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ORIGINAL PAPER

Efficient production of marker-free transgenic 'Tarocco' blood orange (*Citrus sinensis* Osbeck) with enhanced resistance to citrus canker using a Cre/*loxP* site-recombination system

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Abstract Marker-free transgenic plants alleviate concerns regarding the biosafety of genetically modified organisms and promote their commercialization. In this study, a transformation vector pLI35SAAT, harboring a Cre/*loxP*-mediated recombination system combined with the isopentenyl transferase (*ipt*) selectable marker gene and an anti-bacterial peptide gene *AATCB*, was used to produce marker-free transgenic 'Tarocco' blood orange (*Citrus sinensis* Osbeck) with enhanced resistance to citrus canker. Using *ipt* positive selection, a transformation efficiency of 21.4 % was achieved. When the DNA between two *loxP* sites was excised, phenotypically normal shoots gradually appeared from 74.8 % of the transgenic *ipt* shoots. Their marker-free transgenic nature was confirmed using PCR and sequencing analyses. In vitro evaluations of citrus canker disease resistance revealed that marker-free transgenic plants exhibited an enhanced resistance to *Xanthomonas axonopodis* pv. *citri*. The marker-free transgenic plants appeared phenotypically normal under greenhouse conditions. Thus, marker-free transgenic citrus plants with targeted traits can be efficiently produced using a Cre/*loxP*-mediated recombination system combined with *ipt* positive selection.

Keywords Cre/*loxP* recombination system · *Ipt* positive selection · Marker-free transgenic citrus · 'Tarocco' blood orange · Citrus canker

Introduction

In the past 30 years, genetic transformations have been performed on many citrus genotypes, including trifoliate orange (Tan et al. 2009; Fu et al. 2011), sweet orange (He et al. 2011; Yang et al. 2011), sour orange (Gutiérrez et al. 1997), Mexican lime (Peña et al. 1997; Soler et al. 2012), grapefruit (Moore et al. 2000; Cevik et al. 2012), 'Swingle' citrumelo (Molinari et al. 2004), and 'Carrizo' citrange (Caruso et al. 2012). In most citrus transformation systems, selectable marker genes are used to select regenerated transgenic shoots. However, the presence of these marker genes in the transgenic plants becomes undesirable after transformation. Moreover, their presence causes concerns regarding their potential risks to human health and environmental safety.

Theoretically, the use of a selectable marker gene can be avoided and transgenic plants can be directly selected by molecular analyses. Using this strategy, marker-free transgenic Mexican lime (Domínguez et al. 2002) and sweet orange (Ballester et al. 2010) were recovered. However, without selection pressure, transgenic silencing and low target gene expression levels were found in many transformation events. Additionally, the detection of regenerated shoots using PCR was laborious, expensive and time-consuming. Thus, a selectable marker gene is generally required to efficiently recover transgenic plants in citrus transformation.

The removal of the selectable marker gene from transgenic plants after successfully transferring transgenes into host genomes is a more ideal strategy for overcoming the biosafety

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problems associated with selectable marker genes. The feasibility of removing the marker gene from transgenic citrus plants has been proven using site-specific recombination systems. It was first demonstrated by Ballester et al. (2007) using the multi-auto-transformation (MAT) system. The *ipt* gene was successfully removed from 65 % of 'Pineapple' sweet orange transformants. Then, Li et al. (2010) further confirmed that marker-free transgenic plants could be produced by combining the chemically regulated induction system XVE with Cre/*loxP*-mediated site-specific DNA recombination to transform 'Succari' sweet orange. Afterwards, Zou et al. (2013) developed a novel Cre/*loxP* site-specific recombination system in which the *ipt* gene served as the selectable marker and the *Cre* gene was controlled by a constitutively weak nos promoter. This system was efficient in transforming 'Jincheng' orange. These studies revealed the potential of producing marker-free transgenic citrus plants. However, these reports were conducted using reporter genes, not genes controlling targeted traits of interest to genetically engineered citrus breeding. Since no study has been reported on applying these systems to produce marker-free transgenic plants for targeted citrus traits, we have undertaken this goal.

The Asiatic type of citrus canker, caused by *Xanthomonas axonopodis* pv. *citri* (*Xac*), is an important bacterial disease and has caused severe economic damage to the citrus industry worldwide (Graham et al. 2004). 'Tarocco' blood orange, a late-maturing citrus cultivar of great economic importance, is highly susceptible to citrus canker. The transformation of genes that encode antimicrobial peptides has been an important strategy to control bacterial citrus canker. Citrus plants transformed with the *attacin* A gene showed significantly more resistance to citrus canker (Boscariol et al. 2006; Cardoso et al. 2010). Coexpression of *shiva* A and *cecropin* B in transgenic sweet orange can cooperatively inhibit the development of citrus canker (He et al. 2011). The gene *AATCB*, consisting of a *cecropin* B gene and a signal peptide sequence AAT from *Hyalophora cecropia* and the *α-1-antitrypsin* gene (Agarwal et al. 2008), encodes an anti-bacterial peptide that can be secreted to the apoplast (Zou et al. 2014a). The transformation of this gene led to an increase in resistance against citrus canker (Zou et al. 2014a).

Here, using *AATCB* as a gene of interest, we report the production of marker-free transgenic citrus that shows enhanced resistance against *Xac* using a Cre/*loxP*-mediated site-recombination system (Zou et al. 2013).

Materials and methods

Transformation vector

The transformation vector pLI35SAAT (Fig. 1) was derived from pGLINC (Zou et al. 2013). In this vector, the *mCre* gene was controlled by the nos promoter. An intron was inserted into the *mCre* gene to prevent its expression in *Escherichia coli* during cloning, thereby preventing excision of the genes between two *loxP* sites. The *ipt* gene was controlled by the CaMV35S promoter. The *mCre* and *ipt* genes were flanked by two directly oriented *loxP* sites. Outside of the *loxP* sites, the *AATCB* gene served as the gene of interest and was controlled by the CaMV35S promoter. When the T-DNA of the pLI35SAAT plasmid was integrated into the citrus genome, transcription of the *mCre* gene began. Once the intron in the *mCre* gene was correctly spliced, then the recombination function of the Cre recombinase was activated in the transformed cells, and the sequence between two *loxP* sites was removed from transformed cells to produce marker-free transformants. Finally, only the AATCB cassette and one *loxP* site were retained in the genome of transgenic plants. The pLI35SAAT plasmid was transferred into *Agrobacterium tumefaciens* EHA105 by electroporation.

Plant transformation

Transformation experiments were carried out according to a previous protocol (Dutt and Grosser 2009) with minor modifications. Fruit of 'Tarocco' blood orange was collected from the National Citrus Germplasm Repository, Chongqing, China. The fruits were surface sterilized with 75 % ethanol in a laminar flow cabinet. The seeds were collected and cultured on solid MS medium (Murashige and Skoog 1962). The epicotyl segments were inoculated by soaking in an *Agrobacterium* suspension for 15 min, and co-cultivated on MS medium supplemented with 2 mg l^{-1} 6-benzylamino, 0.25 mg l^{-1} indole-3-acetic acid, 1 mg l^{-1} 2,4-dichlorophenoxyacetic acid and 100 μM acetosyringone. The co-cultivation was incubated in the dark for 3 days at 26 °C. Controls were treated in the same way but without *Agrobacterium* infections. After co-cultivation, all of the explants were transferred onto hormone- and antibiotic-free MS medium containing 500 mg l^{-1} cefotaxime to prevent *Agrobacterium* overgrowth, and maintained in the dark at 28 °C for 2 weeks. The cultures were then exposed to a 16-h photoperiod at 28 °C for the regeneration of adventitious shoots. The explants were subcultured every 2 weeks. The shoots that developed at the cut ends of explants were recovered by in vitro grafting of apical portions onto decapitated in vitro-grown citrange seedlings (He et al. 2011). The uninfected controls were maintained under the same growth conditions. Transformation efficiency was defined as the number of transformed shoots out of the total number of inoculated explants. Three independent experiments were performed with 500–600 explants per experiment.

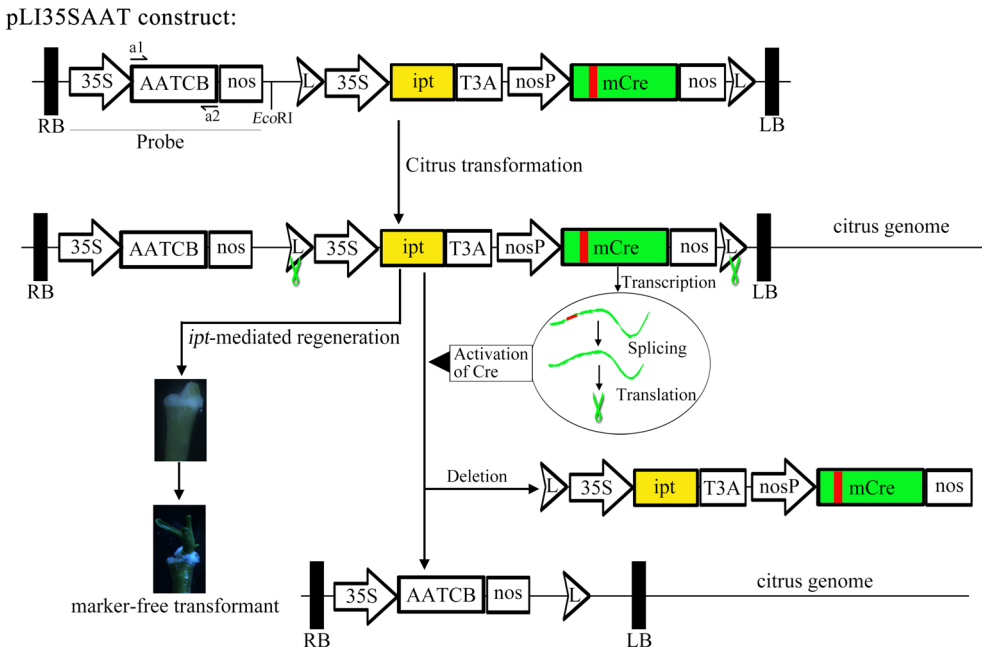

Fig. 1 Schematic diagram of the T-DNA region of the pLI35SAAT vector and the Cre/*loxP*-mediated excision of the marker gene during transformant regeneration. Two sets of 34-bp *loxP* (L) sequences were inserted in a direct orientation to flank the *ipt* and *mCre* genes. When the T-DNA is integrated into the plant genome, the expression of the *ipt* gene, driven by the CaMV 35S promoter, improves transformed cells regeneration. If the correct splicing of the introduced plant intron (shown as the *red box*) in *mCre* gene occurs, then the recombination function of Cre is activated in the plant, and the sequence (including the *mCre* and *ipt* genes) between two *loxP* sites is deleted from the transformed cells, producing marker-free transformants. RB, the right border of T-DNA; LB, the left border of T-DNA; L, *loxP* site; 35S, Cauliflower mosaic virus 35S promoter; nos, nopaline synthase terminator; T3A, the polyA of the small subunit *rbcS-3A* gene of Rubisco (pea ribulose-1,5-bisphosphate carboxylase); nosP, nopaline synthase promoter; *AATCB*, *cecropin* B gene with a signal peptide sequence AAT from the *α-1-antitrypsin* gene; *ipt*, isopentenyl transferase gene; *mCre*, bacteriophage P1 Cre recombinase gene with an intron; *EcoR*I, restriction enzyme site for *EcoR*I; a1 and a2, PCR primer sites for the amplification of the *AATCB* gene. (Color figure online)

Molecular analyses

PCR was used to confirm the presence of the foreign gene in the citrus genome. Genomic DNA was extracted from leaves using a plant genomic DNA extraction kit (Aidlab, Beijing, China). The primer pairs a1/a2 (Fig. 1), i1/i2 and c1/c2 (Fig. 3c) were used to amplify the *AATCB*, *ipt* and *mCre* genes, respectively. The predicted products of these genes were 202-, 500- and 1200-bp long, respectively. PCR reactions were carried out in 30 cycles of 94 °C for 1 min, 58 °C for 45 s and 72 °C for 1 min. The primer pair rb/lb (Fig. 3c) was used to determine whether the excision was efficient and precise. PCR products of the primer pair rb/lb were subjected to sequencing analysis according to Zou et al. (2013). The sequences of the primer pairs are as follows: a1/a2: 5′-AAGGAGATATAACAATGGCTCCATC-3′ and 5′-CTAACCAAGAGCTTTAGCTTCAC-3′; i1/i2: 5′-CTAATACATTCCGAATGGATGACCT-3′ and 5′-AAGCTCATCATAGGCTGATCGAGGA-3′; c1/c2: 5′-CTAATCGCCATCTTCCAGCAGGCGC-3′ and 5′-ATGTCCAATTTACTGACCGTACACC-3′; and rb/lb: 5′-ATCAGCTAGACTGTTGCCCGTCTCA-3′ and 5′-TGTTATCCGCTCACAATTCCACACA-3′.

To investigate the number of insertion events in transgenic plants, Southern blot analysis was performed according to the instructions of the DIG High Prime DNA Labeling and Detection Starter Kit I (Roche, Basel, Switzerland). Genomic DNA (30 μg) from transgenic plants and controls was digested with the *EcoR*I restriction enzyme, separated by 0.8 % agarose gels, UV cross-linked to Hybond-N^+ nylon membrane and hybridized with a DIG-labeled probe. The probe, a DNA fragment of the 35S-AATCB-nos cassette, was amplified from the pLI35SAAT plasmid using the primer pair p5/p3 (5′-TGAATTCGAGCTCGGTACCCGGGGATC-3′ and 5′-AATTGTGAGCGGATAACAATTTCAC-3′) and then labeled with DIG-dUTP according to the supplier's instructions.

Real-time PCR was performed to determine the expression level of the *AATCB* gene. Total RNA was extracted from the leaves of transgenic plants and controls with the TRIzol reagent (Invitrogen, Carlsbad, CA, USA). First strand cDNA was synthesized from 1 μg of total RNA

using the iScript™ cDNA Synthesis Kit (Bio-Rad, Hercules, CA, USA). The *actin* gene was used as an internal control. The primer pairs for the *actin* and *AATCB* genes are af/ar (5′-CATCCCTCAGCACCTTCCAGC-3′ and 5′-CCAACCTTAGCACTTCTCCATGTC-3′) and cf/cr (5′-AGAAAATCGAGAAGATGGGTCGT-3′ and 5′-CCAAGAGCTTTAGCTTCACCC-3′), respectively. Real-time PCR was performed using the iQ™ SYBR® Green Supermix Kit (Bio-Rad, Hercules, CA, USA) according to the manufacturer's instructions. The experiment was repeated three times.

Evaluation of marker-free transgenic citrus plants against citrus canker

An in vitro detached leaf assay was performed to test the resistance of marker-free transgenic plants to citrus canker. The bacterial pathogen *Xac* was isolated according to the report of Zou et al. (2014b). A single colony was selected and cultured in liquid LB medium overnight at 28 °C on a rotary shaker at 220 rpm. The bacterial suspension was diluted with sterile distilled water and adjusted to a concentration of 10^5 cfu ml^{-1}. Then, 3–5 fully expanded leaves from every marker-free transgenic line and control were collected and washed with sterile distilled water. In total, 24 punctures were made per leaf with a needle containing the bacterial suspension. A degreasing cotton with saturated sterile distilled water was placed on the petiole to maintain humidity. The inoculated leaves were incubated in 150-mm petri dishes at 28 °C with a 16-h photoperiod. All of the inoculated leaves were photographed and the leaf disease area was calculated using ImageJ software at 10 days after inoculation. The disease development was observed every day. The relative resistance ratio, indicating the resistance level to *Xac*, was defined as the ratio of the leaf disease area of the marker-free transgenic lines to that of control. The in vitro evaluation analysis was repeated three times.

To measure the number of *Xac* cells in transgenic citrus leaves during inoculation, bacterial growth assays were performed. Punctures were made in leaves with a needle, then 1 µl *Xac* bacterial suspension (0.5×10^8 cfu ml^{-1}) was dropped onto each pin prick. A 5-mm diameter leaf disc, containing only a single puncture site, was excised from inoculated leaves at 0, 1, 3, 5, 7 and 9 days after inoculation using a punch. Three leaf discs were grouped together and then ground in 1 ml sterile distilled water. The suspension was mixed, serially diluted and then plated on LB-agar. After incubation at 28 °C for 48 h, bacterial colonies were counted, and the number of bacterial cells per square centimeter of leaf tissue was calculated. The test was repeated three times.

Phenotype analysis

A phenotype analysis was performed according to the descriptors and data standards for citrus (*Citrus* spp.) (Jiang et al. 2006). Leaves were collected from the third to fifth nodes below the top of the aging spring shoots. The shapes of the leaf lamina, apex, base, petiole wing and lamina margin were investigated. The length and width of the leaf lamina were measured, and then the ratio of leaf lamina length to width was calculated. Five leaves from each plant were analyzed. The experiment was repeated three times.

Statistical analyses

The statistical analysis was performed using the SPSSV13.0 statistical package. Data were presented as the means ± standard deviations (SD). Significant differences were subjected to a Tukey's test. $P < 0.05$ was considered significant.

Results

Production of transgenic citrus plants

Epicotyl segments of 'Tarocco' blood orange were infected with *A. tumefaciens* that harbored the pLI35SAAT vector, which contained the *ipt* gene as a visual positive selectable marker. Adventitious shoots developed at the explants' bases after 3–4 weeks. On average, 436.0 ± 5.9 adventitious shoots were generated from 525.0 ± 4.1 co-cultivated explants after 8 weeks. These shoots developed into three distinct morphological types (Fig. 2a): 319.7 ± 3.9 phenotypically normal (PN) shoots, 37.3 ± 1.7 moderately abnormal *ipt* (MAI) shoots with short, thick internodes and narrower leaves, and 79.0 ± 0.8 extremely abnormal *ipt* (EAI) shoots exhibiting a loss of apical dominance.

Regenerated shoots were recovered by grafting onto in vitro-grown citrange seedlings (Table 1). All of the PN shoots were recovered by in vitro grafting. The MAI shoots grew slowly. Each of them was grafted repeatedly two to four times. During the repeated grafting, the *ipt* phenotype gradually disappeared. Of the MAI shoots, 34.3 ± 1.2 developed the normal phenotype, indicating that repeated grafting facilitated the recovery of the plant morphology. The remaining MAI shoots did not display a normal phenotype after in vitro grafting four times. For the EAI shoots, only those whose stems developed well were recovered by in vitro grafting (Fig. 2b), while the rest were subcultured on fresh solid medium every 2 weeks. After 4 months of further culturing, 53.0 ± 1.2 of the EAI shoots developed the normal phenotype.

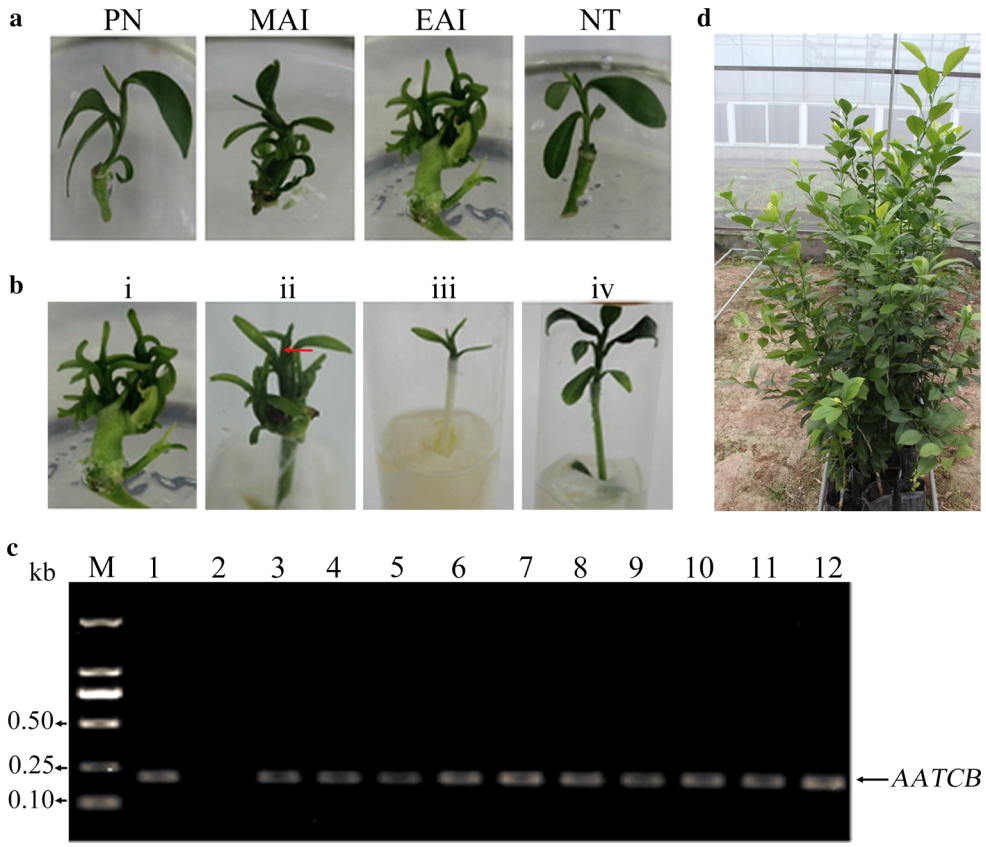

Fig. 2 Production of marker-free transgenic 'Tarocco' blood orange (*Citrus sinensis* Osbeck) plants using the *isopentenyl transferase* (*ipt*) gene as a selectable marker. **a** Three distinct phenotypes of adventitious shoots regenerated from explants 8 weeks after infection. PN, a phenotypically normal shoot developed directly from an explant; MAI, a moderately abnormal *ipt* shoot; EAI, an extremely abnormal *ipt* shoot; NT, nontransgenic control. **b** The process of generating a phenotypically normal plantlet developed from an EAI shoot by repeated in vitro grafting twice. An EAI shoot (*i*) was grafted onto an in vitro-grown citrange seedling. After 15 days, a shoot (*ii*), indicated by a *red arrow*, was cut and re-grafted onto an in vitro-grown citrange seedling (*iii*). After another 15 days of growth, the plantlet exhibited a normal phenotype (*iv*). **c** PCR analysis of the regenerated shoots. *Lane M*, DNA size markers; *lane 1*, the pLI35SAAT plasmid; *lane 2*, control; *lanes 3–4*, PN shoots; *lanes 5–8*, MAI shoots; *lanes 9–12*, EAI shoots. **d** Marker-free transgenic plants at 13 months after being transferred to the greenhouse. (Color figure online)

Table 1 Investigation of the *ipt* phenotype in regenerated transformants of 'Tarocco' blood orange (*Citrus sinensis* Osbeck) after transformation

Phenotype of shoots	No. of shoots	No. of recovered shoots showing normal phenotype	No. of recovered shoots showing *ipt* phenotype	Frequency of positive shoots (%)[a]
PN	319.7 ± 3.9	319.7 ± 3.9	0	1.0 ± 0.1
MAI	37.3 ± 1.7	34.3 ± 1.2	3.0 ± 0.8	89.3 ± 1.9
EAI	79.0 ± 0.8	53.0 ± 1.2	25.3 ± 0.9	100.0 ± 0.0

PN, MAI and EAI indicate phenotypically normal, moderately abnormal *ipt* and extremely abnormal *ipt* shoots, respectively. The data represent three independent transformation experiments

[a] Frequency (%) of positive shoots out of the total shoots analyzed. The positive shoots were determined using PCR

The analysis of the *AATCB* gene's integration events in regenerated shoots was performed using PCR (Fig. 2c; Table 1). Only 1 % of PN shoots showed the presence of the *AATCB* gene. In the MAI shoots obtained, 89.3 % amplified the *AATCB* gene. All of the clones from the EAI shoots were transgenic.

The transformation efficiency was investigated using *ipt* phenotype selection and PCR analysis (Table 2). Visually, 116.0 ± 2.2 adventitious shoots from the 525.0 ± 4.1 co-cultivated explants displayed the *ipt* phenotype. According to PCR analysis, 112.0 ± 1.9 of these shoots were transgenic. Thus, the transformation efficiency was 21.4 %.

Table 2 Comparison of transformation efficiencies in 'Tarocco' blood orange (*Citrus sinensis* Osbeck) obtained by *ipt* phenotype selection and PCR analysis

	Total no. of shoots	No. of positive shoots[c]	Transformation efficiency (%)
ipt selection[a]	116.0 ± 2.2	112.0 ± 1.9	21.4 ± 0.25
PCR analysis[b]	436.0 ± 5.9	115.6 ± 2.4	22.0 ± 0.29

The data represent three independent transformation experiments

[a] Regenerated shoots were identified by *ipt* phenotype screening and were then confirmed using PCR

[b] Regenerated shoots were directly analyzed using PCR

[c] The positive shoots were analyzed using PCR

When the 436.0 ± 5.9 regenerated shoots were subjected to PCR analysis, 115.6 ± 2.4 shoots were AATCB$^+$, indicating a transformation efficiency of 22.0 %. There was no significant difference in the calculated transformation efficiencies between the two screening methods. However, compared with the visual *ipt* selection, the PCR analysis was laborious and time-consuming. These results showed that our Cre/*loxP*-mediated self-excision system combined with a visual *ipt* selection was efficient for transforming 'Tarocco' blood orange.

All of the PCR positive shoots were grafted onto greenhouse-grown citrange seedlings for further experiments (Fig. 2d).

Molecular characteristic of marker-free transgenic citrus plants

To analyze the sequence deletion between two *loxP* sites, the *ipt* and *mCre* genes were investigated using PCR. The predicted 500-bp *ipt* and 1200-bp *mCre* fragments should be detected if there was no deletion in the transgenic lines. Upon excision, the DNA sequence between two *loxP* sites would be deleted. As a result, the *ipt* and *mCre* genes would not be amplified from the genomic DNA. All of the phenotypically normal transgenic lines obtained in this study showed no amplification of the *ipt* and *mCre* fragments (Fig. 3a), verifying that the selectable marker gene had been excised and that these phenotypically normal transgenic lines were marker-free. The *ipt* and *mCre* genes were detected in all of the phenotypically abnormal transgenic lines (Fig. 3a).

To evaluate the complete removal of the *loxP*-comprised fragment, all of the transgenic lines were analyzed using PCR with the rb/lb primer pair. If the *ipt* and *mCre* genes were deleted completely by the Cre/*loxP* recombination system, then an 800-bp fragment containing only one *loxP* site should be amplified (Fig. 3c). If the auto-excision was not completed, then a 4500-bp fragment would be found (Fig. 3c). As shown in Fig. 3b, three amplification patterns were observed. Only the predicted 800-bp fragment was amplified from the marker-free transgenic lines. However, both the 800- and 4500-bp fragments were observed in 14.7 ± 1.2 of the phenotypically abnormal transgenic lines, while only the 4500-bp fragment was found in the remaining phenotypically abnormal transgenic lines. All of the DNA amplicons were sequenced, revealing 774- and 4345-bp fragments that correspond to the lower and the upper PCR bands in Fig. 3b, respectively. The partial nucleotide sequences are shown in Fig. 3c. The nucleotide sequences were in accordance with the theoretical recombination between two *loxP* sites and the T-DNA sequence in the pLI35SAAT plasmid. These results showed that the excision mediated by the Cre/*loxP* recombination system was always precise but not complete in some transgenic lines. According to these data, perfect auto-excision occurred in 74.8 % of the transgenic lines.

To investigate the *AATCB* gene's copy number in the transgenic plants, Southern blot analysis was performed on 25 randomly selected marker-free transgenic lines. The transgene was detected in all of the lines tested (Fig. 4a). One to three copies of the transgene were detected in the transgenic lines, and 76.0 % of the marker-free transgenic lines contained a single copy of the transgene. The hybridization band was not detected in the control.

Expression analyses of the *AATCB* gene at the mRNA level in the above-mentioned 25 marker-free transgenic lines were performed. As shown in Fig. 4b, the expression of the *AATCB* gene was detected in all of the analyzed marker-free transgenic lines.

Evaluation of marker-free transgenic citrus plants to citrus canker disease

To evaluate resistance against citrus canker disease, 19 marker-free transgenic lines with a single T-DNA insertion site, as well as a control, were selected. Fully expanded intact leaves from these lines were inoculated with *Xac*, and 3 days after the pin-prick inoculations, slight lesions with callus-like tissue and a water-soaked margin were

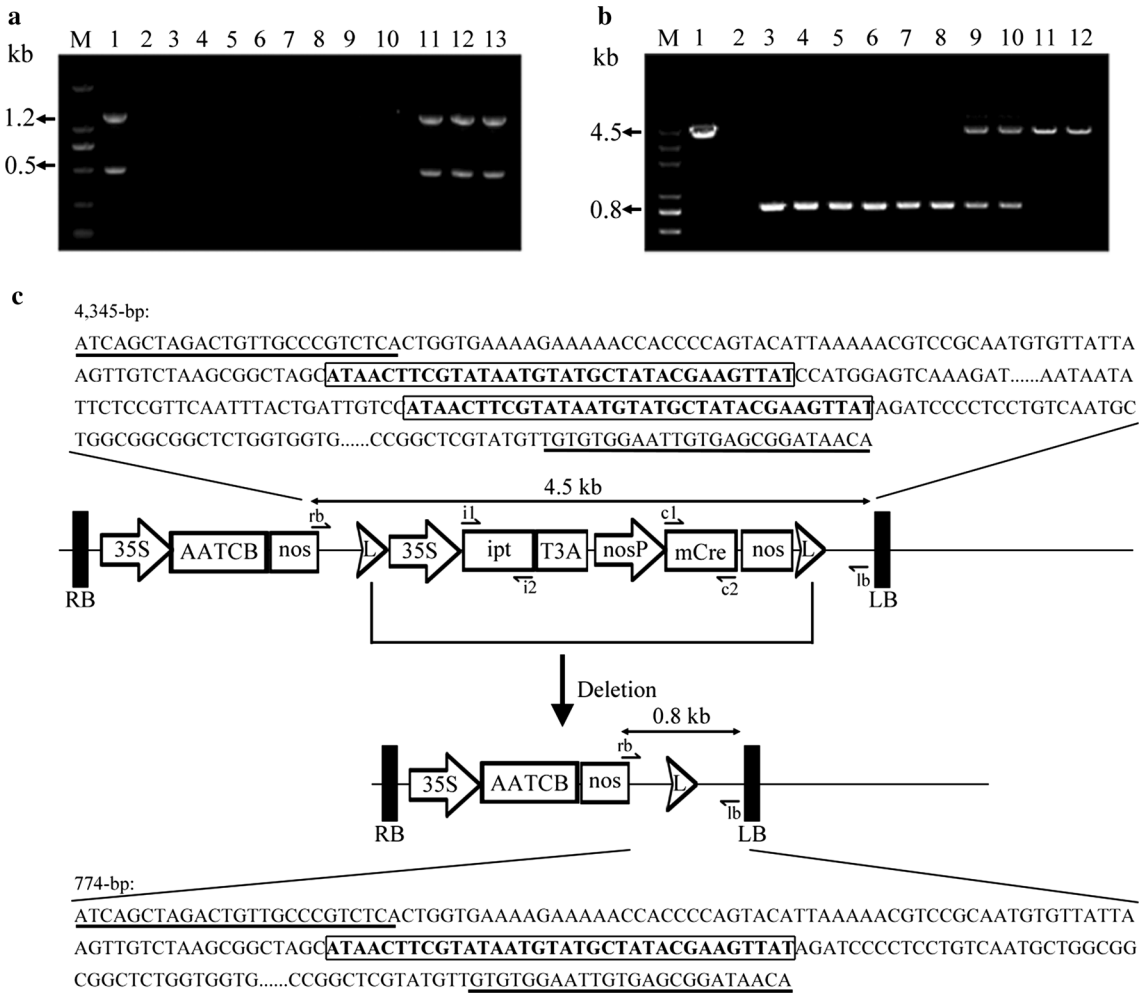

Fig. 3 Excision analysis of marker-free transgenic 'Tarocco' blood orange (*Citrus sinensis* Osbeck) plants. **a** PCR analysis of the selectable marker *isopentenyl transferase* (*ipt*) and *mCre* genes in transgenic plants. *Lane M*, DNA size markers; *lane 1*, the pLI35SAAT plasmid; *lane 2*, control; *lanes 3–10*, phenotypically normal transgenic lines; *lanes 11–13*, phenotypically abnormal transgenic lines. The expected lengths of PCR products are indicated on the *left*. The presence of 1200- and 500-bp fragments indicates the existence of the *mCre* and *ipt* genes in transgenic plants. **b** Excision analysis of the recombination region in transgenic plants. *Lane M*, DNA size markers; *lane 1*, the pLI35SAAT plasmid; *lane 2*, control; *lanes 3–8*, marker-free transgenic lines; *lanes 9–12*, transgenic lines with abnormal phenotypes. Only an 800-bp expected fragment was detected in marker-free transgenic lines. Only a 4500-bp fragment or both the 800- and 4500-bp fragments were detected in transgenic lines with abnormal phenotypes. **c** Nucleotide sequences of the recombination region in transgenic plants. The partial sequences of the 774- and 4345-bp amplicons are shown. The sequences of the rb and lb primers are indicated by underlining, and the *loxP* site is indicated by a *box*. i1 and i2, c1 and c2, and rb and lb represent PCR primer sites for the amplification of the *ipt* gene, *mCre* gene and *loxP*-flanked region, respectively

observed at the puncture sites (Fig. 5). In the following days, the lesions enlarged, and the callus-like tissues extruded over the puncture sites (Fig. 5). The lesion area, as an indicator of disease severity, was calculated at 10 days after inoculation. The lesions of transgenic lines varied from 0.30 ± 0.03 to 1.10 ± 0.12 mm^2 (Table 3). In total, 12 out of 19 transgenic lines showed significantly smaller diseased area than the control. A relative resistance ratio analysis confirmed that 11 out of 19 transgenic lines had significantly increased resistance to *Xac* compared with the control (Fig. 6a). Lines T4, T12, T19 and T22 showed markedly reduced disease severities by 45.5–69.7 % compared with the control.

To further examine the effects of the *AATCB* gene on plant performance after being challenged with *Xac*, the bacterial growth of *Xac* cells in leaves of the 19 tested marker-free transgenic lines was analyzed. The recovery of *Xac* at 1 day after pin-prick inoculation was examined in all transgenic lines and the control. Bacterial growth levels differed significantly between the transgenic lines and control for up to 9 days. The bacterial populations in leaves of transgenic lines T4, T12, T19 and T22 were significantly

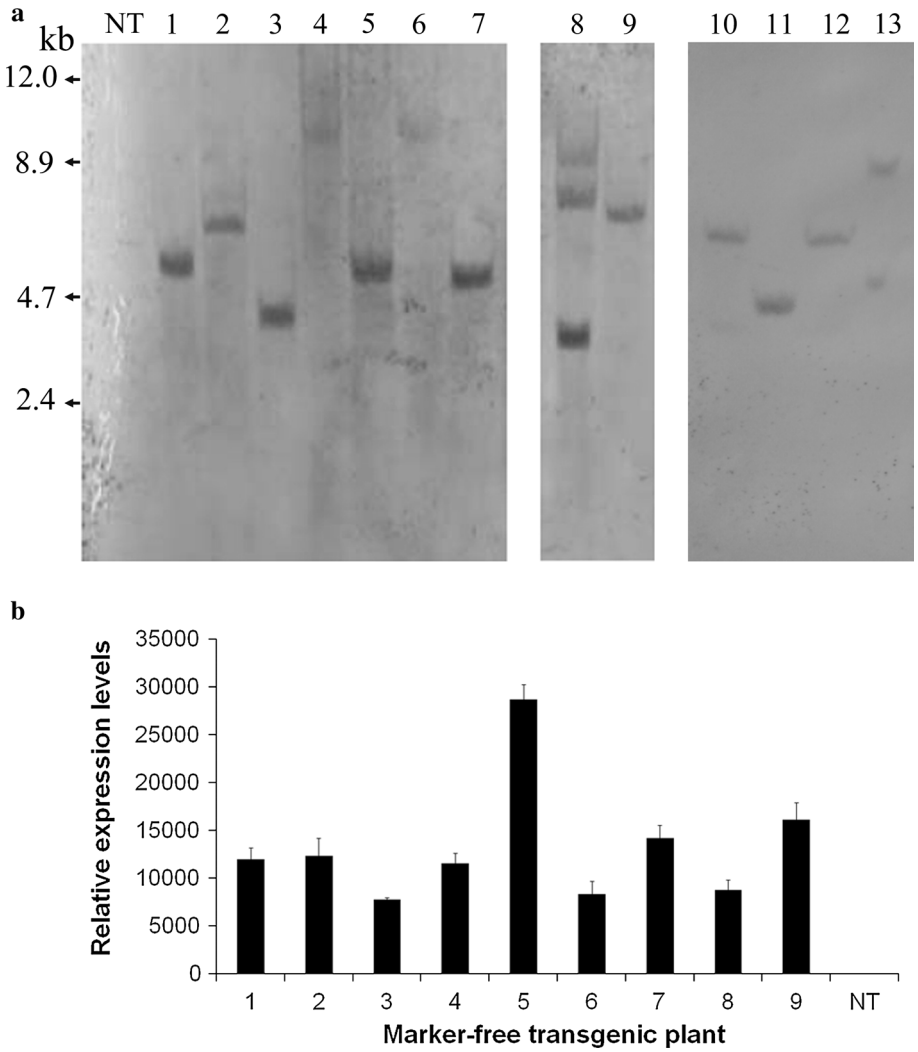

Fig. 4 Molecular analysis of marker-free transgenic 'Tarocco' blood orange (*Citrus sinensis* Osbeck) plants. **a** Southern blotting of marker-free transgenic plants. The total genomic DNA of transgenic plants was digested with *Eco*RI, electrophoresed and probed with a digoxigenin-labeled amplified fragment of the 35S-AATCB-nos cassette (Fig. 1). *Lane NT*, control; *lanes 1–13*, marker-free transgenic lines. **b** Expression analysis of the *AATCB* gene in marker-free transgenic plants. For data analysis, the control was used as a calibrator, and the rest of the samples were assigned to the relative values based on the calibrator. *NT*, control; *1–9*, marker-free transgenic lines

lower compared with that of the control. In particular, transgenic lines T12 and T19 had Log units that were lower by ~1.3 compared with the control (Fig. 6b), indicating that the bacterial growth in these transgenic lines was inhibited.

Phenotype analysis of marker-free transgenic citrus plants

To investigate whether prolonged expression of the *mCre* gene impacted the phenotypes of the transgenic plants, phenotype analyses were performed. As shown in Table 4, every marker-free transgenic line possessed the same morphological characteristics as that of the control. Additionally, compared with the control, the ratio of leaf lamina length to width in the marker-free transgenic lines was not statistically significantly different. These results indicated that the expression of the *mCre* gene controlled by a weak nos promoter would not affect the phenotype of the marker-free transgenic plants.

Discussion

Recently, an efficient Cre/*loxP* site-specific recombination system was developed to generate marker-free transgenic citrus plants (Zou et al. 2013). However, it was not known whether this system was applicable to economically valuable citrus cultivars without a reporter gene. In this study, using the important economic cultivar 'Tarocco' blood orange, we described the application of this Cre/*loxP*-mediated recombination system to produce marker-free transgenic plants with enhanced resistance to citrus canker. Using *ipt* positive selection, a transformation efficiency of 21.4 % was achieved, and the excision between two *loxP* sites was precise and complete in 74.8 % of the transgenic

Fig. 5 Citrus canker symptoms on leaves of marker-free transgenic 'Tarocco' blood orange (*Citrus sinensis* Osbeck) plants after in vitro inoculation of *Xanthomonas axonopodis* pv. *citri* by pin-prick. **a** A leaf of the T12 marker-free transgenic line. **b** A leaf of the nontransgenic line (control). Fully expanded leaves of the transgenic line T12 and control were challenged with 10^5 cfu ml^{-1} *Xac* pathogen. Disease development and symptoms were observed every day after inoculation. The photographs were taken at 0, 3, 5 and 10 days after inoculation

lines. An in vitro disease resistance assay showed that marker-free transgenic lines had enhanced resistance to citrus canker. Our results demonstrated the feasibility of using the Cre/*loxP*-mediated recombination system combined with *ipt* positive selection for engineering marker-free transgenic citrus with targeted traits.

Cre/*loxP*-mediated recombination systems have been applied to produce marker-free transgenic plants with targeted traits (Roy et al. 2008; Zhang et al. 2009). However, unlike with these recombination systems, our system uses the *ipt* gene as the selectable marker (Zou et al. 2013). The overexpression of the *ipt* gene in transformed cells can increase endogenous cytokinin levels, which can lead to the production of *ipt* shoots and promote the organogenesis of transformed shoot, causing an increased transformation efficiency (Smigocki and Owens 1988; Sugita et al. 1999; Saelim et al. 2009; Ballester et al. 2007; Zou et al. 2013). In this study, almost all of the transgenic shoots with functional *ipt* gene exhibited short, thick internodes, narrower leaves and a loss of apical dominance at the early regeneration stage (Fig. 2a), which allowed us to visually select the transformants. Moreover, no significant difference in the calculated transformation efficiencies was found between *ipt*-mediated morphological selection and PCR analysis (Table 2). These results showed that the *ipt* gene is a

Table 3 Leaf disease area (mm^2) of marker-free transgenic 'Tarocco' blood orange (*Citrus sinensis* Osbeck) plants after inoculation with the *Xac* pathogen

Transgenic lines	Leaf disease area (mm^2)	Transgenic lines	Leaf disease area (mm^2)
T1	0.64 ± 0.04^{fgh}	T14	0.93 ± 0.10^{bcd}
T2	1.10 ± 0.12^{a}	T15	0.95 ± 0.04^{bc}
T3	0.75 ± 0.07^{ef}	T16	0.97 ± 0.02^{ab}
T4	0.54 ± 0.09^{h}	T18	0.78 ± 0.06^{ef}
T5	0.81 ± 0.10^{de}	T19	0.38 ± 0.07^{i}
T6	0.97 ± 0.09^{ab}	T21	0.82 ± 0.09^{cde}
T7	0.93 ± 0.13^{bcd}	T22	0.54 ± 0.07^{h}
T9	1.02 ± 0.12^{ab}	T23	0.71 ± 0.05^{efg}
T10	0.63 ± 0.09^{fgh}	T25	0.60 ± 0.04^{gh}
T12	0.30 ± 0.03^{i}	NT	0.99 ± 0.05^{ab}

Different letters on the upper right corner of the leaf disease area represent significant differences from the control plant (NT) at $P < 0.05$ according to Tukey's test. Data were calculated at 10 days after inoculation

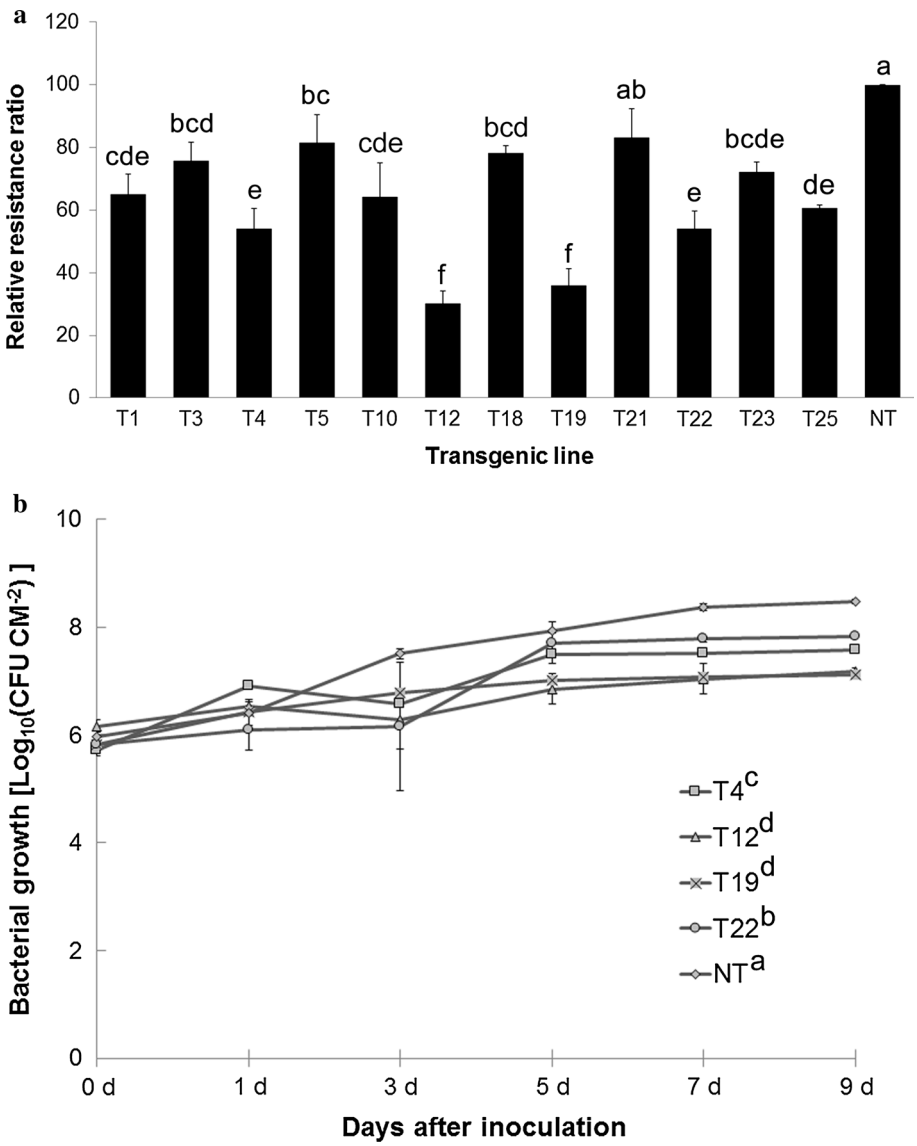

Fig. 6 Evaluation of resistance to citrus canker disease in marker-free transgenic 'Tarocco' blood orange (*Citrus sinensis* Osbeck) plants. **a** Relative resistance ratio of marker-free transgenic lines to citrus canker. Fully expanded leaves of the marker-free transgenic lines and control were treated with 10^5 cfu ml^{-1} *Xac* pathogen. 10 days after inoculation, the lesion area was calculated and the relative resistance ratio was expressed as the lesion area of marker-free transgenic lines to that of control. **b** Growth of *Xac* in leaves of marker-free transgenic citrus plants. Bacterial cells were extracted from leaves at 0, 1, 3, 5, 7 and 9 days after inoculating *Xac* at 0.5×10^8 cfu ml^{-1}. After incubation at 28 °C for 48 h, bacterial populations were counted. T1–T25 and NT represent the marker-free transgenic lines and control, respectively. *Different letters* on the *top of the bars* (Fig. 6a) and on the upper right corner of the transgenic lines and NT (Fig. 6b) represent significant differences from the control plant (NT) at $P < 0.05$ according to Tukey's test at 10 and 9 days after *Xac* inoculation, respectively

Table 4 Phenotype analysis of marker-free transgenic 'Tarocco' blood orange (*Citrus sinensis* Osbeck) plants

Transgenic lines	Shape of leaf lamina	Shape of leaf apex	Shape of leaf base	Shape of petiole wing	Shape of leaf lamina margin	Ratio of leaf lamina length to width
T1	Oblong	Attenuate	Cuniform	Oblanceolate	Dentate	2.3 ± 0.2
T2	Oblong	Attenuate	Cuniform	Oblanceolate	Dentate	2.4 ± 0.1
T3	Oblong	Attenuate	Cuniform	Oblanceolate	Dentate	2.4 ± 0.1
T4	Oblong	Attenuate	Cuniform	Oblanceolate	Dentate	2.4 ± 0.1
T5	Oblong	Attenuate	Cuniform	Oblanceolate	Dentate	2.4 ± 0.1
T6	Oblong	Attenuate	Cuniform	Oblanceolate	Dentate	2.4 ± 0.1
T7	Oblong	Attenuate	Cuniform	Oblanceolate	Dentate	2.4 ± 0.1
T8	Oblong	Attenuate	Cuniform	Oblanceolate	Dentate	2.4 ± 0.1
T9	Oblong	Attenuate	Cuniform	Oblanceolate	Dentate	2.3 ± 0.1
T10	Oblong	Attenuate	Cuniform	Oblanceolate	Dentate	2.4 ± 0.1
T11	Oblong	Attenuate	Cuniform	Oblanceolate	Dentate	2.3 ± 0.1
T12	Oblong	Attenuate	Cuniform	Oblanceolate	Dentate	2.3 ± 0.1
T13	Oblong	Attenuate	Cuniform	Oblanceolate	Dentate	2.3 ± 0.2
NT	Oblong	Attenuate	Cuniform	Oblanceolate	Dentate	2.3 ± 0.1

reliable morphological marker in 'Tarocco' blood orange transformation.

The *ipt* phenotype of regenerated shoots is a typical feature of cytokinin's excessive accumulation in *ipt*-mediated transformation (Smigocki and Owens. 1988; Honda et al. 2011). In our study, many *ipt* shoots and their regenerants were *ipt*-free. Thus, the *ipt* phenotype of these shoots might be caused by the temporal overproduction of *ipt*-mediated cytokinin in the transgenic cells at the early stage of transformation. On the other hand, residual *Agrobacterium* cells in infected explants could secrete cytokinin into the medium because of the microbial overexpression of the *ipt* gene under the CaMV 35S promoter (Fig. 1). This change of cytokinin level might also cause the over-accumulation of the hormone in transformed shoots and could promote the occurrence of the *ipt* phenotype. A hormone content analysis showed that the cytokinin level gradually declined during the regeneration of *ipt* shoots by repeated grafting (Supplementary Fig. 1). Similarly, Honda et al. (2011) showed that grafting decreased the cytokinin level of *ipt* transgenic kiwifruit, leading to the morphological recovery of the scion. These data suggested that the over-accumulated cytokinin in marker-free shoots may be diluted or diffused into the nearby wild type rootstock tissue during repeated grafting. Therefore, the cytokinin level gradually declined to normal levels in these shoots, finally resulting in the morphological recovery of the grafted regenerants (Supplementary Fig. 1).

In our Cre/*loxP*-mediated recombination system, the *mCre* gene was controlled by a constitutively expressed nos promoter. A previous study showed that the nos promoter was a suitable driver for the Cre recombinase expression, which caused 100 % excision in pGLINC transgenic 'Jingcheng' orange plants (Zou et al. 2013). However, in this study, the complete excision of the *loxP*-comprised DNA fragments did not always occur, indicating that the nos promoter was not the most suitable driver for the Cre recombinase in 'Tarocco' blood orange. The sequencing results confirmed that all of the transgenic plants with abnormal phenotypes contained non-recombinant T-DNA (Fig. 3c). The expression level of Cre recombinase might be insufficient to induce the recombination of two *loxP* sites, or a complete excision event might take a longer time to produce marker-free transgenic shoots. We failed to collect enough leaves to analyze the expression levels of Cre recombinase, or a second round deletion analysis of *loxP*-comprised fragments, because the transgenic plants with abnormal phenotypes gradually died after being transferred to the greenhouse. Chimerism and non-excision events are drawbacks of the recombinase-mediated deletion process (Zhang et al. 2006; Bai et al. 2008). However, since the *mCre* gene is controlled by the constitutively expressed nos promoter in the pLI35SAAT vector, it is expected that excision would occur sooner or later in all of the transformed cells. Therefore, the phenotypically abnormal transgenic plants should be kept in vitro rather than moved to the greenhouse until the complete excision event has taken place.

The constitutive expression of the *Cre* gene in transgenic cells caused phenotypic aberrations in a variety of plants (Coppoolse et al. 2003). However, biosafety concerns were minimized because the expression of the *Cre* gene driven by the tissue-specific or inducible promoter was remarkably low (Coppoolse et al. 2003; Cuellar et al. 2006). These results suggested that the preferable expression of the *Cre* gene is best limited to a specific tissue and/

or a short period of time by using tissue-specific or inducible promoters, respectively. Based on this observation, almost all of the Cre/*loxP* recombination systems adopted these strategies (Zhang et al. 2006, 2009; Ma et al. 2009). In our pLI35SAAT vector, the *mCre* gene was driven by a nos promoter, which caused the continuous expression of the *mCre* gene until it was auto-excised. However, when we investigated the phenotypes of the marker-free transgenic plants, no adverse effect from the *mCre* gene's expression was observed (Table 4). Thus, the concern over constitutive expression was minimized in our transformation vector construct because of the use of the weak nos promoter to drive the *mCre* gene' expression.

Genetic engineering of marker-free transgenic plants with important traits, such as biotic-and abiotic-stress resistance, as well as the improved composition and quality of products, has been successfully conducted in some plant varieties (Chakraborti et al. 2008; Darwish et al. 2014; Zhang et al. 2009; Sato et al. 2004; Baisakh et al. 2006). In citrus, Ballester et al. (2007) showed that *R*/RS-mediated recombination could generate mutant sequences in the plant genome near the recombination site, resulting in no or low expression of the *uidA* gene. No adverse effects of the target gene's expression were reported in Cre-mediated production of marker-free transgenic citrus plants (Zou et al. 2013). Here, the high expression level of the *AATCB* gene was detected in most of the marker-free transgenic plants (Fig. 4b), and a sequencing analysis confirmed that the excision mediated by the Cre/*loxP* recombination system was always precise (Fig. 3c). Moreover, our in vitro detached leaf assay revealed that marker-free transgenic plants had enhanced resistance to citrus canker. These results suggested that this Cre/*loxP* recombination system might be more suitable to produce marker-free transgenic plants with targeted traits in citrus genetic breeding than the *R*/RS-mediated recombination system. To our knowledge, this is the first report of marker-free transgenic plants with targeted traits in citrus genetic breeding.

Many antimicrobial peptides, including cecropins, showed activities against bacterial diseases in in vitro and field tests (Jan et al. 2010; He et al. 2011). In this study, when marker-free transgenic plants were treated with *Xac*, the relative resistance ratio analysis revealed that transgenic lines exhibited markedly reduced disease severities compared with the control (Fig. 5a), indicating that the development of citrus canker in these transgenic lines was slowed. This was further confirmed by the subsequent analysis of bacterial growth. The Log units in the transgenic lines T19 and T12 were $\sim$1.30 lower than that of the control. Graham et al. (1992) also reported that citrus varieties with field resistance to *Xac* possessed bacterial populations 1–2 Log units lower than in susceptible varieties. These results indicated that the *AATCB* gene could be used efficiently in citrus genetic breeding for canker resistance. However, further field investigations are needed to test the resistance to citrus canker disease in these marker-free transgenic lines.

In conclusion, this study demonstrated that our Cre/*loxP*-mediated self-excision, combined with *ipt* positive selection, was efficient in producing marker-free transgenic citrus plants. The transgenic 'Tarocco' blood orange acquired enhanced resistance to citrus canker after being infected in vitro with the *Xac* pathogen.

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Conflict of interest The authors declare that they have no conflict of interest.

References

Agarwal S, Singh R, Sanyal I, Amla DV (2008) Expression of modified gene encoding functional human α-1-antitrypsin protein in transgenic tomato plants. Transgenic Res 17:881–896

Bai XQ, Wang QY, Chu CC (2008) Excision of a selective marker in transgenic rice using a novel Cre/*loxP* system controlled by a floral specific promoter. Transgenic Res 17:1035–1043

Baisakh N, Rehana S, Rai M, Oliva N, Tan J, Mackill DJ, Khush GS, Datta K, Datta SK (2006) Marker-free transgenic (MFT) near-isogenic introgression lines (NIILs) of 'golden' indica rice (cv. IR64) with accumulation of provitamin A in the endosperm tissue. Plant Biotechnol J 4:467–475

Ballester A, Cervera M, Peña 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

Ballester A, Cervera M, Peña L (2010) Selectable marker-free transgenic orange plants recovered under non-selective conditions and through PCR analysis of all regenerants. Plant Cell Tissue Org Cult 102:329–336

Boscariol RL, Monteiro M, Takahashi EK, Chabregas SM, Vieira MLC, Vieira LGE, Pereira LFP, Mourão Filho FAA, Cardoso SC, Christiano RSC, Bergamin Filho A, Barbosa JM, Azevedo FA, Mendes BMJ (2006) *Attacin* A gene from *Tricloplucia ni* reduces susceptibility to *Xanthomonas axonopodis* pv. *citri* in transgenic *Citrus sinensis* 'Hamlin'. J Am Soc Hort Sci 131:530–536

Cardoso SC, Barbosa-Mendes JM, Boscariol-Camargo RL, Christiano RSC, Bergamin Filho A, Vieira MLC, Mendes BMJ, Mourão Filho FAA (2010) Transgenic sweet orange (*Citrus sinensis* L. Osbeck) expressing the *attacin* A gene for resistance to *Xanthomonas citri* subsp. *citri*. Plant Mol Biol Rep 28:185–192

Caruso P, Baldoni E, Mattana M, Paolo DP, Genga A, Coraggio I, Russo G, Picchi V, Recupero GR, Locatelli F (2012) Ectopic expression of a rice transcription factor, Mybleu, enhances tolerance of transgenic plants of Carrizo citrange to low oxygen stress. Plant Cell Tissue Org Cult 109:327–339

Cevik B, Lee RF, Niblett CL (2012) *Agrobacterium*-mediated transformation of grapefruit with the wild-type and mutant

RNA-dependent RNA polymerase genes of *Citrus tristeza virus*. Turk J Agric For 36:195–206

Chakraborti D, Sarkar A, Mondal HA, Schuermann D, Hohn B, Sarmah BK, Das S (2008) Cre/*lox* system to develop selectable marker-free transgenic tobacco plants conferring resistance against sap sucking homopteran insect. Plant Cell Rep 27:1623–1633

Coppoolse ER, Vroomen MJ, Roelofs D, Smit J, Gennip F, Hersmus BJ, Nijkamp HJ, Haaren MJ (2003) Cre recombinase expression can result in phenotypic aberrations in plants. Plant Mol Biol 51:263–279

Cuellar W, Gaudin A, Solórzano D, Casas A, Nopo L, Chudalayandi P, Medrano G, Kreuze J, Ghislain M (2006) Self-excision of the antibiotic resistance gene *npt*II using a heat inducible *Cre-loxP* system from transgenic potato. Plant Mol Biol 62:71–82

Darwish NA, Khan RS, Ntui VO, Nakamura I, Mii M (2014) Generation of selectable marker-free transgenic eggplant resistant to *Alternaria solani* using the R/RS site-specific recombination system. Plant Cell Rep 33:411–421

Domínguez A, Fagoaga C, Navarro L, Moreno P, Peña L (2002) Regeneration of transgenic citrus plants under non selective conditions results in high-frequency recovery of plants with silenced transgenes. Mol Genet Genomics 267:544–556

Dutt M, Grosser JW (2009) Evaluation of parameters affecting *Agrobacterium*-mediated transformation of citrus. Plant Cell Tissue Org Cult 98:331–340

Fu XZ, Khan EU, Hu SS, Fan QJ, Liu JH (2011) Overexpression of the *betaine aldehyde dehydrogenase* gene from *Atriplex hortensis* enhances salt tolerance in the transgenic trifoliate orange (*Poncirus trifoliata* L. Raf.). Environ Exp Bot 74:106–113

Graham JH, Gottwald TR, Riley TD, Achor D (1992) Penetration through leaf stomata and strains of *Xanthomonas campestris* in citrus cultivars varying in susceptibility to bacterial diseases. Phytopathol 82:1319–1325

Graham JH, Gottwald TR, Cubero J, Achor DS (2004) *Xanthomonas axonopodis* pv. *citri*: factors affecting successful eradication of citrus canker. Mol Plant Pathol 1:1–15

Gutiérrez-E MA, Luth D, Moore GA (1997) Factors affecting *Agrobacterium*-mediated transformation in citrus and production of sour orange (*Citrus aurantium* L.) plants expressing the coat protein gene of citrus tristeza virus. Plant Cell Rep 16:745–753

He YR, Chen SC, Peng AH, Zou XP, Xu LZ, Lei TG, Liu XF, Yao LX (2011) Production and evaluation of transgenic sweet orange (*Citrus sinensis* Osbeck) containing bivalent antibacterial peptide genes (*Shiva* A and *Cecropin* B) via a novel *Agrobacterium*-mediated transformation of mature axillary shoots. Sci Hort 128:99–107

Honda C, Kusaba S, Nishijima T, Moriguchi T (2011) Transformation of kiwifruit using the *ipt* gene alters tree architecture. Plant Cell Tissue Organ Cult 107:45–53

Jan PS, Huang HY, Chen HM (2010) Expression of a synthesized gene encoding cationic peptide cecropin B in transgenic tomato plants protects against bacterial diseases. Appl Environ Microbiol 10:769–775

Jiang D, Gong GZ, Hong QB, Ye QL, Wang CQ, Zhao XY, Li XQ (2006) Descriptors and data standard for citrus (*Citrus* spp.). Chinese Agricultural Press, Beijing, pp 14–16

Li LJ, Yang L, Li F, Xie YM, Zeng BQ, Deng ZN (2010) Marker-free transgenic system of *Citrus*. J Hunan Agric Univ (Nat Sci) 36:649–652

Ma BG, Duan XY, Niu JX, Ma C, Hao QN, Zhang LX, Zhang HP (2009) Expression of stilbene synthase gene in transgenic tomato using salicylic acid-inducible Cre/*loxP* recombination system with self-excision of selectable marker. Biotechnol Lett 31:163–169

Molinari HBC, Bespalhok JC, Kobayashi AK, Pereira LFP, Vieira LGE (2004) *Agrobacterium tumefaciens*-mediated transformation of Swingle citrumelo (*Citrus paradisi* Macf. × *Poncirus trifoliata* L. Raf.) using thin epicotyl sections. Sci Hort 99:379–385

Moore GA, Febres VJ, Niblett CL, Luth D, McCaffery M, Garnsey SM (2000) *Agrobacterium*-mediated transformation of grapefruit (*Citrus paradisi* Macf.) with genes from *Citrus tristeza* virus. Acta Hort 535:237–243

Murashige T, Skoog F (1962) A revised medium for rapid growth and bio assays with tobacco tissue cultures. Physiol Plant 15:473–497

Peña L, Cervera M, Juárez J, Navarro A, Pina JA, Navarro L (1997) Genetic transformation of lime (*Citrus aurantifolia* Swing.): factors affecting transformation and regeneration. Plant Cell Rep 16:731–737

Roy SD, Saxena M, Bhomkar PS, Pooggin M, Hohn T, Bhalla-Sarin N (2008) Generation of marker free salt tolerant transgenic plants of *Arabidopsis thaliana* using the gly I gene and cre gene under inducible promoters. Plant Cell Tissue Organ Cult 95:1–11

Saelim L, Phansiri S, Suksangpanomrung M, Netrphan S, Narangajavana J (2009) Evaluation of a morphological marker selection and excision system to generate marker-free transgenic cassava plants. Plant Cell Rep 28:445–455

Sato S, Xing A, Ye X, Schweiger B, Kinney A, Graef G, Clemente T (2004) Production of γ-linolenic acid and stearidonic acid in seeds of marker-free transgenic soybean. Crop Sci 44:646–652

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

Soler N, Plomer M, Fagoaga C, Moreno P, Navarro L, Flores R, Peña L (2012) Transformation of Mexican lime with an intron-hairpin construct expressing untranslatable versions of the genes coding for the three silencing suppressors of *Citrus tristeza virus* confers complete resistance to the virus. Plant Biotechnol J 10:597–608

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

Tan B, Li DL, Xu SX, Fan GE, Fan J, Guo WW (2009) Highly efficient transformation of GFP and KNU genes into precocious trifoliate orange (*Poncirus trifoliata* [L.] Raf), a potential model genotype for functional genomics studies in *Citrus*. Tree Genet Genomes 5:529–537

Yang L, Hu CH, Li N, Zhang JY, Yan JW, Deng ZN (2011) Transformation of sweet orange [*Citrus sinensis* (L.) Osbeck] with *pthA-nls* for acquiring resistance to citrus canker disease. Plant Mol Biol 75:11–23

Zhang YY, Li HX, Ouyang B, Lu YG, Ye ZB (2006) Chemical-induced auto excision of selectable markers in elite tomato plants transformed with a gene conferring resistance to lepidopteran insects. Biotechnol Lett 28:1247–1253

Zhang Y, Liu H, Li B, Zhang JT, Li YZ, Zhang HX (2009) Generation of selectable marker-free transgenic tomato resistant to drought, cold and oxidative stress using the Cre/*loxP* DNA excision system. Transgenic Res 18:607–619

Zou XP, Peng AH, Xu LZ, Liu XF, Lei TG, Yao LX, He YR, Chen SC (2013) Efficient auto-excision of a selectable marker gene from transgenic citrus by combining the Cre/*loxP* system and *ipt* selection. Plant Cell Rep 32:1601–1613

Zou XP, Peng AH, Liu QQ, He YR, Wang JZ, Xu LZ, Lei TG, Yao LX, Chen SC (2014a) Secreted expression of *cecropin* B gene enhances resistance to *Xanthomonas axonopodis* pv. *citri* in transgenic *Citrus sinensis* 'Tarocco'. Acta Hort Sinica 41:417–428

Zou XP, Song EL, Peng AH, He YR, Xu LZ, Lei TG, Yao LX, Chen SC (2014b) Activation of three pathogen-inducible promoters in transgenic citrus (*Citrus sinensis* Osbeck) after *Xanthomonas axonopodis* pv. *citri* infection and wounding. Plant Cell Tissue Organ Cult 117:85–98

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<tr><td>委托文献目录</td><td colspan="4">1. Efficient production of marker-free transgenic 'Tarocco' blood orange (Citrus sinensis Osbeck) with enhanced resistance to citrus canker using a Cre/loxP site-recombination system
作者: Peng, Aihong; Xu, Lanzhen; He, Yongrui; 等.
PLANT CELL TISSUE AND ORGAN CULTURE　卷: 123　期: 1　页: 1-13　出版年: OCT 2015</td></tr>
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附件 1：SCI 收录情况

序号	作者排名	题名	检索号	影响因子	中科院 JCR 类别 JCR 分区			出版时间	语种	出版商地址
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					小类	生物工程与应用微生物	3 区			
					小类	植物科学	3 区			

附件 2：SCI 检索结果记录

第 1 条，共 1 条

标题: Efficient production of marker-free transgenic 'Tarocco' blood orange (Citrus sinensis Osbeck) with enhanced resistance to citrus canker using a Cre/loxP site-recombination system

作者: Peng, AH (Peng, Aihong); Xu, LZ (Xu, Lanzhen); He, YR (He, Yongrui); Lei, TG (Lei, Tiangang); Yao, LX (Yao, Lixiao); Chen, SC (Chen, Shanchun); Zou, XP (Zou, Xiuping)

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