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A protocol for *Agrobacterium*-mediated transformation of *Kalanchoë blossfeldiana* with a flavonoid 3',5' hydroxylase *(F3'5'H)* gene

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In the present investigation, explants from *Kalanchoë blossfeldiana* were used for gene transformation. The young leaves were inoculated with *Agrobacterium tumefaciens* LBA4404 strain with a binary vector plasmid pArtblue containing *F3'5'H* gene under control of CaMV35S promoter and *npt*II selectable marker gene. After inoculation, the explants were transferred to the co-cultivation medium. They were then transferred to the selection medium containing kanamycin and were sub-cultured every two weeks. Leaves of the putative transgenic shoots that survived in the selection medium were used in reverse transcription polymerase chain reaction (RT-PCR) analysis to detect gene expression. The RT-PCR analysis showed the presence of 550 bp *F3'5'H* amplification products and had an expression of *F3'5'H* gene. Plants with the introduced *F3'5'H* gene produced totally pale red flowers.

Key words: *Kalanchoë blossfeldiana*, *Agrobacterium*-mediated transformation, young leaf, *F3'5'H* gene, reverse transcription-polymerase chain reaction (RT-PCR).

INTRODUCTION

Kalanchoë blossfeldiana is one of the most attractive representatives of the succulent family. The plant is very common to consumers because of its long lasting flowers and attractive foliage. It blooms during the short days of winters and becomes a popular plant from late falls to late winter. The original colors of the *Kalachoë* are white, orange and red. However, a range of flower color is still insufficient in the present commercial cultivars. The transformation protocol for *K. blossfeldiana* has been already established and described by several independent groups. Breeding new varieties with novel or improved traits will increase economic value of *K. blossfeldiana*. Most of the plant pigments varied in pigmentation of floral parts ranging from white to red and purple colors, and belong to the anthocyanin group of flavonoids (Aizza and Dornelas, 2011). Dihydroflavonol 4-reductase (*DFR*) is one of the enzymes in anthocyanin synthesis pathway, which catalyzes the production of leucoanthocyanidins from dihydroflavonols. It can be hydroxylated on the 3' or 5' position of the B-ring by flavonoid 3'-hydroxylase (*F3'H*) to produce dihydroquercetin or by flavonoid 3',5' hydroxylase

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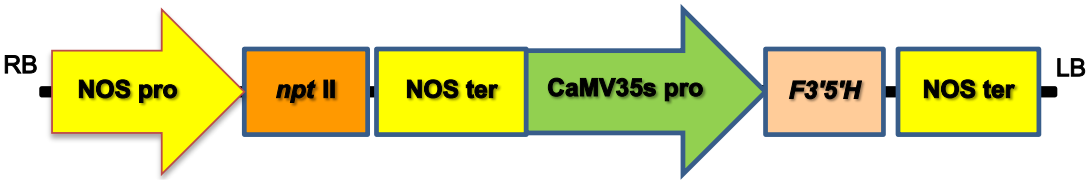

Figure 1. Schematic liner map of the T-DNA region of the pArtblue plasmid. The *F3'5'H* gene is driven by cauliflower mosaic virus promoter (*CaMV*) 35S, and is terminated by nopaline synthase (NOS). *npt* II: neomycine phosphotransferase II, RB: right border, LB: left border.

(*F3'5'H*) to form dihydromyricetin. The last two compounds are involved in the production of flavonoid precursors and in the formation of particular decorated anthocyanin molecules (Holton et al., 1993; Hussein et al., 2013). It is clear that the genes, *DFR* and *F3'5'H*, play important roles in the flavonoid biosynthetic pathway and in floral anthocyanin pathway as well (Zabala and Vodkin, 2007; Hussein et al., 2013).

Plant breeders develop a variety of colors by using traditional time-consuming methods. Recently, genetic modification of plants using *Agrobacterium tumefaciens* has become a very popular gene-transfer technique. *Agrobacterium*-mediated gene transfer has advantages of allowing stable integration of defined DNA into the plant genome, fewer rearrangements and more stable expression over generations than free direct DNA delivery methods (Dai et al., 2001; Hu et al., 2003; Mahadtanapuk et al., 2006). Stable expression of a transgene is necessary for plant breeding by genetic engineering. However, the expression level of transgene may vary among transformants and silencing of a transgene may frequently occur. In this study, the authors introduced a *Agrobacterium*-mediated transformation of *F3'5'H* gene into *K. blossfeldiana*. Moreover, the *F3'5'H* gene isolated from butterfly pea (*Clitoria ternatea* Linn.) was introduced by this method, in an attempt to change the color of *K. blossfeldiana*.

MATERIALS AND METHODS

Plant materials for transformation

The young leaves of *K. blossfeldiana* were sterilized in 15% sodium hypochloride (Clorox) for 15 min, and rinsed four times with sterile distilled water. The young leaves were cultured on MS medium (Murashige and Skoog, 1962) nutrients supplemented with 1.0 mg/L Thidiazuron (TDZ), 0.1 mg/L naphthalene acetic acid (NAA), 3% (w/v) sucrose, 0.8% (w/v) agar, pH 5.8 and adjusted with 1 M NaOH before autoclaving at 121°C for 15 min. The cultures were incubated at 25±2°C, under 16 h/day photoperiod (Buddharak et al., 2012). After 2 weeks of culture, the young leaves produced small shoots.

Bacterial strain and plasmid

A. tumefaciens LBA4404 strain which harbored the pArtblue plasmid, carrying cDNAs encoding butterfly pea *F3'5'H* gene (Thamaragsa et al., 2015) was driven by the cauliflower mosaic virus (*CaMV*) 35S promoter and terminated by nopaline synthase (*NOS*). Neomycin phosphotransferase (*npt*II) gene acting as a plant selectable marker was used (Figure 1).

Transformation and selection of transgenic plants

The leaf explants were incubated in the *A. tumefaciens* suspension for 30 min, and then dried on sterilized filter paper and placed on MS medium solidified with 0.8% agar containing 1.0 mg/L thidiazuron (TDZ), 0.1 mg/L naphthalene acetic acid (NAA) and 100 µM acetosyringon for two days in the dark. After co-cultivation, the explants were transferred to MS solid medium containing 1.0 mg/L TDZ, 0.1 mg/L NAA, 250 mg/L cefortaxime and 50 mg/L kanamycin for regeneration (selection medium). The selection medium was changed every two weeks. Six weeks after infection, explants that formed shoots were transferred to MS solid medium free of hormones containing 250 mg/L cefortaxime and 50 mg/L kanamycin for shoot elongation. After four-weeks culture on elongation medium, only one regenerated shoot was excised from each explant to take an independent plant and planted on MS solid medium containing 0.1 mg/L NAA, 250 mg/L cefortaxime and 50 mg/L kanamycin for roots induction.

RNA isolation and gene expression using RT-PCR analysis

Total RNA from surviving shoots in the selective medium and the control shoots were isolated with the use of easy-RED^{TM} RNA extraction (iNtRON Biotechnology, Korea) and the reverse transcription polymerase chain reaction (RT-PCR) was used to qualitatively detect gene expression through creation of complementary DNA (cDNA) transcripts from RNA. For the RT-PCR, the MyTaq^{TM} One-Step RT-PCR kit (Bioline, USA) was used. The forward primer 5'- AAG TAT CAT AGA GTG GGC AC -3' and reverse primer 5'- TAA CAT TGT AAG CAG TTG GG -3' were used for amplification of the *F3'5'H* gene. All RT-PCR reactions were performed using MyGeneTM Series Peltier Thermal cycler (LongGene, Hangzhou, PRC). The RT-PCR were carried out for 35 cycles: 20 min at 45°C for reverse transcription, 1 min at 95°C for denaturation, 10 s at 55°C for annealing, and 30 s at 72°C for extension, followed by a final 10 min at 72°C. PCR products were visualized on the in 1% agarose gel by electrophoresis using gel red staining. After, the *F3'5'H* gene expression in transgenic plants was confirmed by RT-PCR, rooted plantlets were transferred to soil and acclimatized in the green house.

RESULTS AND DISCUSSION

Selection of kanamycin-resistant regenerated plants

In the primary phase of screening, some of the explants

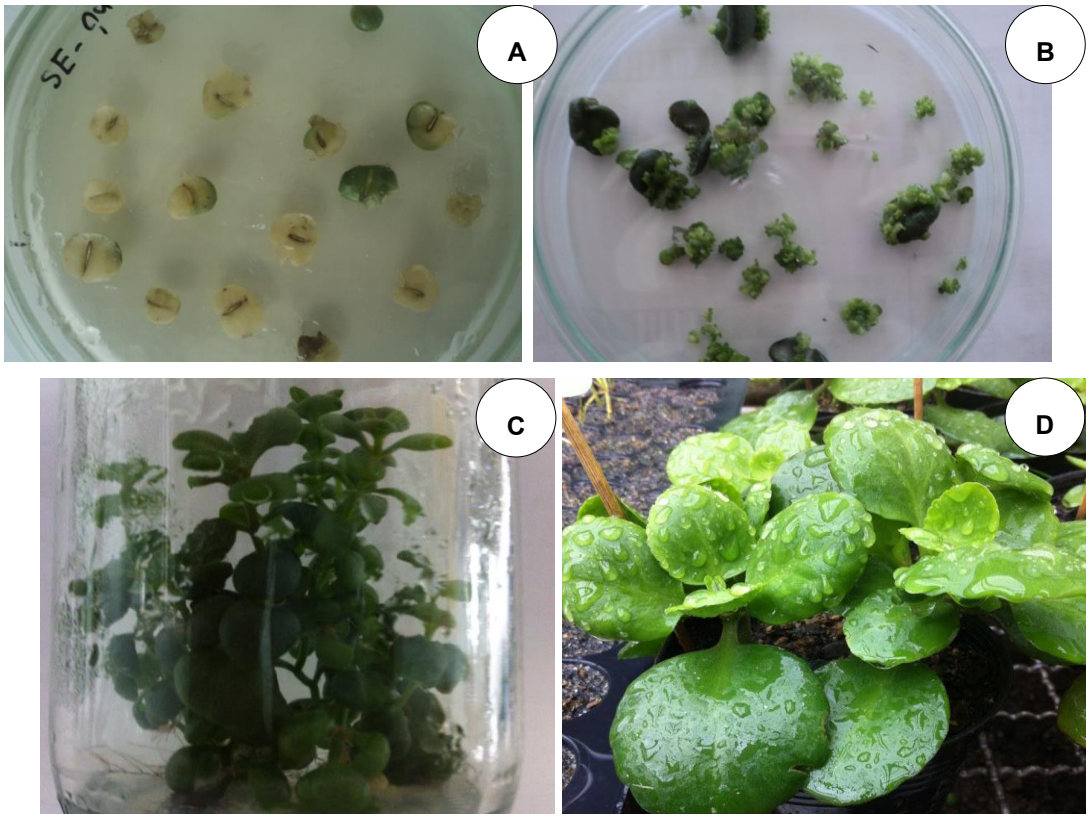

Figure 2. *Agrobacterium*-mediated transformation of *K. blossfeldiana*. A: non-transformation on selection medium, B: transformation on selection medium, C: shoot elongation and root induction on selection medium, D: transgenic *K. blossfeldiana* plants growing in the greenhouse.

initially became dark or bleached after regeneration, while the others regenerated and remained green. Shoots grown from kanamycin resistant explants, were regenerated and survived on selection medium containing 50 mg/L kanamycin suggesting they have received the *F3'5'H* gene. However, approximately 60% of the explants survived and grew normally in the selection medium for a period of 6 weeks (Figure 2) and the survival rates were high. Therefore, it is suggested that 50 mg/L of kanamycin may be used as the optimum concentration for the selection of *K. blossfeldiana* shoot meristem explants transformed with vectors harbouring the *npt*II gene. The amino glycoside kanamycin, acting as a selective agent, has been commonly used in plant genetic engineering (Bao-Hong et al., 2001). The main mode of action or effect of this particular antibiotic was by inhibiting the growth of plant cells by binding to the 30s ribosomal subunit, thereby inhibiting initiation of plastid translation (Moazed and Noller, 1987) and inhibiting ribosomal protein synthesis (Kohanski et al., 2010).

Gene expression using RT-PCR of putative transgenic plants

RT-PCR analysis was carried out on RNA extracted from all the surviving plantlets in the selection medium. During the second round of screening, the samples from the leaves of the putative transformed plantlets showed positive amplification in RT-PCR reaction while using primers specific to *F3'5'H* genes sequence. Five kanamycin resistant plantlets showed the presence of 550 bp *F3'5'H* amplification products (Figure 3). The rooted plantlets were acclimatized, and transferred to the greenhouse successfully (Figure 4). The authors attempted to test expression of the flavonoid 3', 5'-hydroxylase (*F3'5'H*) in pigmented *K. blossfeldiana* petals. Unexpectedly, the introduced gene created a block in anthocyanin biosynthesis. Plants with the introduced *F3'5'H* gene produced totally pale red flowers (Figure 4). Takashi et al. (2010) regulated flower color in blue gentian using RNA interference technology. When the anthocyanin 5, 3'-aromatic acyltransferase gene (*5/3' AT*) was inhibited, the petals became lilac. However, when *5/3' AT* and *F3'5'H* were co-suppressed, the petals were pale blue. Meanwhile, the anthocyanin of the petals contents were changed in all transgenic plants. The mechanism responsible for the reversible co-suppression of homologous genes in transgenic plants is unclear, but the erratic and reversible nature of this phenomenon suggests the possible involvement of methylation (Napoli

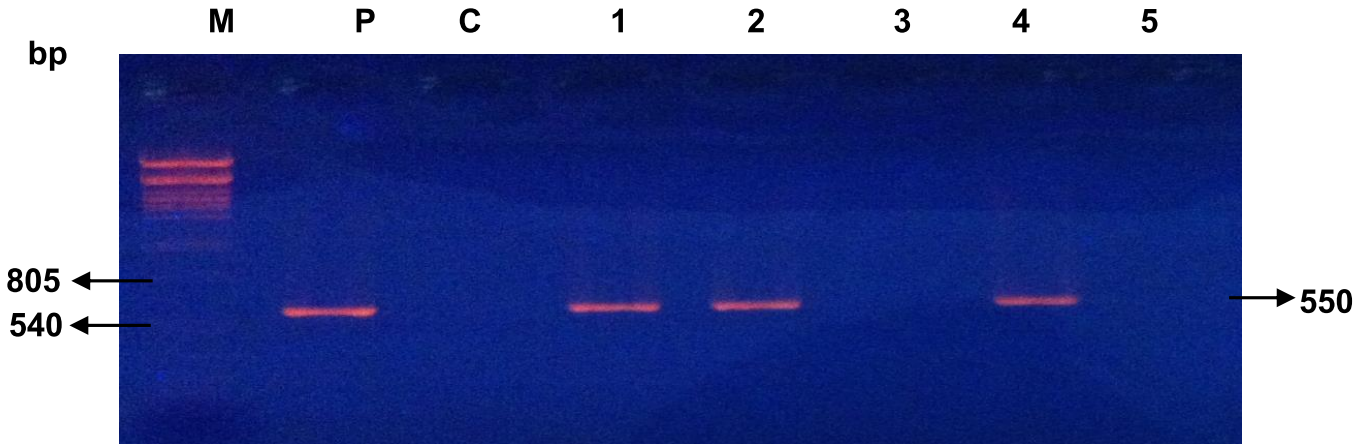

Figure 3. Gene expression using RT-PCR amplification products from RNA of transgenic *K. blossfeldiana* plantlets containing the *F3'5'H* gene. Lane M: marker lambda *pst*I, Lanes 1-5: RT-PCR amplification products from putative transgenic putative plants, Lane C: control plant (non- transgenic plant), Lane P: positive control obtained by amplification of the plasmid DNA.

Figure 4. Flower phenotypes in control and transgenic *K. blossfeldiana* plants. A: Untransformed *K. blossfeldiana* was indicated as "wild-type", which had red-colored flowers. B: Fainter color was observed in a transgenic *K. blossfeldiana*.

et al., 1990). Biologists have made great efforts to study the mechanism of co-suppression in recent years. The results showed that the copy number, DNA methylation and structure of the integrated T-DNA of the transgene may play a role in the process of co-suppression (Stam et al., 1997; Vaucheret et al., 1998). The results also showed that RNA-dependent RNA polymerase may be involved in the RNA degradation (Schiebel et al., 1998; Dalmay et al., 2000). In the study of signal transduction, small signal molecules such as small RNA molecules were detected (Hamilton et al., 1999).

Conclusions

In this investigation, the authors successfully transformed *K. blossfeldiana*, a *F3'5'H* gene via the *Agrobacterium*-mediated transformation system. Transgenic plants obtained in this study were confirmed by RT-PCR analysis. The optimized protocol is simple and reproducible, and may be adapted for other *Kalanchoë* cultivars. Plants with the introduced *F3'5'H* gene produced totally pale red flowers. These results clearly indicate the usefulness of metabolic engineering of the

flavonoid biosynthetic pathway to modify flower color. Only a few of the transgenic *K. blossfeldiana* exhibited phenotypic stability. For commercialization, it was necessary to generate many independent transgenic lines, select elite lines with stable phenotypes and maintain them in tissue culture.

Conflict of interests

The authors did not declare any conflict of interest.

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