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Differential expression of *MYB* gene (*OgMYB1*) determines color patterning in floral tissue of *Oncidium* Gower Ramsey

Chung-Yi Chiou · Kai-Wun Yeh

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Abstract The yellow coloration pattern in *Oncidium* floral lip associated with red sepal and petal tissues is an ideal model to study coordinate regulation of anthocyanin synthesis. In this study, chromatography analysis revealed that the red coloration in floral tissues was composed of malvidin-3-*O*-galactoside, peonidin-3-*O*-glucoside, delphinidin-3-*O*-glucoside and cyanidin-3-*O*-glucoside compounds. By contrary, these pigments were not detected in yellow lip tissue. Four key genes involved in anthocyanin biosynthetic pathway, i.e. *chalcone synthase* (*OgCHS*), *chalcone isomerase* (*OgCHI*), *dihydroflavonol 4-reductase* (*OgDFR*) *and anthocyanidin synthase* (*OgANS*) were isolated and their expression patterns were characterized. Northern blot analysis confirmed that although they are active during floral development, *OgCHI* and *OgDFR* genes are specifically down-regulated in yellow lip tissue. Bombardment with *OgCHI* and *OgDFR* genes into lip tissue driven by a flower-specific promoter, Pchrc (*chr*omoplast-specific *c*arotenoid-associated gene), demonstrated that transient expression of these two genes resulted in anthocyanin production in yellow lip. Further analysis of a R2R3 MYB transcription factor, *OgMYB1*, revealed that although it is actively expressed during floral development, it is not expressed in yellow lip tissue. Transient expression of *OgMYB1* in lip tissues by bombardment can also induce formation of red pigments through the activation of *OgCHI* and *OgDFR* transcription. These results demonstrate that differential expression of *OgMYB1* is critical to determine the color pattern of floral organ in *Oncidium* Gower Ramsey.

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Introduction

Flower pigments, composed of carotenoids, anthocyanins and betalains, are responsible for the natural attractive display of plant colors. These three groups of pigments play important ecological function, such as to attract animal pollinators (Schaefer et al. 2004). Of the three pigments, anthocyanins have the broadest distribution in the flowering plants and their biosynthetic pathway has been well characterized in several plant species (Grotewold 2006). Anthocyanins are derived from the phenylpropanoid pathway. Chalcone synthase (CHS) is the first key enzyme to produce a tetrahydroxychalcone, which acts as the precursor for all classes of flavonoids. A subsequent enzymatic reaction from chalcone to naringenin is catalyzed by chalcone isomerase (CHI), which is further converted to dihydrokaempferol by flavanone 3-hydroxylase (F3H). Finally, three classes of anthocyanidin end products are completed by consecutive enzymatic activities, including flavonoid 3′-hydroxylase (F3′H), flavonoid 3′,5′-hydroxylase (F3′5′H), and dihydroflavonol 4-reductase (DFR). Anthocyanidin synthase (ANS) catalyzes the reaction from the colorless leucoanthocyanidin to the colored anthocyanidin. Almost all anthocyanidins undergo several modifications, such as glycosylation or methylation, by UDP-glucoside:flavonoid 3-*O*-glucosyltransferase (3GT) and anthocyanin methyltransferase (AMT) (Fig. 1a). These water-soluble pigments are eventually accumulated in the vacuoles of epidermal cell and are responsible for color appearance. Delphinidin derivatives usually make the flower color

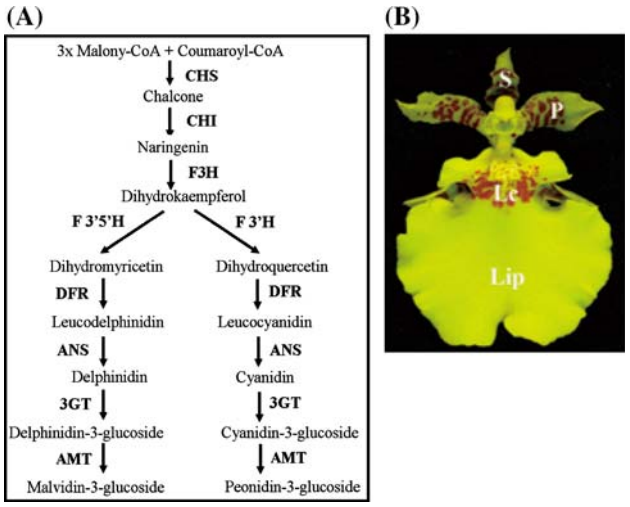

Fig. 1 Flower phenotype of *Oncidium* Gower Ramsey and related anthocyanin biosynthetic pathway. (**a**) Schematic representation of anthocyanin biosynthetic pathway. Names of enzymes are abbreviated in bold capital letters as follows: CHS, chalcone synthase; CHI, chalcone flavanone isomerase; F3H, flavanone 3-hydroxylase; F3′H, flavonoid 3′-hydroxylase; F3′5′H, flavonoid 3′,5′-hydroxylase; DFR, dihydroflavonol 4-redutase; ANS, anthocyanidin synthase; 3GT, UDP-glucoside:flavonoid 3-*O*-glucosyltransferase; AMT, anthocyanin methyltransferase. (**b**) The different positions of flowers are given in white capital letters: S, sepal; P, petal; Lc, Lip crest; Lip, labellum

look purple or dark purple, whereas cyanidin derivatives make the flower present red color.

So far, many studies reported that expression of anthocyanin biosynthetic genes is coordinately controlled by two families of transcription regulators, bHLH and MYB proteins (Holton and Cornish 1995; Koes et al. 2005). bHLH proteins may have overlapping regulatory targets (Zhang et al. 2003; Zimmermann et al. 2004), but MYB proteins are the key components to activate discrete subsets of anthocyanin structural genes (Davies and Schwinn 2003). However, the regulatory function and mechanism of MYB proteins are not fully understood and its related studies in different plant species are inconsistent and variable. Arabidopsis AtPAP1, a MYB protein, up-regulates a number of genes in the anthocyanin biosynthesis pathway from *phenylalanine ammonia-lyase* (PAL) to CHS and DFR (Borevitz et al. 2000; Tohge et al. 2005). By contrary, *MybA* in grape has limited ability to activate genes in the very late steps of anthocyanin biosynthetic pathway, including *3GT* genes (Kobayashi et al. 2002). Interestingly, *FaMYB1* in strawberry activates anthocyanin and flavonol biosynthetic pathways, but it represses these pathways in transgenic tobacco (Aharoni et al. 2001).

Oncidium Gower Ramsey is one of the top-graded cut flowers in orchid industry. Its unique flower shape and predominant yellow color with mosaic red pigmentation make the hybrid flower highly prized in flower market. This orchid is well known as a bi-color mixture of carotenoids and anthocyanins, which are localized in sepal, petal and lip crest tissues (Fig. 1b). The predominant yellow coloration has been characterized, which is comprised of an equal mixture of all-*trans* and 9-*cis* isomers of violaxanthin, with esterification specific to the 9-*cis* isomer (Hieber et al. 2006). Although the red pigments in plant are ascribed to the coexistence of both carotenoids and anthocyanins (Thammasiri et al. 1986; Matsui and Nakamura 1988; Matsui 1994), the property of anthocyanin components in floral tissues of *Oncidium* is still poorly defined. The purpose of this work is to identify the anthocyanin compounds, the expression profiles and coordinate regulation pattern of the relevant biosynthetic genes in the floral tissues of *Oncidium*. Based on transient expression of *OgMYB1*, we demonstrated that red pigments could appear in yellow lip tissue by activating the expression of the silenced *OgCHI* and *OgDFR*. These findings revealed that the color pattern of *Oncidium* floral tissue is determined by differential expression of *OgMYB1*.

Materials and methods

Plant materials

Oncidium Gower Ramsey and *Oncidium* Sharry Baby were obtained from Orchid Nursery Co. Taoyuan, Taiwan. The plant seedlings were grown in green house at a temperature range of 20–28°C. The growing floral organs at different development stages were harvested for RNA extraction and particle bombardment assay.

Anthocyanin analysis

The extraction of anthocyanin pigments was carried out following the method (Goodman et al. 2004). Briefly, *Oncidium* floral tissues were extracted by grinding in appropriate solvent of 0.1 N HCl in methanol. The ground tissues were immediately centrifuged to separated debris for twice. The supernatant was removed, and diluted with 5% acetic acid in ratio ranging from 1:1 to 20:1 depending on the pigment concentration. The final solution was applied to HPLC analysis immediately. HPLC analysis was performed by using a Dionex GP40 gradient pump (Dionex, Sunnyvale, CA) and a Microsorb 100-5 C18 column (Varian, Palo Alto, CA). Pigment separation was carried out by gradient elution with a flow rate of 0.75 ml/min. Solvent A, 5% acetic acid; solvent B, acetonitrile, 1 min at 90% A, 10% B; from 90% A, 10% B to 55% A, 45% B in 17.5 min; to 100% B in 2.5 min, at 100% B for 1 min; to 90% A, 10% B in 3 min; at 90% A, 10% B for 3 min. Absorbance was detected at OD_{520} using a model Dionex

AD20 detector. Data were collected and analyzed by PEAKNET software (Dionex, Sunnyvale, CA). Anthocyanin compounds, such as malvidin-3-*O*-galactoside, peonidin-3-*O*-glucoside, delphinidin-3-*O*-glucoside and cyanidin-3-*O*-glucoside, were purchased from Fluka and monitored by HPLC as standard check.

Cloning of *Oncidium* CHS, CHI, DFR, ANS, *OgMYB1* and *OgMYB33* cDNA

Total RNA was isolated from *Oncidium* by phenol/chloroform extraction and LiCl precipitation method (Chang et al. 1993). Poly(A)$^+$ RNA was purified from total RNA using Oligotex mRNA Kit (Qiagen). Construction of the subtractive EST library from flower bud of *Oncidium* Gower Ramsey was performed as described previously (Tan et al. 2005). The 5′- and 3′-Rapid Amplification of cDNA Ends (5′- and 3′-RACE) on candidate genes was performed (Clontech) according to the manufacturer' instruction. The primer sequences for the amplification of full-length cDNA were as follows:

OgCHS, 5′-ATACCCGGGTGTGTGTTGTTTGGGTAGTGAG-3′, 5′-TATCCCGGGCCATAACATAGCATTACCCACT-3′;
OgCHI, 5′-CAATTAATCATATAGTACTGG-3′, 5′-GACCAGTCTCACCGTACCTC-3′;
OgDFR, 5′-CTCATTGCTCATTCATTGTTCA-3′, 5′-TGGAAAGTGGAGGTGAGGAT-3′;
OgANS, 5′-CAGGAGGAGAAGGATAAGA-3′, 5′-CAGGAGGAGAAGGATAAGA-3′.

The degenerated primers for the amplification of partial *MYB* were designed from conserved R2R3 domain among different plant MYB-related anthocyanin regulators. The degenerated primer sequences were: *parOgMYB1*, 5′-MGNTGYVGNAARWSNTGYMGNYTNMGNTGG-3′, 5′-WRRTKNDTVWTCCARTARTTYTTNAYNTC-3′. The partial MYB cDNA synthesis was performed by One-Step RNA PCR Kit (TaKaRa) according to the manufacturer's instruction. PCR condition was as follow: 50°C/30 min, following 25 cycles with 94°C/30 s, 50°C/30 s, 72°C/1 min. The amplified fragments were subcloned into the pGEM-T Easy vector (Promega) and sequenced. The subcloned fragments of related MYB R2R3 domain were labeled with ^{32}P-dCTP by Random Primer kit (Amersham), and used as probe. The cDNA was synthesized with SMART cDNA library Construction kit (Clontech), according to the supplier's instructions. The ligated DNA was packaged with lambda packaging kit (EPICENTRE), and the library was amplified in the *Escherichia coli* XL-1-Blue before screening. Approximately 5.8×10^5 plaques were transferred onto Hybond-N$^+$ nylon membrane (Millipore), UV cross-linked, and screened by hybridising with the radiolabeled probe described above. Plaques that gave positive signal were purified by two rounds of re-planting and screening. Phagemids were excised from the phages following the manufacturer's instructions (Clontech). The primer sequences for amplification of full-length *OgMYB1* and *OgMYB33* were as follows:

OgMYB1, 5′-GAGAAAAGAAGAAGAAGAAGAG-3′, 5′-TTGCAATGCTATCATATATACTA-3′;
OgMYB33, 5′-TACACTAGACCTTCAGAAGAA-3′, 5′-TGCTGCTCCAGTAATTCTTATT-3′.

The nucleotide sequences reported in this paper have been submitted to GenBank under accession numbers as the followings: *OgCHS*, EF570111; *OgCHI*, EF570112; *OgDFR*, EF570113; *OgANS*, EF570114; *OgMYB1*, EF570115; *OgMYB33*, EF570116.

RNA extraction, Northern blot analysis and RT-PCR

Total RNA was isolated from *Oncidium* by phenol/chloroform extraction and LiCl precipitation method (Chang et al. 1993). About 10 μg RNA was resolved on 1% denatured/formaldehyde agarose gel, transferred onto a Immobilon-N$^+$ membrane (Millipore), and UV cross-linked. The membrane was hybridized (Amersham) at 65°C with α-^{32}P-dCTP -labeling probe. Following hybridization, membranes were washed twice at room temperature in 2 × SSC containing 0.1% SDS for 15 min and once in 0.1 × SSC at 60°C for 10 min. RT-PCR reaction were performed with One-Step RNA PCR Kit (TaKaRa) following the manufacturer's instruction. Primer sequences for PCR were as follows:

rtOgCHI, 5′-AGATCAATAGAACCAGGCCACTGGCT-3′, 5′-CTGTAACCTTCTCAAGTGCTGCTTCT-3′;
rtOgDFR, 5′-TAATGAAAGCTTCGATGACGTGACACGTGG-3′, 5′-TGCCATTTGCTTCGGGATGCTCAAAA-3′;
rtOgMYB1, 5′-CTGCAGATGGAATTGATCAGATAGAAC-3′, 5′-CCCCTTTTTTTTCCTTACATTTCAAA-3′.

PCR conditions were as the follows: 50°C/30 min, 94°C/30 s, 61°C/30 s, 72°C/30 s (30 cycles) for *OgCHI*; 50°C/30 min, 94°C/30 s, 67°C/30 s, 72°C/30 s (30 cycles) for *OgDFR*, and 50°C/30 min, 94°C/30 s, 48°C/30 s, 72°C/30 s (20 cycles) for *OgMYB1*.

Constructs for transient expression using particle bombardment

OgCHI, *OgDFR* and *OgMYB1* gene driven individually by a flower-specific promoter, Pchrc (*chr*omoplast-specific

*c*arotenoid-associated gene), in pCAMBIA1390 was amplified by PCR and subcloned into pGEM-T Easy vector respectively at the compatible *Sph*I restriction site. The recombinant plasmid DNA was transferred and multiplied in *E. coli* XL1-Blue. The recombinant plasmid DNA was extracted, and purified for the preparation of bombardment assay.

The floral tissues of yellow lip were freshly detached from *Oncidium* plants. Bombardment assay was conducted using the instrument of Helium Biolistic particle Delivery System (Model PDS-100, Bio-RAD). Plasmid DNA (1 μg) was precipitated with 0.6 mg gold particles (1.0 μm diameter) through the addition of 10 μl of 2.5 M $CaCl_2$ and 4 μl of 100 mM spermidine. After precipitation, the particles were washed twice with absolute ethanol, and resuspended in 20 μl absolute ethanol. Then, the particles were pipetted onto a microcarries of the Biolistics Device. For bombardment, lip tissue was placed on 0.5× MS medium with 0.75% agar in petri dish, and was bombarded at a distance of 9 cm from the stopping plate using 1,350 psi (1 psi = 6.89 kPa) rupture disks. Bombarded tissues were subsequently incubated on MS medium at 22°C under a 16-h-light/8-h-dark photoperiod condition. After 2 days incubation, tissues were observed for anthocyanin production under a dissecting microscope (Nikon). Pigment analysis and RNA transcript detection were carried out from the anthocyanin-containing red spot tissues.

Phylogenetic analysis

The phylogenetic tree was based on the alignment of the 120 amino acid in the R2R3 domain using the CLUSTAL W alignment program and was then constructed using the neighbor-joining method with PAUP 4.0 (Swofford 2001). One thousand bootstrapped data sets were used to estimate the confidence of each tree clade. Sequences referred to in this article are obtained from GeneBank at the National Center for Biotechnology Information under the following accession numbers: apple, MdMYB1 (DQ886414); gerbera hybrid, GMYB10 (CAD87010); tomato (*Lycopersicon esculentum*), LeANT1 (AAQ55181); petunia (*Petunia hybrida*), PhAN2 (AAF66727), and PhODO1 (AAV98200); capsicum (*Capsicum annuum*) CaA (CAE75745); Arabidopsis, AtPAP1 (ABB03879), AtMYB12 (ABB03913), AtWER (AAF18939), and AtGL1 (AAC97387); strawberry (*Fragaria* spp.), FaMYB1 (AAK84064); grapevine, VvMYBA1 (BAD18977), VvMYBA2 (AB097924), and VvMYB5a (AAS68190); maize (*Zea mays*), ZmC1 (AAA33482) and ZmPl (AAA19821); snapdragon (*Antirrhinum majus*), AmMIXTA(CAA55725), AmROSEA1 (ABB83826), and AmROSEA2 (ABB83827); carrot (*Daucus carota*), DcMYB1 (BAE54312); tobacco (*Nicotiana tabacum*), NtMYB2 (BAA88222); and c-MYB (X52125).

Results

HPLC analysis of anthocyanin in *Oncidium* floral tissues

The yellow pigment composition in *Oncidium* flower has been demonstrated as 9-*cis* violaxanthin (Hieber et al. 2006), one of carotenoid derivatives, but the anthocyanin profile is not clear. To elucidate the anthocyanin components, the extracts from sepal, lip crest, and lip were analyzed separately by HPLC. The results showed that the red portions of sepal and lip crest contain the mixture of peonidin-3-*O*-glucoside, delphinidin-3-*O*-glucoside and cyanidin-3-*O*-glucoside compounds (Fig. 2a, b). Interestingly, no anthocyanins were detected from lip tissue (Fig. 2c). The major peak represents cyanidin-3-*O*-glucoside that accounted for almost 56% of total area in sepal and lip crest. Other peaks revealed delphinidin-3-*O*-glucoside and peonidin-3-*O*-glucoside. These two peaks

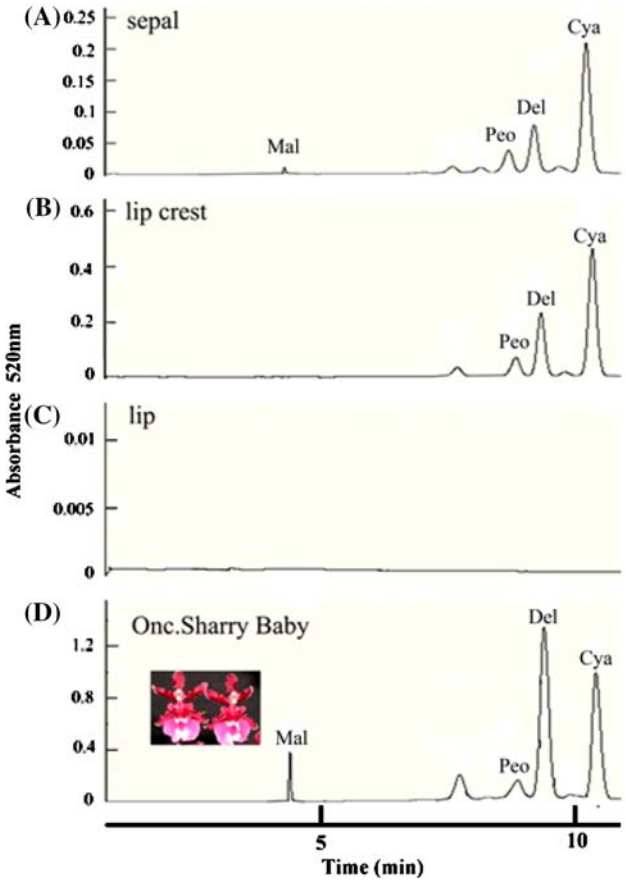

Fig. 2 HPLC profiles of anthocyanin analysis in *Oncidium* Gower Ramsey and *Oncidium* Sharry Baby. Anthocyanins were extracted and analyzed from sepal (**a**), lip crest (**b**) and lip (**c**) of Gower Ramsey and flower bud (**d**) of Sharry Baby. Peaks were identified with commercial standards and traced at 520 nm. Mal: malvidin-3-*O*-galactoside; Peo: peonidin-3-*O*-glucoside; Del: delphinidin-3-*O*-glucoside; Cya: cyanidin-3-*O*-glucoside

accounted for over 30% of total area in the above tissues. Anthocyanin extraction of purple flowers from another *Oncidium* cultivar, called Sharry Baby, was also analyzed by HPLC. Comparing the HPLC profiles of the extracts between cultivar Gower Ramsey and cultivar Sharry Baby, the major peak of the latter was changed to delphinidin-3-*O*-glucoside and the content of malvidin-3-*O*-galactoside increased (Fig. 2d). The total content of anthocyanin in the flowers of Sharry Baby was 53 times to that of the Gower Ramsey. These results explain why the flowers of Sharry baby have a stronger purple color than Gower Ramsey (Fig. 2d).

Cloning and expression patterns of the four anthocyanin biosynthesis genes, *chalcone synthase* (*OgCHS*), *chalcone isomerase* (*OgCHI*), *dihydroflavonol reductase* (*OgDFR*), and *anthocyanidin synthase* (*OgANS*)

To unravel the mechanism of pigmentation pattern in *Oncidium* floral tissue, the expression profiles of related anthocyanin biosynthetic genes were analyzed. We constructed a subtractive EST library of flower bud to clone the candidate genes of anthocyanin biosynthetic pathway, such as *chalcone synthase* (CHS), *chalcone isomerase* (CHI), *dihydroflavonol reductase* (DFR) and *anthocyanidin synthase* (ANS). After partial cDNA sequences of these genes were identified through blast search on NCBI website, 5′-RACE and 3′-RACE were employed to get full-length cDNAs. A 1,488 bp *OgCHS* encoding 394 deduced amino acid residues, a 835 bp *OgCHI* encoding 219 deduced amino acid residues, a 1,241 bp *OgDFR* encoding 282 deduced amino acid residues, and a 968 bp *OgANS* encoding 229 deduced amino acid residues were successfully amplified. The protein sequences of OgCHS, OgCHI, OgDFR, OgANS exhibited 78% and 75%, 62% and 57%, 67% and 55%, 61% and 51% identity to protein sequences of CHS, CHI, DFR, ANS from *Zea mays* and *Arabidopsis thaliana*, respectively.

Northern blot analysis was carried out to examine gene expression pattern in roots, leaves, flower buds at five developmental stages (S1–S5), floral lip tissues and the flower bud of Sharry baby. The results revealed that *OgCHS*, *OgCHI*, *OgDFR*, and O*gANS* were all actively expressed gradually from S1 to S4 and reached maximal expression in S5. On the contrary, both of *OgCHI* and *OgDFR* genes were not expressed in floral lip tissues, but *OgCHS* and *OgANS* remained actively expressed (Fig. 3). The correlation between the activation of *OgCHI* and *OgDFR* genes and HPLC profile in floral lip tissue suggested that anthocyanins could not be synthesized because *OgCHI* and *OgDFR* were not expressed. Interestingly, high

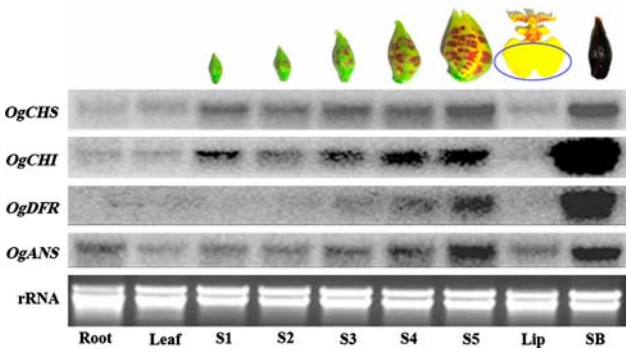

Fig. 3 Northern blot analysis of the four anthocyanin biosynthetic genes during flower development. Ten microgram of total RNA from root, leaf, S1 to S5 (different floral developmental stage), lip, and flower bud of Sharry Baby (SB) were loaded on each lane. In the lower panel, ethidium bromide-stained ribosomal RNA are shown as a control

expression of all the four genes, *OgCHS*, *OgCHI*, *OgDFR*, and O*gANS* in flower bud of Sharry Baby resulted in a high accumulation of anthocyanins based on HPLC data (Fig. 2).

Production of red pigmentation in yellow lip tissues by transient expression of both *OgCHI* and *OgDFR* genes

As Northern analysis shown in Fig. 3, anthocyanin deficiency in floral lip might be resulted from the expression deficiency of both *OgCHI* and *OgDFR* genes. We thought that recovery of these two genes could complete the anthocyanin pathway and produce anthocyanin compounds to display the same red pigment as lip crest tissue. Hence, *OgCHI* and *OgDFR* cDNA genes were together subcloned into a pGEM-T Easy vector backbone driven individually by the Pchrc promoter, which is a 1.7 kb 5′-upstream region of the gene encoding *chr*omoplast-specific *c*arotenoid-associated protein that is specifically expressed in flower (unpublished data). This construct was bombarded into yellow lip tissue. After incubation on a MS agar medium for 2 days, many red color spots were clearly observed in lip tissue (Fig. 4). To identify components in these spots, spots flesh were extracted in methanol solvent and analyzed by HPLC. Our analysis demonstrated that the red color spots contained the same anthocyanin compounds as sepal tissue did, such as peonidin-3-*O*-glucoside, delphinidin-3-*O*-glucoside and cyanidin-3-*O*-glucoside (Fig. 4). These results indicated that expression of *OgCHI* and *OgDFR* together could completely rescue the absence of anthocyanin synthesis in lip tissue. Whereas, the red color spots cannot be observed when lip tissue was bombarded with *OgCHI* or *OgDFR* alone (data not shown).

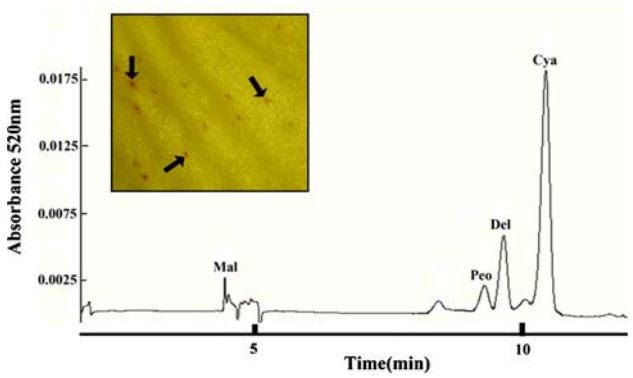

Fig. 4 Development of red spot in yellow lip due to transient expression of *OgCHI* and *OgDFR* genes. Digital image of lip tissue bombarded with *OgCHI* and *OgDFR* under the control of the Pchrc promoter. Black arrow indicated the red spots. HPLC profile of the anthocyanins from lip bombarded with *OgCHI* and *OgDFR* genes. Peaks identified are as the following: Mal: malvidin-3-*O*-galactoside; Peo: peonidin-3-*O*-glucoside; Del: delphinidin-3-*O*-glucoside; Cya: cyanidin-3-*O*-glucoside

Isolation and characterization of *MYB*-related genes from flower bud of *Oncidium* Gower Ramsey

So far, it has been demonstrated that transcription factors, such as MYB and bHLH, are required in anthocyanin biosynthesis to regulate the expression of structural genes. To test whether MYB proteins also regulate structural genes expression in our biological system, we designed degenerated primers corresponding to the most conserved regions of *MYB* genes regulating anthocyanin synthesis in different plant species. RT-PCR was performed to amplify a 214 bp cDNA fragment, which encodes the R2R3 domain (Fig. 5b). This cDNA fragment was then used as a probe to screen a cDNA library generated from the poly (A^+) RNA isolated from flower buds. With this approach, two *MYB* related candidate genes, named *OgMYB1* and *OgMYB33*, were isolated and characterized. The 937 bp full-length *OgMYB1* contains a coding region of 255 deduced amino acid residues, whereas the other 1,428 bp full-length *OgMYB33* encodes 301 deduced amino acid residues.

A phylogenetic analysis of the deduced amino acid sequences of *MYB*s related to anthocyanin regulation or other physiological processes indicated that *OgMYB1* placed within the group of anthocyanin regulators including *ZmPL*, *ZmC1*, *VvMYB5a*. By contrary, *OgMYB33* was not included in this group (Fig. 5a). This result suggests that *OgMYB33* may be involved in other physiological functions in *Oncidium* Gower Ramsey.

Alignment of the deduced amino acid sequences of *MYB*s related to anthocyanin regulation showed high sequence homology within R2R3 domain at the N-terminus (Fig. 5b). The motif [D/E]Lx_2[R/K]x_3Lx_6Lx_3R (from 76 to 95 amino acid) that interacts with R-like bHLH protein is located in the R3 domain (Zimmermann et al. 2004). Takos et al. (2006) indicated that the R2 domain of anthocyanin regulator has an Arg at position 39 and a conserved region in the motif 6 in dicot. By contrary in monocot, Arg is replaced by Gly and the motif 6 is absent. This suggests that the regulatory mechanism of the anthocyanin MYBs is distinct between dicot and monocot.

OgMYB1 expression analysis and regulation of anthocyanin-biosynthetic genes

Transcription level of *OgMYB1* was monitored by RT-PCR using gene specific primers designed to 3′ UTR. The results demonstrated that the expression of *OgMYB1* is highly expressed from S1 to S5, and in lip crest and Sharry baby. However, *OgMYB1* mRNA accumulation was absent in floral lip tissue (Fig. 6a). This suggests that *OgMYB1* expression is responsible for anthocyanin synthesis in floral tissues, and *OgCHI* and *OgDFR* genes may be specifically regulated by *OgMYB1*.

We therefore investigated whether activation of *OgMYB1* expression could induce anthocyanin biosynthesis in yellow lip tissue. In the further step, *OgMYB1* was bombarded into yellow lip tissue. As expected, transient expression of *OgMYB1* resulted in red pigment spots in yellow lip tissue. These results demonstrated that *OgMYB1* could regulate anthocyanin synthesis in lip tissues (Fig. 6b). HPLC analysis of the red pigment spots identified the same anthocyanin compounds as those found in petal and sepal tissues (data not shown).

To examine whether *OgCHI* and *OgDFR* expressions were regulated by *OgMYB1*, RNA samples from the red pigment spots of transformed tissue were extracted and *OgCHI* and *OgDFR* expression levels were identified by RT-PCR. It showed that *OgCHI* and *OgDFR* were found to be more actively expressed in the bombarded tissue comparing to the control tissue bombarded with empty vector only (Fig. 6c). These results suggest that the blockage of anthocyanin synthesis pathway and the absence of anthocyanin compounds in lip tissue are probably caused by the absence of transcriptional activation of *OgMYB1*.

Discussion

Absence of *OgCHI* and *OgDFR* expression causes disproportionate distribution of red pigments in floral tissues

The color pattern in floral tissues of *Oncidium* Gower Ramsey is characteristically yellow pigmentation in lip and

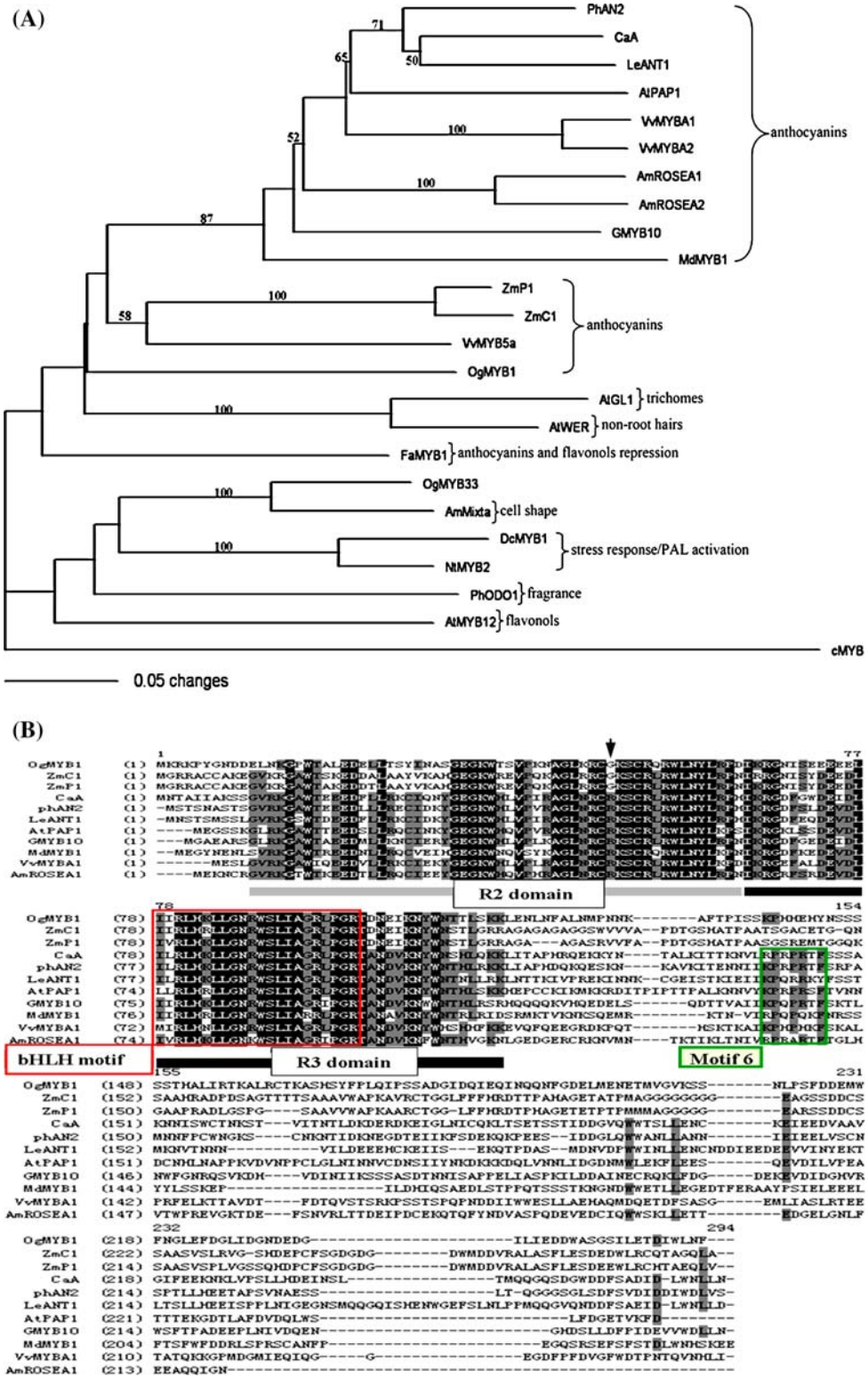

Fig. 5 Comparison of the deduced amino acid sequence of *OgMYB1* with other MYB protein. (**a**) Phylogenetic analysis indicated the similarity of OgMYB1 with other MYBs that have been characterized as regulators of the anthocyanin pathway or other physiological processes. Bootstrap values are shown at nodes as it is greater than 50%. The scale bar represents 0.05 substitutions per site. Accession numbers of proteins are listed in "Materials and methods." Functions of the other MYB proteins are indicated. (**b**) Protein sequence alignment of OgMYB1 with other MYBs involved in the anthocyanin pathway using Vector NTI 9.0. The R2 and R3 domains are underlined in black and gray, respectively. The bHLH binding motif is boxed in purple in the R3 domain. Motif 6 identified in C-terminal is boxed in green. A specific residue of interest that is common to anthocyanin regulator is indicated with a black arrow within the R2 domain

red-yellow mosaic in sepal, petal and lip crest tissues (Fig. 1b). Hieber et al. (2006) reported that 9-*cis* violaxanthin accumulating genes, such as *phytoene synthase* (PSY), *phytoene desaturase* (PDS), and *carotenoid isomerase* genes (crtISO) were active in all tissues. It also revealed that DFR is only expressed in lip crest, sepal and petal, but not in leaf and yellow lip. The lack of DFR expression is recognized as the main factor explaining predominant yellow color in lip tissues. In our work, the investigation of expression level of anthocyanin genes

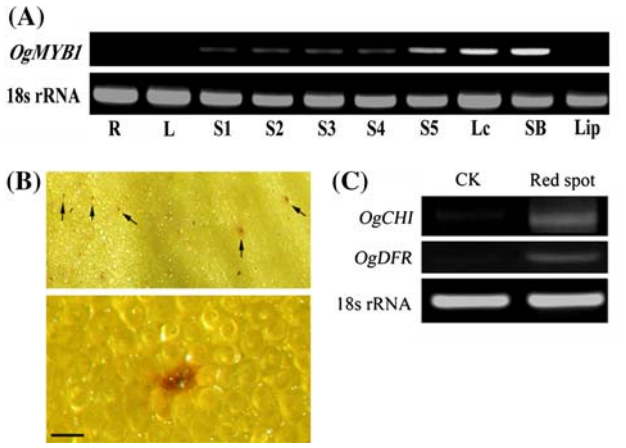

Fig. 6 Expression analysis of the transcripts of *OgMYB1* and transient expression of *OgMYB1* bombarded into lip tissue. (**a**) Transcript detection of *OgMYB1* by RT-PCR with gene-specific primers. Total RNA (1 μg) was isolated from floral stages (S) 1–5, root (R), leaf (L), lip crest (Lc), Sharry Baby (SB) and lip. The 15 cycles of PCR amplification of 18s rRNA was used as an internal control. (**b**) Microscope images of the red spots of a lip tissue was induced to synthesize anthocyanin by transient expression of *OgMYB1* driven by Pchrc promoter at magnification of 20× (left) and 75× (right), respectively. Scale bars = 50 μm. (**c**) RT-PCR analysis of *OgCHI* and *OgDFR* transcripts in red spots of floral lip tissues, after bombarding with *OgMYB1* or empty vector (CK)

further demonstrated that not only *OgDFR* but also *OgCHI* are extremely low expressed in yellow lip tissue (Fig. 3), when compared to other floral parts or floral developmental stages. Due to the down-regulation of both two key genes, the sequence of biochemical reactions in anthocyanin synthesis is blocked. Consequently, anthocyanins are not produced in lip tissue. Therefore, this explains why lip tissue forms a predominantly yellow coloration pattern, and shortage of red pigmentation.

Specific functions of *OgMYB1* in mediating direct activation of *OgCHI* and *OgDFR*

Phenylpropanoid pathway generates various flavonoids in plant tissues. Anthocyanin is one of the most commonly distributed pigments of flavonoid. The genes of anthocyanin biosynthetic pathway have been studied extensively in *Petunia*, *Antirrhinum*, *Maize* and *Arabidopsis* (Dooner et al. 1991; Holton and Cornish 1995; Forkmann and Martens 2001; Winkel-Shirley 2001). The genes involved in flavonoid biosynthesis include CHS, CHI, F3H, F3′H, DFR, ANS, 3GT, glutathione *S*-transferase (GST), and anthocyanin transporter (AT) (Holton and Cornish 1995). An early investigation concluded that the color differences between species in the genus *Ipomoea* were attributable to different expression levels of the anthocyanin biosynthetic genes. Variation in regulatory gene activity may be correlated to variation of expression level of anthocyanin biosynthetic genes (Durbin et al. 2003). Indeed, the variation in anthocyanin accumulation in flower is attributable to differences in *MYB* gene activity in many plants, including *Antirrhinum* (Schwinn et al. 2006), *Petunia* (Quattrocchio et al. 1999), and potato (De Jong et al. 2004). However, the activity of *MYB* gene controlling floral pigmentation in orchid was still poorly understood. A *Dendrobium* orchid *MYB* gene, termed *DwMYB9* (specifically expressed in flower), was reported to involve in anthocyanin biosynthesis based on Northern blot analysis (Wu et al. 2003). However, further functional characterization of *DwMYB9* will be necessary to investigate its role in flower coloration. In our work, we have demonstrated that *OgMYB1* can function as an anthocyanin regulator by bombardment assay (Fig. 6b).

ros^{col} mutant in *Antirrhinum* has no *Ros1* expression (64 bp deletion in R2 domain) but expresses *Ros2*, resulting in weak pigmentation in inner epidermis of corolla lobe. On the contrary, ros^{dor} mutant has no *Ros2* expression (rearranged in R3domain) but expresses *Ros1*, displaying pigmentation only in outer epidermis of the dorsal petals (Schwinn et al. 2006). It suggests that this small family of *MYB*-related genes controls the pattern and intensity of pigmentation of flowers in *Antirrhinum*. However, OgMYB1 protein seems to be a major regulator of *Oncidium* that controls anthocyanin biosynthesis in flower when comparing expression pattern among lip, lip crest and Sharry baby (Fig. 6a). Even intensive screening work done with R2R3 conserve probe, we could not find any other *MYB* gene members related to anthocyanin biosynthesis in phage cDNA library.

A phylogenetic analysis showed that OgMYB1 protein shares homology with *ZmPL*, *ZmC1* and *VvMYB5a* (Fig. 5b). The ZmPL protein activates transcription of genes encoding enzymes in biosynthesis of the anthocyanin pigments including CHS, CHI and DFR, but the ZmC1 protein extensively activates a subset of the genes including CHS, DFR, ANS and GST (Martin and Paz-Ares 1997). The VvMYB5a protein can up-regulate CHS, CHI, F3H and DFR expression by heterologous experiment of overexpression of *VvMYB5a* in tobacco (Deluc et al. 2006). By transient expression of *OgMYB1* gene, we demonstrated that it can activate *OgCHI* and *OgDFR* expression in lip tissue (Fig. 6c). Taken together, the regulatory functions of *MYB* gene members are versatile with diversified target genes.

The regulatory mechanism of *OgMYB1* gene in relation to floral color pattern in *Oncidium* Gower Ramsey is still unknown. It would require isolating genomic DNA clones to study the transcriptional regulation from the hybrid orchid. The likely explanation of the differential expression of *OgMYB1*gene in determining floral color pattern is

allopolyploidy nature (Hieber et al. 2006). It was originated from the interspecific cross of three genetic backgrounds [(*Onc. sphacelatum* × *Onc. flexuosum*) × (*Onc. sphacelatum* × *Onc. varicosum*)]. Allopolyploids are progenitors generated from interspecific cross between polyploidy. It is well known that the polyploidy usually exhibits a range of genetic variation in their progenitors (Osborn et al. 2003). The genetic variation associated with allopolyploid is related to epigenetic change, such as methylation change, gene silencing, and repression of transposable elements (Liu and Wendel 2003). It indicates that gene expression pattern in polyploidy plants varies depending on plant species. For example, a large number of gene transcripts is silencing found in wheat (Feldman and levy 2005), and the organ-specific expression was occurring in cotton (Adams et al. 2004). The finding of *OgMYB1* expression pattern in *Oncidium* Gower Ramsey, preferentially expressed in lip crest but not in lip tissue, suggests that coloration of flower follow a similar scenario as observed in other polyploids. Therefore, the floral color pattern resulting from differential expression of *OgMYB1* likely arises as a result of polyploidization.

In summary, our studies suggest that expression absence of *OgCHI* and *OgDFR* is attributable to the lack of expression of *OgMYB1* in yellow lip, which normally expressed in other floral tissues. Our results demonstrate that the differential expression of *OgMYB1* gene is critical for the unique coloration pattern in *Oncidium* flower.

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