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BRIEF COMMUNICATION

Expression of a *CYP76AB1* correlates with the sequential white-blue-white colour transition of *Vanda coerulea* petals

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Abstract

The blue colour of *Vanda coerulea* petals is slowly produced during flower development but quickly disappears when the flowers are pollinated. To investigate the molecular basis of the phenomenon, we isolated a novel cytochrome P450 gene, *CYP76AB1*, from this plant by polymerase chain reaction (PCR) based on the conserved regions of flavonoid 3´,5´-hydroxylase amino acid sequences. *CYP76AB1* transcripts were detectable at low level in the late phase of flower development when the petals were light blue, but became abundant in the subsequent opening flower stage, as the petals turned blue. When the flowers were pollinated they turned white, and the *CYP76AB1* transcripts returned to low level. This indicates that the expression of *CYP76AB1* may regulate petal colour of *V. coerulea*, and be a target for developing permanently blue flowers.

Additional key words: cytochome P450, flower colour, orchid, RT-PCR, vanda

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Vanda coerulea Griff. is a blue orchid native to Southeast-Asia. It is one of the most rare, expensive, and difficult orchids to find. This blue orchid is not actually true blue in colour, but has hues of light blue and purple in its petals. The blue colour of the petals quickly disappears when the flowers are pollinated. Flavonoids are the most common flower pigments contributing to a range of colours from yellow to orange, red and blue/purple. Cytochromes P450 (P450s) play important roles in flavonoid biosynthesis. The *P450* genes involved in the flavonoid biosynthetic pathway have been cloned and characterized. Molecular markers have also been developed to identify loci for flavonoid accumulation in some plants (Feria-Romero *et al.* 2009). Flavonoid 3´,5´-hydroxylase (F3´5´H) is important for the production of blue/purple anthocyanins. The *F3´5´H* gene was first isolated from petunia and classified into CYP75A (Holton *et al.* 1993). The homologues of CYP75A have subsequently been cloned and identified from various plant species (http://drnelson.utmem.edu/cytochromeP450.html) including *Phalaenopsis* (Su and Hsu 2003, Wang *et al.* 2006) and *Dendrobium* (accession number DQ923127). Flavone synthase II (FNSII) belonging to CYP93B converts flavanones into flavones, which are common copigments that form complexes with anthocyanins and contribute to the bluing of flower colour (Martens and Mithofer 2005). *FNSII* genes have been cloned from several plants (Akashi *et al.* 1999, Martens and Forkmann 1999). Other P450s playing roles in flower colour have not yet been reported. The aim of this study was to investigate the molecular basis of the appearance of blue in the petals of *V. coerulea*. The P450-like gene was isolated from *V. coerulea* using a polymerase chain reaction (PCR) based on F3´5´H amino acid sequences, and its expression pattern was determined in the flowers of *V. coerulea*.

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Abbreviations: cDNA - complementary deoxyribonucleic acid; CYP75A - cytochrome P450, family 75, subfamily A; CYP76AB1 - cytochrome P450, family 76, subfamily AB, isoform 1; CYP93B - cytochrome P450, family 93, subfamily B; dNTP - deoxynucleotide triphosphate; F3´H - flavonoid 3´-hydroxylase; F3´5´H - flavonoid 3´,5´-hydroxylase; FNSII - flavone synthase II; gDNA - genomic deoxyribonucleic acid; P450s - cytochromes P450; ORF - open reading frame; RACE - rapid amplification of cDNA end; RT-PCR - reverse transcriptase polymerase chain reaction.

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Vanda coerulea Griff. plants were grown in a greenhouse at Naresuan university, Phitsanulok province, Thailand. The genomic DNA (gDNA) was extracted from leaves using a modified method of Dellaporta *et al.* (1983). Frozen leaf powder (100 - 200 mg) was homogenized with 0.65 cm^3 of extraction buffer (100 mM Tris-HCl, pH 8.0, 50 mM EDTA, pH 8.0, 500 mM NaCl, 2 % (m/v) sodium dodecylsulphate, 10 mM β-mercapto-ethanol) and then heated at 65 °C for 12 min. RNAs were eliminated by RNaseA at 37 °C. After the phenol/ chloroform extraction, DNA was precipitated with 0.1 volumes of 3 M sodium acetate, pH 5.2, and 0.7 vol. of isopropanol. The partial sequence of a P450 gene was isolated using a PCR-based strategy. The two PCR degenerate primers, 5′-TA(C/T)GG(A/G/T/C)CC (A/G/T/C) GT(A/G/T/C)ATGTA(C/T)(C/T)T(A/G/T/C) AA(A/G)-3′ (forward) and 5′-(A/G/T/C)C(T/G) (A/G/T/C)CC(A/G/T)AT(A/G/T/C)GCCCA(A/G/T)AT (A/G)TT(A/G/T/C)AC-3′ (reverse), were designed to match the conserved regions coding for several published F3′5′H amino acid sequences. The PCR mixture contained 50 - 100 ng of gDNA, 1.875 μM of each primer, 0.2 mM dNTPs, 1.5 mM $MgCl_2$ and 1 unit of Taq polymerase (*Vivantis*, Selangor, Malaysia). The reaction cycling conditions were predenaturation at 92 °C for 2 min, 35 cycles of 92 °C for 30 s, 50 °C for 20 s, 72 °C for 60 s, followed by a final extension at 72 °C for 5 min. The PCR product was cloned into the *pGEM®-T Easy* vector (*Promega*, Madison, WI, USA) and sequenced (DNA sequencing service, *Macrogen Co.*, Seoul, Korea). To obtain the missing 3′ and 5′ sequences of the isolated P450 gene, rapid amplification of cDNA ends (RACE) was carried out according to the protocols of *GeneRacer* kit (*Invitrogen*, Carlsbad, CA, USA). Total RNA was isolated using either the RNeasy *Plant* mini kit (*Qaiagen*, Hilden, Germany) according to the manufacturer's instructions or the LiCl method (Verwoerd *et al.* 1989). The contaminated DNA in RNA samples was removed by digestion with RNase-free DNase I (*Promega*) following the manufacturer's instructions. The mixture of total RNA from flowers at different developmental stages served as template for first strand complementary DNA (cDNA) synthesis. Sequence analysis revealed that the open reading frame (ORF) of the *P450*-like gene of *V. coerulea* is 1 594 bp in length. This gene was firstly designated as *VcF3'5'H-L1* (accession number EU586137 in GenBank/EMBL/DDBJ databases). The pairwise alignment with its cDNA sequence confirmed that it contains an ORF of two exons (876 and 618 bp) encoding a polypeptide of 497 amino acid residues. *BLASTX* analysis revealed that the deduced amino acid sequence of VcF3'5'H-L1 showed highest similarity to CYP76C (41 - 44 % amino acid identity), and less than 40 % amino acid identity to F3′5′H (CYP75A, 32 - 35 %), F3′H (CYP75B, 34 - 36 %) and other P450 families. VcF3'5'H-L1 was classified as a novel CYP76 subfamily and was officially assigned the name CYP76AB1 by Dr. D.R. Nelson (Department of Biochemistry, University of Tennessee, personal communication) and the Committee for Standardized Cytochrome P450 Nomenclature.

RT-PCR was carried out to detect the expression of *CYP76AB1* in petals of the *V. coerulea* flowers at five different developmental stages as follows: stage 1, flower bud with colourless petals; stage 2, flower bud with pale-purple petals; stage 3, flower bud with purple petals; stage 4, bluish-purple opening flower; and stage 5, bluish-purple fully-opened flower (Fig. 1*A*). DNase-treated RNA was reverse-transcribed with random hexamers using *ImProm-II™* reverse transcriptase (*Promega*) according to the manufacturer's instructions. The PCR was performed using *GoTaq® Green Master Mix* (*Promega*) in a final volume of 0.025 cm^3 including the first-strand cDNA and the primer set, F35HVid12F: 5′-GGACCAGTCATGTATCTCA-3′ and cVcXR1: 5′-GCGGCAAAAGAATCTATG-3′, which were designed for detecting the *CYP76AB1* transcripts. The PCR cycling conditions were predenaturation at 92 °C for 2 min, 35 cycles of 92 °C for 30 s, 50 °C for 20 s, 72 °C for 60 s, followed by a final extension at 72 °C for 5 min. To measure the relative amount of cDNA and the samples, the *5.8S rRNA* transcripts were used as an internal control. The PCR was performed using *GoTaq® Green Master Mix* in a final volume of 0.025 cm^3 including the cDNA template and the primer set, Vc-rRNAF1: 5′-CCAAGGTAACTATCGAA AGG-3′ and Vc-rRNAR1: 5′-GCACGAAGGGCGCAATC-3′. The reaction cycling conditions were predenaturation at 92 °C for 2 min, 28 cycles of 92 °C for 30 s, 55 °C for 20 s, 72 °C for 60 s, followed by a final extension at 72 °C for 5 min.

Total PCR products were determined on a 1.5 % agarose gel. A strong signal was detected in the petals of stage 4, whereas the petals of stages 3 and 5 exhibited weaker signals. No signal was observed in petals of stages 1 and 2 (Fig. 1*B*). The expression pattern of the *CYP76AB1* gene in different developmental stages of *V. coerulea* flowers showed that the *CYP76AB1* mRNA was highly transcribed in the late phase of petal development, which was concomitant with the appearance of the blue colour in petal tissue. Expression of different *CYP76* genes occurs in different patterns. The *CYP76A3* gene encoding meristic acid hydrolase was expressed in the leaves and roots of *Petunia hybrida*, but not in the flowers (Imaishi and Ishitobi 2008). The *CYP76B9* gene encoding capric acid *ω*-hydroxylase and lauric acid *ω*-hydroxylase was expressed in the leaves and flower buds of *P. hybrida* (Imaishi and Petkova-Andonova 2007). The unknown-function *CYP76C1* gene was highly expressed in the flowers of *A. thaliana*, and at lower level in the leaves (Mizutani *et al.* 1998). The expression pattern of *CYP76AB1* we describe here in the

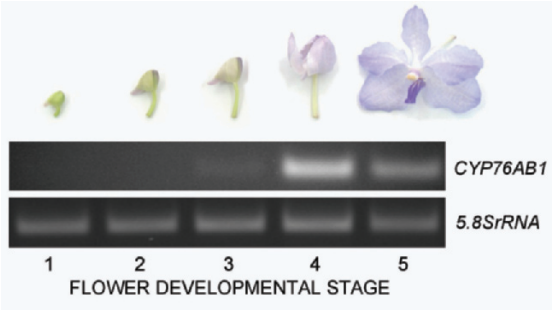

Fig. 1. The flower development and the *CYP76AB1* expression in the petals of *V. coerulea*. Developmental stages of the flower: stage 1 - flower bud with colourless petals; stage 2 - flower bud with pale-purple petals; stage 3 - flower bud with purple petals; stage 4 - bluish-purple opening flower; and stage 5 - bluish-purple fully-opened flower. The expression pattern of *CYP76AB1* in the petals of five developmental stages of the *V. coerulea* flowers.

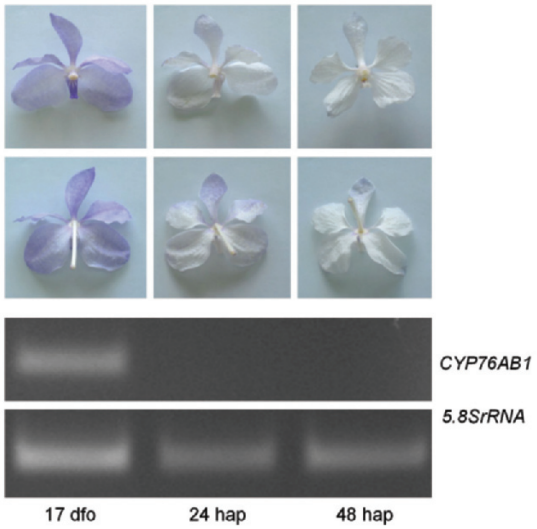

Fig. 2. RT-PCR analysis of *CYP76AB1* in the pollinated flowers of *V. coerulea*. The petal colour alteration in the pollinated flowers (17 dfo: 17-day fully-opened; 24 hap: 24 h after pollination; 48 hap: 48 h after pollination). The expression of *CYP76AB1* in the petals of the pollinated flowers.

petals of *V. coerulea* is similar to the expression patterns of *F3'5'H* in the petals of *Eustoma grandiflorum* (Nielsen and Podivinsky 1997), *Nierembergia* (Yukiko *et al.* 2006) and *Phalaenopsis* (Wang *et al.* 2006). In *Vinca major*, although the transcripts of the *F3'5'H* gene were detected in all five developmental stages of the flower, strong signals were only detected in the petals of flowers in stages 2 - 4 (Mori *et al.* 2004). This indicates that *CYP76AB1* expression in the petals of *V. coerulea* is also developmentally regulated.

A dramatic change from blue to white was noticed in the petals of *V. coerulea* flowers within 24 h of pollination. The 2-d opened flowers of *V. coerulea* were hand-pollinated, the colourless petals were collected at 24 and 48 h after pollination (Fig. 2*A*), and transcripts of *CYP76AB1* in these samples were compared by RT-PCR. No signal was detected in the petals of 24 and 48 h post-pollinated flowers, whereas a signal was clearly detected in the petals of 17-d fully-opened flowers (Fig. 2*B*). This indicates that the *CYP76AB1* gene in the petals of *V. coerulea* was dramatically suppressed when the flowers were pollinated.

In conclusion, CYP76AB1 is a new plant P450 that differs significantly from previously characterized CYP76 in amino acid sequences (a maximum similarity of 44 %). Interestingly, the expression pattern of *CYP76AB1* in the flowers is similar to that of *F3'5'H*. CYP76AB1 is the first reported P450 to be expressed and developmentally regulated in the flower of *V. coerulea*. It seems that the *CYP76AB1* gene is expressed along with the appearance of blue colour in the petals of *V. coerulea*. The *CYP76AB1* gene may be involved in the flower colour development or anthocyanin stabilization. However, the biochemical roles of CYP76AB1 in the flower colour development have not yet been characterized. Further studies of *V. coerulea* CYP76AB1 could lead to a better understanding of its role in the flower colour development. This could be useful for engineering the blue orchid in the future.

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