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 dihidromyricetin (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 finlaysoniana* 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* × 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* × Jaquelyn Thomas ‘Uniwai Prince’ (UH503) and pale orange *Dendrobium* × 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 SuperScriptIII RNase H reverse transcriptase (Invitrogen,
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 mM DTT and 0.5 mM dNTPs, by incubating the reaction mixture at 42 °C for 50 min. Oligo dT (dT₁₆ or dT₃₀–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 with RedTag DNA polymerase (Sigma, St. Louis) in a 1X amplification buffer [0.01 mM Tris (pH 8.3), 0.05 mM 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.

**Gene amplification.**

Amplifications for each reaction. Denaturing of all PCR reactions were performed at 95 °C while extensions were carried out at 72 °C. 

PCR products were separated on agarose gels in 1X TAE buffer (25 mM Na₂EDTA). Amplifications for each reaction. Denaturing of all PCR reactions were performed at 95 °C while extensions were carried out at 72 °C. 

**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 | | |
| dT₁₆ | | |
| T₃₀–T₇ | | |
| Den-CHS-Rout | | |
| Degenerate primers | | |
| CHS-L | gcgaatca(c/t)ca(a/g)ca(a/g)gggt(t/c)tt(c/t)g-3’ | 40 °C for 1 min |
| CHS-R | gcgggattce(a/g)taia(a/g)macieca(c/t)t-3’ | 1.0 mM MgCl₂ |
| DFR-L | gcgaatgcgggcgcgctg(t/g)-3’ | 40 °C for 1 min |
| DFR-R | gcgaatgcgccggtggtgtg(c/t)t-3’ | 1.0 mM MgCl₂ |
| RACE primers | | |
| Den-CHS-6-L | gtcctcgcttcacactga-3’ | 55 °C for 1 min |
| T7 | | 2.0 mM MgCl₂ |
| Den-CHS-Rnest | ctagtagggtaaggtgac-3’ | 54 °C for 45 s |
| AAP | ggcacacgcgctagtaacgccccccgccccccgccccccgcc-3’ | First round 2.1 mM MgCl₂ |
| Den-CHS-5race2 | ggaagagttgaccaaatcag-3’ | Same PCR conditions as the first round amplification |
| AUAP | ggcacacgcgctagtaacgccccccgccccccgcc-3’ | |
| Den-DFR-L | ggggtttggtggcagtcgc-3’ | 55 °C for 1 min |
| T7 | | 1.5 mM MgCl₂ |
| Den-DFR-R | aagtcagttcagttcagtcag-3’ | 55 °C for 1 min |
| AAP | ggcacacgcgctagtaacgccccccgccccccgcc-3’ | First round 1.5 mM MgCl₂ |
| Den-DFR-R | aagtcagttcagttcagtcag-3’ | Same PCR conditions as the first round amplification |
| AUAP | ggcacacgcgctagtaacgccccccgccccccgcc-3’ | |
| RT-PCR primers | | |
| Den-DFR-S’ext | aactggctggtggagagag-3’ | 55 °C for 1 min |
| Den-DFR-3’utr | aacaacctagcatcttctt-3’ | 1.5 mM MgCl₂ |
| Den-CHS-PfxL | gcgaatggaagagatcag-3’ | 55 °C for 1 min |
| Den-CHS-PfxR | gcacagcgaatgcgcgcg-3’ | 1.5 mM MgCl₂ |

zi = 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 α-32P-ATP using random primer labeling (Prime a Gene Kit; Promega). Labeled product was cleaned using Elutip-D column (Schleicher and Shuell, 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 m NaOH). Hybridization and washing was done according to Church and Gilbert (1984) but using 0.25 M Na2HPO4·7H2O in hybridization buffer at pH 7.4. Membranes were hybridized with 32P labeled DNA probes at 60 °C overnight in hybridization buffer [7% SDS, 0.25 M Na2HPO4·7H2O (pH 7.4)]. Blots were washed twice at 60 °C for 10 min, using phosphate wash buffer [0.5% BSA, 1 mM Na2EDTA·2H2O, 5% SDS, 40 mM Na2HPO4·7H2O (pH 7.4)]. The final two washes were done at 60 °C for 20 min using phosphate buffer without BSA [1 mM Na2EDTA·2H2O, 0.25 M NaHPO4·7H2O (pH 7.4)]. We have used high stringency conditions to avoid any cross reaction with BSA [0.5% BSA, 1 mM Na2EDTA·2H2O, 5% SDS, 40 mM Na2HPO4·7H2O (pH 7.4)]. We chose a typical cyanidin accumulating line, Dendrobium x Jaqelyn 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 AAGAATGG which resembles the plant consensus sequence (Joshi, 1987). Apolynadenylation 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 kDa was deduced from the longest open reading frame (ORF) and was designated as Den-DFR-1.

Results

Cloning and sequence analysis of flavonoid biosynthetic genes. Dihydroflavonol 4-reductase. We chose a typical cyanidin accumulating line, Dendrobium x Jaqelyn 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 AAGAATGG which resembles the plant consensus sequence (Joshi, 1987). Apolynadenylation 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 kDa 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 flinlaysioniana 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 Jaqelyn 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 hybrid-DS5102; C. chinensis (L.) Benth.-Z67981; G. hybrid-Z17221; A. majus L.-X15556; T. hybrid-AB10293; L. esculentum Mill.-Z18277; P. x hybrida-AP233639; C. sinensis (L.) Kuntze-AB018686; G. max (L.) Merr.-AF67556; B. hybrida-B. hybrida- AF007096; C. x Jacobsen-CHS-9; L. sativa L.-AB003496; Z. mays L.-X05068; H. vulgare L.-S69616.

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 do 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 Jaques Thomas ‘Univai 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 hybrid 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.
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 closest 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

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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 (%) |
|---------------------------|------------------------|------------------------|--------------|
| *Dendrobium Sw.* (UH503)  | Dendrobium              | 395                    | 100          |
| *Bromheadia finlaysoniana* Rehb. f. | Seraman | 394 | 94 |
| *Lilium L.* hybrid        | Lily                    | AF169798               | 93           |
| *Sorghum bicolor* (L.) Moench | Sorghum | AF152553 | 401 | 84 |
| *Petunia x hybrida* hort. Ex Vilm | Petunia | X14597 | 389 | 84 |
| *Zea mays* L.             | Corn                    | X60205                 | 400          |
| *Oryza sativa* L.         | Rice                    | X89859                 | 398          |
| *Vitis vinifera* L.       | Grape                   | AB066275               | 393          |
| *Torenia L.* hybrid       | Torenia                 | AB012923               | 388          |
| *Gerbera L.* hybrid       | Gerbera                 | Z38096                 | 398          |
| *Nicotiana tabacum* L.    | Tobacco                 | AF11783                | 389          |
| *Camellia sinensis* (L.) Kuntze | Tea | D26593 | 389 | 83 |
| *Catharanthus roseus* (L.) G. Don | Periwinkle | AJ131813 | 389 | 83 |
| *Callistephus chinensis* (L.) Benth. | China aster | Z67988 | 398 | 82 |
| *Petroselinum crispum* (Mill.) Nyman ex A.W. Hill | Parsley | V01538 | 398 | 83 |
| *Daucus carota* L.        | Carrot                  | AJ006780               | 397          |
| *Solanum tuberosum* L.    | Potato                  | U47740                 | 389          |
| *Glycine max* (L.) Merr.  | Soybean                 | L07647                 | 388          |
| *Arthrinium majus* L.     | Snapdragon              | X03710                 | 390          |
| *Rosa L.* hybrid cv. Kardinal | Rose  | AB038246 | 389 | 82 |
| *Lycopersicon esculentum* Mill. | Tomato | X55194 | 389 | 82 |
| *Hordeum vulgare* L.      | Barley                  | X58339                 | 398          |
| *Matthiola incana* (L.) R. Br. | Stock | AJ427536 | 394 | 81 |
| *Brassica napus* L.       | Kale, rape, rutabaga    | AP076335               | 394          |
| *Hydrangea macrophylla* (Thunb.) Ser. | Hydrangea | AF456448 | 389 | 80 |
| *Arabidopsis thaliana* (L.) Heyn. | Arabidopsis | AY0990376 | 395 | 80 |

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

**Literature Cited**

Beld, M., C. Martin, H. Huits, A.R. Stuitje, and A.G.M. Gerats. 1989. Flavonoid synthesis in *Petunia hybrida*: Partial characterization of dihydroflavonol-4-reductase genes. Plant Mol. Biol. 13:491–502.

Bespalova, I.N., S. Adkins, and M. Burmeister. 1998. 3’ RACE: Skewed ratio of specific to general PCR primers improves yield and specificity. BioTechniques 24:575–577.

Champagne, M.M. and A.R. Kuehnle. 2000. An effective method for isolating RNA from tissues of *Dendrobium*. Lindleyana 15:156–168.

Church, G.M. and W. Gilbert. 1984. Genomic sequencing. Proc. Natl. Acad. Sci. 81:1991–1995.

Dooner, H.K. 1983. Coordinate genetic regulation of flavonoid biosynthetic enzymes in maize. Mol. Gen. Genet. 189:136–141.

Dooner, H.K., T.P. Robbins, and R.A. Jorgenson. 1991. Genetic and developmental control of anthocyanin biosynthesis. Ann. Rev. Genet. 25:173–199.
synthase, p. 233–284. In: K.W. Joen and J. Jarwick (eds.). International review of cytology—A survey of cell biology. Academic, New York.

Martin, C. and T. Gerats. 1993. Control of pigment biosynthesis genes during petal development. Plant Cell 5:1253–1264.

Mol, J., E. Grotewald, and R. Koes. 1998. How genes paint flowers and seeds. Trends Plant Sci. 3:212–217.

Mudalige, R.G. 2003. *Dendrobium* flower color: Histology and genetic manipulation. PhD diss., Univ. of Hawaii, Honolulu.

Mudalige, R.G. and A.R. Kuehnle. 2004. Orchid biotechnology in production and improvement. HortScience 39:11–17.

Saito, N., K. Toki, K. Uesato, A. Shigihara, and T. Honda. 1994. An acylated cyaniding glycoside from red-purple flowers of *Dendrobium*. Phytochemistry 37:245–248.

Seki, H., Y. Nagasugi, Y. Ichinose, T. Shiraishi, and T. Yamada. 1999. Changes in *in vivo* DNA-protein interactions in pea phenylalanine ammonia-lyase and chalcone synthase gene promoter induced by fungal signal molecules. Plant Cell Physiol. 40:88–95.

Spribille, R. and G. Forkmann. 1981. Genetic control of chalcone synthase activity in flowers of *Matthiola incana* R. Br. Z. Naturforsch 36c:619–624.

Spribille, R. and G. Forkmann. 1982. Genetic control of chalcone synthase activity in flowers of *Antirrhinum majus*. Phytochemistry 21:2231–2234.

Tamari, G., A. Borochov, R. Atzorn, and D. Weiss. 1995. Methyl jasmonate induces pigmentation and flavonoid gene expression in petunia corollas: A possible role in wound response. Physiol. Plant. 94:45–51.

Thompson, J.D., D.G. Higgins, and T.J. Gibson. 1994. CLUSTAL W: Improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res. 22:4673–4680.

Yong, H.H. and N.M. Chua. 1990. Isolation and characterization of genes involved in pigment biosynthesis of orchids, p. 265. In: D.G. Bonham and J. Kornahan (eds.). Proc. of the 13th World Orchid Conf. 1990. 13th WOC Proc Trust, Auckland, New Zealand (Abstr.).