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Molecular Cloning and Biochemical Analysis of Dihydroflavonol 4-Reductase (DFR) from *Brassica rapa* ssp. *pekinesis* (Chinese Cabbage) using a Heterologous System

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Dihydroflavonol 4-reductase (DFR) is part of an important step in the flavonoid biosynthesis pathway of anthocyanins. We isolated the *DFR* gene from *Brassica rapa* ssp. *pekinesis* (Chinese cabbage), and found that biosynthesis of anthocyanins in tobacco could be modulated by introducing a heterologous DFR_{Bra} gene. RT-PCR showed that transgenic plants had abnormal flower colors compared with the wild-type controls, a response that was correlated with the level of DFR_{Bra} transcription. HPLC analysis revealed that the isolated DFR_{Bra} cDNA mainly produced cyanidin as well as a small amount of pelargonidin in the anthocyanin biosynthesis pathway of those tobacco plants.

Keywords: anthocyanin, Chinese cabbage, DFR, flavonoid

Flavonoids represent a large group of polyphenolic secondary metabolites important for plant biology and human nutrition. Among them, anthocyanins are the most conspicuous because of the wide range of pigments (especially red, blue, and purple) that result from their synthesis (Holton and Cornish, 1995). Because these pigments act as feeding deterrents and protect plants against damage from UV irradiation (Gould, 2004; Zhou et al., 2007), they have garnered much attention (Mol et al., 1998).

Red coloring in flowers comes mainly from two types of pigment, anthocyanins and betacyanins. Three different classes of anthocyanidins are responsible for the most frequent shades -- pelargonidin (orange to brick red), cyanidin (red to pink), and delphinidin (purple to blue) (Tanaka et al., 1998). Their biosynthetic pathways are fairly well established (Paiva, 2000; Winkel-Shirley, 2001). Dihydroflavonol 4-reductase (DFR) is a pivotal enzyme in the flavonoid synthetic pathway, with a crucial role in producing common and condensed anthocyanins (Holton and Cornish, 1995). This enzyme catalyzes the production of flavan-3,4-diols (leucoanthocyanidins) by the reduction of three colorless, corresponding dihydroflavonols--dihydrokaempferol (DHK), dihydroquercetin (DHQ), and dihydromyricetin (DHM) -- that are also intermediates of flavonol biosynthesis through the flavonol synthase reaction. These leucoanthocyanidins are subsequently converted to pelargonidin, cyanidin, and delphinidin, respectively.

Several *DFR* cDNAs have been isolated from plants, and single or multiple genes encoding DFR protein(s) have been reported for a few genomes (Tanaka et al., 1996; Inagaki et al., 1999; Himi and Noda, 2004). In many species, those proteins can accept dihydroflavonols with different hydroxylation patterns as substrates (Heller et al., 1985; Fischer et al., 1998). Therefore, a deeper understanding of the regulation of *DFR* expression in *Brassica rapa* ssp. *pekinesis* (Chinese cabbage) would provide useful information for genetic transformation programs aimed at changing flower color via anthocyanin synthesis in petals.

Here, we isolated that gene from Chinese cabbage, one of the most important vegetable crops in East Asia. To analyze the function of DFR_{Bra}, transgenic tobacco plants that over-expressed DFR_{Bra} were constructed. HPLC analysis was performed to examine the production of floral pigments in the anthocyanin biosynthesis pathway.

MATERIALS AND METHODS

Preparation of Plant Materials

Chinese cabbage (*Brassica rapa* ssp. *pekinesis*) seeds from the Dong-bu Seed Co. (South Korea) were sterilized, then germinated on an MS medium containing 3% sucrose and 0.8% agar (pH 5.8). They were incubated in a culture room for 7 d at 25°C, under a 16-h photoperiod. Transgenic DFR_{Bra} plants and controls were grown in the same greenhouse. Petals were harvested from the flower buds of wild-type and transgenic plants, and all samples were stored at -80°C.

RNA Isolation

Total RNA was extracted from the young leaves of tobacco plants with Trizol reagent (Invitrogen, CA, USA). Excised tissues were ground in liquid nitrogen, and the powder was mixed with 1 mL Trizol reagent per 100 mg of tissue. Total RNA was precipitated by isopropyl alcohol.

Isolation of *DFR*

RT-PCR was conducted to isolate *DFR* from Chinese cabbage, using eight primer sets that were designed based on

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Table 1. The primer sets to isolate DFR gene from Chinese cabbage in this study.

	Primer sequence (5'-3')
dfr F5	aaagagaccgtgtgcctaacc
dfr F6	caacatcttcttgatttgccaaac
dfr F7	tggagttatttcatgtcgaaaacg
dfr F8	gattcattggttcatggctcgt
dfr F9	atggtagctcacaaagagaccg
dfr 10	atggttagtcagaaagagaccgt
dfr R3	tcacacattcataaagacaatagat
dfr R9	ctcgggatcctttgattcaaaat
dfr R10	gggagaaaaccctttggacga

the amino acid residues of *Brassica oleracea* (GenBank Accession No. AY228487) and *Arabidopsis thaliana* (GenBank Accession No. AB033294) (Table 1). One g L^{-1} of total RNA was transcribed with a Reverse-iT™ One-Step RT-PCR kit (ABgene, Surrey, UK). The PCR parameters were 35 cycles at 94°C for 20 s, 55°C for 30 s, and 72°C 1 min; followed by a final extension at 72°C for 10 min. Afterward, 20 µL of the PCR product was separated on a 1% agarose gel. DNA fragments of the expected size were inserted into the pGEM-T vector (Promega, Madison, WI, USA) and sequenced (Macrogen Co. Seoul, Korea).

Southern Hybridization

Genomic DNA was extracted from tobacco (*Nicotiana tabacum* cv. Havana SR1). These transgenic plants had been transformed with C1-DFR according to the method of McCouch et al. (1988). Briefly, 15 g of DNA was digested with the restriction endonuclease *Hin*dIII. The digests were then fractionated on a 1% (w/v) agarose gel and transferred to a Hybond-N^+ membrane (Amersham Pharmacia Biotech, USA) as recommended by the manufacturer. After baking at 80°C for 2 h, the membrane was hybridized at 68°C for 16 h in hybridization buffer containing 1 M Na_2HPO_4, 10% BSA, 20% SDS, and 0.5 M EDTA. Post-hybridization washes were performed twice, for 15 min each, in 20X SSC and 20% SDS at 68°C. The washed membrane was subjected to autoradiography with an intensifying screen. To confirm the copy number of our transformed plants, we used the 1.2-kb full-length cDNA that was inserted into the *Spe*I and *Pml*I sites of the hygromycin resistance sequence for pCAMBIA 1302. This constructed vector was designated pC1-DFR. Primer sequences included hpt primer 1 (5'-ttt cca cta tcg gcg agt ac-3') and hpt primer 2 (5'-tgt cga gaa gtt tct gat cga-3').

DFR Gene Expression Analysis by RT-PCR

RT-PCR was performed to determine the expression levels of *DFR* in transgenic plants. First-strand cDNA was synthe-

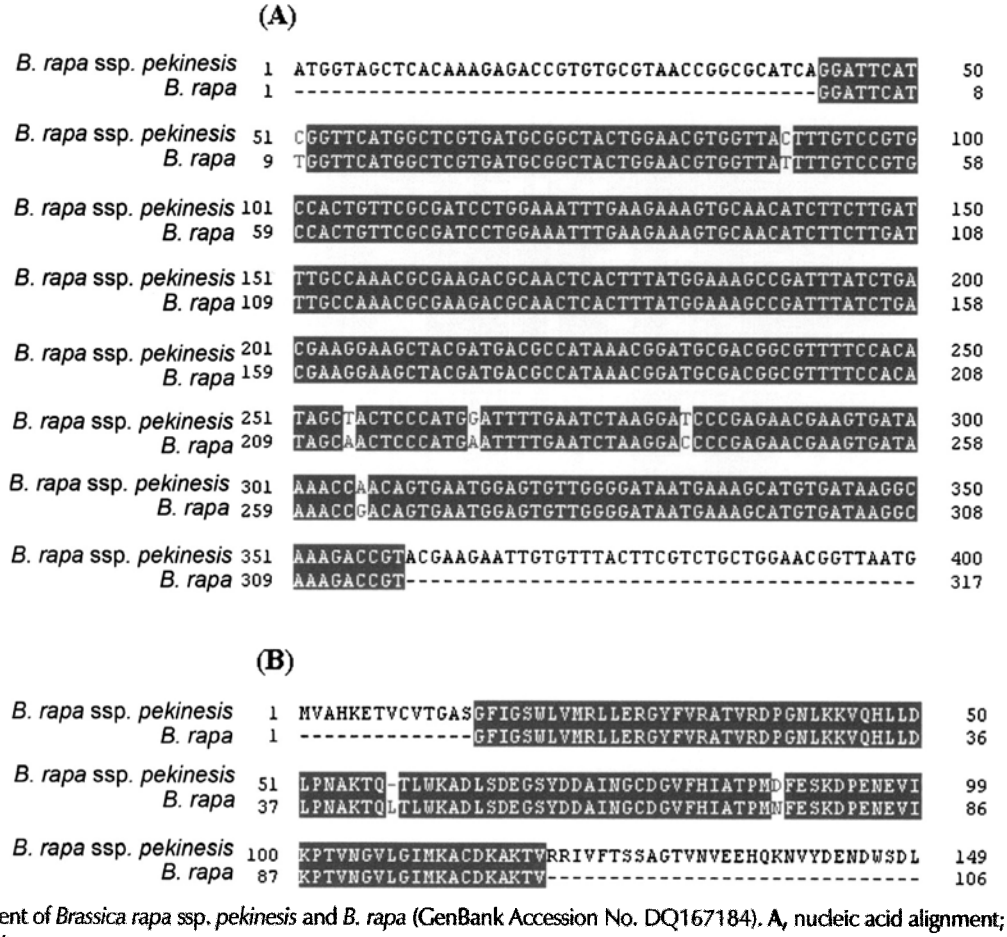

Figure 1. Alignment of *Brassica rapa* ssp. *pekinesis* and *B. rapa* (GenBank Accession No. DQ167184). **A,** nucleic acid alignment; **B,** amino acid residue alignment.

sized at 47°C for 30 min, using a Reverse-iT™ One-Step RT-PCR Kit. The gene-specific primers were dfr F9 (5′ atggtagctcacaaagagaccg 3′) and dfr R11 (5′ tgcacatactgtccttctcttat 3′), and the expected fragment size was 829 bp. PCR parameters were 24 cycles of 95°C for 30 s, 60°C for 1 min, and 72°C for 1 min.

High-Performance Liquid Chromatography Analyses of Anthocyanins

To measure anthocyanin contents, HPLC analysis was performed, using 250 mg of fresh, 5-week-old tobacco flowers that had been macerated overnight in 500 L of 1% (v/v) HCl and 80% (v/v) ethanol at 4°C. After centrifugation, the supernatant was used as the HPLC sample. Separation was carried out at a low rate, i.e., 0.8 mL min^{-1}, on an Atlantis dC18 column (3.0 mm × 205 mm; Waters, Ireland) at room temperature. After equilibration, 20 L was injected into the HPLC apparatus. The elution system consisted of Solvent A -- acetonitrile (MeCN):water:trifluoroacetic acid (TFA) = 10:90:0.1 (v:v:v) -- and Solvent B -- MeCN:water:TFA = 90:10:0.1 (v:v:v). The eluate was monitored for UV absorbance at 520 nm to detect the anthocyanin compounds. Delphinidin, cyanidin, malvinidin, and pelargonidin (Fluka, Buchs, Switzerland) were used as standards during this quantitative analysis.

RESULTS

Isolation of the Dihydroflavonol 4-Reductase (*DFR*) Gene

To obtain *DFR* from *Brassica rapa* ssp. *pekinesis* (Chinese cabbage), we performed RT-PCR with F6 and F9 primer sets to isolate 150-bp cDNA DFR fragments. This product was highly homologous to sequences from *B. rapa* (99%), *B. oleracea* (97%), and *Arabidopsis thaliana* (85%) (Figs. 1, 2). To isolate the entire length of the cDNA DFR, those 150-bp fragments were used as templates. The isolated *DFR* cDNA had a 1206-bp ORF and encoded a protein of 402 amino acid residues (GenBank Accession No. AY 567978).

Expression of Chinese Cabbage *DFR* cDNA in Tobacco

To identify the function of the isolated *DFR* gene from Chinese cabbage, we generated transgenic overexpressing tobacco plants. Using vector pC1-DFR (Fig. 3A), we obtained

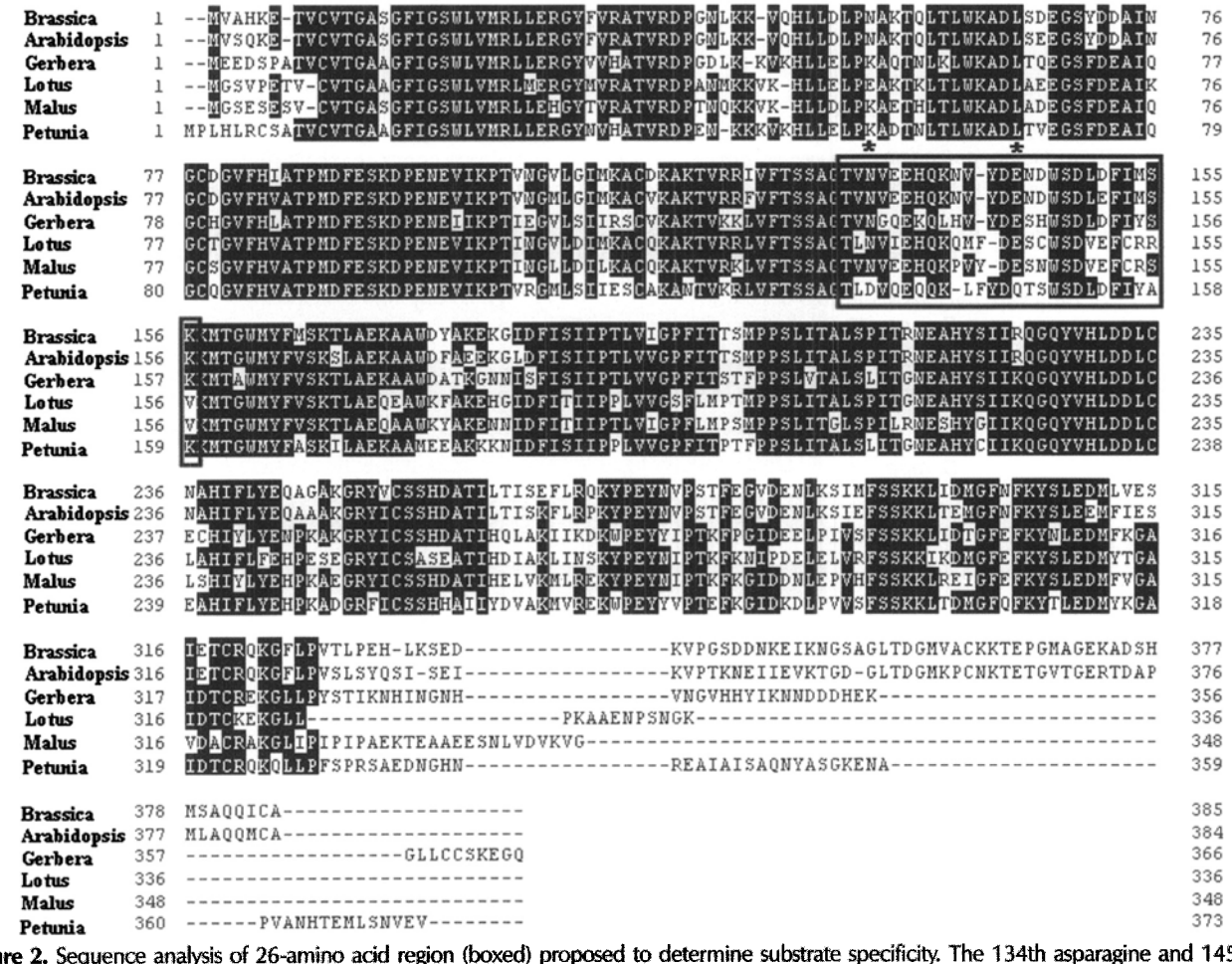

Figure 2. Sequence analysis of 26-amino acid region (boxed) proposed to determine substrate specificity. The 134th asparagine and 145th glutamine are marked with an *, respectively. The former is a critical binding site of DHQ and DHM; the latter is conserved in all DHK-accepting DFR, but not in Petunia DFR (Johnson et al., 2001). Brassica: *Brassica rapa* ssp. *pekinesis* (Chinese cabbage), Arabidopsis: *Arabidopsis thaliana*, Gerbera: *Gerbera hybrida*, Malus: *Malus domestica*, Lotus: *Lotus japonicus*, Petunia: *Petunia hybrida*.

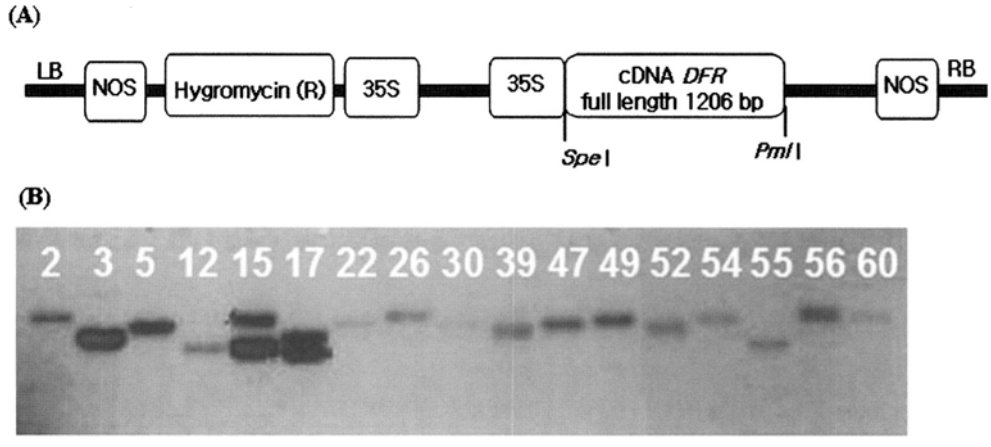

Figure 3. Construct for DFR overexpression and Southern blot analysis. **A,** C1-DFR vector, which was created by inserting isolated full-length DFR_{Bra} cDNA (1206 bp) into pCAMBIA 1302 vector with *Spe* and *Pml*. Nos: nos terminator, LB: left border, RB: right border. **B,** Southern analysis of transgenic tobacco plants with pC1-DFR. Genomic DNA was digested with *Hind* and probed with [^{32}P]-labeled fragment of pC1-DFR DNA. Numerals indicate transgenic lines.

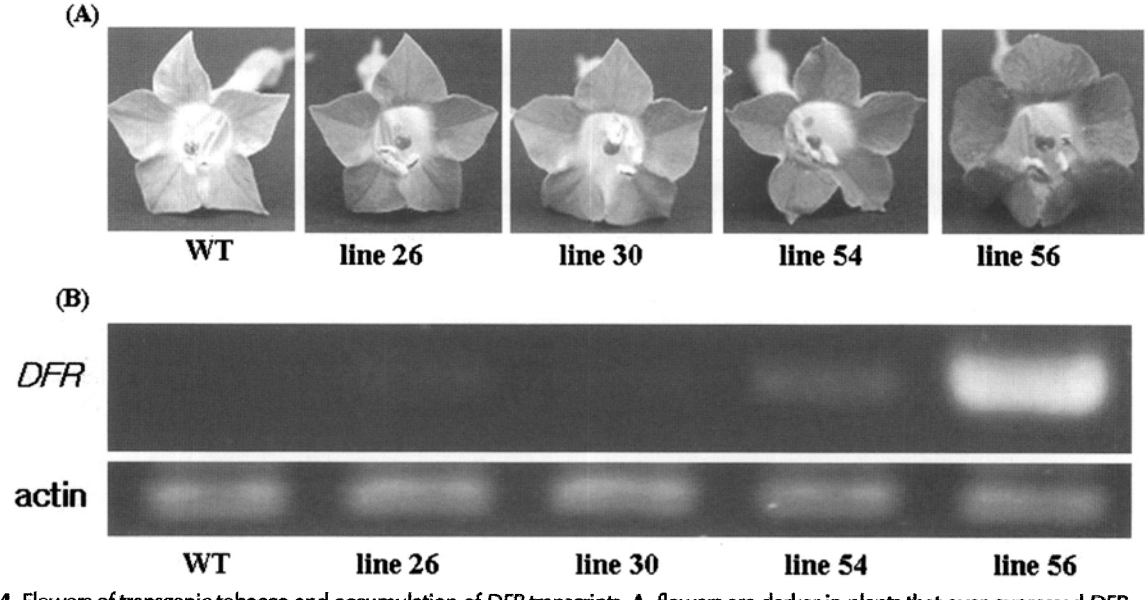

Figure 4. Flowers of transgenic tobacco and accumulation of *DFR* transcripts. **A,** flowers are darker in plants that over-expressed DFR_{Bra}. **B,** DFR_{Bra} was amplified by RT-PCR. SR1: wild type; Lines 26, 30, 54, and 56: transformed plants. Amplification efficiency was considered to be linear during PCR. Expected product size was 829 bp.

17 transgenic plants, all having distinctive petal colors, but showing some variation in their intensity. To confirm this transformation, we performed Southern blot analysis with the hygromycin resistance sequence.

Among these 17 lines, 15 carried 1 copy of the transgene and 2 lines had 2 copies (Fig. 3B). Four of these (Lines 26, 30, 54, and 56) that had the most significantly changed petal color were compared with the wild type (Fig. 4A). RT-PCR analysis revealed that the intensity of red coloring in the transgenic flowers was positively correlated with the level of *DFR* transcription (Fig. 4).

HPLC Analysis and Anthocyanin Contents

Anthocyanins that were the highest and second-highest in abundance had absorptions measured at 520 nm in the petals of the wild-type and transgenic tobacco plants. The major peak was cyanidin, a DHQ derivative (retention time 23.6 min), followed by a product peak that matched the retention time (26.3 min) of pelargonidin, a DHK-derived anthocyanidin (Fig. 5B). When compared with control values, significant increases were observed in the contents of both cyanidin (red to violet; 210%) and pelargonidin (red to orange) from transgenic Line 56, whereas neither delphinidin (violet to blue) nor malvinidin were found (Fig. 5C).

DISCUSSION

We report the isolation of a *DFR* cDNA from *Brassica rapa* ssp. *pekinesis* (Chinese cabbage) and the successful expression of DFR_{Bra} in tobacco plants. The isolated DFR_{Bra} cDNA is 1206 bp long and encodes 402 amino acids. Our comparison of its amino acid sequence showed homologies of 85%

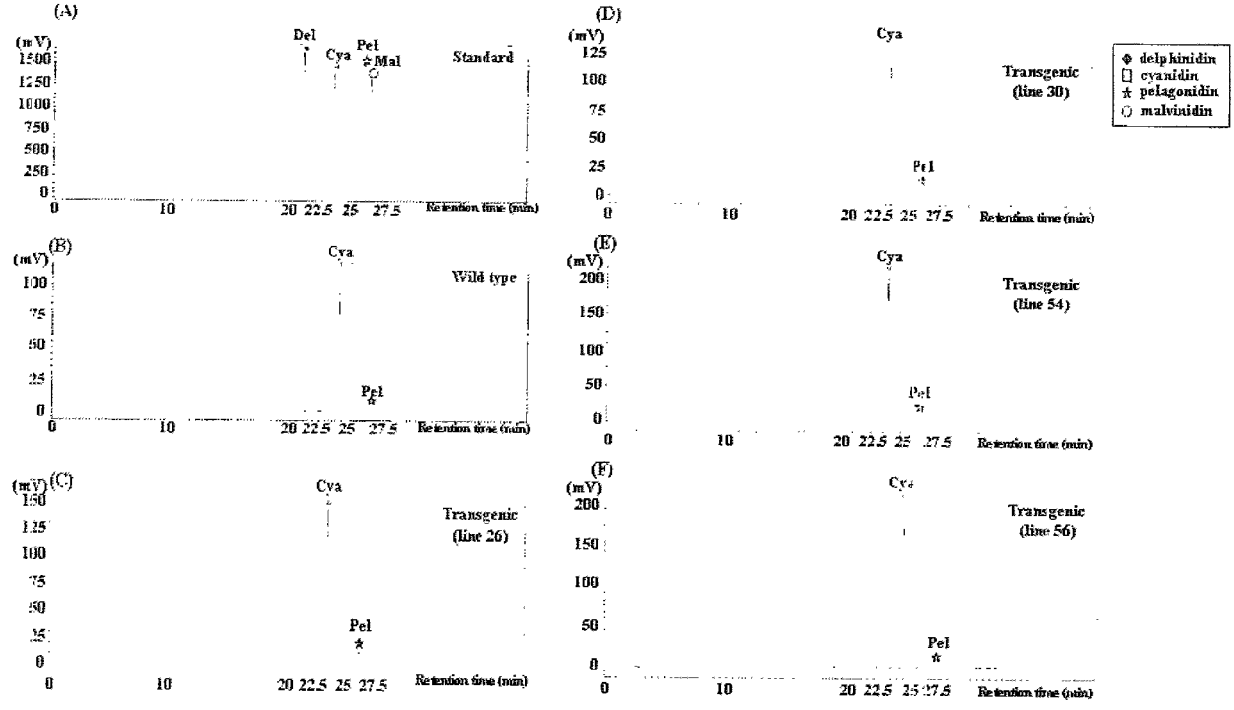

Figure 5. HPLC chromatograms of transformed *Nicotiana tabacum* lines. **A,** anthocyanidin standard samples: delphinidin (Del), cyanidin (Cya), pelargonidin (Pel), and malvinidin (Mal). **B,** DHQ-derived cyanidin and DHK-derived pelargonidin were detected, but DHM-derived delphinidin and malvinidin were not found. **C** through **F,** amounts of cyanidin (red to violet) and pelargonidin (red to orange) were increased in transgenic plants compared with wild type. Transgenic lines are 26 **(C)**, 30 **(D)**, 54 **(E)**, and 56 **(F)**.

to *Arabidopsis thaliana* (GenBank Accession No. AB033294) (Shirley et al., 1995), 69% to *Gerbera hybrida* (Helariutta et al., 1993), 62% to *Hordeum vulgare* (Kristiansen and Rohde, 1991), 69% to *Petunia hybrida* (Beld et al., 1989), and 71% to *Rosa hybrida* (Tanaka et al., 1995). Interestingly, DFR_{Bra} also has high homology (73%) to tree fruits, such as *Citrus sinensis* (Piero et al., 2006), *Malus domestica* (GenBank Accession No. AAD26204), and *Vitis vinifera* (GenBank Accession No. P51110). An N-terminal NADP-binding domain that contains a highly conserved region is also present in the DFR_{Bra} amino acid sequence (Lacombe et al., 1997).

We also generated transgenic tobacco plants that overexpress DFR_{Bra} (Fig. 3A) and demonstrated that *DFR* expression induces and is correlated with anthocyanin accumulations in the petals (Fig. 4A, B). These results indicate that DFR_{Bra} successfully interacts with the endogenous tobacco anthocyanin biosynthetic pathway *in vivo*. Our HPLC analyses showed that the induced anthocyanins are primarily cyanidin, along with a small amount of pelargonidin (Fig. 5B). This suggests that DFR_{Bra} uses DHQ and DHK as substrates to synthesize anthocyanidins. However, it is possible that DFR_{Bra} proteins can catalyze the reduction of all three substrates, as reported by Xie et al. (2004).

It is not known how substrate specificity is determined for DFR or how common it is for DFRs to have altered substrate specificity in nature. Beld et al. (1989) has proposed an insightful hypothesis about the region that determines this specificity, based on the alignment of amino acid sequences with DFRs from petunia, maize, and antirrhinum (Beld et al., 1989). It has been suggested that a stretch of 26 amino acids, between Residues 132 and 157, in the petunia DFR may be related to its substrate specificity (Beld et al., 1989; Johnson et al., 2001; Xie et al., 2004). In Chinese cabbage, the amino acid residues at Positions 134 (Gerbera numbering) and 145 of DFR_{Bra} are asparagine and glutamine, respectively (Johnson et al., 2001; Shimada et al., 2005). Our result (Fig. 2) supports the theory that the 145th glutamine is conserved in all DHK-accepting DFR, but not in either *Petunia* DFR (Johnson et al., 2001) or *A. thaliana* (Shirley et al., 1995). Although the exact molecular mechanism has not yet been found for substrate specificity determination, the hydroxyl groups of the B-ring may play an important role in the specificity of those three DFR substrates (Johnson et al., 2001; Xie et al., 2004). These results imply that, although the biosynthetic pathways share a majority of common reactions in plant species, there are some important differences among the types of anthocyanins produced by each species.

Further molecular study should evaluate DFR activity and identify other biosynthetic genes, such as for anthocyanidin synthase (ANS), in the flavonoid pathway of Chinese cabbage. Expression analyses of those genes would reveal possible competition and/or blocks in the anthocyanin biosynthesis pathway within flower petals.

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