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Transformation RNA interference-mediated silencing of the *Flavanone 3-Hydroxylase* gene in Lotus (*Nelumbo nucifera* Geartn. cv. Buntharik)

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Abstract RNA interference mediated-silencing of the *F3H* gene in the lotus (*Nelumbo nucifera* Geartn.) cv. Buntharik inhibited the anthocyanin pathway after gene transformation. The plasmid pJA8*F3H* contained the inverted repeats of *F3H* fragments, *bar* gene as a selectable marker in plants, which is resistant to basta (glufosinate amonium). The transformation of particle bombardment was obtained with multiple shoots of lotus. *F3H* gene expression analysis showed that expression of *F3H* decreased in transgenic lotus when compared with the control. On the first and the second test, 5 transgenic lotus of the relative expression *F3H* gene was detected with 0.0496 and 0.466 times, respectively. The inhibition of gene expression decreased when they were cultured for a long time. The expression *F3H* gene was detected by RT-PCR, and real-time PCR resulted in consistent decreasing in the transgenic lotus compared with the non-transgenic lotus, which was able to inhibit the expression *F3H* gene.

Keywords: Nelumbo, Particle bombardment, Bar gene

Introduction

Nelumbo nucifera is an aquatic plant native to Asia and Australia and is an important ornamental. The rhizome and seeds of the lotus are edible and are sometimes used in Asian cooking. The flowers are Buddhism religious symbol and are used for decorative purposes. The one of the most important traits for consumer attention is flower color. However, in Thailand there are only four commercial varieties and two flower colors. Therefore, modifying flower color and understanding the function of the gene flovonoid pathway is essential.

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Several genes from the anthocyanin biosynthesis pathway have been used to manipulate flower color in numerous plants such as toronia (Aida *et al.*, 2000) and tobacco (Nishihara *et al.*, 2006). The anthocyanin is the color pigments evident, are present in all plant tissue. The biosynthesis pathway begins with the formation of chalcones by the chalcone synthase enzyme (CHS). Chalcone converted to nariginine by chalcone isomerase (CHI) (Li *et al*., 2006), and this is then hydroxylated at the 3' position of its central ring by F3H to produce dihydrokaempferol (DHK). This pathway is essential for diverse flower color. For example, F3H suppression causes significant flower color reduction in carnations (Zuker *et al*., 2002). One *et al*. (2006) reported the anthocyanin pathway was redirected to the aurone pathway by RNAi silencing of F3H, producing a white flower mutant. This was followed by the overexpression of 4' chalcone-glucosytransferase (AM4'CGT) and aureusidin synthase (AmAS1) from *Antirrhinum majus* L. producing the first aurone-based yellow-colored transgenic Torenia.

There has been improved in lotus breeding through the use of tissue culture, mutagenesis and somatic embryogenesis induction. Molecular breeding using antisense technology is a powerful method of plant breeding. The transformation of sense and antisense *DFR* gene was transferred into *Melastoma malabathricum* and *Tibouchina semidecandra* (Yong *et al.*, 2009), antisense DFR gene was transfered into lotus using particle bombardment (Buathong *et al.*, 2013), and using agrobacterium transformation (Saetiew *et al*., 2014).

RNA interference (RNAi) technology can be used to modify the target flower colors for gene silencing of three anthocyanin biosynthetic genes: CHS, ANS, and F3'5'H. These were reported in gentia (Takashi *et al.*, 2008), torenia (Nishihara *et al.*, 2006), gentia (Nakamura *et al.*, 2006), and torenia (One *et al.*, 2006). RNAi was applied to change flower color of gentia plant sucessfully, and this could be useful for designing novel flower colors and patterns (Takashi *et al.*, 2008). The aim of this study is to examine the suppression of the *F3H* gene in lotus *Nelumbo nucifera* cv. Buntharik by RNAi-mediated gene silencing. The transgenic lotus was obtained and analyzed gene expression by real-time PCR.

Materials and methods

Plant materials, construction and transformation

Lotus seeds cv. Buntharik were washed though running water for 60 min. The seeds were serface-sterilized by immersion in 70% (v/v) ethanol for 1 min,

and then in 3.5% NaOCl (V/V) add two drops of Tween 20 for 20 min, and washed three times in sterile distilled water for 5 min. Callus was initiated by culturing 2 mm of embryo apical buds on Murashige and Skoog (MS) medium contained with 0.5 µM Thidiazuron (TDZ) and 40 µM 1-naphthaleneacetic acid (NAA) for 8 weeks. Calli from these apical buds were cultured on MS medium contained with 50µM BA for 12 weeks and were maintained by sub-culturing every 4 weeks. Cultures were growth in room at 25^{o}C under a 16 hours light. The shoot cluster was developed from calli (Buathong *et al.,* 2013).

Construction of pJA8F3H vector for RNAi

The pJA8F3H vector was constructed by Chaipanya *et al*. (2017). A 308 bp gene fragment was cloned from cDNA of *Nelumbo nucifera* by using specific primer from F3H gene. The size 308 bp of F3H fragment was cloned into pCR8 vector and then transfer into pJAWOHL8 vector using the Gateway system (Invitrogen, USA). The pasmid contained selectable markers ampicillin resistance in bacteria and basta resistance genes in plant. The pasmid was named pJAWOHL8 RNAi (Figure 1).

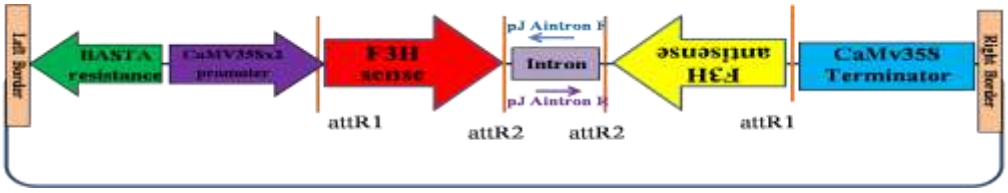

Figure 1. T-DNA of the pJA8F3H RNAi (F3Hi) plasmid

Transformation flavanone-3 hydroxylase (F3H) into shoot clusters by particle bombardment

The shoot clusters were cultured on osmoticum containing medium for 16 hours (Buathong *et al*., 2013). Prior to bombardment, these were transferred to the center of 2.5 cm diameter filter paper dise (Whatman) in 9 cm Petri dishes. The gold particles were coated with pJA8F3H RNAi plasmid were bombarded to explants with condition at 9 cm and 7584.23 kPa (PDS-1000/He, BioRad. The shoot clusters were transferred to culture on osmoticum containing medium for four hours. The tissue was selected on regeneration MS medium supplemented with selective agent 10 mg L^{-1} basta for 16 weeks. The explants were subcultured every 2 weeks.

Real-time PCR analysis of F3H, BAR, and CHS gene expression

The putative transgenic leaf tissue was extracted total RAN using Invitrap Spin Plant RNA Mini Kit (Germany). The first-strand cDNA synthesis was using revertAid First Strand cDNA Synthesis Kit (Fermentas, USA). Primers *18S rRNA* (forward 5'-GGCTCGAAGACGATCAGAT ACCG-3' and *18S rRNA* reverse 5' GTACAAAGGGCAGGGACGTAGTCAA-3'); *F3H* (forward 5'- GAGAAGCTCCGGTTCGACAT-3' and *F3H* reverse 5'-TAGTTCACCACCATCTTCTGG-3'); and *CHS* (forward 5'-AAGAGCTCCCGTCAAGAGACTCA-3' and *CHS* reverse 5'-AAGGATCCCAGAAAATTGAGTTC-3') were designed on the basis of published sequences in the GenBank database to amplify a 600 bp amplification of the *18S rRNA*, a 300 bp of the *F3H*, and 458 bp of the *CHS*. The standard curve produces a linear relationship between C_t and initiation amounts of cDNA of the lotus, allowing the determination of unknown concentration based on their C_t values. The 18S rRNA was used as an internal control, as it constituted 85-90% of total cellular RNA. Real-time PCR reaction was performed using Bio-Rad CFX Manager 3.0. Using SsoAdvanced^{TM} SYBR$^{®}$ Green Supermix (Bio-Rad, USA. Cat. No.172-5260), the reaction contained 4 µL of 4 ng of cDNA, 0.5 µL of 10 µM of the specific primer, 10 µL of SYBR$^{®}$ Green PCR Master Mix, and up to 20 µL of deionized water. The cycling parameters were as follows: pre-incubation one cycle of 95^{o}C for 1 minute, amplification 39 cycles of 94^{o}C for 10 seconds, melting curve 58^{o}C for 30 seconds, and 72^{o}C for 1 minute and 5^{o}C cooling for 5 seconds. Crossing point (Cp) and amplification curve results were analyzed using Bio-Rad CFX Manager 3.0 and calculated relative gene expression (Livak and Schmittgen, 2001). The expression of target gene was $2^{-\Delta\Delta Cp}$.

Results

Shoot cluster induction, plant transformation and selection

The shoot clusters were used as the targeted tissue for particle bombardment (Figure 2). A total of 105 shoot clusters were bombarded with pJA8*F3H* RNAi plasmid. They were cultured on selection medium (MS medium containing 10 mg L^{-1} basta) for 16 weeks. The survival explants decreased every week. The surviver plant number was 28.35%, the number of the shoots was 2.47, and the shoot length was 1.20 cm after selection for 16 weeks (Table 1).

Figure 2. Shoot clusters were used for transformation

Table 1. The average number of shoot, the shoot length, the number of plant survival and the percentage of plant survival after gene transformation by particle bombardment technique on MS supplemented with 50µM BA and 10mg L^{-1} basta

Weeks	4	8	12	16
number of shoots	2.24±0.30	2.18±0.50	2.35±0.63	2.47±0.67
shoot length(cm)	0.83±0.16	0.97±0.13	1.13±0.20	1.20±0.19
plant survival	55	43	27	27
%plant survival	57.77	45.15	28.35	28.35

[1/]total number 105 pieces

Gene expression analysis using semi-quantitative PCR

The results of the RT-PCR analysis of five independent transgenic plants are shown in Figure 3. The *F3H* gene expressed more highly in control than the transgenic plant. The pJA8*F3H* RNAi plasmid was transferred into the plant in order to reduce *F3H* expression. The interference of F3H expression in to the cell was initiated from RNAi-mediated silencing of the pasmid. *BAR* gene expression could be found in only one of three transgenic plants. However, the *BAR* gene could not be found in control (Figure 3A). There is no difference in *CHS* gene expression levels (Figure 3B).

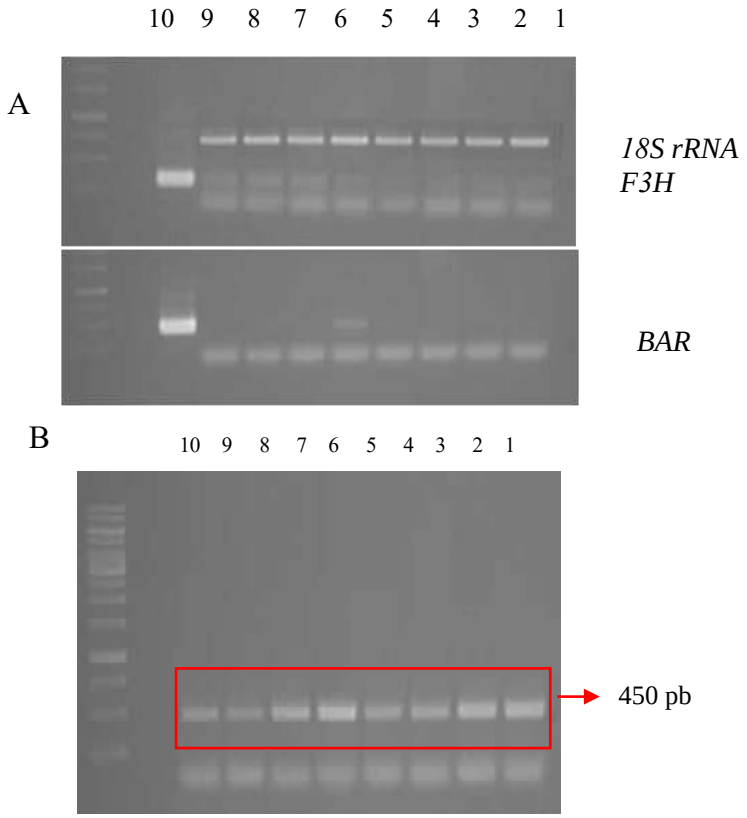

Figure 3. The RT-PCR analysis of the transformed plants using the reference 18S rRNA gene by F3H specific primers (308bp) and bar gene (430bp) (1) marker 1 kb ladder; (2) no template; (3) positive control by plasmid pJA8F3H; (4-6) control with non transformed plants; (7-11) transformed plants (A) and CHS gene specific primers (1) marker 1 kb ladder; (2) no template; (3-5) control with non-transformed plants; (6-10) transformed plants (B)

The gene expression analysis using real-time PCR

The expression of *18S rRNA*, *F3H*, and *CHS* gene were studied using a real-time PCR technique**.** The *F3H* expression was compared between the transgenic and the non-transgenic lotus, while the *CHS* gene is no different between non-transgenic and transgenic lines (Table 2). The *F3H* gene is in the non-transgenic more expressed than transgenic because the pJA8*F3H* RNAi plasmid is an efficient and powerful technique for gene silencing compared

with antisense (Nakatsuka *et al.*, 2008). The correlation coefficients of the standard curves were good (0.97-0.99).

Table 2. The crossing point (Cp) in real-time PCR for expression of *18S rRNA*, *F3H*, *BAR*, and *CHS* genes in lotus

Sample	Crossing point *18S rRNA*	Crossing point *F3H*	Crossing point *Bar*	Crossing point *CHS*
Positive control		13.26	6.63	
control 1/1	22.41±0.12	24.77±0.66	00.00	27.64±0.20
Transgenic No. 1	25.14±0.42	28.55±0.12	32.02±0.61	27.96±0.13
No. 2	22.54±0.12	27.67±0.49	30.44±0.87	30.12±0.18
No. 3	24.91±0.11	29.10±0.19	33.39±0.81	28.29±0.03
No. 4	23.68±0.09	26.68±0.12	31.92±0.57	28.91±0.23
No. 5	24.56±0.12	27.25±0.11	31.46±0.21	29.54±0.14

The relative gene expression of F3H gene using real-time PCR Technique

The relative quantification was done by using comparative ΔΔCp. The transgenic lotus showed the low level of F3H expression when compared with the control. The non-transgenic lotus has a relative gene expression control equal to one. The transgenic lotus showed the relative gene expression of 0.487, 0.155, 0.284, 0.487, and 0.796, respectively (Figure 4).

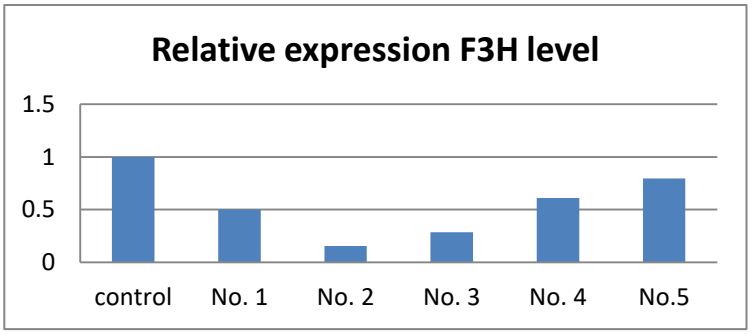

Figure 4. The relative expression level of *F3H* gene of control and the transgenic lotus no.1-5

Discussion

Five clones of independent resistant shoots were obtained for gene expression. The *F3H* expression in the transformed shoot was compared with the non-transformed shoot (control) by using sqPCR. The transformed shoots reduced *F3H* gene expression when compared with the control. The efficient and stable gene silencing by RNAi have more than antisense suppression (Waterhouse *et al.,* 1998). Chaipanya *et al.* (2017) reported RNAi *F3H* gene-silencing encoded a hairpin *F3H* RNA using the *Agrobacterium* infiltration transfored to water lily. The expression of *F3H* was analyzed on the first and the third days in post infiltration (dpi) by sqPCR showed down-regulated at 3 dpi of *F3H* in the red petal and purplish-blue petal varieties compared to control. RNAi technology was used to modify flower colors successfully applied to gentian plant (Nakatsuka *et al.*, 2007) and *Petunia hybrida* the flower color changed from purple to almost white or from purple to red (Tsuda *et al*., 2004). This study, five putative transgenic plant was detected the *BAR* gene and *CHS* gene expression by RT-PCR. The result showed one of the five putative transgenic plants were positive for the *BAR* gene, and that five of the putative transgenic plants were positive for *CHS* gene.

The real-time PCR assay revealed the relative expression level of the lotus transformed by the bombardment method. The transformed lotus revealed reduced expression of the *F3H* gene in comparison to the untransformed lotus. The crossing point (C_p) of relative expression of F3H level in real-time PCR showed that the control was higher than the untransformed, and that the *BAR* gene showed C_p only in the transformed lotus and could not be in the untransformed lotus, while the *CHS* gene showed non-transgenic and transgenic plants to have the same C_p level.

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