**Testing an inducing expression system in transgenic** **Lisianthus(*Eustoma grandiflorum* cv. LisaBlue)**

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**Abstract**

In this present study, we have established an efficient ***Agrobacterium*-transformation** system using an inducible expression vector co-cultivated with leaf explants. To explore the potential application of the inducible systems pJCGLOX in transgenic lisianthus (*Eustoma grandiflorum*cv. LisaBlue). *Arabidopsis* *FLOWERING LOCUS T (FT)* gene was employed as the target gene. Genomic 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.

**Keywords**: Genetic transformation; Inducible gene expression; *Eustoma grandiflorum;* CRE*-lox* recombination

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: Naphthaleneacetic acid, PCR: Polymerase chain reaction

**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. It is economically desirable to breed new cultivars of lisianthus and the most common breeding method so far is sexual hybridization. Genetic engineering can be used to introduce heterogenousgenes that are not present in the gene pool of host plant, thus allowing novel phenotypes to be generated, such as flower, leaf color, shape and flowering time (Thiruvengadam and Yang, 2009).

In the 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, root (Paek and Hahn, 2000). Transformation of lisianthus were reported preliminarily by Handa (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 theoretical research, this is especially true for genes with 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 tacacum* 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* constructs were transformed into lisianthus using *Agrobacterium*-mediated transformation. Expression of the transgene was detected by RT-PCR assay under inducing conditions.

**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 hrs as required by giving a photon flux density of 5000 lux, temperature of 25ºC in a culture room.

**Optimization of regeneration media and kanamycin sensitivity of explants**

To establish an efficient regeneration system, MS media with different concentrations (0.5, 1.0, 1.5 mg l-1) of 6-BA in combination with NAA (0.1, 0,2 mg l-1) were used to test its efficacy in inducing callus and shoot. The leaves were cut into pieces (0.2-0.5 cm2), About 35-40 minced leaves explants were included in each treatment which was triplicated. One months later, the morphogenetic characteristics and callus inducing rate of these explants were recorded and the numbers of adventitious shoots were counted. When the shoots reached 2-3cm, they were cut and placed on a root induction medium with different concentrations of IBA (0, 0.1, 0.5, 1.0, 1.5 mg l-1). All plant material was grown *in vitro* at 25 oC under 16 h photoperiod using 40W cool white fluorescent tubes providing a photon flux density of 5000 lux. MS basal medium was used in all plant culture. The pH of the media was adjusted to 5.7 before autoclaving. After 4 weeks, rooted plantlets were transferred to potting soil. Plants were grown to maturity in greenhouse.

To test the effects of kanamycin on lisianthus, minced leaves (30-40) were cultured in callus inducing medium supplemented with kanamycin at different concentrations (0, 25, 50, 75, 100, 125, 150 mgl-1). Each treatment was repeated 3 times. After 20 days, the explants were transferred onto the same medium. After another 20 days, new calli and adventitious shoots were counted and recovery rate (proportion of explants callusing) was determined.

Data regarding differentiation, recovery rate were analyzed with ANOVA, mean comparisons were made using Duncan’s test (P < 0.05).

**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*IIis flanked by 35S promoter and 35S terminator (Figure 1A). Heat-inducible CRE-GR recombinase would excide 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 gentamicin and 25 mg/ml chloromycin) in a rotating (200 rpm) incubator at 28 oC for about 24 h. When the OD600 value reached 0.6-0.8, the cultures were centrifuged for 10 min at 4000 g and the bacterial pellet was re-suspended in liquid co-cultivation media. Explants from leaf pieces were then submerged in the inoculum for 30 min, then transferred to solid co-cultivation medium. After co-cultivation with *Agrobacterium* for 3 days in the dark, the leaf explants were placed on selection medium. 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.

**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 thetransgenes was tested by primer pairs: 5’-TGTTGGAGACGTTCTTGATCC-3’ and 5’-AGCCACTCTCCCTCTGACAA-3’ for *FT* gene and primer pairs: 5’-ACGTAAACGGCCACAAGTTC-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 *SpeI*, separated electrophoretically on a 1.0% (wt/vol) 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). Probe 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 RM supplemented with 10μmol/L dexamethasone, after a culture period of 24 hours, these plants were subjected to 37 oC for 2 hours per day, applied for 5 consecutive days. Leaves of these plants before and after induction (48 hours after the last treatment) were collected and RNA was isolated. RT-PCR was carried out using *FT* and e*gfp* primer pairs described previously. Tissues form root of each transgenic plantlet was collected and observed for GFP expression using fluorescent microscope before and after induction (48 hours after the last treatment).

**RESULTS AND DISCUSSION**

**Regeneration from leaf explants**

Cytokinin 6-BA was used ( 0.5, 1.0, 1.5 mg l-1) in combination with NAA (0.1,0.2 mg l-1) to test their ability to induce callus and shoot from leaf explants of lisianthus. Calli can be observed about 2 weeks after initiation of the cultures, 4 weeks later, there were great differences in the response among the treatments (Table 1). The highest callus induction rate of 75.5% resulted from MS medium supplemented with 1.0 mg/l 6-BA combined with 0.1 mg/l NAA, this treatment also produced the most shoot number on the callus. Shoot proliferation was observed when transferring these calli onto fresh media with the same hormone composition. 6-BA is beneficial for callus induction and shoot formation. However, an even higher 6-BA was not beneficial for regeneration, instead it appeared to suppress cell division and inhibit shoot formation. The suitable shoots were cut and placed on a root induction medium with different concentrations of IBA (0, 0.1, 0.5 1.0, 1.5 mg l-1), roots appeared from the cut of them within 4 weeks. The highest rooting rate is 86.7% from 0.5mg l-1 NAA (Table 2). Based on the above experiment, we chose 1.0 mg l-1 6-BA with 0.1 mg l-1 2,4-D for callus inducing medium and regeneration medium, and 0.5mg l-1 NAA for root induction in following transformation experiment.

**Kanamycin sensitivity**

Elucidating the effects of kanamycin at various concentrations on the growth of lisianthu**s**is a preliminary work in establishing a reproduction system. Leaf explants that remained green, formed a callus on the wounds and proliferate shoots were considered to be kanamycin resistant. Explants that became chlorotic, blackened at their base and failed to produce resistant callus were considered to be kanamycin sensitive. When nontransformed leaf were maintained on callus inducing medium without kanamycin, 92.62% of the explants produced new calli on the wounds. With increasing concentrations of kanamycin, both percentages decreased dramatically (Figure 2). In media with 100 mg l-1kanamycin, there are only 2.57% of the explants produced calli, however, they were yellow, growing slowly and can not to produce any shoot. No callus growth or shoot regeneration occurred in the medium containing 125 or 150 mg l-1kanamycin for explants from leaf. Therefore, kanamycin 100 mg l-1 was used for selecting transformants.

**Transformation and regeneration of** **lisianthus**

Transformation and regeneration of lisianthus at different stages was shown in Figure 3. 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 547bp 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 is shown in Figure 1C, the presence of a band corresponding to an full length *FT* gene in transgenic lines with no corresponding band present in the non-transgenic lines. Further more, expression of GFP was detected in young calli of the southern-positive transformants with a fluorescent microscope, indicating success of the transformation experiment (Figure 4A).

**Inducing expression of *AtFT* gene in transgenic plants**

In plants transformed with plasmids pJCGLOX, the *HSP 18.2* promoter was used to drive the recombinase gene *CRE*, which can be activated by heat-shock. 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 excide e*gfp* and TOCS in presence of dexamethasone, leading to constitutive expression of *FT* under control of the 35S promoter. To induce expression of *FT* in plants transformed with pJCGLOX, the regenerated plantlets were firstly transferred onto media supplemented with dexamethasone, and then treated with heat shock. RT-PCR assay shows that a 547bp region of the e*gfp* gene was amplified from leaves before induction (Figure. 5) 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 4B).

By RT-PCR analysis, we obtained 11 plantlets with *FT* expression from 3 Out of 7 GFP fluorescence positive stransformant lines. The CRE-lox recombination efficiency is different among the 3 lines (Table 3). Even in a same plantlet, GFP fluorescence was not completely disappeared (Figure 4B), demonstrating incomplete recombination in multi-cell comprised plant. The highest recombination efficiency in this study is 22.7%, which is lower than that in tobacco, rice and banana (Joubes et al., 2004; Chong-Perez et al., 2010; Srivastava et al., 2011). This may 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 study in plant breeding.

**Acknowledgments**

This study was supported by National GMO Major Projects of China: Breeding of Transformed Maize to Produce More Carotenoids (2009ZX08003-019B,450), Establishment and Refinement of Soybean Transformation System with Higher Efficiency and Safety (2009ZX08010-013B), Breeding of Transformed Soybean Resistant to Herbicide (2008ZX08004-001) and Breeding of Transformed Maize with Higher Nutrient Absorption Efficiency (2008ZX08003-005).

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Table 1 Effect of plant regulators on regeneration

|  |  |  |  |
| --- | --- | --- | --- |
| 6-BA （mg l-1） | NAA（mg l-1） | Callus inducing (%) | No. shoots per explant |
| 0.5 | 0.1 | 60.6b | 2.51d |
| 1.0 | 75.5a | 15.01a |
| 1.5 | 54.7c | 10.30b |
| 0.5 | 0.2 | 45.4d | 3.77c |
| 1.0 | 50.3c | 13.11ab |
| 1.5 | 39.6e | 2.22d |
| Each value represent the mean of 3 replicates per treatment. Different letters in the same column indicate significant differences, 5% level, Duncan’s multiple range test. | | | |

Table 2 Effect of IBA on root induction

|  |  |
| --- | --- |
| IBA（mg l-1） | Rooting Rate (%) |
| 0 | 25.5e |
| 0.1 | 47.7d |
| 0.5 | 86.7a |
| 1.0 | 75.7b |
| 1.5 | 50.5c |

Each value represent the mean of 3 replicates per treatment. Different letters in the same column indicate significant differences, 5% level, Duncan’s multiple range test.

Table 3 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% |

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**Figure. 1.** **A.** 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*IIis 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. Lane 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.

**Figure 2.** Callus induction rate decrease with increasing concentrations of kanamycin. Error bars represent standard error (n=3).

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**Figure 3.** 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.

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**Figure 4.** 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.

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**Figure 5.** RT-PCR assay for transgenic lisianthus plants before and after induction. M: DNA marker III; WT: wild type; 1-3: transgenic lines.