

Visible marker excision via heat-inducible *Cre/LoxP* system and *Ipt* selection in tobacco

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Abstract Numerous studies have reported on the heat-inducible deletion of a marker gene in plant transformation. However, these deletions seldom display manifestations that are visible to the naked eye to easily identify whether excision has occurred. In this study, an *isopentenyl-transferase* (*ipt*) gene from *Agrobacterium tumefaciens* was adopted to render abnormal bunched and underdeveloped leaf phenotypes to transgenic buds and/or shoots, and a synthesized heat shock (HS) promoter HSP70m was used to control the *Cre/LoxP* recombination system. Results showed that the *HSP70m* promoter could be highly induced by HS, even reaching the strength of the CaMV 35S promoter. The *HSP70m* was also sufficiently strong to drive *Cre/LoxP* system to obtain marker-free tobacco plants, meanwhile *ipt*-induced abnormal buds and shoots served as a visible selectable marker for transgenics, and normal shoot development after heat shock functioned as a positive selectable marker for removal of marker gene including *ipt*. This screening method is more convenient than the traditional heat-inducible marker excision system. Therefore, the combination of heat-inducible *Cre/LoxP* system and *ipt* selection has the potential to be a reliable tool for generating marker-free transgenic plants in genetic manipulation.

Keywords Tobacco · Heat shock promoter HSP70m · *Cre/LoxP* site-specific recombination system · *Ipt* gene · Marker visible excision

Introduction

Selectable marker genes are necessary for transgenic cells to develop and regenerate under complicated and competitive cell environments during plant transformation process. However, selectable marker genes, such as neomycin phosphotransferase II (*nptII*) gene and hygromycin phosphotransferase (*hpt*) gene, become useless as soon as transgenic plants are obtained. Some people are even worried about the safety of the marker genes (Fuchs *et al.* 1993; Costa-Font *et al.* 2007; Nicolia *et al.* 2014). Therefore, it is desired to have marker genes removed from the transgenic plants (Tuteja *et al.* 2012; Yau and Stewart 2013).

The selectable marker genes in transgenic plants may be eliminated by several methods (Yau and Stewart 2013). One method employs a co-transformation strategy, wherein one plasmid carries a marker gene, another plasmid contains target genes only, and both of them are transformed into a plant simultaneously; thus, the marker gene can be excluded by screening the offspring of the self-crossed generation provides that the marker and target genes are integrated in different chromosomes in the transgenic event (Liang *et al.* 2012). However, this method cannot be used in asexually propagating plants. Another method involves the use of the multi-auto-transformation system, where the T-DNA contains an auto-transposable element *Ac* inserted with an *isopentenyl-transferase* (*ipt*) gene; normal transgenic shoots from a clump of abnormal adventitious buds can be chosen once the *ipt*-containing *Ac* failed to transpose (Ebinuma *et al.* 1997). The third method is the

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deletion of marker genes on the basis of site-specific recombination systems, such as *Cre/LoxP* and *FLP/FRT* or *R/RS*, which are derived from bacteriophage P1 and yeast, respectively; this method is currently the main means of obtaining marker-free transgenic plants (Gibertson 2003; Boszorádová *et al.* 2014; Polóniová *et al.* 2015).

The *Cre/LoxP* recombination system was first established in tobacco to remove the *hpt* gene at a rate of 90.9% (Dale and Ow 1991). The efficiency of this method has been subsequently tested in numerous plants, including potato (Cuellar *et al.* 2006), tomato (Zhang *et al.* 2009), soybean (Li *et al.* 2009), rice (Khattri *et al.* 2011), and other important crops. Moreover, different promoters, such as CaMV 35S constitutive promoter (Liang *et al.* 2012), chemical-inducible promoters (Zuo *et al.* 2001; Petri *et al.* 2012), heat-inducible promoters (Schaart *et al.* 2004; Liu *et al.* 2005; Wang *et al.* 2005; Cuellar *et al.* 2006; Luo *et al.* 2008; Khattri *et al.* 2011), and tissue-specific promoters (Hu *et al.* 2013), were successfully adopted to drive the *Cre/LoxP* system in plants. This technique has been extensively developed in plants. However, extensive molecular methods are necessary to detect excision as there is no evident phenotype in those systems. The current study used the *ipt* gene to induce abnormal bunched leaf phenotypes in transgenic buds or shoots and adopted a synthesized heat shock (HS) promoter to regulate the marker excision in tobacco. This system allows marker excision to be detectable by the naked eye upon heat shock treatment. Thus, marker-free transgenic tobacco can be conveniently obtained without extensive use of molecular analysis.

Materials and methods

Gene cloning and vector construction The *HSP70m* promoter was derived from *AtHSP70b* promoter (Genbank accession no.: At1g16030), wherein the middle imperfect HS element (HSE) in the *AtHSP70b* was eliminated by mutation according to the structure of *AtHSP70* (At3g12580) promoter (Sung *et al.* 2001), and the two other imperfect HSEs were substituted with perfect HSEs (Fig. 1A). The *HSP70m* promoter sequence (Fig. 1B) was synthesized by Shanghai Generay Biotech Co. (China) and was used to substitute the CaMV 35S promoter in pBI121 (AF485783) to control β -glucuronidase (GUS), thereby forming the binary vector pHSP70m-GUS.

Cre (X03453) gene was amplified by PCR from the *Escherichia coli* strain BM25.8 using primer pairs P3 and P4 (Table S1, Fig. 1D and E). The PCR products were cloned into the pMD18-T vector by using the A/T cloning method to obtain the vector pMD18-CRE. An Xba I/Nco I adaptor was produced by annealing the sense sequence 5'-

CTAGAACCATGGCTCCCAAGAAGAAGAGAAAGCTGGT-3' (the underlined portion is a Kozak structure that is efficient for translation) with the antisense sequence 5'-CATGACCAGCTTTCTCTTCTTCTTGGGAGCCATGGTT-3'. The adaptor contains an encoding sequence for the nucleus-targeted signal sequence "PKKKRKL" (JO2400) in the T antigen of the coat protein of SV40 virus, and the adaptor was inserted between the Xba I and Nco I sites in pMD18-CRE. The intron of castor bean catalase gene in the *GUS* gene in pCAMBIA1301 (AF234297) was amplified by PCR using primers P10 and P11 (Table S1) and was inserted into the EcoR V site of the *Cre* gene to prevent its expression in bacteria. This modified *Cre* gene (Fig. 1C) was then used to substitute the GUS in pHSP70m-GUS to construct pHSP70m-CRE. The HSP70m-Cre-Tnos in pHSP70m-CRE was then recombined with pBK-35SeGFP (provided by our laboratory, and it contains *Pnos-nptII* from pBI121 and *eGFP* (coding for enhanced green fluorescence protein) from pEGFP-N1 (U55762) in its T-DNA; the two genes were flanked by two *LoxPs* in direct orientation) to obtain the binary vector pCRE-eGFP (Fig. 1D). The *ipt* (NC_003308) gene, amplified from *Agrobacterium tumefaciens* strain C58 by colony PCR with primers P5 and P6 (Table S1), was inserted between HSP70m-Cre and 35S-eGFP in pCRE-eGFP to obtain the binary vector pCRE-IPT-eGFP (Fig. 1E).

All cloned DNA fragments used in this study were amplified by PCR by using high-fidelity PrimSTAR HS DNA polymerase (TaKaRa, Dalian, China). All constructed vectors, in addition to PCR amplification and enzyme digestion, were confirmed by sequencing in BGI-tech Co. (Beijing, China).

Plant transformation Binary vectors pBI121, pHSP70m-GUS, pCRE-eGFP, and pCRE-IPT-eGFP were separately transformed into *A. tumefaciens* strain LBA4404 by the freeze-thaw method (Chen *et al.* 1994). Aseptic tobacco (*Nicotiana tabacum* L. cv. Wisconsin 38) leaves were cut into square pieces, infected with *A. tumefaciens*, and cultured on solid MS (Murashige and Skoog, 1962) medium supplemented with 2.5 mg l⁻¹ 6-BA, 150 mg l⁻¹ kanamycin (Kan), 300 mg l⁻¹ carbenicillin (Carb), and 3% (w/v) sucrose. The Kan-resistant shoots were rooted on a hormone-free solid MS medium supplemented with 150 mg l⁻¹ Kan and 100 mg l⁻¹ Carb. The rooted plantlets were grown in pots filled with nutrient soil in a greenhouse at 25 ± 2 °C under a 16 h photoperiod and a light intensity of 60 μmol·m⁻²·s⁻¹. Genomic DNA was extracted from the leaf samples of T0 or T1 plants to screen the transgenic lines by PCR using the primers used for the binary vector assays (Fig. 1D and E).

Histochemical staining assay of GUS enzyme activity By using the method described by Jefferson *et al.* (1987), we

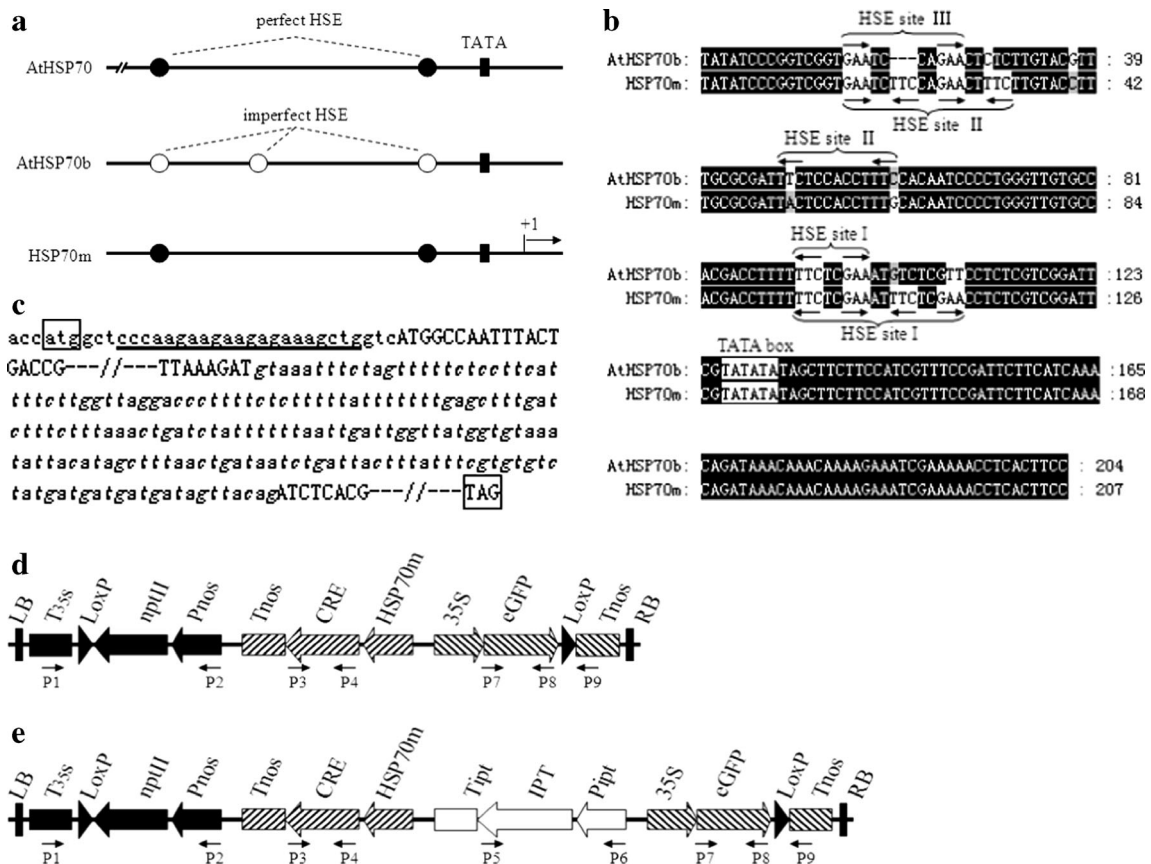


Fig. 1 Structure of the HSP70m promoter, Cre gene, and T-DNAs in binary vectors. (a) Schematic of the heat shock promoters of AtHSP70, AtHSP70b, and HSP70m. (b) sequence alignment between AtHSP70b and HSP70m. (c) structure and sequence of the modified Cre recombinase gene. (d) T-DNA of the vector pCRE-eGFP. (e) T-DNA of the vector pCRE-IPT-eGFP. HSE, heat shock element; TATA, TATA box; ABRE, ABA responsive element; AuxRE, auxin responsive element; +1, transcription start site; [atg], translation initiation codon; Underlined sequence, coding sequence for nuclear location signal (NLS) of the SV40 t-antigen; italicized letters, intron sequence of the castor bean

catalase gene, which was inserted in the EcoR V site of the Cre; Capital letters and ---//---, Cre recombinase gene sequence (X03453); TAG, translation termination codon; LB and RB, left border and right border; T35 s, CaMV 35S terminator; LoxP, Cre recombinase recognition site; nptII, neomycin phosphotransferase gene II; Pnos, promoter of nopaline synthase gene; Tnos, terminator of nopaline synthase gene; CRE, modified Cre recombinase gene; HSP70m, heat shock promoter 70 m; IPT, isopentenyl-transferase gene; Ptipt, promoter of ipt; Tipt, terminator of ipt; 35S, CaMV 35S promoter; eGFP, enhanced green fluorescent protein gene; and P1 to P9, primers used for PCR amplification

performed histochemical staining for GUS activity in tobacco wild type (WT) and transgenic (T1) plants grown in different temperatures ranging from 10 °C to 40 °C.

Heat treatment and marker excision detection The T1 plants regenerated from transformations using binary vectors pCRE-eGFP and pCRE-IPT-eGFP were treated with heat conditions of 37 °C for 16 h and 25 °C for 8 h (day/night) under a light intensity of 20 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$. Marker excision was analyzed in leaves before and after the heat treatment to determine the existence of the genes in the integrated T-DNA of the transgenic plants by PCR amplification using primer pairs P1/P2, P3/P4, P5/P6, and P7/P8 (Table S1, Fig. 1D and E). The remnant sequence flanking the recombination site was amplified by PCR using primers P1 in T35 s and P9 in Tnos (Table S1, Fig. 1D and E) and then sequenced.

Results

HSP70m promoter is highly heat-inducible The tobacco WT and T1 plants separately carrying HSP70m-GUS and 35S-GUS were treated with temperatures ranging from 10 °C to 40 °C for 12 h. The leaf pieces were stained histochemically to determine the GUS enzyme activity. The results showed that the HSP70m-GUS was highly induced by HS temperature of 35 °C and 40 °C, even reaching the intensity of 35S-GUS (Fig. 2). Moreover, the HSP70m promoter could significantly respond to heat at 28 °C and 30 °C, but displayed weak leakage at 10 °C and 25 °C (Fig. 2). Therefore, the synthesized HSP70m promoter was highly heat-inducible and was vigorous under HS treatment.

HSP70m promoter can drive the Cre/LoxP system The HSP70m promoter was utilized to drive the Cre/LoxP

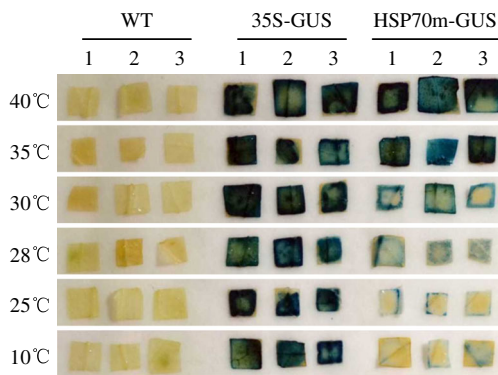


Fig. 2 GUS activities in transgenic tobacco at different temperature. *WT*, wild type plant; *35S-GUS*, transgenic tobacco carrying CaMV 35S-GUS gene; *HSP70m-GUS*, transgenic tobacco carrying HSP70m-GUS gene; 1, 2, and 3, lines that were tested

recombination system in pCRE-eGFP and pCRE-IPT-eGFP (Fig. 1D, E). Each of the T-DNA of the two vectors was transformed into tobacco via *Agrobacterium* mediation (Figs. 3 and 4). More than 20 Kan-resistant plantlets from each vector were obtained and were propagated to lines from each individual transgenic plantlet. The Kan-resistant shoots or plantlets regenerated from pCRE-eGFP transformation were

normal in phenotype (Fig. 3A, B), and the root tip had GFP fluorescence (Fig. 3C). After sufficient HS treatment at 37 °C for 16 h per d (shifting to 25 °C for 8 h at night), the GFP fluorescence disappeared in the roots (Fig. 3D), the shoots could not endure kanamycin and became albino (Fig. 3E), and the *nptII*, *Cre*, and *eGFP* genes in the T-DNA could not be detected in leaf samples by PCR (Fig. 3F–H). In contrast, many abnormal adventitious sprouts were regenerated from the tobacco calli transformed with pCRE-IPT-eGFP because of the expression of the foreign *ipt* gene (Fig. 4A, B). The abnormal sprouts exhibited that their apical dominance was lost, their leaves were unable to fully develop and were bunched close together owing to shortened internodes (Fig. 4A, B). Roots could not be induced from these abnormal shoots, and GFP fluorescence was detected in leaves (Fig. 4C). But after sufficient HS treatment, some normal sprouts grew from the abnormal sprout clumps (Fig. 4D), grew into normal shoots (Fig. 4E), and successfully rooted on the hormone-free medium to produce normal plantlets (Fig. 4F). Furthermore GFP fluorescence was undetectable in the regenerated roots (Fig. 4G), and the normal plantlets died when they were cultured on the kanamycin-contained medium (Fig. 4H). The *nptII*, *Cre*, *eGFP*, and *ipt* genes could

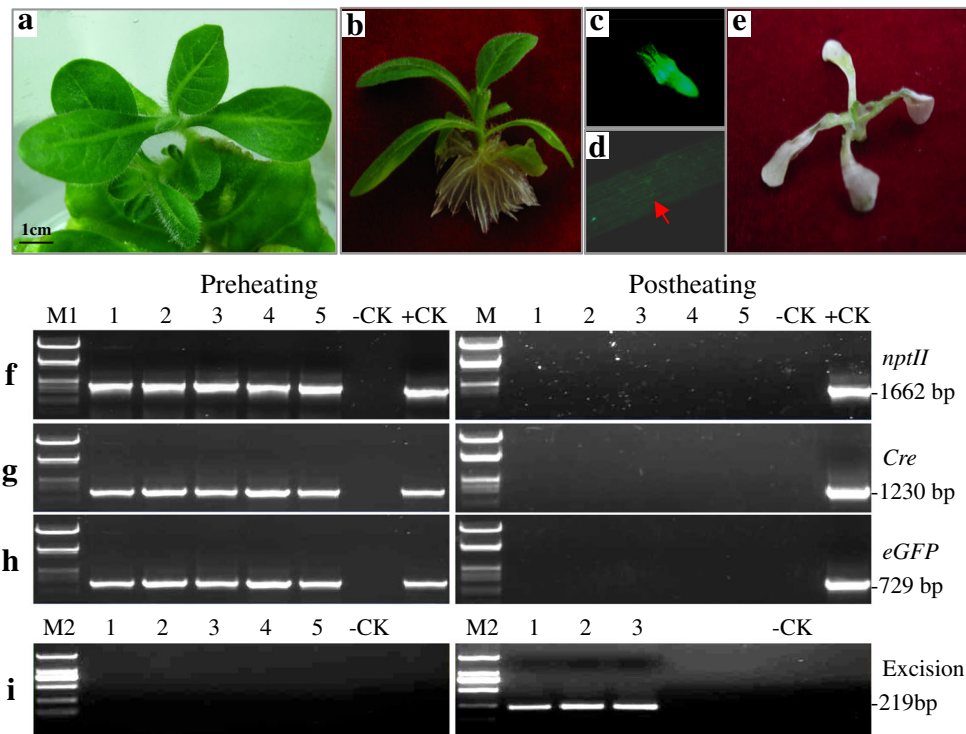
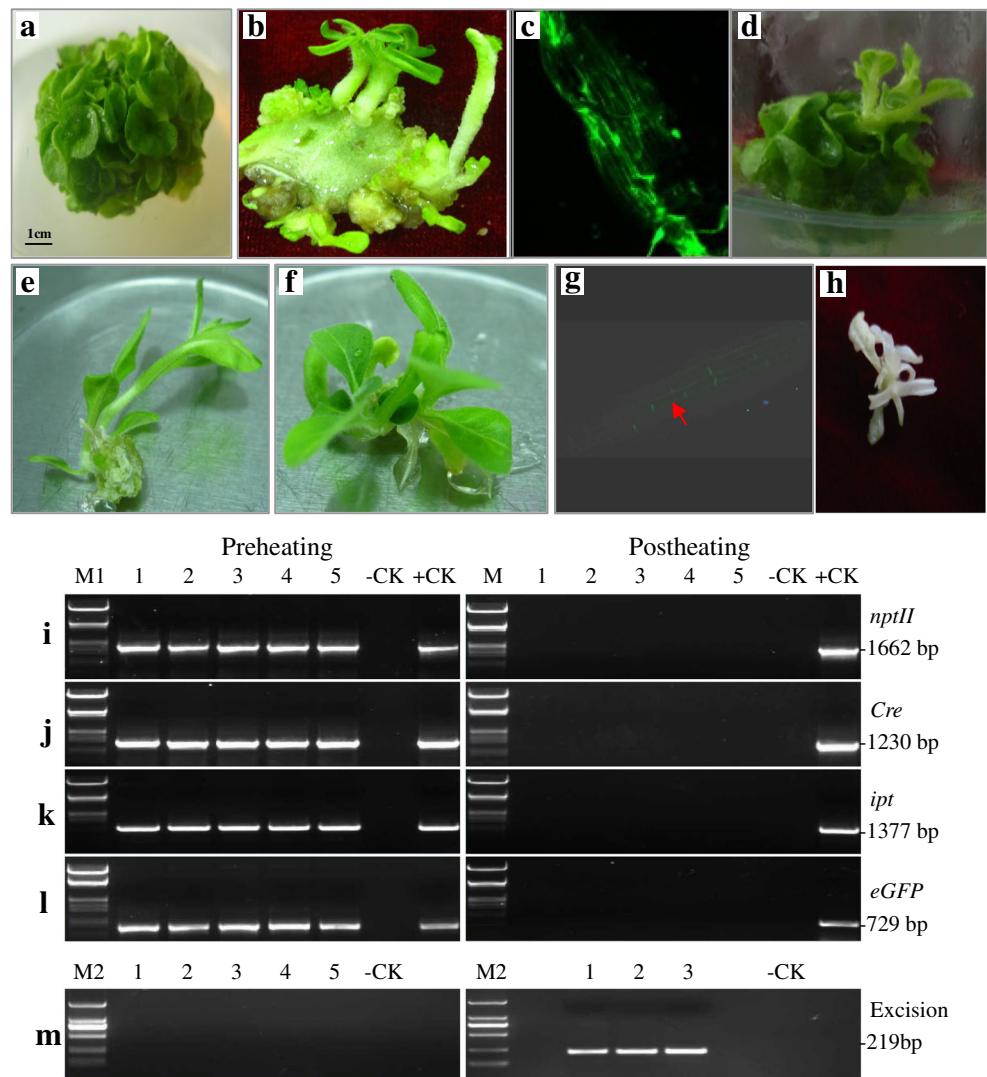


Fig. 3 Heat-inducible excision identified among transgenic tobacco carrying the T-DNA of pCRE-eGFP. (a) Kanamycin-resistant shoots; (b) rooted plantlet; (c) root tip exhibiting eGFP fluorescence; (d) root without eGFP fluorescence in transgenic tobacco after the heat-induced and Cre-mediated excision; (e) dead shoot cultured on kanamycin-containing medium; (f) PCR product of *nptII* using the primers P1/P2; (g) PCR product of *Cre* using the primers P3/P4; (h) PCR product of

eGFP using the primers P7/P8; and (i) PCR product of the sequence flanking the recombined *LoxP* site using the primers P1/P9. *M1*, DNA marker of λ DNA/EcoRI + HindIII; Lanes 1, 2, 3, 4, and 5, transgenic tobacco lines; -CK, negative control with H₂O; +CK, positive control with pCRE-eGFP vector DNA; *M2*, DNA marker of DL2000. The sequences and locations of primers P1 to P9 are listed in Table S1 and on Fig. 1D

Fig. 4 Heat-inducible excision identified among transgenic tobacco carrying the T-DNA of pCRE-IPT-eGFP. (a, b) abnormal buds of kanamycin-resistant transgenic tobacco; (c) abnormal leaf exhibiting eGFP fluorescence; (d, e) normal shoots growing from the abnormal sprout clumps after the heat-induced and Cre-mediated excision; (f) rooted plantlet; (g) root tip without eGFP fluorescence; (h) dead shoot cultured on kanamycin-containing medium; (i) PCR product of *nptII* by using primers P1/P2; (j) PCR product of *Cre* by using primers P3/P4; (k) PCR product of *ipt* by using primers P5/P6; (l) PCR product of *eGFP* by using primers P7/P8, and (m) PCR product of the sequence flanking the recombined *LoxP* site by using primers P1/P9. M1, DNA marker of λ DNA/EcoR I + Hind III; Lanes 1, 2, 3, 4, and 5, transgenic tobacco lines; -CK, negative control with H₂O; +CK, positive control with pCRE-IPT-eGFP vector DNA; M2, DNA marker of DL2000. The sequences and locations of primers P1 to P9 are listed in Table S1 and on Fig. 1E



not be detected in the leaf samples by PCR amplification (Fig. 4I–L). The results indicated that the synthesized HSP70m promoter was insufficient to drive the *LoxP*/*Cre* system to delete the marker genes at room temperature as Kan-resistant plantlets could be regenerated, but it was strong enough to induce sufficient expression of *Cre* recombinase to excise the DNA region between the two *LoxP* sites under HS conditions.

Ipt renders the marker excision visible to the naked eye
Marker excision events could be detected by PCR using primers P1 and P9 (Table S1, Fig. 1E) after HS treatment for two wk, but the complete deletion (Fig. 3F–I) of marker genes from plants containing the T-DNA of pCRE-eGFP required four to eight wk for an approximately 90% efficiency. The transgenic plantlets were grown in pots and not in culture bottles due to long period of HS treatment. A continuous and long treatment with temperatures above 35 °C inhibited tobacco plant growth. Decreasing the temperature

regime to 35 °C for 16 h/25 °C for 8 h (day/night) for longer than three weeks also inhibited tobacco growth, although the plants could grow normally after HS treatment (data not shown). Therefore, the timing of screening for marker-free plants was important to avoid long HS treatment and repetitive analysis. This would make this type of heat-inducible marker excision system inconvenient.

The abnormal buds or shoots containing the T-DNA of pCRE-IPT-eGFP could be directly treated by high temperature in culture bottles. Some of the buds or shoots grew fast and displayed leaf expansion under heat treatment (Fig. 4D, E). This type of shoot was chosen to induce root formation under heat treatment, and could produce roots within 2 to 3 wk. Only the rooted plantlets were further treated with heat for another 2 to 3 wk until their morphology was normal. Only the normal plantlets were screened for the absence of markers. The results showed that the *nptII*, *Cre*, *ipt*, and *eGFP* genes, existed in the abnormal shoots before HS treatment, but could be thoroughly deleted from the genome in

the normal plantlets after heat treatment as revealed by PCR assay (Fig. 4I–M), which reached a deletion rate of 100% in about 20 lines. The remnant DNA sequence flanking the recombined *LoxP* site revealed that the excision occurred accurately (Fig. 5), which also indicated that the modified *Cre* gene inserted with an intron could be spliced correctly. Therefore, the *ipt*-induced abnormality in buds or shoots allowed the visual selection of transgenic shoots and the normal leaf or shoot development after HS treatment would be a positive phenotype for marker excision, thus making the screening for marker-free plants convenient.

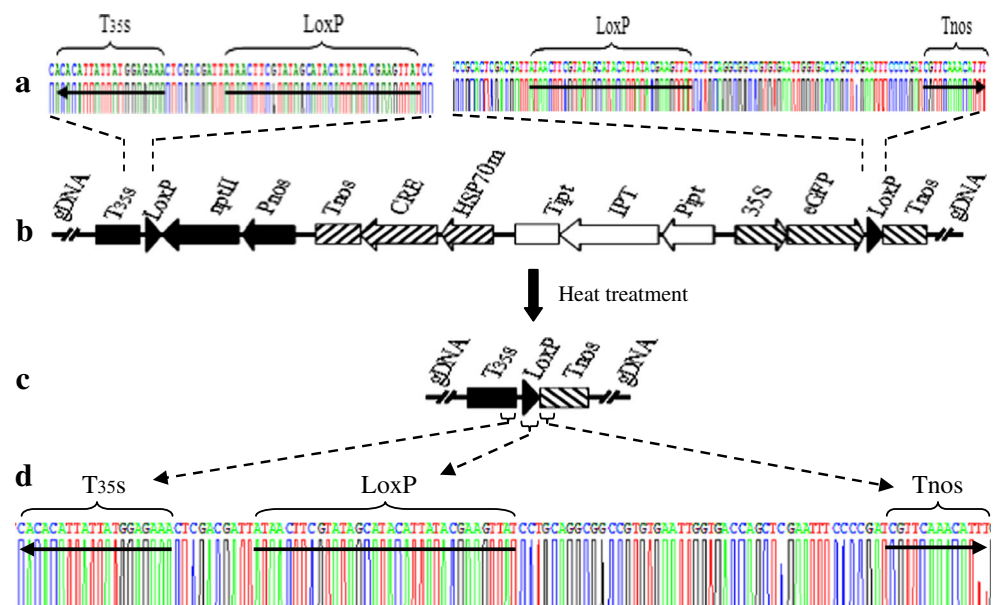
Discussion

HS promoters are widely used to regulate foreign gene expression in transgenic plants owing to their rapid, efficient, and controllable response to HS treatment (Catherine *et al.* 2011). HS promoters contain HSEs that can be bound by HS factors (HSFs) (Qian *et al.* 2014). A perfect HSE is composed of at least three contiguous inverted repeats of core sequences of nGAAn and/or nTTCn, with a structure such as 5'-nnTTCnnGAAnnTTCnn-3' or 5'-nnGAAnnTTCnnGAAnnTTCnn-3' (Santoro *et al.* 1998; Qi *et al.* 2014). A perfect HSE also exhibits higher binding affinity with HSFs than imperfect HSEs (Uffenbeck and Krebs 2006). Moreover, a strong HS promoter consists of two adjacent perfect HSEs, which should be separated with a proper spacing sequence (Cohen and Meselson 1984). AtHSP70b is an HS promoter that is weak under heat and nearly undetectable under non-HS temperature in *Arabidopsis* (Sung *et al.* 2001), but it presents leaks at and below room temperature when transferred into tobacco in our previous study (Pei *et al.* 2007). The HSP70m promoter derived from AtHSP70b

(Fig. 1A, B) showed significant increase in strength as it was as active as the CaMV 35S promoter under HS treatment (Fig. 2). Although weak leakage still existed in the HSP70m promoter below 25 °C (Fig. 2), such a leakage was insufficient to drive the *Cre/LoxP* system to obtain marker-free plants at room temperature (Figs. 3 and 4). This capacity is similar to that of other HS promoters, including the *Arabidopsis* HSP18.2 promoter (Luo *et al.* 2008) and soybean *Gmhsp17.5-E* promoter (Wang *et al.* 2005), but is more efficient than that of the HSP70 promoter from *Drosophila melanogaster* used in potato (Cuellar *et al.* 2006).

Unanticipated recombination in the T-DNA containing the *Cre/LoxP* system caused by the leaky *Cre* expression in bacteria will lead to the failure of vector construction. To prevent this issue from happening, an intron was inserted in *Cre* to ensure that the *Cre* will only perform its function in eukaryotic cells after splicing (Bunting *et al.* 1999). At least three sites of the *Cre* coding sequence (X03453) were reported to successfully insert four types of introns, such as the intron of SV40 T antigen inserted between codons 828 and 829 to construct the vector pACN (AF169416), which was used in human cells (Bunting *et al.* 1999). The intron 1 of *Schizosaccharomyces pombe* rad50 was inserted between codons 39 and 40 to construct pAW8 (EU093095), which was used in yeast (Watson *et al.* unpublished data). The introns of *Arabidopsis* KOR1 gene or castor bean catalase gene were separately inserted between codons 432 and 433 in the binary vectors pX6-GFP (AF330636) and pCre (DQ645631), which were used in plants (Zuo *et al.* 2001; Pogorelko *et al.* 2007). In the current study, we inserted the intron of castor bean catalase gene at the *EcoR* V site or between codons 552 and 553 in the *Cre* (Fig. 1C), and the resulting *Cre* could be spliced correctly as *Cre*-mediated recombination was successfully observed in tobacco (Fig. 5), thus a new site for intron insertion in the *Cre* was demonstrated.

Fig. 5 Sequencing identification of the *Cre*-mediated recombination site after the heat-induced marker gene excision in transgenic tobacco. (a) DNA sequences of two *LoxP* sites and their abutted partial *T35s* and partial *Tnos*; (b) Structure of T-DNA integrated in the transgenic tobacco genomic DNA and derived from pCRE-IPT-eGFP; (c) Structure of the remnant T-DNA after the heat-induced deletion; (d) Sequence of remnant T-DNA after the *Cre*-mediated accurate recombination. gDNA, genomic DNA. The other abbreviations are defined in Fig. 1



The *ipt* gene from *A. tumefaciens* can promote bud differentiation in a medium supplemented with or without low amounts of hormones (Ebinuma *et al.* 1997), but the transgenic plants carrying continuously expressed *ipt* exhibited abnormal phenotypes, including the absence of apical dominance, non-occurrence of full extension of leaves, and inability of root production or root extension (Gan and Amasino 1995), which were also observed in the current study (Fig. 4). These abnormalities could be used as markers that would indicate transgenics, and shoot development into normal phenotype after HS treatment could be an indication of marker excision. In this study, directly determining whether or when the *nptII* marker gene was deleted in the plantlets regenerated from pCRE-eGFP was difficult (Fig. 3), the *ipt* gene transferred from pCRE-IPT-eGFP provided a visible manifestation in discriminating selectable marker gene excision (Fig. 4).

Complete deletion of a marker gene in a transgenic plant is required, but many reports show that marker-chimeric transgenics existed (Dale and Ow 1991; Cuellar *et al.* 2006). Such chimeras were found in some plantlets transformed with pCRE-eGFP as the marker gene could not be thoroughly removed (data not shown), and even some abnormal buds or shoots derived from pCRE-IPT-eGFP transformation retained the abnormal phenotype after HS treatment (data not shown). However, in the visible marker excision system constructed in this study, only the normal, rooted plantlets were chosen to examine the marker deletion events after sufficient HS treatment. Therefore, the visible marker excision system avoided selecting plantlets that resisted Cre-mediated recombination and resulted in all the examined plantlets being marker-free.

In conclusion, an *ipt* gene was adopted to render abnormal phenotypes to transgenic buds and/or shoots, and a synthesized heat shock promoter HSP70m was used to control the *Cre/LoxP* recombination system. The results of phenotype analysis and PCR assays revealed the HSP70m promoter could regulate *Cre/LoxP* to obtain marker free tobacco plants under HS treatment. The study provides a heat-inducible *Cre/LoxP* system linked with *ipt* gene, which can be used to generate marker-free transgenic plants visible to the naked eye.

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