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Full Length Research Paper

Testing an inducible expression system in transgenic lisianthus (*Eustoma grandiflorum* cv. LisaBlue)

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In this present study, we established an efficient *Agrobacterium*-transformation **system using an inducible expression vector co-cultivated with lisianthus leaf explants. To explore the potential application of the inducible system pJCGLOX in transgenic lisianthus (*Eustoma grandiflorum* cv. LisaBlue). *Arabidopsis FLOWERING LOCUS T (FT)* gene was employed as the target gene. Genomic polymerase chain reaction (PCR) and southern blotting confirmed the presence of this transgene in the genomes of several kanamycin-resistant lines. Transcription of *FT* was shown to be activated in these transformants after inducing treatment, demonstrating efficacy of the inducible systems in this kind of flower.**

Key words: Genetic transformation, inducible gene expression. *Eustoma grandiflorum,* CRE-*lox* recombination.

INTRODUCTION

Lisianthus (*Eustoma grandiflorum*), a member of the family *Gentianaceae*, is native to the central and southern United States. It is grown as an increasingly popular cut flower due to its large flowers, long stems and extended vase life. Breeding new cultivars of lisianthus is economically desirable and the common method so far is sexual hybridization. Genetic engineering can be used to introduce heterogenous genes that are not present in the gene pool of host plant, thus allowing novel phenotypes to be generated, such as alteration in flower, leaf color, shape and flowering time (Thiruvengadam and Yang, 2009).

In past decades, micro-propagation systems for lisianthus have been successfully established and transgenic plantlets were regenerated from various somatic tissues such as shoot tips, leaf and root (Paek and Hahn, 2000). Transformation of lisianthus was reported preliminarily by Handa (1992) and since then, transgenic lisianthus were obtained from both microparticle bombardment (Takahashi et al., 1998) and *Agrobacterium*-mediated transformation (Semeria et al., 1996).

Inducible gene expression system is useful for both application and basic research, especially for genes where constitutive expression has detrimental effect. The pJCGLOX vector employs CRE-*loxP* recombination system and the subcellular targeting of proteins by a mammalian glucocorticiod receptor (GR), generating a double-lock conditional induction system. This vector was successfully tested in *Nicotiana tabacum* bright yellow-2 (BY-2) cells (Joubes et al., 2004). However, it is unclear if it has the potential to be suitable for whole plant assay.

In this study, heat-inducible *FT* construct was transformed into lisianthus using *Agrobacterium*-mediated transformation. Expression of the transgene was detected by reverse transcriptase-polymerase chain reaction (RT-PCR) assay under inducing conditions. All the result suggests the inducible expression system may have wide potential application in lisianhus breeding.

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Abbreviations: 6-BA, **6**-Benzylaminopurin; **AS,** Acetosyringone; **CTAB,** hexadecyltrimethylammonium bromide; **GFP,** green fluorescent protein; **HSP,** heat shock protein; **IBA,** indole-3-butyric acid; **MS** medium, Murashige and Skoog medium**; NAA,** naphthalene-acetic acid; **PCR,** polymerase chain reaction.

*Both authors contributed equally to the work.

MATERIALS AND METHODS

Plant materials

The axenic lisianthus (*Eustoma grandiflorum* cv. LisaBlue) plants were obtained from surface-sterilized seeds and maintained in MS media under photoperiod of 16 h as required by giving a photon flux density of 5000 lux, temperature of 25°C in a culture room.

Bacterial strains and plasmids used for transformation

Agrobacterium tumefaciens strain GV3101 (pMP90RK) was used as the T-DNA donor. In plasmid pJCGLOX, the e*gfp* gene is under the control of 35S promoter and terminated by OCS terminator, the *npt*II is flanked by 35S promoter and 35S terminator (Figure 1A). Heat-inducible CRE-GR recombinase would excise e*gfp* and TOCS when exposed to dexamethasone, thus leading to constitutive expression of the *FT* under the control of the 35S promoter (Joubes et al., 2004).

Transformation of leaf explants

The *Agrobacterium* cells carrying the binary vectors were grown in YEP liquid medium supplemented with the corresponding antibiotics (50 mg ml^{-1} gentamicin and 25 mg ml^{-1} chloromycin) in a rotating (200 rpm) incubator at 28°C for about 24 h. When the OD_{600} value reached 0.6 to 0.8, the cultures were centrifuged for 10 min at 4000 g and the bacterial pellets were re-suspended in liquid co-cultivation media (MS media, 6-BA 1.0 mg ml^{-1}, NAA 0.1 mg ml^{-1}). Explants from leaf pieces were then submerged in the inoculum for 30 min, then transferred to solid co-cultivation medium with 100 µmol l^{-1} AS (Saharan et al., 2004). After co-cultivation with *Agrobacterium* for 3 days in the dark, the leaf explants were placed on selection medium (MS media, agar 7 g L^{-1}, 6-BA 1.0 mg ml^{-1}, NAA 0.1 mg ml^{-1}, cefotaxime 400 mg ml^{-1}, kanamycin 100 mg ml^{-1}). The explants were transferred to fresh selection medium every 3 weeks, until resistant calli formed. Then the kanamycin-resistant calli were cut from explants and transferred to fresh selection medium for shoot induction. During this stage, leaves of the little shoots were cut and subjected to molecular analysis. Elongated shoots (about 2 cm) were rooted in rooting medium (MS media, agar 7 g L^{-1}, IBA 0.5 mg ml^{-1}, cefotaxime 400 mg ml^{-1}).

Genomic-PCR assay Southern blotting

Genomic DNA was isolated from putative transformants and wild type plants using the CTAB method (Chaudhry et al., 1999). Presence of the transgenes was tested by primer pairs: 5'-TGTTGGAGACGTTCTTGATCC-3' and 5'-AGCCACTCT-CCCTCTGACAA-3' for *FT* gene and primer pairs: 5'-ACGTA-AACGGCCACAAGTTC-3' and 5'-TAGCTCAGGTAGTGGTTGTCG-3' for e*gfp* gene, respectively. PCR products were electrophoresed in agarose gel, stained with ethidium bromide and visualized under ultraviolet light (Sambrook and Russell, 2001). For Southern blotting, 10 µg genomic DNA was digested with *Spe*I, separated electrophoretically on a 1.0% (w/v) agarose gel and transferred onto Hybond N+ blotting membrane (Roche Diagnostics) under alkaline conditions, following the manufacturer's instructions. Southern blotting was carried out according to Sambrook (Sambrook and Russell, 2001). Probes were produced using the DIG Easy Hyb kit (Roche Diagnostics).

Inducing expression of *FT* and RT-PCR assay

To induce expression of *FT* in plants transformed with pJCGLOX, the regenerated plants of each transgenic line and wild type plantlets at rooting stage *in vitro* were transferred into fresh rooting media supplemented with 10 µmol L^{-1} dexamethasone, after a culture period of 24 h, these plants were subjected to 37°C for 2 h per day, applied for 5 consecutive days. Leaves of these plants before and after induction (48 h after the last treatment) were collected and RNA was isolated. RT-PCR was carried out using *FT* and e*gfp* primer pairs as described previously. Tissues from root of each transgenic plantlet was collected and observed for GFP expression using fluorescent microscope before and after induction (48 h after the last treatment).

RESULTS AND DISCUSSION

Transformation and regeneration of lisianthus

Transformation and regeneration of lisianthus at different stages is shown in Figure 2. After co-cultivation with *Agrobacterium*, the leaf explants were placed on selection media to induce callus. About 3 weeks later, some green-yellow, newly formed calli appeared from the wounded leaf pieces while other explants became yellow, dark and gradually died. These resistant calli were transferred to fresh SM for shoot induction and regenerated shoots were rooted in rooting medium when they reached 2 to 3 cm. Inducing roots is a time-consuming period during the whole transformation process, 8 weeks was needed for the regenerated shoots to form systematic roots in this study. Then, the rooted plantlets were transferred to pots, acclimated for 2 weeks and were moved to the greenhouse. Overall, a total of 6 months is necessary from co-cultivation to transplantation.

Molecular analysis of putative transgenic lines

The presence or absence of target genes in all putative transformants was determined by genomic PCR analysis. PCR products of the expected size (467 bp) corresponding to *FT* were amplified from some of the kanamycin-resistant plants. Whereas, DNA samples from non-transformed plants did not yield such a PCR product (Figure 1B). A 547 bp band of the e*gfp* gene was also amplified from the *FT*-positive genomes transformed with the plasmid (Figure 1B). Southern DNA analysis was employed to confirm the presence of the *FT* transgene in the genomes of these transgenic lines. As shown in Figure 1C, the presence of specific band corresponds to the full length of *FT* gene in transgenic lines and no corresponding band present is in non-transgenic lines. Furthermore, expression of GFP was detected in young calli of the southern-positive transformants with a fluorescent microscope, indicating success of the transformation experiment (Figure 3A).

We got 8 transgenic lines approved by PCR and southern analysis. However, 3 of them displayed ideal GFP expression, then micro-propagation was carried out for each of the lines and thus many individual plantlets

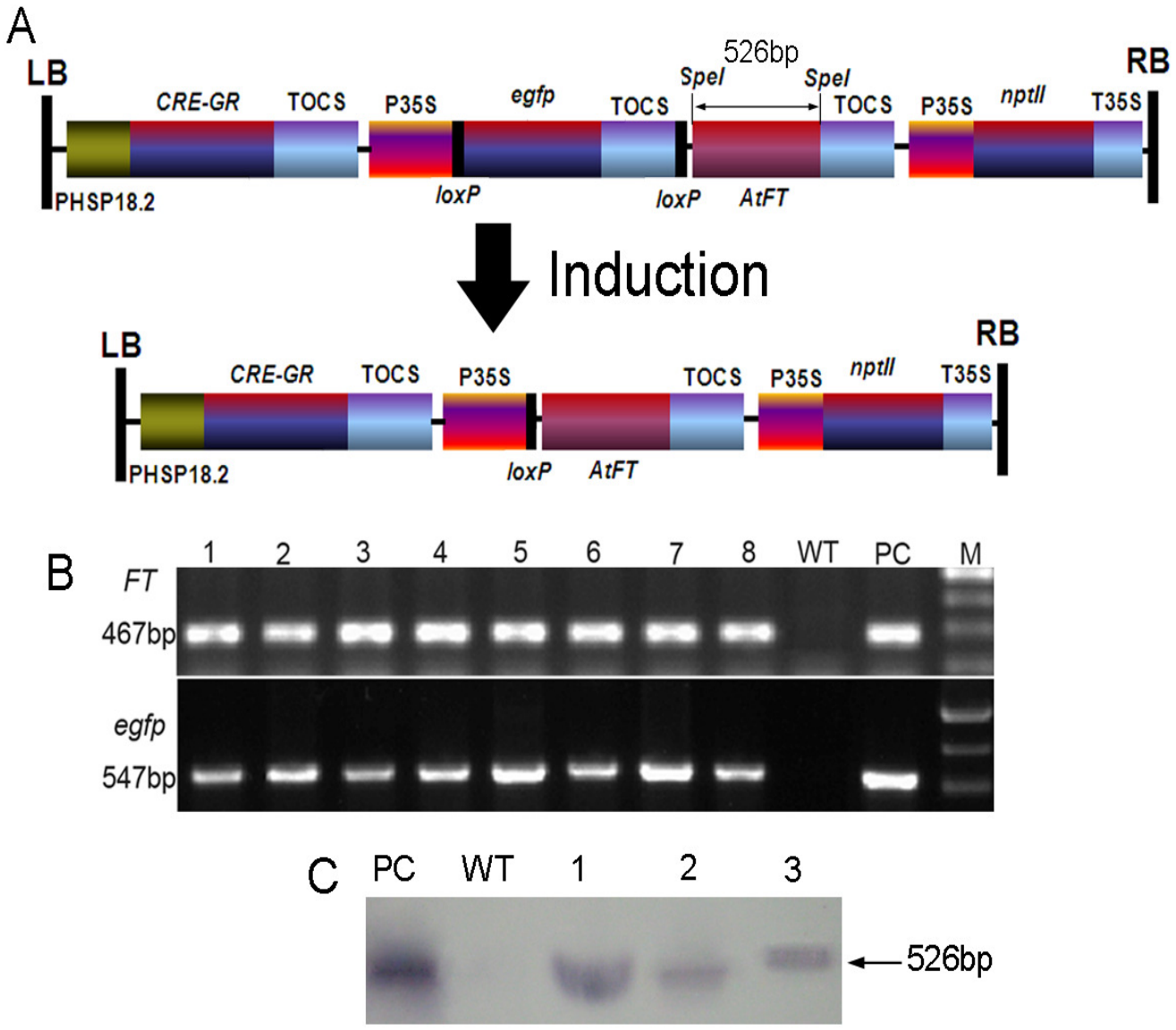

Figure 1A. Schematic diagram of the T-DNA regions pJCGLOX. PHSP18.2, promoter of the Arabidopsis HSP18.2; *CRE-GR*, *CRE* recombinase and fused to the *GR* sequence. TOCS, octopine synthase terminator; P35S, 35S promoter of cauliflower mosaic virus; e*gfp*, enhanced green fluorescent protein reporter gene, the e*gfp* ended by the TOCS is flanked by two *loxP* sites; *FT*, the coding sequence of the Arabidopsis *FLOWERING LOCUS T* gene; T35S, terminator of cauliflower mosaic virus; The *npt*II is flanked by P35S and T35S. Excision of the *egfp* and TOCS flanked by the two *loxP* sites by the induced ligand-inducible CRE-GR recombinase, thereby generating the constitutive expression of the *FT* under control of the P35S. B. Electrophoresis of genomic-PCR products. Genomic PCR transformed with pJCGLOX. Lanes 1-8, transformed lines. PCR products corresponding to *FT* and *egfp* were amplified. WT, non-transgenic lisianthus. PC, positive control, PCR using plasmid. M, DNA marker III. C. Southern blotting of 10 µg *SpeI* –cut genomic DNA probe with *FT* fragment.

from them were obtained. Recombination efficiency was evaluated based on these plants.

Inducing expression of *AtFT* gene in transgenic plants

In plants transformed with plasmids pJCGLOX, the heat-shock inducible promoter *HSP 18.2* was used to drive the recombinase gene *CRE*. To further lock the system, the CRE was fused to the hormone-binding domain of the rat glucocorticoid receptor GR. Thus, heat inducible CRE-GR recombinase could only excise e*gfp* and TOCS in the presence of dexamethasone, leading to constitutive expression of *FT* under the control of the 35S promoter. To induce expression of *FT* in plants transformed with

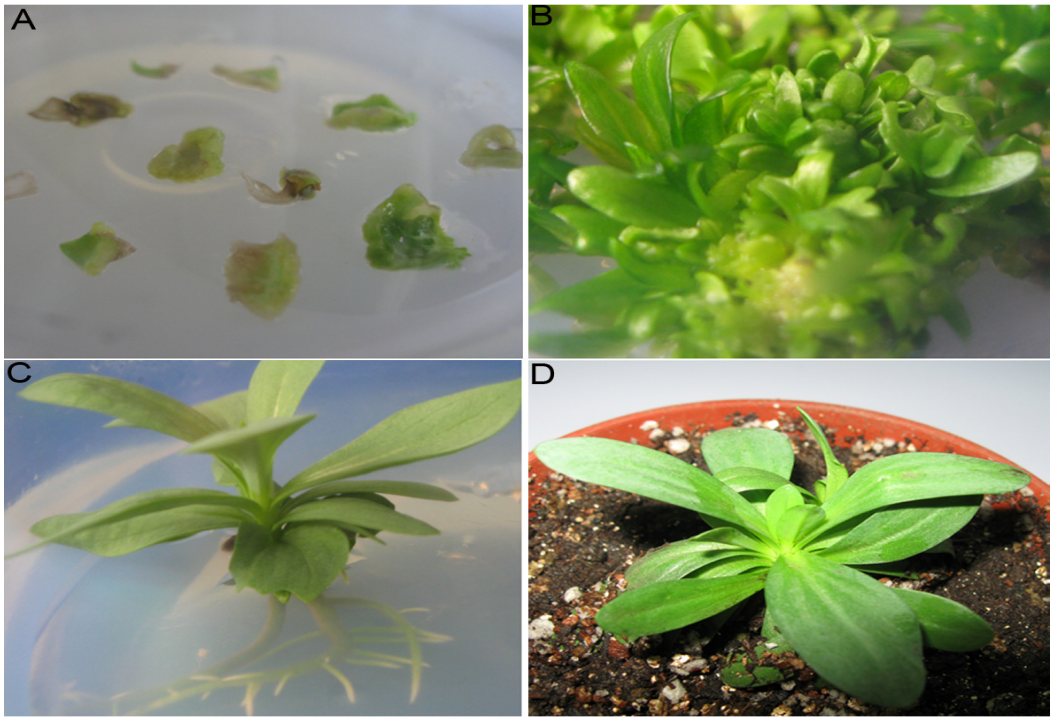

Figure 2. Regeneration of lisianthus from leaf explants. A. Callus formation from leaf explants. B. Multiple shoots induction from callus. C. Roots development from shoots. D. Rooted plantlet transferred in soil.

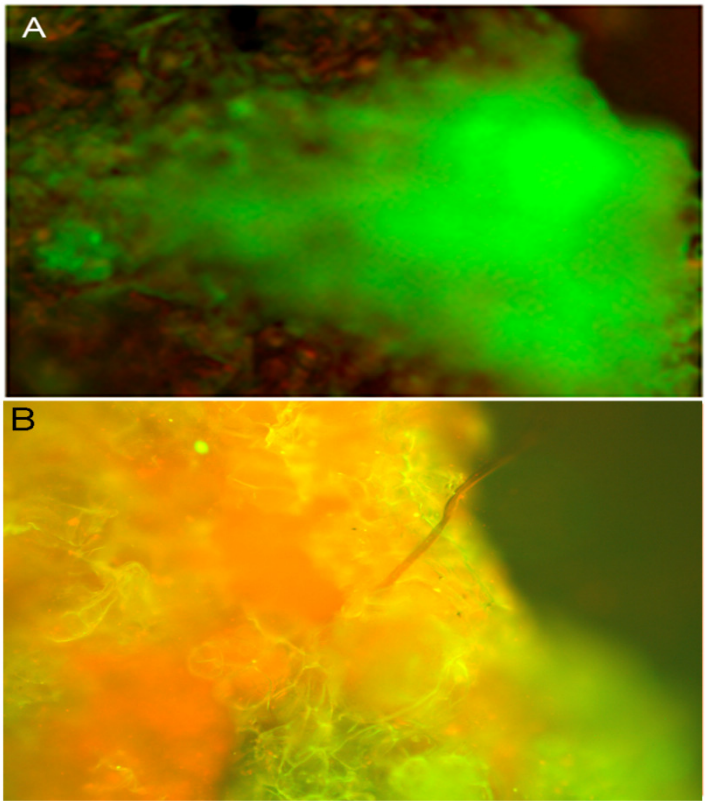

Figure 3. GFP observation in lisianthus transformed with pJCGLOX before and after induction. A. Expression of eGFP in transformed callus. B. Green fluorescence was attenuated after induction.

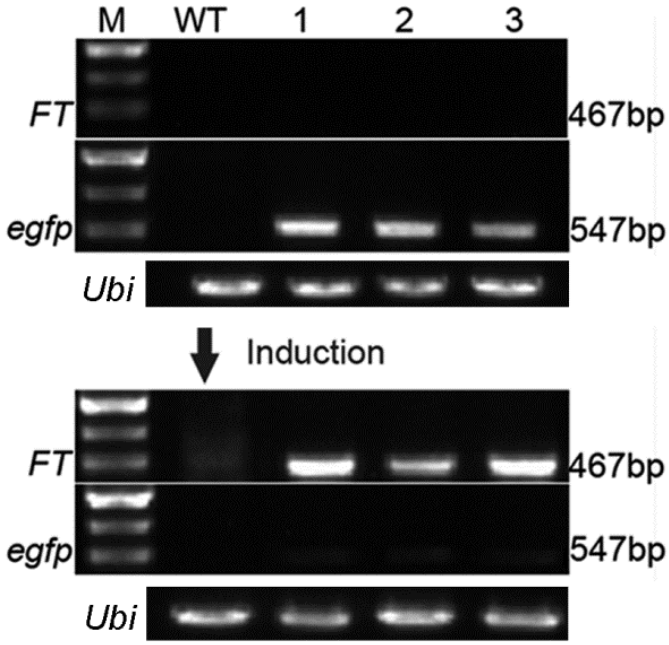

Figure 4. RT-PCR assay for transgenic lisianthus plants before and after induction. Lane M: DNA marker III; WT: wild type; lane 1-3: transgenic lines.

Table 1. Recombination efficiency in 3 transgenic lines.

Transgenic line	Southern /GFP	Number of plantlets induced	FT-RTPCR positive	Efficiency
T1	+/+	25	4	16%
T4	+/+	22	5	22.7%
T8	+/+	15	2	13.3%

pJCGLOX, the regenerated plantlets were firstly transferred onto media supplemented with dexamethasone, and then treated with heat shock. RT-PCR assay shows that a 547 bp region of the e*gfp* gene was amplified from leaves before induction (Figure 4) and no DNA fragment was obtained from the same materials using primer pairs corresponding to *FT*. After induction treatment, a 467bp fragment of *FT* gene appeared with attenuation of e*gfp* fragment in 3 transgenic lines, which indicated the successful recombination in these lines. Furthermore, the presence or absence of the GFP allowed the transgenic cells to be traced and the recombination event to be monitored easily by fluorescent microscope observation (Zhao et al., 2010). The GFP fluorescence was weakened after induction to great extent, leaving red auto-fluorescence of chlorophyll (Figure 3B).

The CRE-lox recombination efficiency is different among the 3 lines (Table 1). GFP fluorescence was not completely disappeared even in a same plantlet (Figure 3B), demonstrating incomplete recombination in multi-cell comprised tissues. The highest recombination efficiency in this study was 22.7%, which is lower than that in tobacco, rice and banana (Chong-Perez et al., 2011; Joubes et al. 2004; Srivastava et al., 2011). This may be due to species variation or different developmental stages of the inducing materials. However, we realized inducing expression of the target gene, which is useful in many application studies in plant breeding.

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