Full Length Research Paper

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

Phopgao Buddharak¹, Ruttaporn Chundet² and Warut U-kong²*

¹Department of Biology, Faculty of Science, University of Phayao, Phayao 56000, Thailand.  
²Program in Biotechnology, Faculty of Science, Maejo University, Chiang Mai 50290, Thailand.

Received 14 July, 2015; Accepted 23 September, 2015

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 nptII 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 Kalanchoë 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.
(F3’5’H) to form dihydrorhizomicrin. 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; Mahadtaanapuk 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), and 100 µM acetosyringone for 2 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™ 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™ 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 TG -3’ were used for amplification of the F3’5’H gene. All RT-PCR reactions were performed using MyGene™ Series Pettier 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
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 *nptII* 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...
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.

**REFERENCES**


