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ORIGINAL ARTICLE

Methylation effect on chalcone synthase gene expression determines anthocyanin pigmentation in floral tissues of two *Oncidium* orchid cultivars

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Abstract The anthocyanin-biosynthetic pathway was studied in flowers of *Oncidium* Gower Ramsey with yellow floral color and mosaic red anthocyanin in lip crests, sepals and petals, and compared with the anthocyanin biosynthesis in flowers of *Oncidium* Honey Dollp, a natural somatoclone derived from tissue culture of Gower Ramsey, with a yellow perianth without red anthocyanins in floral tissues. HPLC analysis revealed that the red anthocyanin in lip crests of the Gower Ramsey cultivar comprised peonidin-3-*O*-glucoside, delphinidin-3-*O*-glucoside and cyanidin-3-*O*-glucoside, whereas Honey Dollp was devoid of anthocyanin compounds. Among the five anthocyanin-biosynthetic genes, *OgCHS* was actively expressed in lip crests of Gower Ramsey flowers, but no transcripts of *OgCHS* were detected in Honey Dollp floral tissues. Transient expression of *OgCHS* by bombardment confirmed that recovery of the *OgCHS* gene expression completed the anthocyanin pathway and produced anthocyanin compounds in lip crests of Honey Dollp flowers. Transcription factor genes regulating anthocyanin biosynthesis showed no distinctive differences in the expression level of *OgMYB*1, *OgbHLH* and *OgWD*40 between the two cultivars. A methylation assay revealed that the promoter of *OgCHS* was not methylated in Gower Ramsey, while a positive methylation effect was present in the upstream promoter region of *OgCHS* in Honey Dollp. Overall, our results suggest that the failure of anthocyanin accumulation in Honey Dollp floral tissues may be attributed to inactivation of the *OgCHS* gene resulting from the epigenetic methylation of 5′-upstream promoter region.

Keywords Anthocyanin synthesis · Chalcone synthase · DNA methylation · Flower · *Oncidium*

Abbreviations

GR Gower Ramsey
HD Honey Dollp
CHS Chalcone synthase
CHI Chalcone isomerase
F3H Flavanone 3-hydroxylase
DFR Dihydroflavonol reductase
ANS Anthocyanidin synthase

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Introduction

Anthocyanins, a kind of flavonoids, are the largest group of water-soluble pigments in the plant kingdom. They are responsible for most of the red, blue, and purple colors in flowers, fruits, leaves, seeds and other tissues. Hundreds of different anthocyanidins have been identified (Ghosh and Konishi 2007), and some of the most common classes are pelargonidin, cyanidin, delphinidin, peonidin, petunidin and malvidin. They have many biological functions, which include a role in attracting insects for pollination, UV protection and plant-pathogen interactions (Gronquist et al. 2001).

The biosynthesis of anthocyanins has been well established, and related genes have been identified in *Arabidopsis thaliana* (Burbulis and Winkel-Shirley 1999), maize (*Zea mays*; Winkel-Shirley 2001; Grotewold 2006), *Petunia hybrida* (Quattrocchio et al. 1999) and morning glories (*Ipomoea nil*; Morita et al. 2006; *Ipomoea purpurea*; Park et al. 2007). The first key enzyme of anthocyanins biosynthesis is chalcone synthase, which catalyzes the condensation reaction to yield chalcones. Subsequent enzymatic reaction from chalcones to naringenins is catalyzed by chalcone isomerase (CHI). Oxidation of naringenins by flavanone 3-hydroxylase (F3H) yields dihydrokaempferols, which can be hydroxylated on 3′ or 5′ position of the B-ring by flavonoid 3′-hydroxylase (F3′H) or flavonoid 3′, 5′hydroxylase (F3′, 5′H). The anthocyanidin precursors are further converted by dihydroflavonol reductase (DFR). Anthocyanidin synthase (ANS) catalyzes the penultimate step in anthocyanin biosynthesis, from colorless leucoanthocyanidins to colored anthocyanidins. Anthocyanidins are finally modified with acyl, glycosyl or methyl groups, catalyzed by acyltransferases (AT), glucosyltransferases (GT) and methyltransferases (MT). This final step is essential for the stable storage of anthocyanin complexes, and the modification is species-dependent and full of diversity.

Although some regulations of anthocyanin-related genes occurred at posttranscriptional level (Pairoba and Walbot 2003), most regulation of anthocyanin-biosynthetic genes was subject to transcriptional control (Koes et al. 2005). In all species analyzed to date, anthocyanin-biosynthetic genes are mainly regulated by three types of transcription factors, MYB, basic helix–loop–helix (bHLH) and WD40 proteins (Ramsay and Glover 2005; Bai et al. 2011; Hichri et al. 2011). In addition, members of the LBD gene family of transcription factors are reported to negatively regulate the anthocyanin biosynthesis in *Arabidopsis* (Rubin et al. 2009). Combined interactions or individual regulations of these transcription factors in activation of structural pigmentation genes had been reported in *Arabidopsis* (Zhang et al. 2003), petunia (*Petunia hybrida*; Spelt et al. 2000), maize (*Zea mays*; Lesnick and Chandler 1998; Grotewold et al. 2000) and Japanese Morning Glory (*Ipomoea nil*; Morita et al. 2006). An interaction model described that MYBs, which contact the promoter region of anthocyanin genes, control transcription of HLH factors, and subsequently form a complex that also involves the WD40 protein to activate the expression of structural anthocyanin genes (reviewed by Koes et al. 2005). The model indicated a significant role of these transcriptional factors in the activation of structural anthocyanin genes.

Some phenotypic consequences of plants show a close association with epigenetic changes, particularly DNA methylation (Zhu 2008). The reaction of DNA methylation is catalyzed by the enzymes DNA methyltransferases, and genes encoding the methyltransferases, the *METI* and *CMT* family, have been isolated from *Arabidopsis* (Cao and Jacobsen 2002). In diverse biological processes, DNA methylation is essential for genome management and gene transcription (Finnegan and Kovac 2000). The most common enzymatic DNA modification is methylation of the 5-position of cytosine, and the proportion of methylated cytosine residues varies widely, ranging from about 6% in *Arabidopsis* to 33% in rye (*Secale cereale*; Finnegan and Kovac 2000). Cytosine-5-methylation typically occurs at CpG sites (cytosine-phosphate-guanine sites) and the trinucleotide CpNpG sites in plants. Methylation in the vicinity of the promoter region is often associated with the absence of transcription (Chen et al. 2001).

Oncidium Gower Ramsey is one of the most popular cultivars in tropical orchid industry. This commercial cultivar is characterized by its brilliant yellow floral color with mosaic red pigmentation. A novel cultivar, *Oncidium* Honey Dollp, selected from the original cultivar of *Oncidium* Gower Ramsey, has been asexually propagated by orchid nursery. Honey Dollp has an entirely yellow perianth without the red portions in floral tissues. It had been reported that in Gower Ramsey the pigments of red floral tissues were coexistence of carotenoids and anthocyanins (Matsui 1994), and three kinds of anthocyanins were detected, including peonidin-3-*O*-glucoside, delphinidin-3-*O*-glucoside and cyanidin-3-*O*-glucoside (Chiou and Yeh 2008).

The purpose of the present work was to identify the variation of anthocyanin pigmentations between the two *Oncidium* cultivars, the expression profiles and coordinated regulatory mechanism of the relevant genes in *Oncidium* floral tissues. Our work disclosed that the epigenetic methylation effect of *chalcone synthase* was the determining factor for the failure of anthocyanin biosynthesis in floral tissues of *Oncidium* Honey Dollp. We address the molecular mechanism about the anthocyanin-related gene expression and the variation of pigmentation pattern in floral tissues of two *Oncidium* cultivars.

Materials and methods

Plant materials

Oncidium cv. Gower Ramsey and *Oncidium* cv. Honey Dollp were obtained from Orchid Nursery Co., Taoyuan, Taiwan. Plant seedlings were grown in the green house at a temperature range from 20 to 28°C. Floral organs at different developmental stages were harvested for RNA extraction, HPLC analysis, and particle bombardment assay.

HPLC analysis of anthocyanin

The extraction of anthocyanin pigments was carried out following the method described by Goodman et al. (2004). In brief, the *Oncidium* floral tissues were ground in appropriate solvent of 0.1 NH_4Cl in methanol. Then the ground tissues were centrifuged at 20,000*g* for 20 min at 4°C to separate the debris. The supernatant was removed, and diluted with 5% acetic acid in ratios ranging from 1:1 to 20:1 (v/v) depending on the concentration of the pigments. The final solution was immediately applied to HPLC analysis. HPLC analysis was performed by using a Dionex GP40 gradient pump (Dionex, Sunnyvale, CA, USA) and a Microsorb 100-5 C18 column (Varian, Palo Alto, CA, USA). 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 recorded and analyzed by PEAKNET software (Dionex). Anthocyanin compounds, such as cyanidin-3-*O*-glucoside, peonidin-3-*O*-glucoside, delphinidin-3-*O*-glucoside and malvidin-3-*O*-galactoside, were purchased from Fluka and monitored by HPLC as standard check.

Cloning of *OgF3H* and the 5′-promoter region of *OgCHS* in *Oncidium*

Total RNA was isolated from floral tissues of *Oncidium* by pine tree extraction method (Chang et al. 1993). One microgram of total RNA was used for synthesizing the cDNA using Powerscript reverse transcriptase (Clontech, Palo Alto, CA, USA). cDNAs were then amplified with degenerated primers, 5′-GCGARGRSTGGGGCATCTTC CAGGTGGT-3′, and 5′-GTTCACCACCGCCTGGTGR TCCGCRTTC-3′ to obtain the partial *OgF3H*. To isolate the 5′-upstream DNA region of *OgCHS*, genomic DNA was isolated from lip crest of *Oncidium* Gower Ramsey and *Oncidium* Honey Dollp cultivars using Tri-Plant Genomic DNA Reagent Kit (Geneaid, Taipei, Taiwan). Then, the promoter sequences were amplified using the Genome Walking Kit (Clontech). DNA sample was subsequently digested by restriction endonuclease, and then ligated to a cassette DNA. A primary PCR to amplify the 5′ upstream region of *OgCHS* was performed with a gene-specific primer, GSP1, and a cassette primer, AP1. The primary PCR products were then diluted and amplified using a nested gene-specific primer, GSP2, and a nested cassette primer, AP2. Finally, the 5′-upstream promoter regions were isolated from the two orchid cultivars. They were further used for DNA methylation analysis. The primer sequences of GSP1 and GSP2 were as follows:

GSP1: 5′-CTTCTCCTTGAGGTCGGTGAGATGC-3′
GSP2: 5′-GGCGGCGTCGAGGTCCCGATGGCCAG-3′

Northern-blot analysis and RT-PCR

Total RNA was isolated from floral tissues at various developmental stages of *Oncidium* cultivars by pine tree extraction method (Chang et al. 1993). About 10 μg total RNA was resolved on 1% denatured/formaldehyde agarose gel, transferred onto an Immobilon-N^+ membrane (Millipore), and UV crosslinked. 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 buffer containing 0.1% SDS for 15 min, then once in 0.1× SSC at 60°C for 10 min.

RT-PCR was performed with One-Step RNA PCR Kit (TaKaRa) following the manufacturer's instructions. The primer sets used in the RT-PCR analysis were as follows:

*OgMYB*1:
5′-TTATAATTCTTCTTCTTCCTCCACG-3′
5′-CCAATCCATTGAACCACATTTCA-3′
*OgWD*40:
5′-CTTTAGGAATTGAGGAAGATGTCACT-3′
5′-ACCAGCGATTTGAGAGTGAGGAAC-3′
OgbHLH:
5′-ACAGTGGTTCCTGCGTTTGAGAAATAT-3′
5′-GGCCACTCAATGGGCCATTGCT-3′

Constructs for transient expression using particle bombardment

OgCHS gene driven by 35S promoter in PBI121 was amplified by PCR and subcloned into pGEM-T Easy vector. 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 lip crests were freshly detached from *Oncidium* Honey Dollp. Bombardment assay was conducted using the instrument of Helium Biolistic Particle Delivery System (Bio-Rad, Hercules, CA, USA). 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. The particles were pipetted onto microcarriers of the Biolistics Device. For bombardment, lip crest 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 1,100 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 with red spot production were photographed under a dissecting microscope (Nikon).

Methylation analysis using the bisulfite sequence method

Two DNA samples containing 5′-upstream promoter region of *OgCHS* isolated, respectively, from lip crests of *Oncidium* Gower Ramsey and *Oncidium* Honey Dollp cultivars were employed for DNA methylation analysis. A total of 1.5 µg DNA was subjected to bisulfate modification with the EpiTect Bisulfite Kit (Qiagen, Hilden, Germany) following the manufacturer's instruction. The following methylation and unmethylation primers were designed based on MethPrimer website (http://www.urogene.org/methprimer). Then primers were used to amplify the *OgCHS* promoter region. PCR condition for methylation region of *OgCHS* promoter was as following: 94°C/10 min, following 25 cycles with 94°C/30 s, 55°C/30 s, 72°C/30 s. Primers used for methylation-specific PCR were as the following:

M-methprimer-*OgCHS*:
5′-AGTTTTGAATTTATTAAGAAGGCGT-3′
5′-ACTATAAAACGAACTCGATACCCG-3′
U-methprimer-*OgCHS*:
5′-AGTTTTGAATTTATTAAGAAGGTGT-3′
5′-CACTATAAAACAAACTCAATACCCAAA-3′

Results

Analysis of anthocyanin composition in floral tissues of *Oncidium* Gower Ramsey (GR) and *Oncidium* Honey Dollp (HD)

The flower of GR has a yellow color with mosaic red pigmentation in lip crests, sepals, and petals (Fig. 1a). Cultivar HD, a somatoclonal variant, has an entirely yellow perianth, without any red pigmentation (Fig. 1b). Apart from the lack of red portions, the cultivar HD was indistinguishable from GR in floral morphology.

However, high sectional magnification of lip crests showed that red anthocyanin was localized in the conical-papillate cells in GR, whereas no anthocyanin pigments were observed in HD papillate cells (Fig. 1c, d). To investigate the components of the red pigments, the extracts from lip crests were analyzed by HPLC. As shown in Fig. 2a, the red portion of GR lip crests contains a mixture of peonidin-3-*O*-glucoside, delphinidin-3-*O*-glucoside and cyanidin-3-*O*-glucoside compounds. The major peak represents cyanidin-3-*O*-glucoside that accounted for almost 56% of the total area. However, no anthocyanin compounds were detected in HD (Fig. 2b). Based on HPLC profiles, the lack of anthocyanin in HD suggested that some of the anthocyanin-biosynthetic genes may be inactive, or anthocyanins may be degraded by some unknown factors.

Characterization of anthocyanin biosynthetic genes

In our previous work, we have constructed an EST library of *Oncidium* flower buds and isolated four anthocyanin-

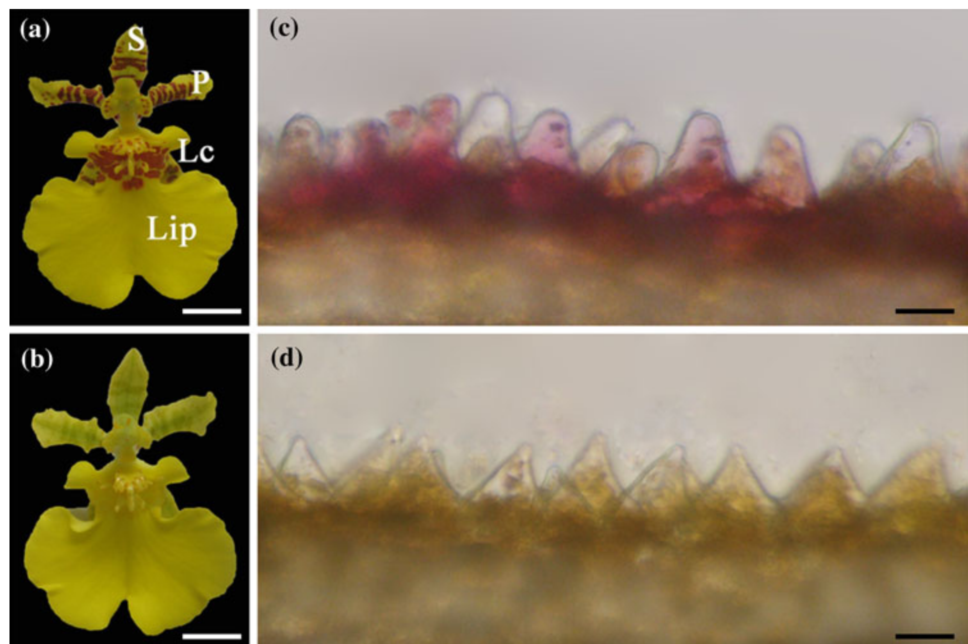

Fig. 1 Tissue sections of *Oncidium* Gower Ramsey and *Oncidium* Honey Dollp flowers showing the different color pattern. **a** Floral pigmentation of *Oncidium* Gower Ramsey. The floral tissues: *S* sepal, *P* petal, *Lc* Lip crest, *Lip* labellum. *Scale bar* = 8 mm. **b** Floral pigmentation of *Oncidium* Honey Dollp. *Scale bar* = 8 mm. **c** Anatomic structure of the conical-papillate cells from Lc of *Oncidium* Gower Ramsey. 200×, *scale bar* = 15 µm. **d** Anatomic structure of the conical-papillate cells from Lc of *Oncidium* Honey Dollp. 200×, *scale bar* = 15 µm

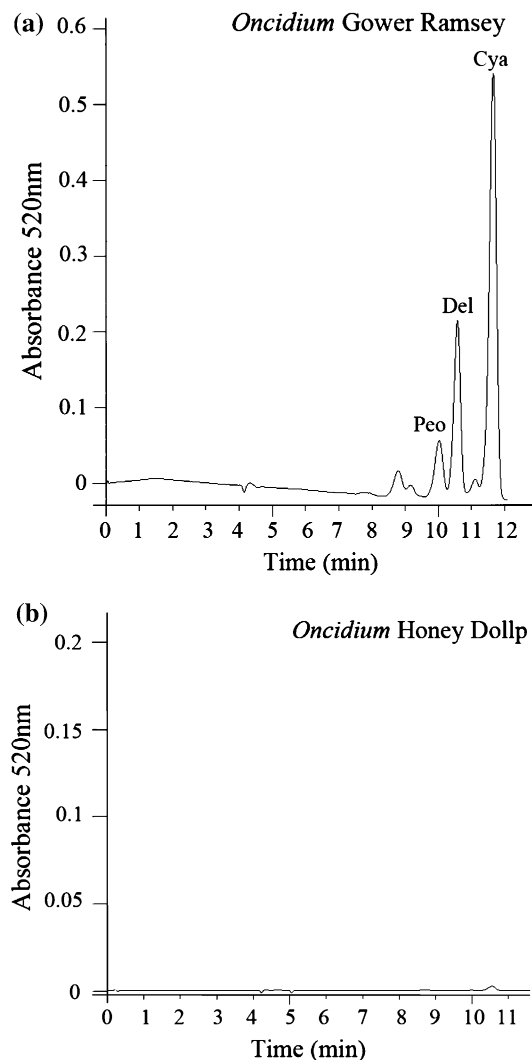

Fig. 2 HPLC profiles of anthocyanins in *Oncidium* Gower Ramsey and *Oncidium* Honey Dollp flowers. **a** Anthocyanins profile of lip crest (Lc) tissues in *Oncidium* Gower Ramsey. *Peo* peonidin, *Del* delpinidin, *Cya* cyanidin. **b** Anthocyanins profile of Lc tissues in *Oncidium* Honey Dollp

biosynthetic genes, such as *OgCHS* (EF570111), *OgCHI* (EF570112), *OgDFR* (EF570113) and *OgANS* (EF570114) (Chiou and Yeh 2008). In the present study, we have isolated an additional anthocyanin-biosynthetic gene named *OgF*3*H* (accession number JQ081067, NCBI databank) for fully monitoring the gene expression profiles in anthocyanin-biosynthetic pathway (Fig. 3a).

To unravel the absence of anthocyanin accumulation in HD floral tissue, Northern-blot analysis was carried out to examine the gene expression pattern in leaves, flower buds at different developmental stages (S1, S3, S5), floral lip crests, lips, sepals and petals from two *Oncidium* cultivars. The results showed that *OgCHS* was actively expressed in the floral red portions of GR, such as the developmental stages of flower buds (S1 to S5), floral lip crests, sepals, and petals. On the contrary, *OgCHS* transcripts were almost undetectable in HD floral tissues. No distinctive difference was found in the expression pattern of *OgCHI*, *OgF*3*H*, *OgDFR* and *OgANS* between the two cultivars (Fig. 3b). To further confirm the *OgCHS* expression level, RT-PCR was performed by gene-specific primers, yet the expression was also not detectable in lip crests of HD flowers. The correlation between the gene expression patterns and HPLC profiles suggested that the anthocyanin deficiency in HD floral tissues resulted from the transcription silence of *OgCHS* (Fig. 3c).

Transient expression of *OgCHS* gene in floral lip crests of *Oncidium* Honey Dollp

In order to confirm the function of *OgCHS* gene expression for anthocyanins production in *Oncidium* floral tissues, the recombinant *OgCHS* DNA construct and an empty construct (pGEM-T vector only) were used for bombardment into lip crests of HD cultivars. After incubation on MS agar medium for 36 h, many red color spots were visible in the transformed region (Fig. 4a–c); however, no visible red spots were found in tissues of the empty vector transformation (Fig. 4d). Thus, the results confirmed that active expression of *OgCHS* gene possibly rescue the missing anthocyanin synthesis in floral tissue of HD flowers.

Characterization of transcription factor genes regulating flavonoid biosynthesis in lip crests

Three types of transcription factors, *Og*MYB, *Og*basic helix–loop–helix (bHLH) and *Og*WD40, were known to activate transcription of anthocyanin-biosynthetic genes in many plant species (Koes et al. 2005; Morita et al. 2006). To determine whether the transcription silence of *OgCHS* was caused by a defective transcription factor function, RT-PCR analysis was performed to monitor the expression levels of *OgMYB*1 (EF570115), *OgWD*40 and *OgbHLH* in lip crest tissues. The results demonstrated that no distinctive differences were found in the expression level of *OgMYB*1, *OgWD*40 and *OgbHLH* in lip crests of GR and HD (Fig. 5). Therefore, the deficiency of *OgCHS* gene expression in HD cultivar was not due to the transcription silence of *OgMYB*1, *OgbHLH* and *OgWD*40.

Methylation analysis on the *OgCHS* 5′-upstream promoter region

To investigate the regulatory mechanism of *OgCHS* in the two *Oncidium* cultivars, we isolated a 1,500 bp promoter sequence of *OgCHS* from HD, and a 2,044 bp size

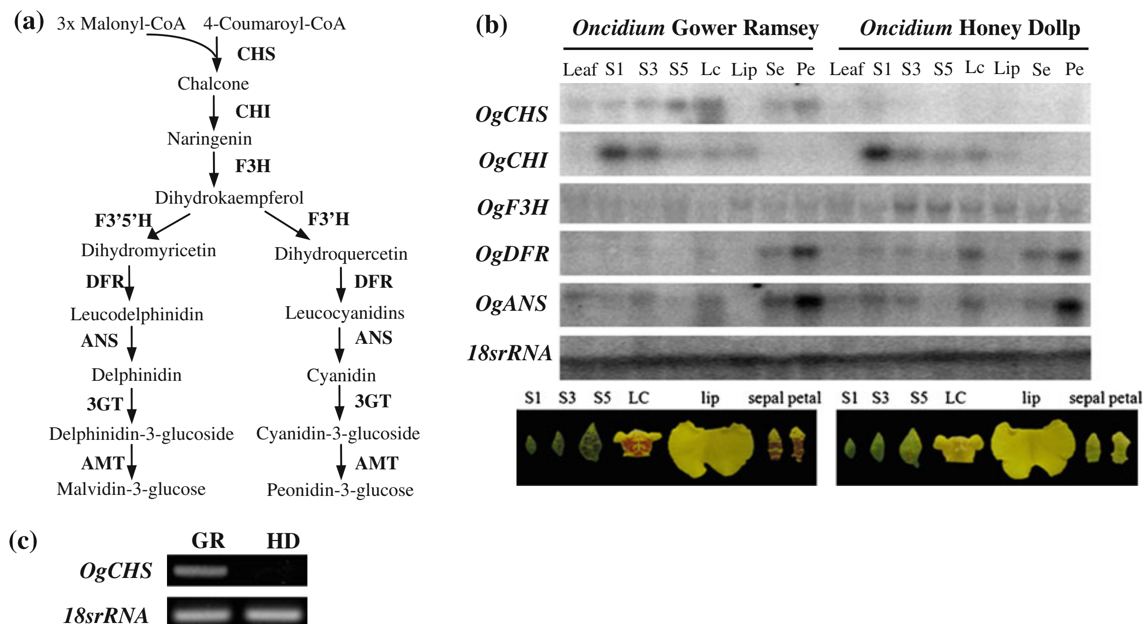

Fig. 3 Expression analysis of anthocyanin-biosynthetic genes in *Oncidium* Gower Ramsey and *Oncidium* Honey Dollp during floral development. **a** A simplified schematic pathway of the anthocyanin biosynthesis. **b** Northern analysis of five anthocyanin-biosynthetic genes in the two *Oncidium* cultivars. Ten microgram of total RNA from leaves; S1, S3, S5, different floral developmental stages; lip crests (Lc), lip, sepal, petal of GR and HD were, respectively, used for RNA gel blot. *Se* sepal, *Pe* petal. 18 s rRNA is the loading control. **c** RT-PCR analysis of *OgCHS* gene in lip crests of the two *Oncidium* cultivars

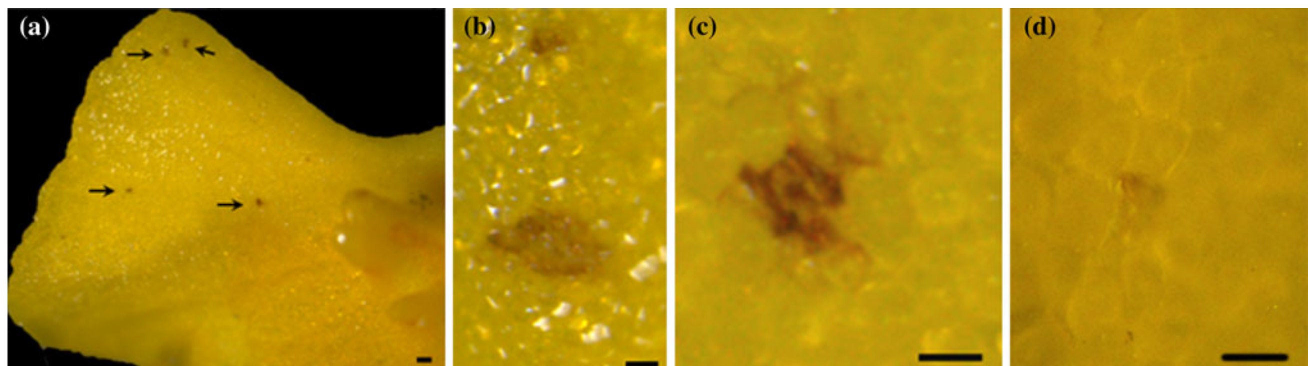

Fig. 4 Transient expression assay of *OgCHS* on floral lip crests of *Oncidium* Honey Dollp. Synthesis of anthocyanin was induced by transient expression of *OgCHS* driven by 35S promoter. Microscopic images of the *red* spots of a lip crest tissue at magnification of 20× (**a**), 50× (**b**), and 75× (**c**), respectively. Bombardment of particles with the empty vector was used as a negative control, 75× (**d**). *Scale bars* = 50 μm

promoter region from GR. Both nucleotide sequences were identical (Supplemental Fig. S1). CpG islands within *OgCHS* promoter were predicted using the MethPrimer design program (MSP; http://www.urogene.org/methprimer/) (Fig. 6a). The MSP results showed that the promoter of *OgCHS* was unmethylated in GR flowers, whereas HD showed a positive methylation effect (Fig. 6b). Thus, methylation of the 5′-upstream region may play a critical role for *OgCHS* gene inactivation, thus resulting in the absence of anthocyanin biosynthesis in HD floral tissues.

Discussion

The cultivar *Oncidium* Honey Dollp (HD) is deficient in anthocyanin in floral lip crests, petals and sepals, and displays a different pigmentation pattern compared with the parental cultivar GR. Our present work revealed that the color variation is a result from the epigenetic modification in the vicinity of *OgCHS* promoter of HD.

Chalcone synthase (CHS) is the first key enzyme in phenylpropanoid biosynthetic pathway. It catalyzes the

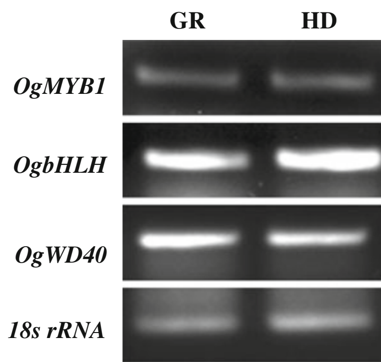

Fig. 5 Expression level of *OgMYB*1, *OgbHLH* and *OgWD*40 detected by RT-PCR with gene-specific primers in the lip crest tissues of two *Oncidium* cultivars. Total RNA (1 μg) was isolated from lip crests of *Oncidium* Gower Ramsey and *Oncidium* Honey Dollp. 18 s rRNA was used as an internal control

condensation of one molecule of *p*-coumaroyl-CoA and three molecules of malonyl-CoA to form one molecule of naringenin chalcone. It is the intermediate for the biosynthesis to a diverse set of secondary metabolites, including isoflavones in seed cotyledons, defense compounds in leaves, phenolic exudates in roots, and anthocyanins in hypocotyl, pod, trichome, especially in flower. *p*-Coumaroyl-CoA is also catalyzed by hydroxycinnamoyl-CoA shikimate/quinate hydroxycinnamoyl transferase (HCT) leading to the biosynthesis of two major lignin units, namely the guaiacyl and syringyl unit. Thus far, *CHS* genes have been isolated from a number of plant species. As well known, the expression of each member of the genes is regulated spatially and temporally. However, each gene is responsible for the production of specific metabolites and for a unique function. In grapevine, *CHS*2 and *CHS*3 are responsible for anthocyanins accumulation in berry skins, but not *CHS*1 (Goto-Yamamoto et al. 2002; Jeong et al. 2004). In petunia, *CHS* comprises a multigene family in which only one gene is expressed to high levels in petal tissues (Koes et al. 1989). Although, in *Oncidium* GR and HD, four to five gene copies of *CHS* in haploid genome were predicted based on Southern-blot analysis (data not shown), only one gene was identified from twenty cDNA clones, which were amplified by RT-PCR in floral tissues. Therefore, we suggest that *OgCHS* (EF570111) is the sole floral-specific gene in *Oncidium CHS* family, and is differentially expressed in floral tissues for anthocyanin synthesis. The nucleotide sequences of the 5′-upstream promoter region of *OgCHS* (EF570111) from both GR and HD are identical (Supplemental Fig. S1). It is suggested that both cultivars comprise the same genomic DNA composition. A possible mechanism for the transcription silence of *OgCHS* in HD is that the methylated sequence impedes the specific binding of transcription factors (Juven-Gershon et al. 2008). The other possible mechanism might be caused by the function of methyl-binding domain (MBD) proteins, which are able to recruit transcriptional repressors. MBD proteins read the epigenetic signals, and recruit the enzymatic machinery to establish a repressive chromatin environment (Springer and Kaeppler 2005; Hu et al. 2011). Based on our work, it is interesting to note that factors, which activate DNA methyltransferase and demethylation, were present in HD cultivars, while not in GR cultivars.

It has been reported that flavonoid-deficient *Arabidopsis* plants with mutation in *CHS* (*transparent testa*4, *tt*4), displayed delayed root gravitropism and defective lateral

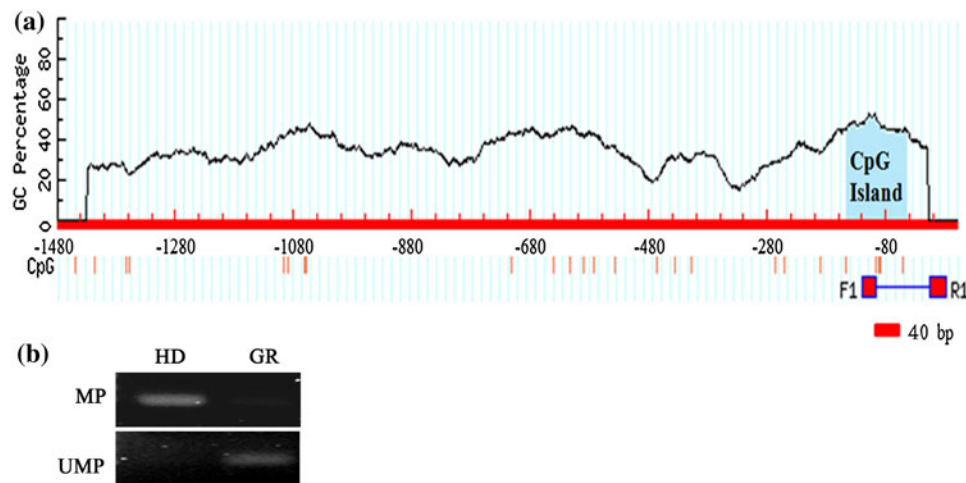

Fig. 6 Methylation assay of the 5′-upstream DNA region of *OgCHS*. **a** Schematic distribution of CpG sites and CpG island in *OgCHS* promoter region. CpG sites are represented by *red straight line*. *Blue area* indicates CpG island. The *scale bar* indicates 40 bp. **b** Gel analysis of PCR-amplified DNA products showing methylation on HD cultivar. The genomic DNA of lip crest tissues from HD was treated with sodium bisulfate, and the MSP method was performed by polymerase chain reaction using methylation primers (*MP*) and unmethylation primers (*UMP*)

roots due to changing IAA distribution (Buer and Muday 2004). Auxin transport was prominently elevated in the *transparent testa*4 (*tt*4). Therefore, plant flavonoids were considered as negative regulators of cellular auxin efflux and of auxin polar transport (Taylor and Grotewold 2005). Although the *CHS*-deficient *Arabidopsis* mutant had a decrease in flavonoid accumulation and an increase in the lignin content, the growth rate and development were similar to that one of the wild type (Besseau et al. 2007). In contrast, silencing of HCT, a gene for lignin biosynthesis, resulted in flavonoid accumulation and repression of lignin production, with a consequence of a strong growth reduction. The reduction of plant growth and development was correlated with the extent of flavonoid accumulation and with the level of auxin transport inhibition (Besseau et al. 2007).

Our morphological observations showed that transcription silence of *OgCHS* in floral tissues of HD caused no visible phenotypic difference between the two *Oncidium* cultivars, except the flower pigmentation (Fig. 1). This phenomenon is similar to the *CHS*-silenced *Arabidopsis* mutant, which showed the same phenotype as the wild type (Besseau et al. 2007). Basically, the redirection of the phenylpropanoid metabolic flux caused by *OgCHS* silence could result in accumulation of lignin compounds in floral tissues. Although no detrimental effect on floral growth and development was observed, the possible tolerance against environmental stresses, enhanced by the accumulated secondary metabolites, raises up our interest in future research.

It was also suggested that the downstream enzymes, such as DFR, likely were controlling the anthocyanin pathway (Rausher et al. 1999; Lu and Rausher 2003). In petunia and *Cymbidium* flowers, defective DFR enzyme activity was mainly responsible for the lack of an orange/brick red color (Forkmann and Ruhnau 1987; Johnson et al. 1999). In Gower Ramsey, it was found that the down-regulation of *OgDFR* and *OgCHI* blocked anthocyanin synthesis in lip tissue and resulted in shortage of red pigmentation (Chiou and Yeh 2008). However, in the present work, no significant difference was found in downstream gene expression of the two *Oncidium* cultivars. Complex regulation model and metabolic networks of structural genes might be involved in anthocyanin pathway.

We showed here that the transcription silence of *OgCHS* leading to anthocyanin deficiency in lip crest/sepal/petal of Honey Dollp was not caused by nucleotide sequence mutation, since both *OgCHS* cDNAs of GR and HD confirmed sequence identity (Supplemental Fig. S2). In addition, no significant difference of expression level of transcription factor genes regulating flavonoid biosynthetic pathway was found between the two cultivars (Fig. 5). The transcription factors genes R2R3-MYB, basic helix-loop–helix (bHLH) and WD40 repeats (WDRs) (Koes et al. 2005; Quattrocchio et al. 2006) regulating anthocyanin structural genes and the MYB-bHLH-WDR protein complex play a role in anthocyanin biosynthesis. In our previous study, we have demonstrated that the inactive expression of *OgMYB*1 in lip tissues caused the transcription silence of *OgCHI* and *OgDFR* and thus resulted in anthocyanin deficiency in yellow lip of Gower Ramsey (Chiou and Yeh 2008). However, in the present study, no distinctive difference was found in the expression level of *OgMYB*1, *OgWD*40 and *OgbHLH* in lip crest of GR and HD. It indicates that the expression of *OgMYB*1 was highly differentially regulated, active in lip crest but not in lip tissues.

In conclusion, the present study demonstrates the differential expression pattern of *OgCHS* in two *Oncidium* cultivars. The anthocyanin absence in HD cultivar caused by the epigenetic modification is addressed. Thus, the knowledge of DNA methylation affecting floral pigmentation in *Oncidium* orchids would be of significance for the breeding program of the flower industry for the generation of novel cultivars.

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