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The Expression of Egg Plant Flavonoid 3',5'-Hydroxylase Gene in Tobacco Plants (*Nicotiana tabacum* cv. Xanthi)

Sun Young Park, Younghee Kim*

Department of Horticultural secience, Sangmyung University, Anso-Dong, San 98-20, Cheonan, 330-180, Korea

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Abstract

The anthocyanin gene encoding flavonoid 3',5'-hydroxylase(F3,5H) was normally expressed in *Nicotiana tobacco* (Xanthi) plants cocultivated with *Agrobacterium tumefaciens* LBA4404 carrying egg plant flavonoid 3',5'-hydroxylase cDNA. Northern blot analysis showed the normal expression of *F3', 5'H* gene from transgenic plants. Here we found the phenotypic differences between transgenic plants and wild-type plants. The petal shape of transgenic plants showed more round shape and around petal tube area was compared to that of wild-type tobacco plants. And the petal color of transgenic plants was much lighter than that of wild-type tobacco plants.

Introduction

Anthocyanins are soluble pigments existing in the petal, leaf, and stem of plants. Synthesis of anthocyanin in the petal is undoubtedly intended to attract pollinators, whereas anthocyanin synthesis in seeds and fruits may aid in seed dispersal(Holton and Cornish, 1995). Anthocyanins and other flavonoids can also be important as feeding deterrents and as protection against damage from UV irradiation (Toguri et al., 1993). Blue and violet flowers generally contain derivatives of delphinidin, and red and pink flowers generally contain derivatives of cyanidin or pelargonidin. Differences in hydroxylation patterns of these three major classes of anthocyanidins are controlled by the cytochrome p 450 enzymes, flavonoid 3'-hydroxylase(F3H) and 3',5'-hydroxylase (F3',5'H). F3',5'H can also convert dihydrokaempferol and dihydroquercetin to dihydromyricetin(Menting et al., 1994; Toguri et al., 1993). In particular, hydroxyl group incorporation at the 3', 5' position of the flavonoid B-ring gives rise to flavonoids that, with further enzymic modifications, form purple anthocyanidins(Figure 1). To understand the biosynthesis of delphinidin-3-glucoside in terms of molecular biology and biochemistry(Goto et al., 1982), we are extensively studying the pathway. We report here the molecular biology of *F3', 5'H* gene in transgenic *Nicotiana tobaccum* plants and phenotypic differences of flower in terms of color and shape.

Materials and Methods

Plant materials

Nicotiana tabacum(cv. Xanthi) seeds were soaked in a 70% ethanol solution for 1 min and sterilized the surface in a 2% sodium hypochlorite solution for 15 mins, washed with sterilized distilled water three times. The seedling plants were subcultured every week in MS (Murashige and Skoog, 1962) solidified medium without plant growth regulators under the continuous flourescent light (3,000 Lux) at 25℃.

Construction of F3',5'H gene in pBI121

pBS SK- carrying egg plant *F3',5'H* cDNA was digested by *Bam*HI and *Kpn*I restriction enzymes. The 1.7 kb insert was subcloned into pUC18 (*Bam*HI/

* Corresponding author, E-mail; ykim@smuc.sangmyung.ac.kr
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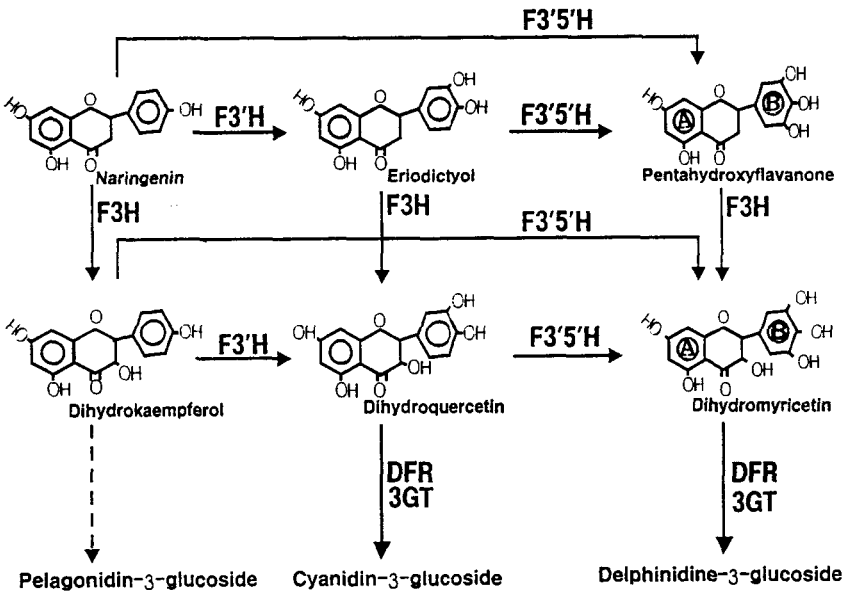

Figure 1. Anthocyanin biosynthesis pathway.
Enzymes involved in the synthesis of anthocyanidin 3-glucosides are indicated as follows: F3H, flavanone 3-hydroxylase; F3'H, flavonoid 3'-hydroxylase; F3'5'H, flavonoid 3',5'-hydroxylase; DFR, dihydroflavonol 4-reductase; 3GT, UDP-glucose; flavonoid 3-O-glucosyltransferase. Further modifications of cyanidin and delphinidin-3-glucosides are possible depending on the genotype.

*Kpn*I), which is called pYB3. And then, *Xba*I/*Sac*I fragment of pYB3 was eluted and inserted into corresponding site of pBI121 (Jefferson et al., 1987) (Figure 2).

Transformation of A. tumefaciens into Nicotiana tabacum

The tobacco leaves were cut into 25 mm^2 sections, and then the sections were cocultured with *Agrobacterium tumefaciens* in liquid MS medium (MS medium, 2.0mg/L NAA, and 0.5mg/L BAP) for 3 days (An et al., 1988). The *Agrobacterium* cells were washed out with sterilized liquid MS medium, and then transformed tobacco cells were selected and regenerated into shoots on solidified shoot regeneration medium (MS medium, 1mg/L BAP, 100mg/L kanamycin, 100mg/L cefotaxime and 500mg/L carbenicillin). Shoots were transferred to root induction medium (hormone free).

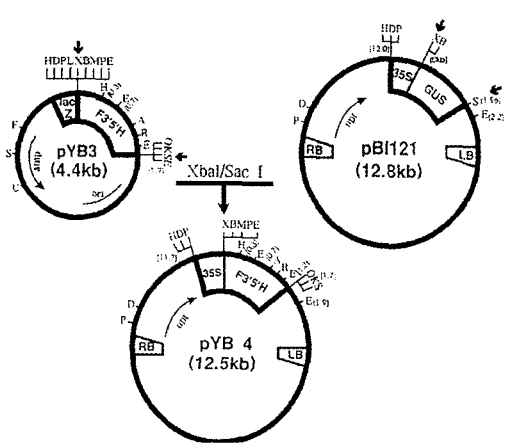

Figure 2. Construction of binary vector pYB4. A : *Apa*I, B : *Bam*HI, D : *Sph*I, E : *Eco*RI, F : *Ssp*I, H : *Hin*dIII, K : *Kpn*I, M : *Sma*I, O : *Xho*I, P : *Pst*I, R : *Eco*RV, S : *Sac*I, X : *Xba*I U : *Pvu*I

Identification of transgenic plants by polymerase chain reaction(PCR)

Two specific oligonucleotides derived from *npt*II gene were used as primers for PCR. One primer(5'-GAGGCTATTCGGCTATGACTG-3') is located in the position from 201 to 221 of the *npt*II gene, and the other primer(5'-ATCGGGAGCGGCGATACCGTA-3') from 900 to 880 (Beck et al., 1982). Genomic DNA from transgenic seedlings was isolated and amplified by the modified procedure of Edwards et al. (1991). PCR reaction was carried out in 20 μL solution containing 2 μL of 10 x buffer, 1 μL of 2.5 mM dNTP, 5 units of Taq polymerase and 50 pM of each oligonucleotide primer. PCR was performed in a thermal cycler(Takara) for 30 cycles, with each cycle consisting of 94℃ for 1 min to denature the template, 60℃ for 30 sec to anneal primer and 72℃ for 1 min for polymerization. Prior to cycles, the samples were heated at 95℃ for 10 min for denaturation and at

the end of cycles, samples were incubated at 72℃ for 10 min and kept at 4℃ prior to agarose gel analysis.

Northern blot analysis

Total RNAs were isolated from transgenic plants by the guanidine thiocyanate-CsCl purification method according to Maniatis et al. (1989). Poly $(A)^+$ mRNAs were isolated by two cycles of oligo (dT) cellulose affinity chromatography. Total mRNAs were denatured and subjected to eletrophoresis in a formaldehyde 1.2% agarose gel and transferred to Hybond-N nylon membranes by capillary blotting according to the manufacturer's protocol. RNA was quantified spectrophotometrically before loading. Hybridization was done with ^{32}P -labelled *F3',5'H* cDNA as a probe.

Results and Discussion

Transformation of F3',5'H gene into Nicotiana tabacum plants.

pYB 4 plasmids were transformed into *Agrobacterium tumefaciens* LBA 4404. About 1.2kb length band was identified from *Agrobacterium tumefaciens* which carried pYB 4 plasmids. *Agrobacterium tumefaciens* was infected into wounding sites of leaf tissue of *Nicotiana tobaccum* plants. Transgenic plants were selected on shoot induction media with antibiotics (An et al., 1988). After 5 weeks, approximately 60% of infected leaves generated shoots at wounding sites and some shoots reached 1.2 cm in height(data not shown). They were transferred on root induction media and after two weeks roots were formed(data not shown).

Identification of transgenic plants through PCR and Northern hybridization.

Genomic DNA was isolated from individual transgenic plants and used as template for PCR. Two specific sequences from *npt*II gene were used as primers. The PCR products were electrophoresed on 1% agarose gel. It showed that the 700 bp PCR fragment was observed from *npt*II gene as a positive control(lane 3 of Figure 3) and transgenic plants (lanes 4-10 of Figure 3), whereas a expected fragment was not observed from a negative control(lane 2 of Figure 3) which is a wild-type *Nicotiana tabacum* plant. All transgenic plants that we observed on this study carried *npt*II gene(lanes 4, 5, 6, 7, 8, 9 and 10 of Figure 3). It suggested that *npt*II gene was stably integrated into *Nicotiana tabacum* genomic DNA (Bakkeren et al., 1989). We also confirmed that transformed *F3',5'H* gene was integrated into plant genome by northern hybridization. Two micrograms of mRNA from different tissues of transgenic plant were isolated and used in northern analysis for *F3', 5'H* gene expression. About 1.2kb band was identified from transgenic plants from tissues(Figure 4). The level of *F3',5'H* gene expression was higher in petal and stem tissue whereas it was low in leaf tissue. In general, *F3',5'H* gene expression was not observed from petals of wild-type plants such as tobacco, *Chrysanthemum*, and petunia by northern hybridization because of the low frequency of gene expression. In *Chrysanthemum F3',5'H* gene expression was observed by PCR (Master's thesis by Sunyoung Park). In Gentiana triflora, full length cDNA clone of flavonoid 3,5 hydroxylase was cloned from petals and recombinant gentian *F3,5H* was expressed in the yeast expression system. But in this system only biochemical aspects for substrate specificity which it have a broad substrate specificity was observed

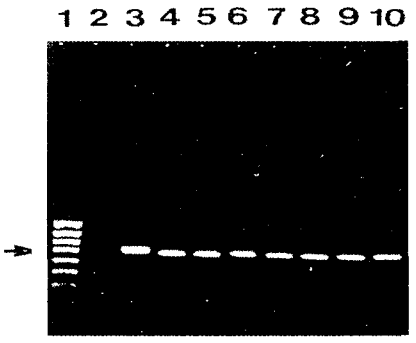

Figure 3. Identification of transgenic plants by polymerase chain reaction (PCR) for *npt*II gene. Lane 1: DNA ladder marker, lane 2: non-transgenic plant, lane 3: positive control, lane 4-10: transgenic plant.

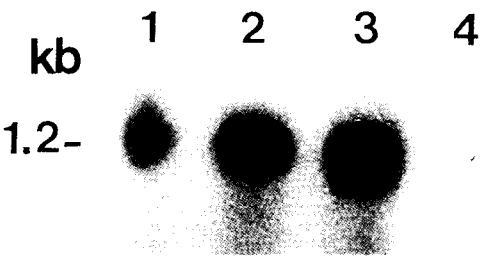

Figure 4. Northern hybridization of transgenic plants. Lane 1:leaf, lane 2:petal, lane 3:stem, lane 4:petals of non transgenic plants

(Tanaka et al., 1996). Therefore, we confirmed that both *nptII* gene and *F3',5'H* gene was stably integrated into plant genome and attempted further experiment.

Flower phenotypes and color patterns produced by transgenic plants

In the previous figure(Figure 4), we presented an evidence which indicates that excess mRNA of integrated *F3',5'H* gene was expressed relative to the endogenous *F3',5'H* mRNA. Therefore, flower phenotypes and color pattern in transgenic plants was observed(Figure 5). The transgenic plants showed many differences in their petals(Table 1).

Table 1. Phenotypic differences between wild-type (non-transgenic plant) and transgenic plants

	Wild-type	Transgenic plant
Petal Shape	Sharp	Round
Petal Color	Pink	Light purple, Light pink
Blooming period	10 days	6 days

The petal shape of transgenic plants showed more round shape and around petal tube area compared to that of wild-type tobacco plants. And the blooming period of transgenic plants were shorter than that of wild-type plants. One distinctive effect was that the flower pigmentation varies from completely colored flowers(E of Figure 5; no visible effect), reduced pigmentation pattern to very thin pinkish flowers(B, C, and D of Figure 5). This is an unexpected result compared to the result by Krol van der et al. (1988) whom got pigmentation patterns with colored rings and sectors by insertion of antisense chalcone synthase gene.

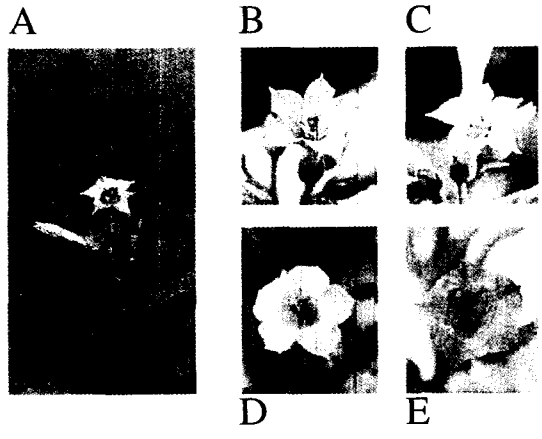

Figure 5. Phenotypic difference between wild-type plant and transgenic plants. A: wild-type plant, B,C,D and E: Transgenic plants

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