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Analysis of Anthocyanins and the Expression Patterns of Genes Involved in Biosynthesis in two *Vanda* Hybrids

NITTAYA JUNKA, SIRICHAI KANLAYANARAT†, MANTANA BUANONG†, SHERMARL WONGCHAOCHANT‡ AND CHALERMCHAI WONGS-AREE[1]‡
Postharvest Technology Program, School of Bioresources and Technology, King Mongkut's University of Technology Thonburi, Bangkok 10140, Thailand
†*Postharvest Technology Innovation Center, Commission on Higher Education, Bangkok 10400, Thailand*
‡*Department of Horticulture, Faculty of Agriculture, Kasetsart University, Bangkok 10900, Thailand*
[1]Corresponding author's e-mail: chalermchai.won@kmutt.ac.th

ABSTRACT

Pigment accumulation, anthocyanin structure and the expression of floral anthocyanin genes were evaluated in anthocyanin-based colored florets of a pale-mauve *Vanda* hybrid (*V. teres* x *V. hookeriana*) and compared to those of a white *Vanda* hybrid (*V. teres* var. alba x *V.* Cooperi var. White Wing). In the pale-mauve *Vanda* hybrid, anthocyanins gradually accumulated during all of the three developmental stages of the florets, whereas no anthocyanins detected in the florets of the white *Vanda* hybrid at any of the stages. The chlorophyll *a* content accumulated to higher levels in the white *Vanda* hybrid florets and slightly decreased from the unopened-floret stage (B) to the full-bloom floret stage (FB) in both hybrids. In the pale-mauve *Vanda* hybrid, the anthocyanin contents in each of the floral parts revealed a direct correlation to the a^* values (R^2=0.7276) and an inverse relation to the L^* values (R^2=0.9023). Based on HPLC-PDA and LC-ESI-MSn analyses, the anthocyanins in the pale-mauve hybrid were only derived from cyanidin molecules, which were diversely conjugated with some hexose sugars and organic acids, such as ferrulic acid, sinapic acid and malonic acid. In addition, the semiquantitative expression of four genes involved in the anthocyanin biosynthetic pathway, including phenylalanine ammonia-lyase (*Va-PAL1*), chalcone synthase (*Va-CHS1*), flavanone 3-hydroxylase (*Va-F3H1*) and dihydroflavonol 4-reductase (*Va-DFR1*), of the pale-mauve *Vanda* hybrid showed that all of the genes, except *Va-CHS1*, were highly expressed in all of the three stages of the florets, whereas *Va-F3H1* was rarely expressed in the vegetative organs. When *Va-PAL1* was expressed at high levels in the florets at all of the floral stages, *Va-F3H1* expression was very low only in the developing florets of the white hybrid. Therefore, *Va-F3H1* could play an important role in controlling the biosynthesis of anthocyanin in the *Vanda* hybrids. © 2011 Friends Science Publishers

Key Words: *Vanda* hybrid; Anthocyanins; CIELAB; Cyanidin derivatives; Gene transcript; RT-PCR

INTRODUCTION

The Orchidaceae is a large family of angiosperms that includes various genera, which plentifully produce colored flowers (Bechtel *et al*., 1992). *Vanda* is an important commercial orchid in Thailand and is the subject of conventional breeding program for the cultivation of orchids. The *Vanda* genus comprises multiple colors and forms and is a major source of the cut flowers and potted plants in the orchid market. In this study, we investigated the different floral colors of two *Vanda* hybrids. One, a pale-mauve hybrid (*V. teres* x *V. hookeriana*) which produces a rosy-violet color in the dorsal sepal and petals. The lateral sepals are paler violet, and the broad lip forms a pale-yellow throat (Fig. 1a). The other hybrid, a white *Vanda* hybrid which was bred by twice backcrossing the pale-mauve hybrid with *Vanda* (*Papilionanthe*) *hookeriana* var. alba. The white hybrid produces crystalline-white florets that are composed of a broad, flat lip and a throat that is yellowish at the centre (Fig. 1b). In addition, the lateral sepals and floret size of the white hybrid are larger than those of the pale-mauve hybrid.

Flower color formation is caused by the accumulation of pigment compounds and the investigation of this process is required for breeding programmes of orchid plants for improving quality of existing colors. Anthocyanins, the main pigment in the floral color of orchids, are a subgroup of flavonoid compounds that appear red to purple in color. Acylated cyanidins have been found to be the main pigment in the red-purple florets of *Dendrobium* 'pramot' (Saito *et al*., 1994), whereas the hybrid, *Dendrobium* x Icy Pink 'Sakura', accumulates high pelargonidin and a few cyanidins (Kuehnle *et al*., 1997). The biosynthesis of anthocyanin via the phenylpropanoid pathway begins with the conversion of phenylalanine to *trans*-cinnamic acid by phenylalanine ammonia lyase (PAL) (Andersen & Jordheim, 2006). Additional major genes in the pathway include

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chalcone synthase (*CHS*), chalcone isomerase (*CHI*), flavanone 3-hydroxylase (*F3H*), dihydroflavonol 4-reductase (*DFR*) and anthocyanidin synthase (*ANS*). The *CHS* gene has been shown to be expressed in all of the floral-bud stages and vegetative tissues (except the pseudobulbs), whereas the *DFR* gene has been shown to be expressed in all of the developmental stages of the buds in lavender *Dendrobium* x Jaquelyn Thomas 'Uniwai Prince' and pale orange *Dendrobium* x Icy Pink 'Sakura' hybrids (Mudalige-Jayawickrama *et al*., 2005). Furthermore, the floral color of 'White Mind' carnation is nearly pure-white due to lack of some flavonoid compounds such as flavanone (naringenin) and flavonol (kaempferol) in its petal (Onozaki *et al*., 1999). Deficiency of some enzymes involved in the anthocyanin biosynthesis such as DFR and ANS of 'White Mind' flower at stage 1 and 4 affects the colorful development (Mato *et al*., 2000). Apart from the *ANS*, the expression trends of various anthocyanin genes of ivory white-flowered gentian cv. 'Homoi' are similar to 'Miciry' cultivar, which contains anthocyanins in the colorful petal. On the other hand in white 'Polano' cultivar, many expression patterns of anthocyanin genes are contrast with those in white 'Homoi' cultivar (Nakatsuka *et al*., 2005).

Different levels of anthocyanins accumulated in florets might cause diverse colors during developmental stages of *Vanda* hybrids. However, although the pale-mauve *Vanda* hybrid florets would contain high anthocyanins, the expression of key anthocyanin biosynthetic genes may be independent on floral colors. In the present study, we analyzed the accumulation of anthocyanins in the pale-mauve *Vanda* hybrid and investigated the expression of the *Va-PAL1*, *Va-CHS1*, *Va-F3H1* and *Va-DFR1* genes in various parts, including unopened, half full-bloom and full-bloom florets, as compared to the white *Vanda* hybrid. Preliminary study of the anthocyanin structure was also included.

MATERIALS AND METHODS

Plant materials: The inflorescences of the two *Vanda* hybrids were collected from the 'Yen' orchid farm in the 'Nakhon Pathom' province of Central Thailand (13°48′4″N, 100°16′18″E) between January 2010 and March 2011. Within 1 h the plant materials were packed into a cold box and transferred to the Postharvest Technology Laboratory, King Mongkut's University of Technology Thonburi, Bangkok. The strawberries and grapes (as the aglycone references) were purchased from a local supermarket in Bangkok. The floral organs of pale-mauve *Vanda* hybrid (*V. teres* x *V. hookeriana*) (Fig. 1a) and white *Vanda* hybrid (*V. teres* var. alba x *V.* Cooperi var. White Wing) (Fig. 1b) and the strawberry and grape exocarps were used for the analysis of anthocyanins. The orchid florets at the three developmental stages of buds (B), half full-bloom florets (HB) and full-bloom florets (FB) and the vegetative parts, were frozen in liquid nitrogen and stored at -80°C until used for RNA extraction.

Physical attributes of *Vanda* hybrid florets: The appearances of the orchid florets at the three developmental stages, B, HB and FB, of both cultivars were captured using a digital camera (Sony, Model DSC-P52). The weight and size of the florets at each stage were determined from 10 replicates; the weight was determined and the flower dimensions were measured on a centimetre scale (Table I).

The CIE L^* and a^* values of the floret parts at FB, including the petals, dorsal sepals, laterals sepals, and lips, from the two *Vanda* hybrids (Fig. 1a & b) were measured directly with 10 replicates (1 floret/replicate) using a colorimeter (Minolta model CR 300, Japan). The results are expressed as lightness (L^*) and red intensive values (a^*).

Floral pigment accumulation during floral development of *Vanda* hybrids: The frozen materials of three developmental stages of B, HB and FB were ground to a fine powder and mixed with 0.01% HCl in methanol. The samples were then sonicated until colorless. The supernatant was recovered after centrifugation at 12,000 rpm for 30 min at 4°C, and the anthocyanin content was measured using the pH differential technique (Giusti & Wrolstad, 2005). Potassium chloride buffers (0.025 M KCl, pH 1.0) and sodium acetate (0.4 M $CH_3CO_2Na{\cdot}3H_2O$, pH4.5) were used for examining the monomeric anthocyanin contents. A mixture of 900 μL of either pH 1.0 or pH 4.5 buffer and 100 μL of the extracted samples was incubated for 15 min at room temperature (25°C) and then measured by spectrum scanning (320-700 nm) with a UV-visible spectrophotometer (Shimadzu model 1601, Japan) controlled by the UV-Probe program (Shimadzu, Japan). The absorbance of the diluted sample was calculated as follows:

$$A = (A_{\lambda\ vis\text{-}max} - A_{700})_{pH\ 1.0} - (A_{\lambda\ vis\text{-}max} - A_{700})_{pH\ 4.5}$$

The anthocyanin pigment concentration in the sample was calculated using the following formula:

$$\text{anthocyanin pigment (mg/L)} = (A \times MW \times DF \times 1000)/(\varepsilon \times 1)$$

Where the cyanidin-3-glucoside molecular weight (MW=449.2) and the molar absorptivity (ε = 26,900) constant was used.

For the chlorophyll *a* content, the absorbance at 665.2 and 652.4 nm was measured and then calculated using the formula of Lichtenthaler and Buschmann (2005), as follows:

$$Ca\ (\mu g/mL) = 16.72\ A_{665.2} - 9.16\ A_{652.4}$$

Analysis of anthocyanins: Extraction of anthocyanins: Anthocyanins of the pale-mauve *Vanda* hybrid at FB and the strawberry and grape peels were extracted using the method from Rodriguez-Saona and Wrolstad (2005). Frozen samples were homogenized with acidified methanol (0.01% HCl) and then filtered through Whatman paper No. 1 until the solution was colorless. The methanol was removed via rotary evaporation at 40°C and the extract was adjusted to a known volume with acidic deionised water (0.01% HCl).

Table I: Floral size attributes of the pale-mauve and white *Vanda* hybrids

	Floret size[1]					
	pale-mauve *Vanda* hybrid			white *Vanda* hybrid		
	B	HB	FB	B	HB	FB
Floret weight (g)	0.60 ± 0.00	1.61 ± 0.02	2.247 ± 0.11	0.7 ± 0.08	1.70 ± 0.08	2.3 ± 0.11
Floret dimension (cm)	1.3 x 1.6 ± 0.04	1.8 x 2.4 ± 0.05	5.7 x 5.6 ± 0.08	1.3 x 1.7 ± 0.08	1.8 x 2.4 ± 0.12	6.3 x 6.6 ± 0.09

[1] Means (n=10) with the standard deviation are presented

Table II: Color occurrence in the floral parts of the pale-mauve and white *Vanda* hybrids

Floral organs	CIE colors[1]			
	Pale-mauve *Vanda* hybrid		white *Vanda* hybrid	
	L^*	a^*	L^*	a^*
Dorsal sepal (DS)	52.82[b]	27.70[c]	79.64[a]	0.41[a]
Petal (P)	50.11[b]	35.43[b]	79.61[a]	0.28[c]
Lateral sepal (LS)	65.92[a]	13.27[d]	72.18[b]	0.59[a]
Lip (L)	33.85[c]	44.15[a]	78.27[a]	0.13[d]
F-test	**	**	**	**

[1] Means (n=10) with different letters within the same column are significantly different
** Significantly different at $P<0.01$

Table III: LC-ESI-MSn ion mass of anthocyanins (530 nm) of standard cyanidin 3-glucoside and purify from the pale-mauve *Vanda* hybrid

Cyanidin 3-glucoside			Anthocyanins standard		
			RT (min)	MS, M^+ (m/z)	MS/MS (m/z)
			29.96	449	287
sample					
Peak no.	RT (min)	MS,M^+ (m/z)	MS/MS (m/z)		MS^3 (m/z)
1	40.9	1065.5	903, 859, 817, 655, 449		[655] > 449, 287
2	44.4	817	655, 449, 287		[655] > 449, 287
3	45.3	1185.5	1023, 817, 655, 449		[655] > 449, 287
4 (1)	47.3	1242	1155, 993, 873, 829, 655, 449		[655] > 449, 287
4 (2)	47.5	1272	1227, 1023, 903, 858, 655, 449		[655] > 449, 287

Saponification of purified anthocyanins: The extracted anthocyanins were then processed to remove any acylation followed by the protocol of Durst and Wrolstad (2005). The anthocyanin extracts were purified using a C_{18} mini-column (Waters Sep-Pak®). After absorption, the compounds were washed with a column volume of acidic distilled water (0.01% HCl) and another column volume of ethyl acetate was then applied for the removal of the major free phenolic compounds. The purified anthocyanins were then eluted by acidic methanol (0.01% HCl). The residual methanol was removed via rotary evaporation at 40°C and the pellet was dissolved with acidic deionised water. The purified anthocyanins were mixed in 10 mL of 10% KOH and incubated for 15 min in the dark, after which the mixture was neutralised by adding 15 mL of 2N HCl. The hydrolysate was then purified by passing through a C_{18} mini-column, as described above.

Acid-hydrolysis of anthocyanins: Ten millilitres of 2 N HCl were added to the saponified anthocyanins, and the mixture was transferred to a screw-cap test tube. After flushing with nitrogen gas, the mixture was incubated in boiling water (97-99°C) for an h and then placed on ice. A C_{18} mini-column was again used for the anthocyanidin purification.

Analysis of anthocyanidins by high-performance liquid chromatography - photodiode array: The anthocyanidins separation was performed following the method of Junka *et al*. (2007). The identification of anthocyanidins from the purified acid-hydrolysate was carried out using a LC-20AT Prominence liquid chromatograph (Shimadzu, Japan) equipped with a SPD-M20A diode array detector (PAD) and an Inertsil ODS-3 column (5 m 4.6 x 250 mm, S/N 5FI86218), controlled by the LC Solution program (Shimadzu, Japan). Each anthocyanin residue was eluted using gradient solutions of solvent A (4% phosphoric acid) and solvent B (100% acetonitrile) under 3000 psi at a 0.7 mL/min flow rate.

Analysis of anthocyanins by liquid chromatography-mass spectrometry: For individual anthocyanins, the purified anthocyanin method was modified from the method of Rodriguez-Saona and Wrolstad (2005). Frozen samples were finely homogenized with acidified methanol (0.01% HCl) and then filtered through Whatman paper No. 1 until colorless. The sample was transferred to a separatory funnel, and two volumes of chloroform were added to the sample and gently mixed a few times. The samples were kept overnight at 4°C until a clear partition between the two phases appeared. The upper phases were transferred to a boiling flask; the methanol was removed via rotary

Fig. 1: Visual appearance of the floral parts of the pale-mauve (a) and white (b) *Vanda* hybrids at three developmental stages, showing the petals (P), dorsal sepal (DS), lateral sepals (LS) and lip (L)

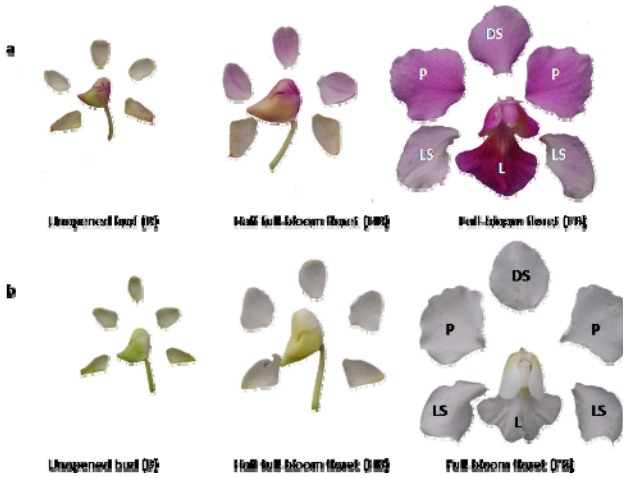
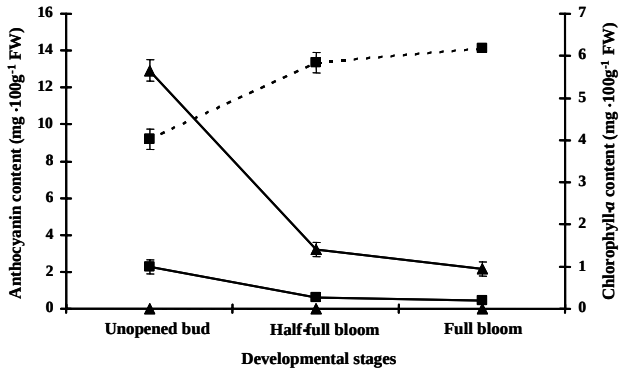


Fig. 2: Accumulation of chlorophylls (bold lines) and anthocyanins (dashed lines) during floret development of pale-mauve (■) and white (▲) *Vanda* hybrids. Vertical bars indicate standard deviation of means (n=6)



evaporation at 40°C and the sample was adjusted to a known volume with acidic deionised water (0.01% HCl). The extracted anthocyanins were flowed through a C_{18} mini-column (Waters Sep-Pak®) after which, the absorbed anthocyanin samples were washed twice with acidic distilled water (0.01% HCl) and then with ethyl acetate. The purified anthocyanins were eluted by acidic methanol (0.01% HCl). The remaining methanol was removed by flushing with nitrogen gas until nearly dried. The anthocyanin compounds were separated and detected using a Bruker Daltonics Esquire 3000 plus ion trap mass spectrometer (Bruker Daltonics, Bremen, Germany) connected to an Agilent 1100 HPLC system (Agilent Technologies) equipped with a binary pump and a variable wavelength detector. The components were separated with a Hypersil Gold C_{18} column (150 mm in length x 4.6 mm in

Fig. 3: Scanning spectra from extracts of the pale-mauve (bold lines) and white (short dashed lines) *Vanda* hybrids at the three floral developmental stages of unopened floret (a), half full-bloom floret (b), and full-bloom floret (c). The black arrows (↑) indicate anthocyanin maximum absorption; chlorophyll peaks are indicated by the dashed arrows (↑)

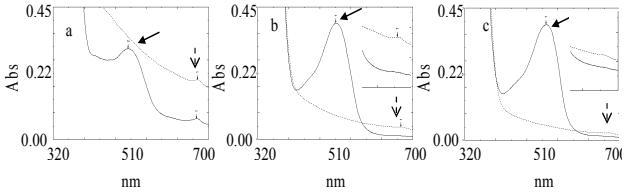
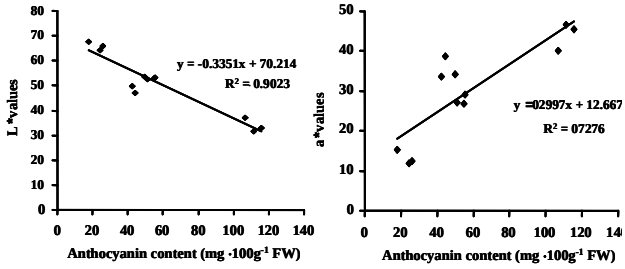


Fig. 4: Correlation of anthocyanin content and CIE L^* (a) and a^* values (b) of the floral parts of the pale-mauve *Vanda* hybrid



i.d., particle size of 3 µm). The LC-ESI-MSn operation and analysis were performed by the Central Instrument Facility, Faculty of Science, Mahidol University, Bangkok.

Total RNA extraction: Total RNA was extracted from florets of both *Vanda* hybrids using the $LiCl_2$ method described by Smith *et al.* (1986). RNA concentration was measured using a UV-visible spectrophotometer (Shimadzu model 1601, Japan) controlled by the UV-Probe program (Shimadzu, Japan). The concentration of RNA was calculated using the absorbance at 260 nm by the following formula:

$$\text{RNA concentration } (\mu g/\mu L) = A_{260} \text{ x dilution factor x } 40.$$

Reverse transcriptase polymerase chain reaction (RT-PCR): The first strand cDNA and second strand DNA synthesis was performed using the Revet Aid™ H Minus First Strand cDNA Synthesis kit (Fermentas). The cDNAs of *PAL*, *CHS*, *F3H*, *DFR* and the *18S rRNA* of the *Vanda* hybrids, designated as *Va-PAL1*, *Va-CHS1*, *Va-F3H1*, *Va-DFR1* and *Va-18S rRNA*, respectively were used as templates for semiquantitative RT-PCR using the following primer sets: 5′ACTACCAACTCACTCGCCTCCT′3 and 5′GAAGGCCTCAAGGAGACATTTT′3 for *Va-PAL1*; 5′ACTACCAACTCACTCGCCTCCT′3 and 5′GAAGGCCTCAAGGAGACATTTT′3 for *Va-CHS1*; 5′GCGCGTTGTTGTAGAGCAGTA′3 and 5′TCACACAAGCACACTAATCGTTC′3 for *Va-F3H1*; 5′CCCATGAATTTTCAATCCGAAG′3 and

5′CGAGTAATGAGCTTCATTTCCTG TA′3 for *Va-DFR1*; and 5′AAGCATTTGCCAAGGATGTT′3 and 5′ACCACCACCCATAGAATCAAGA′3 for *Va-18S rRNA* (as the internal reference). These specific primers have been used to clone the genes from the pale-mauve hybrid, and the genes have been previously sequenced and checked for the corresponding genetic alignment (Junka *et al*., 2011).

The PTC-200 Peltier Thermal Cycler (MJ Research, USA) used for the reactions was set to the following PCR program: a hot start at 94°C for 3 min; 35 cycles of 94°C for 0.3 min; primer annealing of *Va-PAL1*, *Va-CHS1*, *Va-F3H1*, *Va-DFR1*, and *Va-18s rRNA* primer at 58, 56, 58, 55, and 54°C, respectively then 72°C for 0.45 min and a final step of 72°C for 7 min.

Statistical analysis: Measurements of color changes, anthocyanin and chlorophyll *a* contents of the orchid florets were managed in a completely randomized design (CRD). All treatments were subjected to an analysis of variance (ANOVA) with 10 replicates. The treatment means were separated using the least significant difference (LSD) method at the differences of $P \leq 0.01$. The data are presented as means ± standard deviation (SD).

RESULTS AND DISCUSSION

Physical characteristics of orchid florets: An inflorescence of the pale-mauve *Vanda* hybrid contained approximately 30-35 florets, whereas there were 25-30 florets per inflorescence for the white *Vanda* hybrid. The commercial requirement of a *Vanda* inflorescence for cut flowers in orchid markets features the following stages: 20% unopened florets (B), 20% half full-bloom florets (HB), and 60% full-bloom florets (FB). The floral weight of developing florets of both *Vanda* hybrids increased from the B to FB which increased 2.6-fold from the B to HB stage and 3.7-fold from the B to FB stage in the pale-mauve *Vanda* whereas it was 2.42-fold from the B to HB stage and 3.28-fold from the B to FB stage in the white *Vanda* hybrid (Table I).

A *Vanda* floret is composed of 5 parts: 2 petals, 1 dorsal sepal, 2 lateral sepals and 1 lip (Fig. 1). The petals and dorsal sepal of the pale-mauve *Vanda* hybrid expressed a light violet color. The lateral sepals exhibited the highest level of the lightness (65.92) and the least *a** values (13.27), whereas the lip part, which contributed the red color, provided the highest *a** (44.15) and the lowest *L** (33.85). The dorsal sepal, the petals and the lip of the white *Vanda* hybrid presented higher *L** values than the lateral sepals, whereas all of the parts of the white *Vanda* hybrid florets displayed a pure white color, showing low *a** values (Table II).

Distribution of pigmentation during floret development: The spectrum of the extracts from the florets showed a maximum absorption for chlorophylls at 671 nm. Chlorophylls accumulate to high levels at the early stage and reduce at the later stages of floret development. It was found that the white hybrid contained higher chlorophyll *a* content in the florets, as compared to the pale-mauve hybrid at the same floral stage. The chlorophyll *a* content in the white hybrid was the highest at the B stage (5.65 mg/100 g FW) and highly reduced at the HB (1.4 mg/100 g FW) and FB stages (0.95 mg/100 g FW), whereas the contents were 2.26 mg/100 g FW, 0.63 mg/100 g FW and 0.45 mg/100 g FW in the pale mauve florets, respectively (Fig. 2). In contrast, the anthocyanin contents of the B, HB and FB florets of the pale-mauve hybrid progressively increased from 9.19, 13.35 to 14.09 mg/100 g FW, respectively. These

Fig. 5: HPLC chromatograms of the absorbance at 515 nm of acid-hydrolysed anthocyanins from a: grape skin containing delphinidin, cyanidin, petunidin, peonidin and malvidin (Durst & Wrolstad, 2005), b: strawberry flesh containing cyanidin and peonidin and malvidin (Durst & Wrolstad, 2005), and c: florets of the pale-mauve *Vanda* hybrid

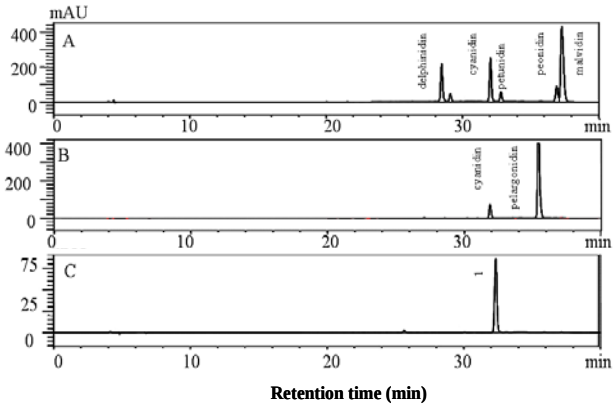


Fig. 6: Spatial expressions of anthocyanin biosynthetic genes (a = *Va-PAL1*, phenylalanine ammonia lyase; b = *Va-CHS1*, chalcone synthase; c = *Va-F3H1*, flavanone-3-hydroxylase; d = *Va-DFR1*, dihydroflavonol 4-reductase; e = *Va-18S rRNA*) of the pale-mauve *Vanda* hybrid (*V. teres* x *V. hookeriana*) roots (R), leaves (L), stems (S), unopened buds (B), half full-bloom florets (HB) and full-bloom florets (FB) using semiquantitative RT-PCR (at 35 cycles)

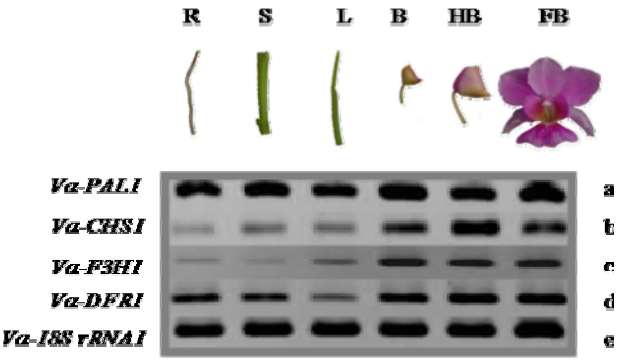
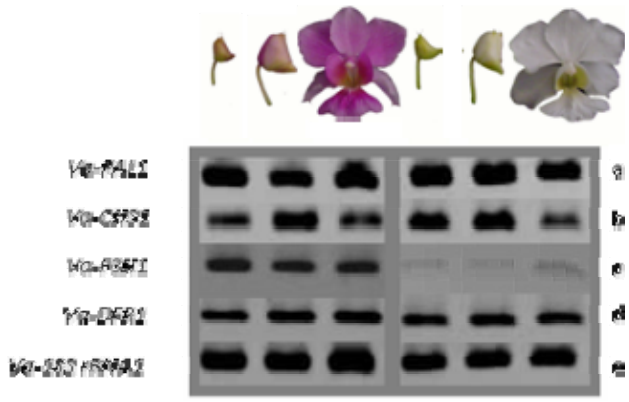


Fig. 7: Expression of anthocyanin biosynthetic genes (a= *Va-PAL1*, phenylalanine ammonia lyase; b = *Va-CHS1*, chalcone synthase; c = *Va-F3H1*, flavanone-3-hydroxylase; d = *Va-DFR1*, dihydroflavonol 4-reductase; e = *Va-18S rRNA*) of the pale-mauve (left-box) and white (right-box) *Vanda* hybrids in unopened florets (B), half full-bloom florets (HB), and full-bloom florets (FB) using semiquantitative RT-PCR (at 35 cycles)



results are similar to those that have been reported for *Campanula isophylla* Moretti during bud and floret development, where the anthocyanin and flavone contents were very low in the buds until the anthesis stage, after which, they increased rapidly (Justesen *et al*., 1997). The maximum absorption spectra of the extracted anthocyanins were 503, 512 and 519 nm in the florets at the B, HB and FB stages, respectively (Fig. 3a & c). The changes in the anthocyanin maximum wavelength indicated that the anthocyanin molecules could be modified by side-chain conjugation during floral development. Regarding pigment distribution during floret development, Stickland (1971) has reported that the anthocyanin concentration in purple *Fandango* florets increased to a maximum in the half-opened floret stage and then decreased sharply, whereas carotenoid and chlorophyll concentrations declined continuously from the bud stage of the florets in ornamental chrysanthemums. The color changes of the pale-mauve *Vanda* petal from the B to FB stage were closely related to decreasing L^* values and increasing a^* values. As a result, the floral-color values of the pale-mauve hybrid demonstrated a strong relationship with the accumulation of anthocyanin. Increase in anthocyanins was related to decreasing L^* values (R^2=0.9023) (Fig. 4a), whereas they were directly related to the increasing a^* values attributed to the red color (R^2=0.7276) (Fig. 4b). Furthermore, anthocyanins were not detected in any of developing florets of the white hybrid (Figs. 2 & 3). This would imply that even though the gene may be weakly expressed, the function of the enzyme is absent. For instance, it has been reported that the white flowers of *Gentiana triflora* cv. Homoi did not accumulate anthocyanin yet showed weak *ANS* transcripts in the petals (Nakatsuka *et al*., 2005), whereas white morning glory (*Ipomoea purpurea*) flower resulted due mainly to a lack of *CHS* expression (Durbin *et al*., 2000).

Identification of anthocyanins: The HPLC chromatograms of acid-hydrolysed anthocyanins from the pale-mauve hybrid florets in visible spectral region of 515 nm were compared to the known references from grape (Fig. 5a) and strawberry (Fig. 5b). The floral anthocyanins from the pale-mauve hybrid were only derived from a cyanidin backbone because there was only one acid-hydrolysed anthocyanin peak released at a retention time (RT) of 31.83 (Fig. 5c), as compared to a cyanidin peak from the grape (Fig. 5a, peak 2) and from the strawberry (Fig. 5b, peak 1) at RTs of 32.06 and 31.90, respectively. A further analysis of the anthocyanin components in the florets of the pale-mauve hybrid was performed using liquid chromatography mass spectrometry (LC-ESI-MSn). The ion mass spectrum in Table III shows that the orchid florets contained 5 types of anthocyanins (peaks 1-5) and confirmed that all 5 of the anthocyanins in the pale-mauve hybrid florets were derived from cyanidin ($[M]^+$ of 287). The total molecular ions $[M]^+$ in the 5 peaks were observed at 1065.5, 817, 1185.5, 1242, and 1272 m/z (Table III). The anthocyanin at peak 1 (RT: 40.9) was composed of a cyanidin base conjugated with three molecules of hexose, a molecule of sinapic acid and a molecule of malonic acid, whereas at peak 2 (RT: 44.4), the molecule was composed of a cyanidin base linked with two molecules of hexose and a molecule of sinapic acid, and at peak 3 (RT: 45.3), the molecule was composed of a cyanidin base joined with three molecules of hexose and two moieties of sinapic acid. The anthocyanin at peak 4 (RT: 47.3) was composed of a cyanidin base conjugated with three molecules of hexose, a molecule of ferrulic acid and two molecules of malonic acid, whereas at peak 5 (RT: 47.5), the molecule was composed of a cyanidin base with three molecules of hexose, a molecule of sinapic acid and two molecules of malonic acid. Lowery and Keong (1973) were the first to report that *Dendrobium cornutum* and *D. crocatum* accumulated cyanidin -3- glucosides. Furthermore, Saito *et al*. (1994) and Williams *et al*. (2002) have found acylated cyanidin and peonidin glycosides in the flowers of *Dendrobium*. A flower color often related to types of anthocyanidin, such as polyacylated delphinidin and cyanidin, has also been found in the violet-blue and red-purple colors of *Vanda* (Tatsuzawa *et al*., 2004). In addition, *Aerides multiflora* and *A. rosea* orchids, which are the same subgroup with the pale-mauve hybrid (*V. teres* x *V. hookeriana*) have violet-pink florets, showing an accumulation of cyanidin derivatives (Junka *et al*., 2007).

Semi-quantitative expression of the anthocyanin genes: The spatial expression of anthocyanin genes in the vegetative organs and florets of the pale-mauve hybrid showed a sharp increase in the expression of *Va-PAL1* in all of the plant organs, including the roots, stems, leaves, floral buds and half full-bloom and full-bloom florets (Fig. 6a). From a metabolic perspective, *Va-PAL1* expression is

ubiquitous throughout the plant because it is involved in many secondary metabolic pathways in the plant. PAL, the first enzyme of the phenylpropanoid pathway, catalyses the first step for the synthesis of various phenylalanine derivatives and affects the productions of lignin, flavonoids, chlorogenic acid, salicylic acid and catecholamines (Aksamit-Stachurska *et al*., 2008). The *PAL* expression observed in this study was similar to what has been reported for many plant species (Dixon *et al*., 2002), such as carnation (Mato *et al*., 2000) and grape (Boss *et al*., 1996). The *Va-CHS1* gene was distinctively expressed in the floral parts, including the B, HB and FB stages, but weakly expressed in the vegetative organs, such as the roots, stems and leaves (Fig. 6b). Similarly, the *Va-F3H1* gene was not constitutively expressed in all of the vegetative organs, but appeared to be markedly induced in the florets, beginning in the young buds and extending through the full-bloom flower stage (Fig. 6c). The higher expression of this gene in the reproductive parts is consistent with other plants cultivated for their flowers, such as *Antirrhinum majus*, *Dahlia variabilis*, *Dianthus caryophyllus*, and *Petunia hybrida*, in which it has been reported that *F3H* expression was more plentiful in the flowers than in other parts of the plant (Britsch & Grisebach, 1986). The *Va-DFR1* gene was ubiquitously expressed in all parts of the hybrid plants (Fig. 6d); however, the expression was high in the florets but slightly lower in the leaves. The gene expressions of *DFR* and anthocyanin pigmentation have been correlated in the flower petals of five *Gerbera hybrida* varieties with variegated anthocyanin pigmentation (Helariutta *et al*., 1995). Even though there are multiple *DFR* genes present in *G. hybrida*, only *DFR1* has been shown to be catalytically active and expressed in the flowers (Helariutta *et al*., 1993). The red color appearing in the different organs of the pale-mauve hybrid was correlated to anthocyanin accumulation and the expression of the *Va-CHS1* and *Va-F3H1* genes. The florets appearing pale-mauve in color exhibited expression of both of the genes, whereas the expression patterns were dramatically reduced in the roots, stems and leaves, which contain no anthocyanins.

In our comparative study of gene expression during floral development between the *Vanda* hybrids, the mRNA of *Va-PAL1* was strongly expressed in all of the three floral stages (B, HB & FB) (Fig. 7a), whereas *Va-CHS1* was weakly expressed in the FB stages of both *Vanda* hybrids (Fig. 7b). The *Va-F3H1* gene was infrequently expressed in the white hybrid, whereas it was weakly expressed in the pale-mauve hybrid during the developmental stages of the florets (Fig. 7c). Because of its floral specificity in the pale-mauve hybrid, *Va-F3H1* could play a role in controlling anthocyanin production in these *Vanda* hybrids. The anthocyanin expression in the white hybrid may be similar to the expression phenomenon in the 'White Mind' carnation flower, where the expression of *F3H* was blocked at the transcriptional level: by RT-PCR, *F3H* transcription in "White Mind" was reduced in the petals at stage 4 (5-mm long petal) (Mato *et al*., 2000). However, other plants bearing white flowers, such as *G. triflora* cv. Homoi (Nakatsuka *et al*., 2005) and *Antirrhinum majus* mutant (Spribille & Forkmann, 1982) have been reported to lack expression of *ANS* and *CHS*, respectively. The expression of *Va-DFR1* was ubiquitous in the florets of both *Vanda* hybrids; however, the expression was higher in the pale-mauve hybrid (Fig. 7d). Indeed, the expression of *DFR* has been highly correlated to anthocyanin pigmentation in the petals of five *G. hybrida* varieties (Helariutta *et al*., 1995).

CONCLUSION

Chlorophylls accumulated to high levels at the early stage and were reduced in the late stages of floral development of both *Vanda* hybrids included in this study. Anthocyanins increasingly accumulated in the developing florets of the pale-mauve hybrid; in contrast, anthocyanins were not detected in any of the developing stages of the white hybrid. There were 5 types of cyanidin derivatives conjugated with hexose sugars and some organic acids in the pale-mauve hybrid florets, in which the anthocyanin content showed a strong correlation with the L^* values. Expression of anthocyanin biosynthetic genes between the two hybrids indicated that the *Va-PAL1*, *Va-CHS1* and *Va-DFR1* genes were highly expressed in the three developmental stages of the florets, whereas the expression of *Va-F3H1* was infrequent in the florets of the white hybrid. Therefore, we conclude that *Va-F3H1* could play an important role in controlling anthocyanin production in these *Vanda* hybrids. Further work on understanding the control of color development would be beneficial for commercial breeding programs of *Vanda* spp.

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