. Shoots with true leaves from induced and uninduced transgenic shoots were assayed for GUS activity, which is expressed in quantity (pmol) of 4-methylumbelliferyl-b-Dglucuronide (MUG) per minute per milligram protein. (a) GUS activities of five pBLF-FLP transgenic lines. (b) GUS activities of five pLF-Cor15-FLP transgenic lines. Error bars indicate SE values (n = 3).

amplified from Line C1□C17, C19 with primers L1/L2 were sequenced (the partial sequence was not shown here).

DNA sequencing analysis revealed that the 1.1 kb product consisted of the left and right borders, a single LF site (86 kb) and pBin19 skeleton sequence of vectors. These results further confirmed that this cold inducible system uninstalled all transgenes from the plant regenerated from tobacco plants.

DISCUSSION

Accounts of the economic, environmental and health benefits of genetically modified (GM) crops have been published, but safety of selectable marker genes is a major limitation of commercial use of GMO. It is very necessary for developing efficient molecular technologies to eliminate the selectable marker genes after transformation is accomplished. In this paper, we use cold

inducible promoter to control the expression of Flp gene in 'Gene deletor', and successfully uninstalled all selectable marker genes of plants regeneration from transgenic tobacco leaves. The excision rate of marker genes by cold treatment was determined to be 75 - 94% of regenerated shoots, a litter higher than that heat shock treatment (Wang et al., 2005), which was only 70 - 80%. Compared with the heat shock inducible system used to eliminate selection markers in tobacco (Wang et al., 2005), when FLP recombinase was driven by a cold inducible promoter, recombinase-mediated marker gene excision only occurred in transgenic plants after cold treatment. This may indicate there no potentially serious problems with maintenance transformation efficiency (Wu et al., 2001; Wang et al., 2005).

The low excision efficiency (28.0%) in C-12 maybe caused by chromosomal position of the transgenic loci in or the difference in recombinase activity in plants (Gilbertson, 2003). Because of apparent inactivity of the cor15 promoter in the roots of tobacco plants (Baker et al., 1994), so the GUS activity still appeared in roots of transgenic plants hosting plasmid pLF-Cor15-FLP after cold treatment. In the process of the transgenic shoots produced via a bacterium, leaf disc and shoots were usually used as the explants, once the transgenic callus and shoots were induced from the explants, the cold inducement would be performed, then the selectable marker genes can be deleted and the marker-free transgenic shoots would be produced.

There are three strategies to eliminate marker genes through site-specific recombination methods: retransformation, crossing and inducible auto-excision. However, in the former two methods, 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). These two strategies are quite laborious and time-consuming, especially for forest trees. Therefore, the inducible auto-excision strategy can be used for vegetative propagated plants, such as potato, or plants for which crossing is very difficult to carry out, such as soybean and wheat (Wang et al., 2005). Similar auto excision strategies have been demonstrated in tobacco (Sugita et al., 2000; Wang et al., 2005; luo et al., 2008; woo et al., 2008; zhang et al., 2009), Arabidopsis (Zuo et al., 2001), Brassica napus (Lilya et al., 2009), hybrid aspen (Matthias et al., 2009) by the different inducible promoters. Luo et al. (2008) reported excision of selectable marker gene from transgenic tobacco using the gene-deletor system regulated by a heat-inducible promoter. But in this system, the shoots should be heated under high 37℃ temperature. For some high temperature sensitive plant species, high temperature altering lipid properties, causing membranes to become more fluid and thereby disrupting membrane processes. Thus, some plant species would be injured or even die after heat treatment. In the process of agriculture production, some cultivar should be grown under low temperature especially winter annual plant and some horticultural plants products also should be kept in low temperature to postpone deterioration. With the advantages of saving time and avoiding the crossing procedure, when this types of crops are grown under low temperature, the selectable marker genes can be auto-uninstall from transgenic plant. So this cold inducible auto-uninstall system provides an easier way to eliminate selection markers compare to other systems. The overall results presented in this article demonstrate that cold inducible auto excision strategy provides a very useful tool in transgenic manipulation to produce marker-free transgenic plants.

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REFERENCES

Araki H, Jearnpi T, Muta Y, shima O (1985). Molecular and functional organization of yeast plasmid. pSR1 J. Mol. Bio., 182: 191-203.

Bai X, Wang Q, Chu C (2008). Excision of a selective marker in transgenic rice using a novel Cre/loxP system controlled by a floral specific promoter." Transgenic Res., 17(6): 1035-1043.

Ballester A, Cervera M, Pena L (2007). Efficient production of transgenic citrus plants using isopentenyl transferase positive selection and removal of the marker gene by site-specific recombination. Plant Cell Rep. 26: 39-45.

Chen Y, Rice PA (2003). New insight into site-specific recombination from Flp recombinase-DNA structures. Annu. Rev. Biophys. Biomol. Struct., 32: 135-159.

Dale PJ, Clarke B, Fontes EMG (2002). Potential for the environmental impact of transgenic crops. Nat. Biotech., 20: 567-574.

Daley M, Knauf V, Summerfelt KR, Turner JC (1998). Co-transformation with one Agrobacterium tumefaciens strain containing two binary plasmids as a method for producing marker-free transgenic plants. Plant Cell Rep., 17: 489-496.

De Block M, Debrouwer D (1991). Two T-DNA's co-transformed into Brassica napus by a double Agrobacterium tumefaciens infection are mainly integrated at the same locus. Theor. Appl. Genet., 82: 257-263.

Depicker A, Herman L, Jacobs A, Schell J, Van Montagu M (1985). Frequencies of simultaneous transformation with different T-DNAs and their relevance to the Agrobacterium/plant cell interaction. Mol. Gen. Genet., 201: 477-484.

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

Gregory D. Van Duyne (2001). A structural view of cre-loxP site-specific recombination. Annu. Rev. Biophys. Biomol. Struct., 30: 87-104.

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

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: b-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. EMBO J., 6: 3901-3907.

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.
Luo, K, Duan H, Zhao D, Zheng X, Deng W, Chen Y, Stewart CN, Jr McAvoy R, Jiang X, Wu Y, He A, 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(2): 263-274.
McCormac AC, Fowler MR, Chen DF, Elliott MC (2001). Efficient co-transformation of Nicotiana tabacum by two independent T-DNAs, the effect of T-DNA size and implications for genetic separation. Transgenic Res., 10: 143-155.
Meyer-Leon L, Senecoff JF, Bruckner RC, Cox MM (1984). "Site-specific genetic recombination promoted by the FLP protein of the yeast 2-micron plasmid in vitro." Cold Spring Harb. Symp. Quant. Biol., 49: 797-804.
Khodakovskaya MR, McAvoy JH, Peters Wu H, Li Y (2006). Enhanced cold tolerance in transgenic tobacco expressing a chloroplast omega-3 fatty acid desaturase gene under the control of a cold-inducible promoter. Planta., 223(5): 1090-1100.
Khodakovskaya M, Li Y, Li J, Vankova R, Malbeck J, McAvoy R (2005). Effects of cor15a-IPT gene expression on leaf senescence in transgenic Petunia x hybrida and Dendranthema x grandiflorum. J. Exp. Bot., 56: 1165-1175.
Ow DW (2002). Recombinase-directed plant transformation for the post-genomic era. Plant Mol. Biol., 48: 183-200.
Odell JT, Nagy F, Chua N H (1985). "Identification of DNA sequences required for activity of the cauliflower mosaic virus 35S promoter." Nature 313(6005), pp. 810-812.
Puchta H (2000). Removing selectable marker genes: taking the shortcut. Trends Plant Sci. 5: 273-274.
Baker SS, Wilhelm KS, Thomashow MF (1994). The 5'-region of Arabidopsis thaliana corl5a has cw-acting elements that confer cold-, drought- and ABA-regulated gene expression, Plant Mol. Biol., 24: 701-713.
Sambrook J, Fritsch EF, Manistis T (2001). Molecular cloning: A laboratory manual, 3rd edn, Cold Spring Harbor Laboratory Press, New York.
Stricklett PK, Nelson RD, Kohan DE (1998). "Site-specific recombination using an epitope tagged bacteriophage P1 Cre recombinase." Gene, 215(2): 415-423.
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.
Wang Y, Chen B, Hu Y, Li J, Lin Z (2005). Inducible excision of selectable marker gene from transgenic plants by the cre/lox site-specific recombination system. Transgenic Res., 14(5): 605-614.
Hee-Jong Woo, Hyun-Suk Cho, Sun-Hyung Lim, Kong-Sik Shin, Si-Myung Lee, Ki-Jong Lee, Dong-Hern Kim, Yong-Gu Cho (2009). Auto-excision of selectable marker genes from transgenic tobacco via a stress inducible FLP/FRT site-specific recombination system. Transgenic Res., 18(3): 455-465.
Xing A, Zhang Z, Sato S, Staswick P, Clemente T (2000). The use of the two T-DNA binary system to derive markerfree transgenic soybeans. *In vitro* Cell Dev. Biol. Plant, 36: 453-456.
Yoder JI, Golgsbrough AP (1994). Transformation system for generating marker-free transgenic plants. Biotechnology, 12: 263-267.
Zelasco S, Ressegotti V, Confalonieri M, Carbonera D, Calligari P, Bonadei M, Bisoffi S, Yamada K, Balestrazzi A (2007). Evaluation of MAT-vector system in white poplar (Populus alba L.) and production of ipt marker-free transgenic plants by single-step transformation. Plant Cell Tiss. Org. Cult., 91: 61-72.
Zubko E, Scutt C, Meyer P (2000). Intrachromosomal recombination between attP regions as a tool to remove selectable marker genes from tobacco transgenes. Nat. Biotechnol., 18: 442-445.
Zuo J, Niu QW, Moller SG, Chua NH (2001). Chemical-regulated site-specific DNA excision in transgenic plants. Nat. Biotechnol., 19: 157-161.