Note

Sequence variations in the *flavonoid 3′,5′-hydroxylase* gene associated with reddish flower phenotypes in three delphinium varieties

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Abstract   Although delphiniums are famed for their blue flowers, a few varieties display reddish flowers, such the pale-pink garden varieties 'Ehime Kou 9 (Kou 9)' and 'F$_1$ Super Happy Pink (SHP),' and the orange-red flowered species *Delphinium nudicaule* (NDC). Blue delphinium flowers contain a delphinidin-derived anthocyanin, whereas the three varieties mentioned above have anthocyanins derived from the aglycone pelargonidin in their sepals. As flavonoid 3′,5′-hydroxylase (F3′5′H) is known to be the key enzyme in biosynthesis of delphinidin, we compared the structure and function of the *F3′5′H* gene in three varieties of delphiniums with reddish flowers to one that has blue flowers. We found that the *F3′5′H* gene of 'Kou 9' had a point mutation that generated a stop codon in the first exon. Genomic PCR analysis indicated that the 'SHP' variety lacked *F3′5′H*. Although the nucleotide sequence of the *F3′5′H* open reading frame was identical in 'NDC' to that of the wild type, it lacked an intron and no *F3′5′H* transcripts could be detected in this variety. We conclude that the red flower phenotypes of these delphiniums derive from independent mutations of the *F3′5′H* gene. This is the first report on the delphinium *F3′5′H* gene. At a practical level, these mutations should be of value for breeding new pink and red flower varieties.

Key words: Anthocyanin, delphinium, flavonoid 3′,5′-hydroxylase, pelargonidin.

Ornamental plant species with blue flowers are particularly popular, a fact that has stimulated considerable commercial breeding for varieties with such coloration. Recently, the application of transgenic technology has produced blue carnations and roses (Katsumoto et al. 2007; Mol et al. 1999; Tanaka et al. 1998). The development of these new blue varieties was achieved through transfection of the anthocyanin biosynthesis gene *flavonoid 3′,5′-hydroxylase* (*F3′5′H*), which is central to the synthesis of the blue pigment. *Delphinium* species naturally have blue flowers that range from blue-violet to light blue (e.g. Figure 1A). These colors are produced by anthocyanins that are derived from the aglycone delphinidin following modification with sugars and *p*-hydroxybenzoic acid. In delphiniums, the main pigments are called violdelphin and cyanodelphin (Hashimoto et al. 2002; Kondo et al. 1990; Kondo et al. 1991). Although blue flowers are the norm for most delphiniums, some varieties with red

flowers have been identified during breeding or have been found in wild plant populations. Plants with these novel flower colors have been used to generate further varieties with different depths and tones of red, such as the 'University Hybrid' varieties (Legro 1961). The University Hybrid variety 'Princess Caroline' has been reported to have six pelargonidin derivatives (Saito et al. 1998). To date, however, the underlying cause of this red coloration in some varieties of delphinium has not been explained. Here, we investigated the question of the identity of the anthocyanidin molecules in three different red varieties of delphinium and also examined the structure and function of the gene required for delphinidin biosynthesis in these varieties.

For the purposes of this study, we selected three delphinium cultivars with reddish flowers. First, a variety of *Delphinium grandiflorum* called 'Ehime Kou 9 (Kou 9)' (Figure 1B); second, 'F$_1$ Super Happy Pink (SHP)' (Figure 1C); and third, the wild species *D.*

Figure 1.   Flower colors in four delphinium varieties. (A) 'F$_1$ Super Platinum Blue (PB),' (B) 'Ehime Kou 9 (Kou 9),' (C) 'F$_1$ Super Happy Pink (SHP)' and (D) *D. nudicaule* (NDC).

nudicaule (NDC) (Figure 1D). A blue flower variety, 'F$_1$ Super Platinum Blue (PB)' (Figure 1A), was used for isolation of *Delphinium* (*Dp*) *F3′5′H* cDNA. 'Kou 9' was established by the Department of Agricultural Research at the Ehime Research Institute of Agriculture, Forestry and Fisheries. 'SHP' was purchased from Miyoshi & Co. Ltd., Tokyo, Japan. 'NDC' was cultivated from seeds at the Department of Agricultural Research at the Ehime Research Institute of Agriculture, Forestry and Fisheries. 'PB' was purchased from Miyoshi & Co. Ltd. 'Kou 9' and 'SHP' have pale pink sepals, 'NDC' has orange-red sepals, and 'PB' has pale blue sepals (Figure 1A–D). We prepared anthocyanin aglycones, RNA and genomic DNA from sepals of these four varieties. The sepals were harvested from freshly opened flowers, immediately frozen in liquid nitrogen, and stored at −80°C until required.

High-performance liquid chromatography (HPLC) analysis was used to confirm the anthocyanin aglycone of the pink and red varieties. For blue flower control of HPLC analysis, the variety 'Blue Candle' established by Sakata Seed Co. was used as we had no frozen samples of 'PB' sepals for pigment extraction. Frozen samples (0.5 g fresh weight) of sepals were soaked in 100 ml of 80% aqueous methanol containing 0.1% trifluoroacetic acid (TFA) for 2 h at 4°C to extract the pigments. The absorbance at 510 nm (A$_{510}$) per μg fresh weight of sepals were 19.4±3.70 for 'NDC,' 1.53±0.52 for 'Kou 9,' and 2.53±0.17 for 'SHP.' Thus, the levels of accumulated total anthocyain in 'NDC' sepals were approximately 12 and 8 times higher than in 'Kou 9' and 'SHP,' respectively. The pigments in the total extract were hydrolyzed by evaporating and redissolving the extracts in a few ml of 12 N HCl, then incubating at 80°C for 1h. The aglycones in the crude hydrolyzed extract were separated by HPLC and compared to aglycone standards (delphinidin, cyanidin, and pelargonidin) purchased from Extrasynthese Co. Genay, France. HPLC analysis was carried out using an LC-20AT Prominence liquid chromatograph, an SPD-10Avp UV-VIS detector, and a DGU-20A3 degasser (Shimadzu Co., Kyoto, Japan) equipped with a Handy ODS (i.d. 4.6×250 mm, Wako Pure Chemical Industries, Ltd., Osaka, Japan) column. Detection was performed at 510 nm. The analysis used linear gradient elution at a flow rate of 1 ml·min^{-1} from 40 to 100% methanol in 1.5% phosphoric acid in water for 15 min.
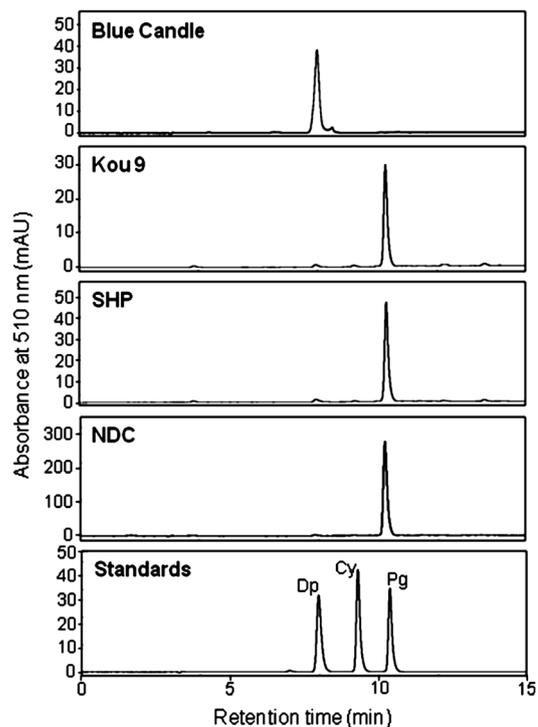

Figure 2.   Determination of anthocyanin aglycones by high-performance liquid chromatography (HPLC). Dp, delphinidin; Cy, cyanidin; Pg, pelargonidin.

The HPLC analysis of the extracts from the 'Blue Candle' variety showed that the aglycone was delphinidin (Figure 2). By contrast, the three red varieties showed the same retention time, which corresponded to the standard peak for pelargonidin. Thus, all three red varieties had pelargonidin as an aglycone; however, the amount of pelargonidin aglycone in the total extract from 'NDC' was approximately 13 and 6 times higher than in 'Kou 9' and 'SHP,' respectively (Figure 2; the peak area of 'NDC,' 'Kou9' and 'SHP' is 7,160 mAUsec, 555 mAUsec and 1,165 mAUsec, respectively). The biosynthetic pathway for aglycones includes a step in which dihydromyricetin is synthesized from dihydrokaempferol by F3′5′H to produce delphinidin (Menting et al. 1994). If F3′5′H is non-functional, dihydrokaempferol is expected to be metabolized to pelargonidin. Therefore, the results of our aglycone analysis in these three varieties suggested that F3′5′H might not function in their petals. To test whether this interpretation was correct, we focused on the structure and expression of the *F3′5′H* gene in these

varieties.

First, we isolated a cDNA for the *DpF3′5′H* gene. Total RNAs were extracted from sepals of the light-blue variety 'PB' and first strand cDNAs were synthesized as described previously (Nishizaki et al. 2011). Two degenerate primers, DpDgF and DpDgR (Supplementary Table S1), were designed using the sequences of the *F3′5′H* gene of *Camellia sinensis, Gentiana triflora, Petunia×hybrida*, and *Torenia×hybrida* (DDBJ database accession numbers AY945842, D85184, AY245545, and AB012925, respectively). Degenerate RT-PCR using the first strand cDNAs prepared above as the template was performed by 3 min denaturation at 96°C, then 30 cycles of 10 s denaturation at 96°C, 15 s annealing at 45°C, and 20 s extension at 68°C using PrimeSTAR GXL DNA Polymerase (Takara Bio Inc., Shiga, Japan). The sequence of the initial *DpF3′5′H* cDNA fragment obtained by degenerate PCR was used to design the Dp5RACEF and Dp3RACER primers (Supplementary Table S1). The 5′ cDNA end of *DpF3′5′H* cDNA was obtained using a SMARTer RACE cDNA Amplification Kit (Clontech, Mountain View, CA, USA) with the Dp5RACER primer and 5′-RACE PCR Primer included in the kit, followed by amplification of the 3′ ends of *DpF3′5′H* cDNA using the 3′-Full RACE Core Set (Takara Bio) with the Dp3RACER primer and 3-sites Adaptor Primer. The PCR conditions were: 3 min denaturation at 96°C, then 38 cycles of 10 s denaturation at 96°C, 15 s annealing at 58°C, and 30 s extension at 68°C. This cDNA sequence was used to design the forward primer, 5′UTRF, that included the 5′ untranslated region and the ATG codon for the 1st methionine. It was also used for the antisense primer, 3′UTR, that corresponded to the antisense strand of the 3′ untranslated region 45 bp downstream from the stop codon. These primers were used for RT-PCR to detect *F3′5′H* transcripts and for genomic PCR. The expression of the *F3′5′H* gene was demonstrated by RT-PCR using these primers and cDNAs prepared from sepals of flowers of the four varieties. The PCR conditions were the same as those for 5′ and 3′ RACE PCR except that 32 cycles of amplification were employed. RT-PCR amplification of *Delphinium actin* gene transcripts using the DpActinF and DpActinR primers and the four cDNAs as templates (Supplementary Table S1) served as the control (Figure 3A). Genomic DNAs were extracted from *Delphinium* varieties using a DNeasy Plant Mini Kit (QIAGEN, Hilden, Germany). Genomic PCR was performed with PrimeSTAR GXL DNA Polymerase (Takara Bio) using genomic DNAs from the four varieties as templates and the primers indicated above. The PCR conditions were: 3 min denaturation at 96°C, then 35 cycles of 10 s denaturation at 96°C, 15 s annealing at 60°C, and 30 s extension at 68°C. The DNA fragments amplified by PCR were introduced into T-vector pMD20 (Takara
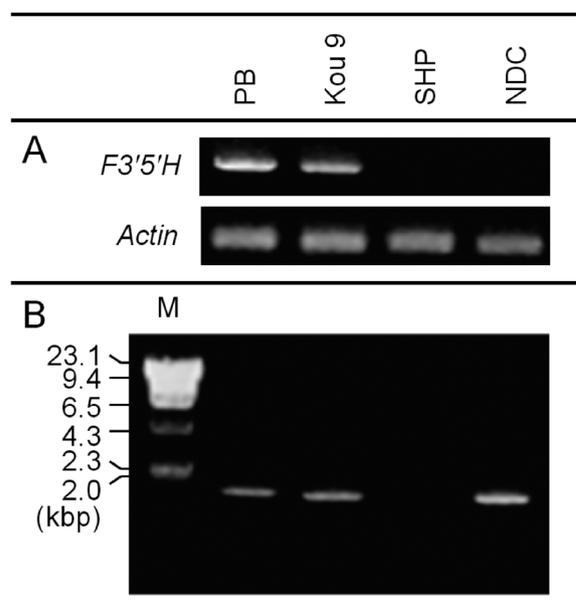

Figure 3. RT-PCR analysis of expression and genomic PCR analysis of the *F3′5′H* gene in four delphinium varieties. (A) RNAs were prepared from sepals and first strand cDNAs were synthesized; RT-PCR was performed using the 5′UTRF and 3′UTRR primers. *Actin* was used as an RNA extraction, cDNA synthesis, PCR amplification and loading control. (B) Genomic PCR was performed using the 5′UTRF and 3′UTRR primers. An explanation of the abbreviations 'PB,' 'Kou 9,' 'SHP' and 'NDC' is given in the legend to Figure 1. M, molecular marker.

Bio) and the nucleotide sequences of a minimum of six independent clones of *Escherichia coli* transformants for each genotype were determined using a 3130xl Genetic Analyzer (Applied Biosystems Inc, Carlsbad, CA, USA).

The 'PB' *F3′5′H* cDNA (accession no. AB818394) contained a single open reading frame (ORF) of 1,518 bp, without a stop codon, that encoded a 506 amino acid sequence. The genomic sequence of *DpF3′5′H* of 'PB' (accession no. AB818395) was 1,603 bp and consisted of a first exon of 891 bp, an 82 bp intron, and a second exon of 630 bp. The position of the intron was identical to that reported in other plant species, such as *Saintpaulia* sp. (accession no. AB596830, AB596831), *Pisum sativum* (accession no. GU596478, GU596479) and *Gentiana triflora* (accession no. AB586142).

We examined expression of *F3′5′H* in sepals by RT-PCR using cDNAs from each of the four varieties. *F3′5′H* transcripts were detected in sepals of the 'PB' and 'Kou 9' varieties but not in the 'SHP' or 'NDC' varieties (Figure 3A). We retested expression of *F3′5′H* in 'SHP' and 'NDC' using another primer set, 5′UTRF and Oligo dT Adaptor Primer (Takara Bio), and again failed to detect amplified products in either variety (data not shown).

The genomic PCR amplified an approximately 1,700 bp fragment from 'Kou 9,' 'NDC,' and 'PB,' but not from 'SHP' (Figure 3B). The 'Kou 9' genomic sequence was determined and compared to that of 'PB'; a T/A transition was present 380 bp downstream of the ATG
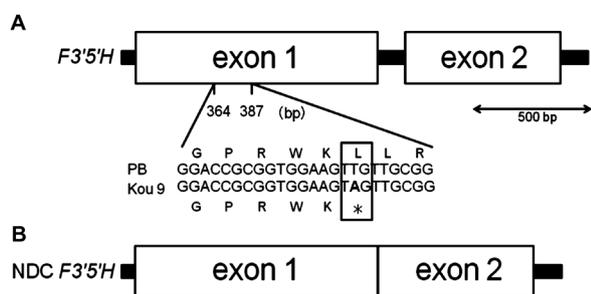
Figure 4. Exon-intron structure of the *F3′5′H* gene. (A) Structure of *F3′5′H* in the blue flowered variety 'PB,' which has a single 82 bp intron. Comparison of the sequences in 'PB' and 'Kou 9' showed a single nucleotide substitution 380 bp downstream of the ATG. This T/A transition generated a stop codon. (B) 'NDC' has no intron present; the ORF potentially encodes the complete amino acid sequence without any internal stop codon.

corresponding to the first methionine in 'Kou 9' cDNA, resulting in replacement of a codon for lysine with a stop codon (Figure 4A). Since the *F3′5′H* transcripts in sepals of 'Kou 9' contained a point mutation generating in a stop codon, the resulting polypeptide did not express F3′5′H activity, leading to the production of a pink flower due to accumulation of pelargonidin derivatives.

The *F3′5′H* sequence of 'NDC' did not have the two exons and one intron structure as in the 'PB' variety; rather, the exons were directly connected without any intronic sequences (Figure 4B). This *F3′5′H* gene structure showed no evidence of being expressed in sepals (Figure 3A). Although introns are important for gene expression in many eukaryotes (reviewed by Hir et al. 2003), the chalcone isomerase (*CHI*) gene offers an example of an intron-less gene that can be expressed. In some species, the *CHI* gene has four exons and three introns (Itoh et al. 2002; Kuittinen and Aguadé 2000; Shimada et al. 2003). In *P. hybrida*, *CHI* gene B has three introns and is expressed in immature anthers, however, the *CHI* gene A has no introns and is expressed in the corolla (van Tunen et al. 1988). *CHI* genes that lack introns, but which are expressed in plantlets, have also been reported of *Saussurea medusa* (Li et al. 2006) and *Deschampsia antarctica* (Zamora et al. 2013). These observations indicate that the lack of an intron in 'NDC' need not necessarily prevent expression of *F3′5′H*; the ORF nucleotide sequence in 'NDC' was perfectly identical to that of the wild type *F3′5′H* gene, suggesting that a transcribed and translated protein would show F3′5′H activity. As *F3′5′H* transcripts were either not present or below the level of detection (Figure 3A), it will be necessary to examine the promoter and terminator sequences of the gene in 'NDC' to elucidate the cause of the apparent failure of expression.

Identification of sequences corresponding to the *F3′5′H* gene of 'SHP' was carried out by genomic PCR using a combination of the 5′UTRF, F1, F2, R1, R02, 3′UTRR sense and antisense primer sets (Supplementary

Table S1, Figure S1). Primers F1, F2, R1, and R02 were based on the ORF sequence of the *F3′5′H* gene. Although amplified products were observed using these six primer sets with 'PB' genomic DNA as the template, no DNA fragments were amplified from 'SHP' genomic DNA. Our analysis indicates that the *F3′5′H* gene in 'SHP' might have either a substantial alteration or deletion of the ORF sequence. It has been reported that the deletion of a large part of the ORF region of *F3′5′H* gene causes loss of F3′5′H activity in a neutron beam induced mutant of *Pisum sativum* that has pink flowers (Moreau et al. 2012). The 'SHP' variety was developed by hybridization of a parental line with pink sepals that arose spontaneously in a variety with blue flowers (Sakaguchi (Miyoshi & Co. Ltd.), personal communication). It will be interesting to determine the scale of any deletion or alteration to the *F3′5′H* gene in this variety.

There have been no previous reports on changes in sepal color in delphiniums as a result of mutation of the *F3′5′H* gene. Our analysis here shows that three varieties of delphiniums with reddish flowers have different underlying mechanisms for the loss of F3′5′H activity and the associated accumulation of pelargonidin in sepals. Although pelargonidin is the aglycone in all three varieties, they nevertheless showed clearly different phenotypes: the sepals of 'Kou 9' and 'SHP' are pale pink; those of 'NDC' are deep orange-red. Our results indicate that the difference in color density between 'NDC' and 'Kou 9' or 'SHP' is caused by differences in the amounts of accumulated pigment in the varieties, with 'NDC' having 8 to 12 times more than the others. It is possible that different color hues of the three varieties, i.e., orange-red in 'NDC' and pink in 'Kou 9' and 'SHP,' might result from differences in the modification of their pelargonidin aglycones. The one of the anthocyanins of 'Princess Caroline,' a pink delphinium variety, is pelargonidin 3-(6-(malonyl)glucoside)-7-glucoside; additionally, five other anthocyanins are modified by glucosyl and acyl moieties at the 7 position of pelargonidin (Saito et al. 1998). We have carried out a preliminary analysis of the structures of the major anthocyanins of 'Kou 9' and 'SHP' and found that they accumulate pelargonidins modified by two glucoses and two organic acids at the 7 position. An electrospray ionization mass spectrometry analysis of the partially purified anthocyanin from sepals of 'NDC' suggested that the pigment is pelargonidin 3-malonylglucoside that has not been modified at the 7 position (data not shown). This suggestion of a difference in the pattern of modification of the pelargonidin aglycone between 'NDC' and 'Kou 9' or 'SHP' may provide an explanation for their different flower colors. It is also possible that other factors, such as the presence of flavonoid co-pigments in the vacuole and vacuolar pH, may also

contribute to the final color of the flower. Future investigations on the mechanisms that modify color tone and depth and their interactions with anthocyanin molecules will provide further insights of value to the production of a wider variety of red flower colors in delphiniums.

Acknowledgements

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SUPPLEMENTARY DATA:

Supplementary Table S1. Oligonucleotide sequences of the primers used for amplification of *F3′5′H* gene.

primer name	sequences (5′ to 3′)
for degenerate PCR	
DpDgF	YTNCAYATGYTNGGNGGNAARGCN
DpDgR	DATNCCCATNCKNGTNCCNGCRCA
for RACE PCR	
Dp3RACEF	GGAGGGATGAGGTGGGGTAT
Dp5RACER	TCGCCGACATTAAACAACCCCGCCGACG
for RT-PCR, genomic PCR	
5′UTRF	ATGCACCTTTCACCATGTCTACAAGCTTG
3′UTRR	CCAAACACCTTATAGTCCACGACTCC
for RT-PCR	
DpActinF	TGCTGACCGTATGAGCAAG
DpActinR	CCACATCTGTTGGAATGTGC
for genomic PCR	
F1	TACAACTCGCAGGACATGGTGTTTGC
F2	CAGCTGGAACAGACACATCCTCAAGC
R1	CAAGCTACTCAGCTTCCGCAACAACT
R02	GGTTCTTTATCATTTCGGTCAGTGCC

Supplementary Figure S1.   The position of SHP genomic PCR primers.