Cloning and Characterization of Two Anthocyanin Biosynthetic Genes from *Dendrobium* Orchid

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ABSTRACT. Two full-length cDNA clones, Den-CHS-4 and Den-DFR-1, encoding chalcone synthase (CHS) and dihydroflavonol 4-reductase (DFR) were obtained from flower bud RNA of a lavender cyanidin-accumulating *Dendrobium* Sw. hybrid using reverse transcription-polymerase chain reaction (RT-PCR). Northern analyses indicated that both genes are expressed in all developmental stages of buds, with highest expression in the medium-sized buds. RT-PCR analyses showed that DFR expression was confined to floral tissue while CHS was expressed in floral and vegetative tissues but not in pseudobulbs. The nucleotide sequence of a DFR clone isolated from a pale orange pelargonidin-accumulating *Dendrobium* hybrid was exactly the same as Den-DFR-1, ruling out the substrate specificity of DFR as a possible cause of the color difference.

Flavonoids, a diverse group of phenolic compounds, play a wide variety of roles in plants such as pollinator attraction, protection from stress and pathogens, and cell signaling in plant-microbe interactions (Koes et al., 1994). Anthocyanins are colored flavonoid glycosides, which accumulate in vacuoles giving characteristic colors to flowers and fruits. Molecular, genetic, and enzymatic aspects of anthocyanin biosynthesis by the phenylpropanoid pathway are notably well characterized in *Petunia x hybrida* Hort. Ex Vilm., *Zea mays* L., and *Antirrhinum majus* L. (Dooner et al., 1991; Holton and Cornish, 1995; Mol et al., 1998). Two key anthocyanin biosynthetic enzymes are chalcone synthase (CHS) and dihydroflavonol 4-reductase (DFR).

The first committed step of flavonoid biosynthesis is the formation of chalcone catalyzed by CHS. In many plants, several CHS genes constitute a small multi-gene family. Promoter regions of these genes are activated by different environmental stimuli (Martin, 1993), such as light and abiotic stress allowing the plant to accumulate anthocyanins in responsive tissues. DFR catalyzes the first specific step of anthocyanin production in which dihydroflavonols are reduced to leucoanthocyanidins, the immediate precursors of anthocyanins (Heller et al., 1985). One interesting aspect of the DFR enzyme is its substrate specificity. DFR can accept dihydrokaempferol (DHK), dihydroquercetin (DHQ), or dihydromyricetin (DHM) to form orange pelargonidin, purple cyanidin or blue delphinidin, respectively. In *Petunia* Juss., a well-characterized example, DFR does not reduce DHK at all, explaining the lack of pelargonidin-accumulating orange flowers (Forkman and Ruhnau, 1987; Gerats et al., 1982). Unlike CHS, DFR is represented by only a single gene in many plant species, including the two orchids *Bromheadia finlaysonian* Rchb. f. and

Cymbidium Sw. (Johnson et al., 1999; Liew et al., 1998a).

In *Dendrobium*, analysis of floral flavonoids found in species and hybrids identified 3'-hydroxylated cyanidin as the major pigment aglycone while pelargonidin was found to be rare and blue delphinidin was not detected (Kuehnle et al., 1997). This study identified *Dendrobium* x Icy Pink 'Sakura' as a unique line that accumulates 98% pelargonidin and only 2% cyanidin. This hybrid has only kaempferol derivatives as the major flavonol while 3'-hydroxylated quercetin derivatives were not detected. An acylated cyanidin glycoside was isolated as the major pigment found in a red-purple flower of *Dendrobium* 'Pramot' (Saito et al., 1994). Acyl group is suggested to increase the stability of the structure and the color of this anthocyanin. Nevertheless, the core anthocyanidin structure of cyanidin still remains to be the most predominant in most commercial hybrids and species.

Nucleotide sequences for any flower color genes of *Dendrobium*, a major commercial orchid, are lacking in accessible databases [CHS was reported as cloned in abstract only in (Yong and Chua, 1990)]. Genes encoding CHS, DFR and flavanone 3-hydroxylase have been isolated from other orchids (reviewed in Mudalige and Kuehnle, 2004). Our objective was to isolate the genes encoding CHS and DFR from *Dendrobium* and to characterize their expression patterns. This information will be useful in determining the molecular basis of flower color, including the most probable cause for the scarcity of orange-pelargonidin accumulation in *Dendrobium* flowers, and in identifying possible candidates for perianth-specific promoters for use in genetic engineering.

Materials and Methods

RNA EXTRACTION AND cDNA SYNTHESIS. Inflorescences and vegetative parts of lavender *Dendrobium* x Jaquelyn Thomas 'Uniwai Prince' (UH503) and pale orange *Dendrobium* x Icy Pink 'Sakura' (K1224) were harvested from greenhouse-grown plants at the Univ. of Hawaii at Manoa. Total RNA was extracted from unopened buds, flowers, pseudobulbs, leaves, and roots according to the method of Champagne and Kuehnle (2000).

cDNA was synthesized from 5 µg of total RNA using 200 units of SuperScriptII RNase H⁻ reverse transcriptase (Invitrogen,

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Carlsbad, Calif.) in 1X first strand synthesis buffer (50 mM Tris-HCl, 75 mM KCl, 3 mM MgCl₂, pH 8.3), supplemented with 0.01 M DTT and 0.5 mM dNTPs, by incubating the reaction mixture at 42 °C for 50 min. Oligo dT (dT₁₆ or dT₂₀-T7) primers or the gene specific primers were used for first strand cDNA synthesis (Table 1). The reaction was stopped by incubation of the mixture at 70 °C for 15 min. The RNA template was removed by incubating the reaction mixture with 2 units of RNase H (Promega, Madison, Wis.) at 37 °C for 20 min. The samples were stored at -20 °C until utilized in PCR amplifications.

PCR WITH DEGENERATE PRIMERS TO AMPLIFY FLAVONOID GENES. Degenerate oligonucleotide primers were designed (Table 1) and synthesized (IDT, Coralville, Iowa), using the conserved regions of the GenBank DNA sequences of orthologous genes. For *DFR* and *CHS*, PCR reactions were carried out in a total volume of 50 µL using RedTaq DNA polymerase (Sigma, St. Louis) in a 1X amplification buffer [0.01 M Tris (pH 8.3), 0.05 M KCl, 0.01% gelatin, 0.2 mM dNTPs, 1.0 mM MgCl₂] using the iCycler thermal cycler (Bio-Rad, Hercules, Calif.). Flower bud cDNA made from 250 ng of total RNA was used with 200 nM primer concentrations for each reaction. Denaturing of all PCR reactions were performed at 95 °C while extensions were carried out at 72 °C. Sequences of each primer and annealing temperatures used are listed in Table 1.

PCR products were separated on agarose gels in 1X TAE buffer (40 mM Tris-acetate, 1 mM Na₂EDTA). Amplified DNA fragments with expected molecular weight were excised and purified using GENECLAN II kit (QBiogene, Carlsbad, Calif.) and ligated into pCR II-TOPO (Invitrogen).

Ligation and transformation was carried out according to the manufacturer's instructions using chemical competent *Escherichia coli* TOP 10 cells (Invitrogen). PCR product inserts were fully sequenced at the Univ. of Hawaii Biotechnology Core Facility.

RACE (RAPID AMPLIFICATION OF cDNA ENDS) REACTIONS. UH503 and K1224 flower bud cDNA was synthesized according to the reverse transcription protocol as described above, using dT₂₀-T7 primer (Table 1). A gene specific primer and the T7 adapter primer were used in subsequent 3' RACE reactions. Primers and the PCR conditions used in each reaction are listed in Table 1. For all RACE reactions denaturations of DNA were carried out at 95 °C and extensions were carried out at 72 °C for all *Taq* DNA polymerase enzymes and 68 °C for *Pfx* DNA polymerase (Invitrogen). A skewed ratio of 10:1 gene-specific primer to adapter primer (T7) was used in all 3' RACE reactions (Bespalova et al., 1998), which was crucial for the successful amplification of the gene. Isolated PCR products were ligated into pCRII-TOPO (Invitrogen) or pGEM-Teasy (Promega) and fully sequenced.

For the 5' RACE reaction of *DFR*, synthesized cDNA was cleaned in order to remove the extra dNTPs and other impurities using GENECLAN II kit. Cleaned cDNA was boiled for 5 min and quickly chilled on ice. A poly-C tail was added to the single-stranded cDNA using terminal deoxyribonucleotide transferase (TdT) (Promega) in 1X tailing buffer (10 mM Tris-HCl, pH 8.4, 25 mM KCl, 1.5 mM MgCl₂) supplemented with 0.8 mM dCTP at 37 °C for 40 min. Enzyme was inactivated by incubation at 65 °C for 10 min. A polyG primer with an adaptor and a gene specific primer (Table 1) were used in amplification of the 5'

Table 1. Primers, reverse transcription conditions, and annealing temperatures used for amplification of flavonoid genes from *Dendrobium*.

Primer designation	Sequence	Annealing temps/ reaction conditions
Reverse transcription primers		
dT ₁₆	ttttttttttttttt-3'	42 °C for 50 min
T ₂₀ -T7	taatacgactcactatagggttttttttttttttt-3'	42 °C for 50 min
Den-CHS-R _{out}	ttagagatcaagcctgggac-3'	42 °C for 50 min
Degenerate primers		
CHS-L	cggaattca(c/t)ca(a/g)ca(a/g)ggitg(c/t)tt(c/t)g-3'	40 °C for 1 min
CHS-R	cgggatccc(a/g)aaia(a/g)naciccca(c/t)t-3'	1.0 mM MgCl ₂
DFR-L	cgggaattcgggncigntgtgtn-3'	40 °C for 1 min
DFR-R	cgggatcctacatccancngtcat(c/t)tt-3'	1.0 mM MgCl ₂
RACE primers		
Den-CHS-6-L	gtccctgttccaactcgta-3'	55 °C for 1 min
T7	taatacgactcactata-3'	2.0 mM MgCl ₂
Den-CHS-R _{nest}	cagtaggtggaaggttagtcc-3'	54 °C for 45 s
AAP	ggccacgcgtcgactagtagcggggggggggggggg-3'	First round 2.1 mM MgCl ₂
Den-CHS-5 _{race2}	ggaaggttagtccatctcg-3'	Same PCR conditions as the first round amplification
AUAP	ggccacgcgtcgactagtagc-3'	
Den-DRF-L	gggttatgtgtcagggcta-3'	55 °C for 1 min
T7	taatacgactcactata-3'	1.5 mM MgCl ₂
Den-DFR-R	agtcaaggtcactccagcag-3'	55 °C for 1 min
AAP	ggccacgcgtcgactagtagcggggggggggggggg-3'	First round 1.5 mM MgCl ₂
Den-DFR-R	agtcaaggtcactccagcag-3'	Same PCR conditions as the first round amplification
AUAP	ggccacgcgtcgactagtagc-3'	
RT-PCR primers		
Den-DFR-5'ext	aactggcgttgaggagagag-3'	55 °C for 1 min
Den-DFR-3'utr	aacaacaacattagcttctcttt-3'	1.5 mM MgCl ₂
Den-CHS-PfxL	ggcaatggaagagatcagg-3'	55 °C for 1 min
Den-CHS-PfxR	gcaccagcaatcgggaacgc-3'	1.5 mM MgCl ₂

i = inosine, a substitute for (a/c/g/t) to reduce degeneracy in primers, n = a/c/g/t.

end of the gene. The primary amplification was re-amplified in a second PCR with the same primer combination to generate a sufficient amount of products for cloning and sequencing. For the 5' RACE reaction of *CHS* gene the RACE kit from Invitrogen was used with the primers and PCR conditions listed in Table 1. Later, full clones were amplified using high fidelity Platinum *Pfx* DNA polymerase and fully sequenced. A phylogenetic tree was drawn from Phylogenetic Inference Package (PHYLIP version 3.5c; Felsenstein, 1993) showing relationships among the nucleotide sequences of *DFR* genes from different plant species. Sequence alignment was performed with CLUSTAL W version 3.2 (Thompson et al., 1994) with a gap penalty of 3.

RADIOACTIVE PROBE SYNTHESIS FOR NORTHERN HYBRIDIZATION. The cDNA inserts of the flavonoid genes were isolated by restriction digestion of the clones with *EcoRI* enzyme and separation on 1% agarose gel. DNA fragments were purified (GENECLEAN II kit) and 20 ng were labeled with $\alpha^{32}\text{P}$ -ATP using random primer labeling (Prime a Gene Kit; Promega). Labeled product was cleaned using Elutip-D column (Schleicher and Schuell, Keene, N.H.). Cleaned probe was added to the hybridization buffer at a concentration of 1×10^6 counts/min per mL of buffer.

NORTHERN BLOT HYBRIDIZATION. Floral buds and flowers from harvested inflorescences of UH503 and K1224 were divided into 10 different developmental stages, stage 1 being the most immature (Table 2). RNA was extracted from bud/flower stages and mature leaves according to Champagne and Kuehnle (2000). Total RNA (10 μg) from each stage was size fractionated by a standard 2% formaldehyde agarose (0.9%) gel electrophoresis (1X MOPS buffer). RNA was transferred overnight onto Nytran Supercharge nylon membrane (Schleicher and Schuell) by downward capillary transfer using alkaline transfer buffer (3 M NaCl, 0.01 N NaOH). Hybridization and washing was done according to Church and Gilbert (1984) but using 0.25 M $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$ in hybridization buffer at pH 7.4. Membranes were hybridized with ^{32}P labeled DNA probes at 60 °C overnight in hybridization buffer [7% SDS, 1% fatty acid free bovine serum albumin (BSA) (Sigma), 1 mM $\text{Na}_2\text{EDTA} \cdot 2\text{H}_2\text{O}$, 0.25 M $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$ (pH 7.4)]. Blots were washed twice at 60 °C for 10 min, using phosphate wash buffer with BSA [0.5% BSA, 1 mM $\text{Na}_2\text{EDTA} \cdot 2\text{H}_2\text{O}$, 5% SDS, 40 mM $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$ (pH 7.4)]. The final two washes were done at 60 °C for 20 min using phosphate buffer without BSA [1 mM $\text{Na}_2\text{EDTA} \cdot 2\text{H}_2\text{O}$, 1% SDS, 40 mM $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$ (pH 7.4)]. We have used high stringency conditions to avoid any cross reaction of *Den-CHS-4* probe with other transcripts with high similarity

to *CHS*. Signals were detected after 3 d of exposure by autoradiography for UH503 or using a Bio-Rad Personal Molecular Imager FX for K1224.

RT-PCR ANALYSES (REVERSE TRANSCRIPTION-PCR). cDNA samples synthesized from total RNA of roots, pseudobulbs, leaves, flowers, and buds of UH503 and K1224 *Dendrobium* orchids were used to amplify *DFR* and *CHS* transcripts using gene-specific primers and PCR conditions listed in Table 1. The resulting products were ligated to pGEM-T easy vector and sequenced to verify their identity.

Results

CLONING AND SEQUENCE ANALYSIS OF FLAVONOID BIOSYNTHETIC GENES. Dihydroflavonol 4-reductase. We chose a typical cyanidin accumulating line, *Dendrobium* x Jaquelyn Thomas 'Uniwai Prince' (UH503), and a rare pelargonidin accumulating line *Dendrobium* x Icy Pink 'Sakura' (K1224), for isolation of the *DFR* gene. Two partial cDNA clones were isolated from UH503 and K1224 flower bud cDNA and amplified using 5' RACE and 3' RACE techniques. Overlapping regions (400 bp) of the two partial clones were identical to each other. Later, a full-length clone of 1320 bp was amplified with the high fidelity *Pfx* DNA polymerase and fully sequenced (GenBank accession No. AY741319). The nucleotide sequence of the *DFR* clone isolated from *Dendrobium* Icy Pink 'Sakura' (K1224) was identical to the clone isolated from UH503 despite their flower color difference. The first ATG found 30 nucleotides downstream from the 5' end of the clone is the most probable start codon since it is immediately preceded by AAAGAAATGG which resembles the plant consensus sequence (Joshi, 1987). A polyadenylation signal, AATAAA, was found 159 nucleotides downstream from the TGA stop codon. A polypeptide of 352 amino acid residues with a molecular mass of 39.7 kD was deduced from the longest open reading frame (ORF) and was designated as *Den-DFR-1*.

The nucleotide sequence of *Den-DFR-1* revealed a similarity ranging from 65% to 86% when compared to nineteen other plant *DFR* sequences available in GenBank. A phylogenetic tree based on CLUSTAL W alignment shows that *Den-DFR-1* is closest to the *DFR* clones from the two orchids, *Bromheadia finlaysonian* and *Cymbidium*, with 86% and 83% identity, respectively (Fig. 1). The closest nonorchid sequence, from *Lilium* L. hybrid (Liliaceae), was 56% identical to *Den-DFR-1*. The phylogenetic tree shows all monocotyledonous sequences cluster into a single branch

showing their common ancestry, while dicotyledonous sequences clustered into two clades.

Comparison of the deduced amino acid sequence of *Den-DFR-1* with those of the two orchids, *Bromheadia* and *Cymbidium*, showed 86% and 82% identity, respectively. *Lilium* hybrid and *Zea mays* (Poaceae) shared 72% and 67% identity with the *Dendrobium*. We have compared the putative region that determines the substrate specificity of *DFR* as proposed by Beld et al. (1989) and Johnson et al. (1999), among eight different *DFR* enzymes including *Dendrobium* (Fig. 2). *DFR* of five species, *Rosa* L. hybrid, *Dianthus*

Table 2. Description of the bud and flower stages of *Dendrobium* x Jaquelyn Thomas 'Uniwai Prince' used in temporal expression of flower color genes.

Bud/flower stage	Length (cm)	Description and position on the raceme
1	1.3–1.5	Most immature buds, light green adaxial surface with a little purple color on the abaxial surface.
2	1.5–1.7	Small buds, adaxial surface still green, most of the abaxial surface turned purple.
3	1.7–1.9	Medium-small buds, adaxial surface of perianth turning dark purple.
4	1.9–2.1	Medium size buds, dark purple perianth on both surfaces.
5	2.1–2.3	Medium-large unopened buds, dark purple.
6	2.4–2.8	Most mature buds, unopened, dark purple on both adaxial and abaxial surfaces of perianth.
7	Not measured	Flowers just opened (half open).
8	Not measured	Flowers fully opened, dark purple perianth, one position below stage 7 flower.
9	Not measured	Open flower, two flowers below stage 7 on the raceme, dark purple.
10	Not measured	Older flower, three flowers below stage 7, dark purple.

Fig. 1. Phylogenetic tree showing relationships among the nucleotide sequences of *DFR* genes from different plant species. GenBank accession numbers for each species are listed below: *A. thaliana* (L.) Heynh.-M86359; *D. caryophyllus* L.-Z67983; *V. vinifera* L.-X75964; *J. nigra* L.-AJ278459; *Rosa hybrida*-D85102; *C. Chinensis* (L.) Benth.-Z67981; *G. hybrida*-Z17221; *A. majus* L.-X15536; *T. hybrida*-AB012924; *L. esculentum* Mill.-Z18277; *P. x hybrida*-AF233639; *C. sinensis* (L.) Kuntze-AB018686; *G. max* (L.) Merr.-AF167556; *B. filaysoniana* Rchb.f.-AF007096; *Cymbidium hybrida*-AF017451; *Lilium hybrid*-AF169801; *O. sativa* L.-AB003496; *Z. mays* L.-X05068; *H. vulgare* L.-S69616.

	133	168
<i>Rosa hybrid</i>	SVNVEETQKPVYNESNWS	DVEFCRRVKMTGWMYFAS
<i>Dianthus caryophyllus</i>	TVNVEATQKPVYDETCWS	DLDFIRSVKMTGWMYFVS
<i>Gerbera hybrid</i>	TVNGQEKQLHVYDESHWS	DLDFIYSKMTAWMYFVS
<i>Antirrhinum majus</i>	TVNVEEHQKPVYDETDSS	DMDFINSKMTGWMYFVS
<i>Zea mays</i>	TVNLEERQRPVYDEESWT	DVDFCRRVKMTGWMYFVS
<i>Petunia x hybrida</i>	TLDVQEQQLFYDQTSWS	DLDFIYAKMTGWMYFAS
<i>Cymbidium</i>	TVNVEEHQATVYDESSWS	DLDFVTRVKMTGWMYFVS
<i>Dendrobium</i>	TVNVEEHQAAYYDESCWS	DLDFVNRVKMTGWMYFVS

Fig. 2. Region of substrate specificity of dihydroflavonol reductase enzyme from six different plant species compared with the two orchid sequences. The first five enzymes listed are known to accept DHK as a substrate. *Petunia x hybrida* and *Cymbidium* DFR do not accept DHK as a substrate. Underlined amino acid residues are different in *Petunia* but conserved in all other enzymes including *Cymbidium* despite the similarity of substrate specificity of these two enzymes. *Dendrobium* DFR is also similar to *Cymbidium* in this critical region.

caryophyllus L., *Gerbera* L. hybrid, *Antirrhinum majus*, and *Zea mays* were already known to accept DHK as a substrate while *Petunia* and *Cymbidium* does not accept DHK (Johnson et al., 1999, 2001). Our comparison showed that the four amino acid residues that are unique to *Petunia* DFR are not shared by *Dendrobium* or *Cymbidium* DFR sequences (Fig. 2). In fact, these four amino acid residues are conserved among the five DHK accepting enzymes

and the two orchid DFR sequences despite the common substrate specificity shared by *Cymbidium* and *Petunia*.

Southern analysis of genomic DNA from young leaves of the two *Dendrobium* hybrids, UH503 and K1224, indicated that there is only a single copy of *DFR* gene present in both hybrids (data not shown). This result is similar to the other two orchids, *Cymbidium* and *Bromheadia* (Johnson et al., 1999; Liew et al., 1998a). However, we cannot eliminate the possibility of the presence of more than one copy since both these hybrids are polyploids.

Chalcone synthase. PCR with degenerate primers resulted in the isolation of two partial clones from *Dendrobium* x Jaquelyn Thomas 'Uniway Prince', *CHS-6* and *CHS-9*, with significant similarity to a *Phalaenopsis* Blume 'True Lady' *CHS* homolog (A.R. Kuehnle and M.M. Champagne, unpublished data). Gene-specific primers designed from the sequence data of *CHS-6* and *CHS-9* were used to isolate the 3' ends of each clone. We were able to obtain the 3' end of a chalcone synthase gene using the primers designed from the *CHS-6* clone. Surprisingly, this partial clone (*Den-CHS-11*) was only 70% similar to the *CHS-6* partial clone. A different set of primers designed from *Den-CHS-11* enabled isolation of a full-length *CHS* clone *Den-CHS-4* (GenBank accession No. AY741319).

The 3' RACE performed with primers designed from the *CHS-9* sequence resulted in a 500 bp fragment (Mudalige, 2003), with high similarity to bibenzyl synthase, a close relative of *CHS* that catalyzes a similar chemical reaction. Bibenzyl synthase participates in synthesizing a repertoire of chemical defense compounds known as phytoalexins and hence may be a valuable tool in disease resistance. A closer inspection of the *CHS* homologue from *Phalaenopsis* sp. 'True Lady' (Hsu et al., 1997) (GenBank No. U88077, protein ID AAB650941) with BLAST searching revealed this *Phalaenopsis* cDNA clone is indeed more similar to the bibenzyl synthase genes from *Bromheadia* and other *Phalaenopsis* hybrids than to *CHS* from these two orchids.

The most closely related *CHS* sequence, from the orchid *Bromheadia finlaysoniana*, is 81% and 94% identical to *Den-CHS-4* at the nucleotide and amino acid levels, respectively (Table 3). The closest nonorchid sequence is that of *Lilium hybrida* with 85% identity (93% similarity) in deduced amino acid sequences. Amino acid sequences of *CHS* from 25 other plant species have similarly high identity, indicating that chalcone synthase is highly conserved among different plant species (Table 3).

GENE EXPRESSION BY NORTHERN ANALYSES. Temporal expression of *DFR* and *CHS* in the developing inflorescences of UH503 showed that both transcripts are most abundant in medium sized flower buds (stages 3 and 4) with levels declining to a nearly undetectable level as the flowers open (Fig. 3). A similar temporal expression pattern was observed for *DFR* and *CHS* in the developing flowers of K1224 (Fig. 4). We have used high stringency washes to minimize any cross reactions of *Den-CHS-4* with other *CHS*-like mRNAs. The mRNA of the two other *Dendrobium* clones, *CHS-6* and *CHS-9*, hybridized at a higher molecular weight than *Den-CHS-4* (data not shown).

RT-PCR analysis has shown that *DFR* transcripts could be amplified from the total RNA of open flowers as well as unopened buds in both genotypes (Fig. 5). The *DFR* transcripts could not be amplified from vegetative parts such as leaves, pseudobulbs or roots in UH503 and K1224 (Fig. 5). Contrary to *DFR*, *CHS* transcripts could be amplified from all vegetative tissues except pseudobulbs.

Table 3. Comparison of deduced amino acid sequence of *Dendrobium* chalcone synthase clone (*Den-CHS-4*) with chalcone synthase from other plants at the amino acid level.

Plant species	Common name	GenBank accession no.	Length of the sequence	Identity (%) ^z
<i>Dendrobium</i> Sw. (UH503)	Dendrobium	-----	395	100
<i>Bromheadia finlaysonian</i> Rchb. f.	Seraman	AF007097	394	94
<i>Lilium</i> L. hybrid	Lily	AF169798	393	85
<i>Sorghum bicolor</i> (L.) Moench	Sorghum	AF152553	401	84
<i>Petunia xhybrida</i> hort. Ex Vilm	Petunia	X14597	389	84
<i>Zea mays</i> L.	Corn	X60205	400	83
<i>Oryza sativa</i> L.	Rice	X89859	398	82
<i>Vitis vinifera</i> L.	Grape	AB066275	393	85
<i>Torenia</i> L. hybrid	Torenia	AB012923	388	84
<i>Gerbera</i> L. hybrid	Gerbera	Z38096	398	83
<i>Nicotiana tabacum</i> L.	Tobacco	AF11783	389	83
<i>Camellia sinensis</i> (L.) Kuntze	Tea	D26593	389	83
<i>Catharanthus roseus</i> (L.) G. Don	Periwinkle	AJ131813	389	83
<i>Callistephus chinensis</i> (L.) Benth.	China aster	Z67988	398	82
<i>Petroselinum crispum</i> (Mill.) Nyman ex A.W. Hill	Parsley	V01538	398	83
<i>Daucus carota</i> L.	Carrot	AJ006780	397	82
<i>Solanum tuberosum</i> L.	Potato	U47740	389	83
<i>Glycine max</i> (L.) Merr.	Soybean	L07647	388	83
<i>Antirrhinum majus</i> L.	Snapdragon	X03710	390	82
<i>Rosa</i> L. hybrid cv. Cardinal	Rose	AB038246	389	82
<i>Lycopersicon esculentum</i> Mill.	Tomato	X55194	389	82
<i>Hordeum vulgare</i> L.	Barley	X58339	398	81
<i>Matthiola incana</i> (L.) R. Br.	Stock	AJ427536	394	81
<i>Brassica napus</i> L.	Kale, rape, rutabaga	AF076335	394	80
<i>Hydrangea macrophylla</i> (Thunb.) Ser.	Hydrangea	AF456448	389	80
<i>Arabidopsis thaliana</i> (L.) Heynh.	Arabidopsis	AY090376	395	80

^zSequences are aligned with CLUSTAL W (Thompson et al., 1994) program via San Diego Supercomputer Center Biology Workbench.

Fig. 3. Northern blots showing temporal expression of *DFR* and *CHS* mRNA in the developing inflorescence of *Dendrobium* x Jaquelyn Thomas 'Uniwai Prince' (UH503). Upper panel shows the unopened buds and flowers used in RNA extraction, sorted according to size and the position on raceme (smallest bud is at the end of raceme). Total RNA (10 µg/lane) was hybridized with ³²P-labeled denatured probes of *Den-DFR-1* or *Den-CHS-4*. rRNA bands shown in the bottom panel indicate the loading levels of RNA.

Discussion

We have successfully isolated full-length cDNA clones encoding *DFR* and *CHS* from *Dendrobium*. Analysis of deduced amino acid sequence of *Den-CHS-4* shows that it is highly conserved across 25 plant species. *Bromheadia* *CHS*, the clos-

est to *Dendrobium* sequence, demonstrates a 94% identity at the amino acid level. However, the presence of other enzymes with sequence similarity such as bibenzyl synthase, stilbene synthase and acridone synthase makes it difficult to identify the genes encoding *CHS* by sequence similarity alone (Helariutta et al., 1995; Liew et al., 1998b). Stilbene synthase uses exactly the

Fig. 4. Northern blots showing temporal expression of *DFR* and *CHS* in the developing inflorescence of *Dendrobium* x Icy Pink 'Sakura' (K1224). Total RNA (10 μ g/lane) from 10 bud and flower stages were hybridized with 32 P-labeled denatured cDNA probes of *Den-DFR-1* or *Den-CHS-4*. The bottom panel indicates the loading levels of RNA in ethidium bromide stained rRNA bands. 1 to 6 = flower buds from youngest to most mature; 7 to 10 = open flowers.

Fig. 5. Agarose gel of RT-PCR products showing the spatial expression of *DFR* and *CHS* in different tissues of *Dendrobium* hybrids UH503 and K1224. cDNA synthesized from 200 ng of total RNA was amplified with gene specific primers for *DFR* and *CHS* to detect the presence of transcripts in roots (R), pseudobulbs (P), leaves (L), open flowers (F) and Buds (B). M = molecular weight marker.

same substrates as CHS to form a different product, resveratrol, through a different cyclization reaction. Bibenzyl synthase utilizes *m*-hydroxyphenylpropionyl-CoA instead of coumaryl CoA to synthesize 3,3',5-trihydroxybibenzyl by a similar reaction to CHS. Genes encoding all three enzymes share a high homology due to the similarity of their catalysis.

Northern analysis supplements our sequence data, with the strong expression of *Den-CHS-4* in flower buds. This is consistent with the results of *Bromheadia* (Liew et al., 1998b). In many

plants *CHS* is represented by a multi-gene family, with different members of the gene family responding to different environmental stimuli such as UV light (Hirner et al., 2001; Jenkins et al., 2001; Loyall et al., 2000), low temperature (Hasegawa et al., 2001), pathogen attack (Seki et al., 1999), wounding and phytohormones (Tamari et al., 1995). Although the clone we have isolated seemed to be strongly expressed in floral buds, the presence of transcripts in all other tissues indicates that this enzyme is not exclusively expressed in floral tissue. This is not surprising since CHS is the primary enzyme that provides the building blocks of all flavonoids and is expected to be active in most tissues. In contrast, *DFR* expression is restricted only to floral tissue at a specific developmental stage of the flower bud.

Regulatory genes directly control CHS activity in many plants (reviewed in Martin and Gerats, 1993; Mol et al., 1998). Recessive alleles of the C2 gene of *Zea mays* (Dooner, 1983), *F* in *Matthiola incana* (L.) R. Br. (Spiribille and Forkmann, 1981), and *Niv* in *Antirrhinum majus* (Spiribille and Forkmann, 1982), are unable to activate the transcription of CHS gene and block the CHS activity and anthocyanin synthesis in the respective plant. These color regulatory genes encode flower specific bHLH, Myb and Myc type transcription factors that activate the transcription of anthocyanin biosynthetic genes (reviewed in Mol et al., 1998). Genetic studies in our classical breeding program suggest that both structural and regulatory genes may be involved in breeding of white *Dendrobium*, producing color by complementary gene action when certain whites are crossed with other whites (Kamemoto et al., 1999). We hypothesize that when a white flowered structural mutant is crossed with any other white flowered (different) structural mutant will result in complementation to form color; likewise any regulatory mutant with any other white regulatory mutant or a combination amongst them will lead to color complementation. The identification of the key genes *CHS* and *DFR* will now allow the breeder to screen white phenotypes for the expression profile of these biosynthetic genes, thereby determining the role these genes play in the complementary gene action of white x white crosses.

Sequence analysis revealed *Den-DFR-1* shares 83% identity with the *Cymbidium* *DFR* gene, whose corresponding enzyme does not efficiently reduce DHK to form orange pelargonidin (Johnson et al., 1999). A putative region that determines the substrate specificity of DFR based on sequence alignment of petunia, maize and snapdragon, was proposed by Beld et al. (1989). Johnson et al. (2001) further identified four unique amino acid residues from this region that determine the substrate specificity

of *Petunia* DFR. However, these four amino acids are not shared by *Cymbidium* and *Petunia*, despite their similarity in substrate specificity ruling out the possibility of these residues as the region of substrate specificity for the *Cymbidium* DFR. These four residues are conserved among all DHK accepting enzymes as well as the *Dendrobium* and *Cymbidium* DFR. Therefore, our results agree with the hypothesis that the substrate specificity of orchid DFR may be determined by another region of the enzyme (Johnson et al., 2001), different from that has been described in *Petunia*.

Most *Dendrobium* hybrids contain cyanidin and peonidin as their major anthocyanidin (Kuehnle et al., 1997). Two unique lines, *D. x* Icy Pink 'Sakura' (K1224) and *D. x* Waianae Blush, have pelargonidin as their major anthocyanidin and the amount of cyanidin is limited to only 2% of total anthocyanidin content. The pedigree of K1224 shows that this unique color phenotype occurred in the progeny of a cross between white and two-tone lavender parents (Kamemoto et al., 1999). It is theorized that such a color change could occur due to two reasons: 1) a mutation of the DFR enzyme could change its substrate specificity from dihydroquercetin to dihydrokaempferol, or 2) a mutation of the flavonoid 3'-hydroxylase (F3'H) enzyme could reduce the amount of available DHQ making DHK the most abundant substrate available for DFR. Sequence comparison of *DFR* from a typical lavender phenotype, UH503, with that of K1224 (pelargonidin accumulating line) has shown that the two sequences are identical, eliminating the substrate specificity of DFR as the reason for color difference. Chemical analysis of K1224 has detected only kaempferol derivatives as the major flavonols, with no detectable levels of 3'-hydroxylated quercetin derivatives, suggesting very low or no activity of F3'H in flowers (Kuehnle et al., 1997). Therefore, the most probable reason for the pelargonidin accumulation in K1224 is a mutation of F3'H leading to a reduction of F3'H activity, promoting *Dendrobium* DFR to accept DHK, the less preferred substrate, resulting in low levels of pelargonidin synthesis. Interestingly, spontaneous mutations in the F3'H gene in three species of *Ipomoea* L. confer reddish color due to the accumulation of pelargonidin instead of cyanidin in their flowers (Hoshino et al., 2003).

The spatial and temporal expression patterns of *Den-DFR-1* revealed that it is highly specific to floral tissue and coincides with the color development in buds. Therefore, the promoter region of *Den-DFR-1* may be an ideal candidate for a perianth-specific promoter for flower color manipulation. Furthermore, a potential for the increase of pelargonidin accumulation in *Dendrobium* flowers with the addition of a DFR enzyme that readily accepts DHK to form true orange flowers might provide an opportunity to increase the color range for this important ornamental.

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