DENDROBIUM FLOWER COLOR:

HISTOLOGY AND GENETIC MANIPULATION

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By

Rasika Geethanjali Mudalige

Dissertation Committee:

Adelheid R. Kuehnle, Chairperson
Robert E. Paull
Richard M. Manshardt
Chung-Shih Tang
David T. Webb

We certify that we have read this dissertation and that, in our opinion, it is satisfactory in scope and quality as a dissertation for the degree of Doctor of Philosophy in Horticulture.

DISSERTATION COMMITTEE

Adelheid R. Kuehle
Chairperson

Robert E. Paull

Richard Marchand

I dedicate this dissertation to my most wonderful mother, who taught me courage, resilience and dedication, and to the memory of my father, my biggest admirer and the strongest critic, who convinced me luck is in favor of those who strive the most.

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ABSTRACT

Dendrobium is the most important cut flower orchid in the world. Understanding the chemical, histological and molecular aspects of flower color is crucial for the development of breeding strategies for novel colors. The objectives of this research were to examine the histology of flower color, cloning and characterization of flavonoid biosynthetic genes, and metabolic engineering of *Dendrobium* flavonoid pathway to obtain new colors.

In *Dendrobium,* anthocyanins can be confined to a single layer of cells (epidermal or suepidermal) in pale flowers. More intensely colored flowers had anthocyanin in several cell layers. Striped patterns on the perianth were due to the restriction of pigment to cells surrounding the vascular bundles. Color perception is markedly influenced by the presence or absence of carotenoids.

Four types of epidermal cells were found in *Dendrobium*: flat, dome, elongated dome, and papillate. Epidermal cell shape and cell packing in the mesophyll affected the visual texture. Perianth parts with flat cells and a tightly packed mesophyll had a glossy texture, whereas dome cells and loosely packed mesophyll contributed a velvety texture. The labella in the majority of flowers examined had a complex epidermis with more than one epidermal cell shape, predominantly papillate epidermal cells.

We were able to isolate a full clone of *Dendrobium* dihydroflavonol 4-reductase (*dfr*), and partial clones of chalcone synthase (*chs*), flavonoid 3'-hydroxylase (*f3'h*) and flavonoid 3', 5'-hydroxylase (*f3'5'h*), from *Dendrobium*

Jaquelyn Thomas 'Uniwai Prince' (UH503). Expression data indicated that *dfr* and *chs* were expressed to the greatest degree in unopened buds. Amount of *f3'h* and *f3'5'h* mRNA was too small to detect. Southern analysis has shown that *f3'h* and *f3'5'h* is represented by 2 copies each in UH503. These clones will be extremely useful in future for flower color manipulation.

Two different color genes, *dfr* and *f3'5'h* from two non-orchid plants, under the constitutive promoter ubiquitin3, were inserted into *Dendrobium* Icy Pink 'Sakura' with the intention of creating orange-red and blue shades, which are absent in commercial *Dendrobium*. Presence of the transgene in two sets of transformants was confirmed by PCR. Expression of the transgene from a few plants was indicated by RT-PCR and northern analyses.

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LIST OF ABBREVIATIONS

Enzyme and chemical abbreviations

Anthocyanidin synthase ..... ANS
Chalcone isomerase ..... CHI
Chalcone reductase ..... CHR
Chalcone synthase ..... CHS
Dihydroflavonol 4-reductase ..... DFR
Dihydrokaempferol ..... *DHK*
Dihydromyricetin ..... *DHM*
Dihydroquercetin ..... *DHQ*
Firefly luciferase ..... LUC
Flavonoid 3'-hydroxylase ..... F3'H
Flavonoid 3', 5'-hydroxylase ..... F3'5'H
Flavonol synthase ..... FLS
Flavanone 3-hydroxylase F3H
Flavanone reductase ..... FNR
β Glucuronidase ..... GUS
Hygromycin phosphotransferase ..... HPT
Neomycin phosphotransferase ..... NPT II
Nopalene synthase ..... nos
Phenylalanine ammonia lyase ..... PAL
Terminal deoxyribonucleotide transferase ..... TdT
UDPG:flavonoid-3-*o*-glucosyl transferase ..... UFGT

Other technical terms and abbreviations

Base pairs ..... bp
Basic Local Alignment Search Tool ..... BLAST
Cauliflower mosaic virus ..... CaMV
Complementary DNA ..... cDNA
Cymbidium mosaic virus ..... CymMV
Deoxyribonucleotides ..... dNTP
Kilo base pairs (1000 base pairs) ..... kb
KiloDaltons ..... kD
Nanogram (10^{-9} of a gram) ..... ng
Open reading frame ..... ORF
Protocorm-like-bodies ..... PLBs
Phylogenetic Inference Package ..... PHYLIP
Polymerase Chain Reaction ..... PCR
Reverse Transcription – Polymease Chain Reaction ..... RT-PCR
Rapid Amplification of cDNA ends ..... RACE
Ubiquitin 3 promoter UBQ3

CHAPTER 1

ORCHID BIOTECHNOLOGY IN PRODUCTION AND IMPROVEMENT

1.1 INTRODUCTION

The Orchidaceae is one of the largest families of flowering plants, with several genera being used in cut flower and potted plant production. *Dendrobium*, a member of the Orchidaceae, is one of the largest genera with approximately 1400 species (Dressler, 1990) and many man-made hybrids. Classical breeding techniques have given rise to many commercially successful hybrids with attractive flower colors and forms, long vase life, fragrance, seasonality and desirable spray length. However, some colors such as orange-red and blue are missing from *Dendrobium* flower color spectrum (Kuehnle et al., 1997). Genetic modification of pigment biosynthesis through biotechnology is becoming an integral part of breeding new colors in ornamental plants (Davies et al., in Press). A few transgenic ornamentals (carnations) are already available in the market and are well received by consumers. A detailed understanding of the flower color is important in developing strategies to modify flower color.

The overall objectives of this research were to obtain a detailed knowledge on *Dendrobium* flower color by examining the histology of pigment distribution, cloning and characterization of flavonoid biosynthesis and to modify flower color through genetic manipulation of flavonoid synthesis through biotechnology.

The molecular biology of orchids, last reviewed in Kuehnle (1997), covered research in phylogeny and systematics, floral physiology, and plant breeding. Only ten orchid genes were cloned at that time. Since then, the total number of genes cloned from orchids has expanded considerably. The objective of this chapter is 1) to provide an overview of the function of some recently cloned genes, and 2) to review advances made in other applications of biotechnology in orchid production and improvement.

1.2 FUNCTIONS OF SOME CLONED ORCHID GENES

Approximately 70 genes have been cloned from seven orchid genera, namely *Dendrobium, Phalaenopsis, Doritaenopsis, Aranda, Bromheadia, Vanilla* and *Cymbidium.* These genes can be divided into seven categories based on their presumptive functions (Table 1.1). Here we review genes affecting flower induction, flower color, flower senescence, and disease resistance. Other genes have been cloned that affect primary metabolism, ovule development, cell division and cell structure (Table 1.1). At this time, the latter categories have had less direct impact on commercial orchid production and improvement.

1.2.1 Flower Induction

The ability to time flowering for holidays and to hasten flowering in otherwise slow-maturing orchids is of keen interest to growers of commercially cropped orchids. Several research groups are examining the genetic aspects of flower induction and the transition of the apical meristem from a vegetative shoot apical meristem (VSAM) through a transitional shoot apical meristem (TSAM) to a reproductive meristem.

Table 1.1. Isolated orchid genes or cDNA clones and their presumptive functions.

Gene or cDNA Designation	Orchid genus	Presumptive function	Reference and GenBank accession no.[a]
Flower Induction			
ovg2/DOH1	*Dendrobium*	Homeobox gene, class 1 knox genes	Yu and Goh, 2000a; Yu et al., 2000. AF100326, AJ276389
om1	x *Aranda*	Flower specific MADS box gene	Lu et al., 1993. X69107
otg7	*Dendrobium*	MADS box gene	Yu and Goh, 2000a. AF107588
DOMADS1, 2 and 3	*Dendrobium*	MADS-box genes in AP1/AGL9 subfamily	Yu and Goh, 2000b. AF198174
ovg27	*Dendrobium*	Transcriptional repressor	Yu and Goh, 2000a. AF100331
otg 16	*Dendrobium*	Casein kinase1, a homolog of serine/threonine protein kinase.	Yu and Goh, 2000a. AF107592
Cell Division and Cell Structure			
ovg14	*Dendrobium*	Transcriptional regulator of cell cycle regulators.	Yu and Goh, 2000a. AF100328
otg4	*Dendrobium*	Cell division control protein, a homolog of yeast NDA4	Yu and Goh, 2000a. AF107586
ovg30	*Dendrobium*	DNA binding protein, regulate cell cycle progression	Yu and Goh, 2000a. AF100333
ovg29	*Dendrobium*	Putative 21D7 protein. Degradation of cell cycle regulatory proteins	Yu and Goh, 2000a. AF100332
otg2	*Dendrobium*	Myosin heavy chain	Yu and Goh, 2000a. AF107585
P-ACT1	*Phalaenopsis*	Actin partial clone	Nadeau et al., 1996. U18102
ACT2	*Phalaenopsis*	Actin-like protein	Huang et al., 2000. AF246715
----------	*Phalaenopsis*	Profilin, an actin binding protein	Lee et al., 2000. AF126263
Flower Senescence			
Ds-ACS1, Ds-ACS2	x *Doritaenopsis*	ACC synthase	O'Neill et al., 1993. L07882, L07883
ACS2, ACS3	*Phalaenopsis*	ACC synthase	Bui and O'Neill, 1998. AF007213, AF00721
pOACS10/77	*Phalaenopsis*	ACC synthase	Do and Huang, 1998. Z77854
DCACS	*Dendrobium*	ACC synthase	Yang et al., 1996. U64031
OAO1	x *Doritaenopsis*	ACC oxidase	Nadeau et al., 1993. L07912
D-ACO2	x *Doritaenopsis*	ACC oxidase	Nadeau and O'Neill, 1995. L37103
pPEFEA	*Phalaenopsis*	ACC oxidase	Lee and Huang, 1995.
Petr1	*Phalaenopsis*	Homolog of ethylene receptor	Do et al., 1999. AF055894
Per1	*Phalaenopsis*	Ethylene response sensor	Chai et al., 1999. AF113541
POAC031/ PACO1	*Phalaenopsis*	Homolog of human Acyl-CoA oxidase	Do and Huang, 1996. U66299

Table 1.1. (Continued) Isolated orchid genes or cDNA clones and their presumptive functions.

Gene or cDNA Designation	Orchid genus	Presumptive function	Reference and GenBank accession no.[a]
Ovule Development			
o39	*Phalaenopsis*	Homeobox protein	Nadeau et al., 1996. U34743
o40/ CYP78A2	*Phalaenopsis*	Putative cytochrome p-450 monooxygenase	Nadeau et al., 1996. U34744
o108	*Phalaenopsis*	Homolog of SKP1, a cell cycle regulator gene.	Nadeau et al., 1996. U34745
o126	*Phalaenopsis*	Glycine rich cell wall structural protein	Nadeau et al., 1996. U34746
o141	*Phalaenopsis*	Cysteine proteinase	Nadeau et al., 1996. U34747
o138	*Phalaenopsis*	A new gene involved in embryo formation	Wang et al., 1999
o38	*Phalaenopsis*	Specific function unknown	O'Neill et al., 1996. U78100
Flower Color			
OCHS3,4, and 8	*Bromheadia finlaysoniana*	Chalcone synthase	Liew et al, 1998a. AF007097
pOCHS 01	*Phalaenopsis*	Chalcone synthase	Hsu et al., 1997. U88077
Fht/pCF1	*Bromheadia finlaysoniana*	Flavanone 3 hydroxylase	Liew et al., 1995. X89199
ODFR	*Bromheadia finlaysoniana*	Dihydroflavonol 4-reductase	Liew et al., 1998b. AF007096
Dfr gene	*Cymbidium* hybrid	Dihydroflavonol 4-reductase	Johnson et al., 1999. AF017451
AM 1-3	*Phalaenopsis equestris*	Gerenyl-Geranyl pyrophosphate synthase	Liu and Chen 1999.
Disease Defense/ Stress Response			
Pal/OPAL1	*Bromheadia finlaysoniana*	Putative phenyl alanine ammonia lyase (PAL).	Liew et al., 1996. X99997
ovg43	*Dendrobium*	PAL enzyme	Yu and Goh, 2000a. AF100336
pBibSy811, pBibSy211	*Phalaenopsis*	Bibenzyl synthase	Preisig-Müller et al., 1995. X79904, X79903
pBBS1	*Bromheadia finlaysoniana*	Bibenzyl synthase	Lim et al., 1999a. AJ131830
pAHH511	*Phalaenopsis*	S-adenosyl homocysteine hydrolase	Preisig-Müller et al., 1995. X79905
ovg23	*Dendrobium*	Putative copper chaperone of copper/zinc superoxide dismutase.	Yu and Goh, 2000a AF100330
Primary Metabolism			
otg11	*Dendrobium*	Putative fructose-bis-phosphate aldolase.	Yu and Goh, 2000a. AF107590
otg9	*Dendrobium*	Alternative oxidase	Yu and Goh, 2000a. AF107589
otg6	*Dendrobium*	NADH dehydrogenase intron region	Yu and Goh, 2000a. AF107587
ovg41	*Dendrobium*	formate dehydrogenase	Yu and Goh, 2000a. AF100335

Table 1.1. (Continued) Isolated orchid genes or cDNA clones and their presumptive functions.

Gene or cDNA Designation	Orchid genus	Presumptive function	Reference and GenBank accession no.[a]
Primary Metabolism (continued)			
Pepc gene, isoform 1-4	*Vanilla planifolia*	Phoshoenol pyruvate (PEP) carboxylase	Gehrig et al., 2002a. AJ312624, AJ312625, AJ312626, AJ312627
Ppc3	*Vanilla planifolia*	PEP carboxylase	Gehrig et al., 1999. AJ249988, AJ249989
Mdh1/pVM7	*Vanilla planifolia*	NADP malate dehydrogenase	Gehrig et al., 2002b. AJ306489
--------	*Phalaenopsis*	Putative chlorophyll a/b binding protein	Lee et al., 1999. AF133340
DcrIc1	*Dendrobium crumenatum*	Isocitrate lyase	Vellupillai et al., 1999., AF193815
ovg37	*Dendrobium*	Putative acyl carrier protein,	Yu and Goh 2000a. AF100334
-------	*Dendrobium crumenatum*	Vacuolar H^+ ATPase proteolipid subunit	Liew et al., 1999. AF193814
Other Genes			
ovg11, ovg15, ovg50, otg14	*Dendrobium*	Function unknown, involved in floral transition	Yu and Goh, 2000a. AF100327, AF100329, AF100337, AF100337, AF107591
cko1	*Dendrobium*	Cytokinin oxidase	Yang et al., 2001. AJ294542

[a] Some genes are directly submitted to the GenBank and the research is published later in journals. For these records, the year of publication does not reflect the date of submission to the GenBank. For the sequences that are only in GenBank, the date of publication reflects the published date on GenBank database.

Specifically, several orchid genes have been identified to be so-called homeobox and MADS box genes, which encode transcription factors and are preferentially expressed in vegetative or transitional stages (Table 1.1).

Homeobox genes. Five clones were identified by mRNA differential display as transcription factors involved in floral induction (Yu and Goh, 2000a). Of these, the partial cDNA clone *ovg2* showed significant similarity to homeobox genes, which are a universal group of transcription factors important in development. The full-length single copy clone, *DOH1*, which was subsequently isolated from a *Dendrobium* cDNA library of VSAM, shared considerable similarity to a class of homeobox genes known as class I *knox* (knotted-like) genes (Yu et al., 2000). Northern analysis and *in situ* hybridization revealed that *DOH1/ovg2* mRNA accumulated in all meristem-rich tissues and its expression was down regulated at the beginning of floral transition (Yu and Goh, 2000a; Yu et al., 2000). A *Dendrobium* hybrid transformed with sense constructs of *DOH1* gene driven by the Cauliflower Mosaic Virus (CaMV) 35S promoter revealed that the overexpression of *DOH1* completely suppresses shoot organization and development (Yu et al., 2000). Anti-sense plants formed multiple shoot meristems and precociously flowered. With these results, the authors concluded that *DOH1* is involved in controlling the formation and identity of the shoot apical meristem, and is thereby involved in maintaining the basic plant architecture.

Another floral homeotic gene was isolated from red and white *Phalaenopsis* using Amplified Fragment Length Polymorphism (Liu and Chen, 1999). A fragment

that was differentially amplified, AM1-3, has shown 87% similarity to the floral homeotic gene *AGL5* from *Arabidopsis thaliana* and 90% similarity to the *AG* gene of *Brassica napus* at the deduced amino acid sequence. *AGL5* is known to act downstream of *AG* in programming normal carpel and ovule development.

MADS box genes. MADS-box genes comprise another important family of transcription factors that regulate the transition of meristem identity. They are characterized by the presence of a highly conserved DNA binding domain (MADS-box) and a second conserved domain (K-box) involved in protein-protein interactions. The first flower-specific MADS-box gene from an orchid was isolated from the mature flower cDNA library of *Aranda* Deborah hybrid using an *agamous* cDNA from *Arabidopsis* as the probe (Lu et al., 1993). A single clone, *om1* was isolated, with an open reading frame (ORF) of 750 base pairs (bp) encoding a polypeptide of 250 amino acids. Expression of *om1* was limited to the petals and sepals of mature flowers but was not found in the column, early inflorescence, or floral buds, suggesting that *om1* might be playing an important role in petal development.

Another orchid MADS-box gene, *otg7*, expressed only in the TSAM was identified from *Dendrobium* (Yu and Goh, 2000a). Clone *otg7* was used later to isolate three new MADS box genes, *DOMADS1*, *DOMADS2*, and *DOMADS3*, all of which were expressed exclusively in floral tissues (Yu and Goh, 2000b). The *DOMADS1* gene was expressed early in the developing inflorescence and in all floral organ primordia. Its high expression in pollinaria suggested it could be an early

regulator of development of pollen mother cells. *DOMADS 2* was expressed early in the VSAM and increased in expression during the transition of the shoot apical meristem from the vegetative to reproductive phase, which might indicate a role in vegetative to reproductive phase change. The expression pattern of *DOMADS3*, as revealed by *in situ* hybridization, suggested that it might function as a regulatory factor in the formation of the TSAM and in the development of pedicel tissue.

Another transcription factor, *ovg27*, a homologue of a *Drosophila shuttle-craft* gene, was isolated from the VSAM of *Dendrobium* and appears to be important in maintaining the vegetative state of shoot apical meristem in plants (Yu and Goh, 2000a). Also, a cDNA clone, *otg16*, expressed only in the TSAM has shown significant similarity to an *Arabidopsis* casein kinase gene involved in protein phosphorylation and signal transduction (Yu and Goh, 2000a). The complete signal transduction pathway in floral induction of orchids is yet to be discovered.

1.2.2 Flower Color (Flavonoid Biosynthesis)

The magnificent spectrum of colors found in orchids is mainly due to the accumulation of anthocyanins in their flowers (Arditti and Fisch, 1977). Although carotenoids and chlorophylls contribute to flower color in many orchids, there are no detailed reports of cloned orchid genes associated with the biosynthesis of these compounds except for a brief mention of an amplified restriction fragment of *Phalaenopsis,* AM4-1, which showed similarity to the geranyl-geranyl pyrophosphate synthase gene (Liu and Chen, 1999). The biosynthesis of anthocyanins is well characterized in many ornamental plants. The synthesis of

flavonoids, including anthocyanins, occurs through the phenylpropanoid pathway, which starts with the stepwise condensation of three acetate units from malonyl-coA with 4-coumaroyl CoA to yield tetrahydroxychalcone (Heller and Forkmann, 1988). This reaction is catalyzed by the enzyme chalcone synthase (CHS), which is attractive to researchers for flower color manipulation since this is the first step in anthocyanin biosynthesis. Genes for other important enzymes cloned in orchids are flavanone 3-hydroxylase (F3H) and dihydroflavonol 4-reductase (DFR).

Chalcone synthase genes. The earliest report of the isolation of flower color genes in orchids appeared as an abstract only by Yong and Chua (1990), in which four cDNA clones encoding CHS were isolated from a cDNA library of a *Dendrobium* hybrid. These cDNA sequences are absent in all the searchable databases. Later, three *chs* cDNA clones, *OCHS3*, *OCHS4*, and *OCHS8*, isolated and characterized from the cDNA library of *Bromheadia finlaysoniana* flowers (Liew et al., 1998a), all were shown to contain a single ORF (Open Reading Frame) of 1185 bp (base pairs) encoding a protein of 42.9 kD. CHS is encoded by a small multigene family and is expressed in high levels in flower buds and other tissues containing anthocyanin such as leaves with pigmented edges and roots (Yong and Chua, 1990). Another cDNA clone encoding CHS, *pOCHS01*, was isolated from a *Phalaenopsis* hybrid (Hsu et al., 1997). However, a recent BLAST search revealed that this cDNA clone is in fact 98% similar to bibenzyl synthase clones of *Phalaenopsis* and *Bromheadia*. The authors noted that there are at least 10 other sequences that hybridized with *pOCHS01* in Southern analysis. It is difficult to

distinguish genes encoding CHS, bibenzyl synthase and stilbene synthase due to their close similarity at the nucleotide and amino acid sequence level since all three enzymes catalyze very similar chemical reactions.

Flavanone 3-hydroxylase genes. Another key enzyme in flavonoid biosynthesis, flavanone 3-hydroxylase (F3H), catalyzes the formation of dihydroflavonols from (2S)-flavanones in plants. A cDNA clone encoding F3H was isolated from *Bromheadia finlaysoniana,* with the intent of producing transgenic plants to study the role of this enzyme in orchid flower color (Liew et al., 1995). A full-length clone of 1393 bp encoding a protein of 41.5 kD with 464 residues was isolated. It shared 52-59% and 70-76% homology with other plants at the nucleotide and amino acid levels, respectively.

Dihydroflavonol 4-reductase genes. The conversion of dihydroflavonols such as dihydrokaempferol (*DHK*), dihydroquercetin (*DHQ*), and dihydromyricetin (*DHM*) to the corresponding leucoanthocyanidin is the first committed step in anthocyanin biosynthesis and is catalyzed by the enzyme, Dihydroflavonol 4-reductase (DFR). The color of the anthocyanin produced depends on the dihydroflavonol substrate that is reduced by DFR enzyme. The substrate specificity of DFR explains the absence of certain colors from some ornamental plants, e.g. *Petunia hybrida* which does not catalyze the conversion of *DHK* to orange-colored pelargonidin (Meyer et al., 1987). In orchids, *Cymbidiums* are noticeably devoid of orange colored flowers (Johnson et al., 1999) and only a few pelargonidin accumulating flowers are found in *Dendrobium* (Kuehnle et al., 1997). This

important feature of DFR makes this enzyme an important target for flower color manipulation through genetic engineering (Johan et al., 1995; Meyer et al., 1987).

In order to characterize DFR in orchids, full-length clones encoding DFR were isolated from petal cDNA libraries of *Bromheadia finlaysoniana* (Liew et al., 1998b) and a *Cymbidium* hybrid (Johnson et al., 1999). Southern analysis revealed that DFR is represented by a single copy gene in both orchid genera. A full-length DFR cDNA clone isolated from *Dendrobium* Jaquelyn Thomas 'Uniwai Prince' using a PCR-based technique showed 87% homology to *Bromheadia* and 84% homology to *Cymbidium* (Kuehnle and Mudalige, 2002). The substrate specificity of *Cymbidium* DFR was investigated by transforming a mutant *Petunia* line accumulating *DHK* as the major flavonol (Johnson et al., 1999). Chemical analysis of transformed lines revealed that *Cymbidium* DFR did not efficiently catalyze the reduction of *DHK* and preferred *DHQ* as a substrate, resulting in the production of pink cyanidin instead of orange pelargonidin. Introduction of a DFR from a plant that efficiently reduces *DHK* to pelargonidin (*Antirrhinum majus* and *Zea mays*) might be an attractive alternative to introduce orange color into orchids such as *Cymbidium* and *Dendrobium*.

1.2.3 Flower Senescence

In the absence of pollination, many orchid flowers are known to have a very long life, some lasting up to six months (Goh and Arditti, 1985). This long life span may increase the chance of pollination by a highly specific insect pollinator. Pollination triggers a series of physiological and developmental changes, known

collectively as "post-pollination syndrome," which mobilize nutrients out of non-essential parts (perianth) into essential parts, the developing ovule and ovary. The hormone ethylene plays a key role in the transduction of the pollination signal and the coordination of post-pollination development in many flowers including orchids (Porat et al., 1994a & 1994b). The production of ethylene starts with the synthesis of S-adenosyl methionine (SAM) catalyzed by SAM synthetase. Conversion of SAM to 1-aminocyclopropane-1-carboxylate (ACC) is catalyzed by ACC synthase, followed by the oxidation of ACC to ethylene by ACC oxidase. The rate- limiting step is believed to be the conversion of SAM to ACC. The physiology of post-pollination syndrome in terms of ethylene production and sensitivity in *Phalaenopsis* flowers (Nadeau et al., 1993; Porat et al., 1994b) and *Dendrobium* flowers (Porat el al., 1994a) has been well characterized. The exact nature of the primary pollination signal is still uncertain and speculated to be pollen-borne ACC or auxin (Bui and O'Neill, 1998; Wang et al., 2001).

ACC synthase genes. A total of six cDNA clones have been obtained from the pollinated flowers of different species of orchids (Table 1.1). Two ACC synthase clones, *Ds-ACS1* and *Ds-ACS2*, were isolated from *Doritaenopsis* (O'Neill et al., 1993). Northern analysis revealed that *Ds-ACS1* and *Ds-ACS2* mRNA accumulated in the stigma, ovary, and labellum (modified petal or lip) but not in the perianth (petals and sepals). Both ACC synthase genes were shown to respond to ethylene produced in the stigma in response to the primary pollination signal. Three cDNA clones involved in regulation of the initiation and propagation of ethylene

biosynthesis, *Phal-ACS1*, *Phal-ACS2*, and *Phal-ACS3* were isolated from stigma and ovary tissue of pollinated *Phalaenopsis* flowers (Bui and O'Neill, 1998). *Phal-ACS2* mRNA was apparent in stigma while *Phal-ACS3* mRNA accumulated in the ovary as a response to pollination. *Phal-ACS1* was believed to be involved in amplification and transmission of the pollination signal among different floral organs.

Yang et al. (1996) isolated a clone, *DCACS*, from the senescing flowers of *Dendrobium crumenatum* with the intent of extending flower longevity by producing transgenic plants over-expressing an antisense form of this gene. Two additional clones (*pOACS10* and *pOACS77*) were isolated from wilting petals of *Phalaenopsis* for the same purpose (Huang et al., 1996).

ACC oxidase genes. Three ACC oxidase genes have been reported as cloned (Table 1.1). Two genes, *OAO1* and *D-ACO2*, were isolated from cDNA libraries of gynoecia and the senescing petals of pollinated *Doritaenopsis* flowers, respectively (Nadeau et al., 1993; Nadeau and O'Neill, 1995). Another ACC oxidase cDNA clone was isolated from senescing petals of *Phalaenopsis* (Lee and Huang, 1995). ACC oxidase activity increased significantly in the stigma of *Phalaenopsis* after pollination due to *de novo* synthesis of ACC oxidase mRNA and presumably the protein (Nadeau et al., 1993). Unlike ACC synthase, ACC oxidase mRNA accumulated in the perianth in response to pollination. Emasculation, or treatment with auxin and/or ethylene was found to simulate the pollination signal, increasing the expression of the ACC oxidase gene. Application of ACC to petals of pollinated

flowers also increased the ACC oxidase expression in petals, suggesting that ACC itself also acts as a secondary signal in *Phalaenopsis*.

Other senescence-related genes. The binding of ethylene to its receptor is the first step in hormonal signal perception and transduction. Ethylene receptors are members of a multigene family, with highly conserved domains in the N-terminus of their deduced amino acid sequence and their putative histidine kinase domains (Do et al., 1999). A putative ethylene receptor cDNA clone was isolated from the pollination induced senescent petals of *Phalaenopsis* (Table 1.1). It encodes a 71 kD polypeptide reported to be most similar to banana and rice (monocotyledonous) ethylene receptors (75-79%) in deduced amino acid sequences. Another important gene associated with pollination-induced flower senescence, *PACO1*, was isolated from senescing *Phalaenopsis* petals (Do and Huang, 1996). It appeared to be a single copy gene whose expression is induced by exogenous application of ethylene. A genomic clone of *PACO1* was isolated from a genomic DNA library of *Phalaenopsis* with the intention to analyze the promoter sequence and activity of this gene in orchids (Do and Huang, 1997).

1.2.4 Disease Defense

The production of polyphenols is a defense response of plants towards wounding, microbial infection and stress. The first step in biosynthesis of polyphenols is the production of cinnamate from phenylalanine, catalyzed by the enzyme phenylalanine ammonia-lyase (PAL). A full length cDNA clone, *OPAL1*, was isolated from the orchid *Bromheadia finlaysoniana* in order to understand the

regulatory response of PAL towards wounding and fungal elicitors and to produce transgenic plants with variations in the level of PAL activity (Liew et al., 1996). The deduced amino acid sequence was 78-83% similar to PAL of other plant species. Another cDNA clone encoding PAL, *ovg 43*, was isolated from the vegetative shoot apical meristem (Yu and Goh, 2000a). Northern analysis indicated that expression of *ovg43* is down-regulated in the transition of the shoot apical meristem from a vegetative to a reproductive phase. However the exact reason for this down regulation and its function in floral transition is unknown.

Phytoalexin production genes. In many orchid plants, the invasion of fungal parasites induces the synthesis of specific chemical defense compounds, 9,10-dihydrophenantherenes, through the dihydrophenantherene pathway. A cDNA library prepared from mRNA of *Phalaenopsis* leaves inoculated with the conidia of *Botrytis cinerea* was used to isolate clones encoding for the enzyme bibenzyl synthase (Preisig-Müller et al., 1995). Two full-length cDNA clones encoding bibenzyl synthase, *pBIbSy811* and *pBibSy212*, were isolated, as well as a clone encoding s-adenosylhomocysteine hydrolase (*pAHH511*). The bibenzyl synthases, *pBibSy811* and *pBibSy212,* were verified by expression and production of enzymatically active recombinant proteins in *Escherichia coli* with the same substrate specificity *in vitro* as that of the plant enzyme. In young *Phalaenopsis* plants, fungal infection induced a transient increase in expression of both bibenzyl synthases and s-adenosylhomocysteine hydrolase by 100 fold with a peak at 20 hours after infection. A concomitant increase of PAL expression and the production of the

phytoalexin hircinol were also observed with fungal infection (Reinecke and Kindl, 1994). Another full-length cDNA clone encoding bibenzyl synthase was isolated from a petal cDNA library of *Bromheadia finlaysoniana* (Lim et al., 1999a, GenBank no. AJ131830).

1.3 ORCHID GENETIC ENGINEERING AND COMMERCIALLY APPLICABLE TRAITS

1.3.1 Methods of Transgenesis

Production of new varieties with improved characters such as new flower colors, flower morphology, plant stature, fragrance and increased vase-life is crucial for the growth of the floriculture industry. Plant breeding by sexual hybridization, selection of variants and polyploids has been an essential part of developing many improved traits and a large number of commercial orchid varieties. Interest in molecular breeding of orchids has increased considerably during the recent years to hasten selection and expand the gene pool available for improvement. Methods used in transformation of *Phalaenopsis, Dendrobium, Cymbidium* and *Calanthe* have been extensively reviewed by Kuehnle (1997) and Chen et al. (in Press). Here we discuss additional papers appearing since these reviews on gene transfer methods (Table 1.2) and commercially applicable traits (Table 1.3).

The most widely used method of gene transfer in orchids is particle bombardment, with protocorms and protocorm-like-bodies (PLBs) being the most popular choices as target tissues (Table 1.2).

Table 1.2. Examples of genetically engineered orchids: Means of transgenesis.

Method of Transformation Orchid genus	Gene(s) used[a]	Reference
Particle Bombardment		
Dendrobium protocorms	NPT II, GUS, Papaya ringspot virus coat protein	Kuehnle and Sugii, 1992
Dendrobium	GUS, NPT II	Nan and Kuehnle, 1995a
Dendrobium PLB[b]s	LUC	Chia et al., 1994
Dendrobium PLBs	LUC	Chia et al., 2001
Dendrobium protocorms	GUS, HPT	Yu et al., 1999
Cymbidium PLBs	GUS, NPT II	Yang et al., 1999
Cymbidium petals	GUS	Boase et al., 2001; Peters et al., 2001
Phalaenopsis x *Doritaenopsis*, *Phalaenopsis*	Bar, NPT II, GUS, Soybean β-1,3-endogluconase	Anzai and Tanaka, 2001
***Agrobacterium*-mediated**		
Doritaenopsis x *Phalaenopsis* callus	GUS, NPT II, HPT	Belarmino and Mii, 2000
Dendrobium	GUS, HPT	Nan et al., 1998
Pollen Tube Pathway		
Dendrobium	GUS, NPT II	Nan and Kuehnle, 1995b
Phalaenopsis	GUS, NPT II	Hsieh and Huang, 1995
Seed Imbibition; Electroporation		
Dendrobium	GUS, NPT II	Nan and Kuehnle, 1995b
Calanthe	GUS NPT II	Griesbach, 1994

[a] NPT II=neomycin phosphotransferase; GUS=β-glucuronidase; LUC=firefly luciferase; HPT=hygromycin phosphotransferase; Bar=Bialophos resistance gene.

[b] PLB=protocorm-like-bodies

Table 1.3. Examples of genetically engineered orchids: Potential commercial traits.

Purpose of Transformation	Genus	Gene(s) used	Reference
Disease Resistance			
Virus	*Dendrobium*	CymMV coat protein	Chia, 1999
Virus	*Dendrobium*	CymMV movement protein	Kuehnle, Hu, et al., (unpublished)
Fungus	*Phalaenopsis*	β-1,3-endoglucanase	Anzai and Tanaka, 2001
Bacteria, fungus	*Dendrobium*	Lytic peptides	Kuehnle et al., (unpublished)
Color Change			
Not specified	*Dendrobium* *Oncidium*	Sense and antisense CHS	Chia, 1999
Red/Orange	*Dendrobium*	*Antirrhinum* DFR	Kuehnle and Mudalige, 2002
Flower Wilting			
Long vase-life	*Dendrobium*	Sense and antisense ACC oxidase	Chia et al., 2001
Other Interests			
Function of DOH1	*Dendrobium*	Sense and antisense DOH1 gene	Yu et al., 2001
Glow in the dark	*Dendrobium*	Firefly luciferase	Chia et al., 2001

Evaluation of antibiotic selective agents for *Dendrobium* transformation indicated hygromycin to be excellent and geneticin also effective (Ong et al., 2000). The herbicide bialophos was proven to be a viable selection agent in transformation of *Brassia, Cattleya* and *Doritaenopsis* (Knapp et al., 2000).

Agrobacterium-mediated transformation is also proving applicable to some orchid genera (Table 1.2). Wounded PLBs of *Dendrobium* were inoculated with *Agrobacterium* strain LBA4301 harboring a binary vector system (Nan et al., 1998), containing the HPT (hygromycin phosphotransferase) gene under the control of a nopaline synthase (*nos*) promoter and the uidA gene encoding β-glucuronidase (GUS) enzyme under the CaMV 35S promoter. Southern analysis and PCR confirmed the successful transformation and recovery of transgenic plants. Parallel work has shown the presence of coniferyl alcohol as the virulence gene inducer in *Dendrobium* (Nan et al., 1997). *Agrobacterium*-mediated gene transfer was also used to elucidate the function of class 1 *knox* gene, *DOH1*, in *Dendrobium* (Table 1.3; Yu et al., 2001). Selectable marker NPTII under the control of a *nos* promoter, and sense as well as antisense *DOH1* genes under the CaMV 35S promoters were used to transform thin sections of *Dendrobium* PLBs. Molecular analysis by PCR and Southern hybridization revealed the successful integration of both sense and antisense constructs of the *DOH1* gene. Analysis of results and discussion of the role of *DOH1* gene in shoot apical meristem is discussed previously under floral induction.

1.3.2 Engineering Disease Resistance

Two common, readily transmitted viruses impacting cultivated orchids are Cymbidium mosaic virus (CymMV) and Odontoglosum ring spot virus (ORSV). Viral genomic RNA was isolated from a field strain of CymMV, and a cDNA clone encoding the viral coat protein (CP) was synthesized from it (Chia et al., 1992a). This cDNA clone driven by CaMV 35S promoter was used to transform the model plant *Nicotiana benthamiana* to test the effectiveness of the CP gene in conferring viral resistance. At a low level of inoculum, transformed *Nicotiana* plants showed resistance to CymMV by preventing systemic infection completely and reducing the viral titer in the inoculated leaf. A coat protein gene was also used in antisense orientation under the CaMV 35S promoter to transform *Nicotiana occidentalis* (Lim et al., 1999b). Transformed *Nicotiana* plants were found to be highly resistant to CymMV virus. However, *Dendrobium* plants transformed with the CymMV CP gene showed only partial resistance; plants were susceptible when challenged with high viral titer (Chia, 1999). In Hawaii, *Dendrobium* plants transformed with a CymMV cDNA clone encoding a mutated movement protein (mut 11) under the control of an *Arabidopsis* ubiquitin promoter were identified by PCR analysis and are being grown in the greenhouse for virus challenge (Kuehnle, Hu and Obsuwan, unpublished data).

Plants show various defense mechanisms to prevent infection from fungal pathogens. Release of elicitors from fungal cell walls by the β-1,3-endogluconase enzyme of the host plant is considered to be one of the earliest processes in disease

defense mechanisms such as phytoalexin production. Anzai and Tanaka (2001) produced transgenic *Phalaenopsis* plants transformed with soybean β-1,3-endogluconase (EG) gene under the control of a maize ubiquitin promoter. Two transformants showed incorporation of EG by Southern hybridization. However, western analysis failed to detect the EG enzyme in either of the transformants.

Another strategy in plant disease defense is to transform the plants with magainin genes to produce antibiotic polypeptides isolated from the skin of African clawed frog (*Xenopus laevis*). These peptides have the ability to insert into lipid bilayers of microbes, thereby disrupting the membrane integrity. *Dendrobium* plants have been transformed with lytic peptide genes (Kuehnle et al., in collaboration with Sanford Scientific; unpublished results) and await challenge with the most common orchid microbial pathogens.

1.3.3 Engineering Novel Flower Colors

Chia (1999) reported the use of sense and antisense orientations of chalcone synthase genes from *Dendrobium* and *Oncidium* to modify flower color of these orchids. The transformants are still at the vegetative stage and flower color changes are not determined yet (Chia, 2001; personal communication). PCR analysis indicates that we have introduced the *Antirrhinum* dihydroflavonol 4-reductase gene (courtesy of Dr. Cathie Martin, John Innes Centre, obtained via Crop and Food Research Institute, New Zealand), under the control of a ubiquitin promoter, into a *Dendrobium* hybrid believed to be deficient in flavonoid 3' hydroxylase activity (Kuehnle and Mudalige, 2002). We intend to increase and intensify the production

of pelargonidin to produce a new orange/red phenotype. Presence of the transgene is indicated by PCR analysis while RT-PCR confirmed the transcription of the foreign gene in vegetative tissue (Chapter 4). Currently, transformed plants are being grown in the greenhouse for color change and growth observations.

Chia et al. (2001) reported successful production of transgenic plants carrying the firefly luciferase gene. When these plants are sprayed with the substrate luciferin, the plants emit a soft light (glow-in-the-dark). This could be used as a commercially attractive novelty in orchids.

1.3.4 Engineering Increased Vase Life

As discussed under flower senescence, flower wilting of orchids is mediated by the phytohormone ethylene. An endogenous ACC oxidase gene cloned in sense and antisense orientation was used to transform *Dendrobium* plants to increase the vase-life of flowers (Chia, 1999). Transgenic plants showed normal morphology and growth without any aberrations due to the ACC oxidase insert. Authors reported a significant extension in vase-life in 1 out of 4 transformed lines. These transformants are currently being tested in commercial farms (Chia et al., 2001).

1.4 OTHER BIOTECHNOLOGY APPLICATIONS IN PRODUCTION AND IMPROVEMENT

1.4.1 Virus Detection

Cultivated orchid plants that are infected by CymMV and ORSV can remain symptomless, making it difficult to diagnose and control the spread of the virus. A simple and sensitive technique known as nucleic acid spot hybridization (NASH)

was used by Chia et al. (1990 & 1992b) for the detection of these two viruses in picogram amounts. Two cDNA probes specific to CymMV and ORSV were used for hybridization and detection of viral nucleic acid in crude extracts from infected plants spotted onto a membrane. Barry et al. (1996) used two other methods, immunocapture-PCR (IC-PCR) and direct binding PCR (DB-PCR), for the detection of these two viruses. In IC-PCR, microcentrifuge tubes coated with polyclonal antibodies developed against the virus were used to capture viral particles from a crude plant extract. PCR was carried out directly in these tubes to detect the captured viral particles. In DB-PCR, crude plant extracts were incubated in tubes without the antibody trapping and subsequent PCR was performed to identify viral particles directly bound to the tubes. Both methods were shown to be easy, rapid, and reliable, and to eliminate the necessity of time-consuming nucleic acid extractions.

1.4.2 Antiviral Strategies

A new molecular biology based technique known as population cloning was used to synthesize biologically active cDNA clones from a CymMV genomic RNA isolate (Yu and Wong, 1998). Three populations of overlapping RT-PCR products were ligated to form full-length cDNA clones. Infectious clones were identified by inoculating each clone onto *Nicotiana benthamiana* leaves. The infectivity of these clones was verified by western blotting, electron microscopy, and PCR techniques. The authors have successfully demonstrated the appropriateness of this technique in synthesizing biologically active viral clones from positive-strand RNA viruses.

1.4.3 Flower Color Gene Activity Test

There are several regulatory genes that can affect flower pigmentation. Three regulatory genes, C1, B, and R, from *Zea mays* are suggested to encode transcriptional activators of pigment biosynthetic genes. Effects of B and C1 genes on the biosynthesis of anthocyanin in *Doritis pulcherrima* were investigated using particle bombardment (Griesbach and Klein, 1993). Petals from three different floral phenotypes, purple, white (alba), and white with a light purple lip (albescent), were used in the study. The authors concluded that the alba phenotype was due to a mutation in an anthocyanin biosynthetic gene (structural gene) while the albescent form is due to a mutation in the anthocyanin regulatory genes. Testing of anthocyanin biosynthetic and regulatory genes using transient expression is an attractive rapid procedure for assessment of color gene complementation via sexual hybridization.

Transient expression was also used to assess the effectiveness of petal-specific promoters to drive the expression of reporter genes in *Cymbidium* petals compared to the most commonly used constitutive promoter, CaMV 35S (Peters et al., 2001). Promoter regions from the *chs* gene and ketolase (AKET) gene isolated from *Antirrhinum* and *Adonis aestivalis* respectively, were used to drive the beta-glucuronidase (GUS) reporter gene. Results indicated that the 35S-GUS construct produced the highest level of expression followed by CHS-GUS and AKET-GUS constructs.

Another short-term alternative to test color genes is to transform model crops with defined color mutations, such as transforming *Petunia* with orchid genes (Johnson et al., 1999; see section 1.2.2 on cloned color genes).

1.4.4 DNA Markers to Improve Breeding Strategy

Orchid breeding is a lengthy, time consuming process due to the long life cycle of many commercially important genera. In order to understand the genetic basis underlying the quantitative traits (QTL) and to select for these traits at an early seedling stage, it is important to develop a DNA-based marker system. Chia (1999) communicated the identification of RAPD markers (Random Amplified Polymorphic DNA) for inflorescence length, flower shape and size in *Dendrobium* orchids. With such marker systems, breeders will be able to identify the superior offspring and predict the productivity and flower qualities when the plants are still at the seedling stage.

Another DNA marker system known as DNA Amplification Fingerprinting (DAF) was developed by Chen et al. (1994) to identify different varieties developed at the Taiwan Sugar Corporation to protect the patent rights of their *Phalaenopsis* hybrids. Genomic DNA was amplified by PCR using different sets of primers to distinguish the clonal variation. In addition, authors discussed the importance of establishing a molecular marker system to identify important horticultural traits such as flower color, fragrance and disease resistance. DAF analysis was used to identify DNA markers associated with red flower color in *Doritis pulcherrima* and

Phalaenopsis equestris. In addition to plant breeding, DNA markers can also be used in plant systematics, evolution and determining phylogenetic relationships.

A PCR-based DNA fingerprinting technique, Amplified Restriction Fragment length Polymorphism (AFLP), has been successfully used in the identification of different cultivars of *Aranda* and *Mokara* hybrids (Lim et al., 1999c). In AFLP, the genomic DNA is digested with restriction enzymes and the fragments are ligated to adapters. These fragments are amplified with different sets of standard primers, resulting in a distinct banding pattern. Reproducibility of AFLP was tested with DNA from different tissues, and same tissues collected at different times, and the results were shown to be consistent. The authors also showed that the range of polymorphic bands/total number of bands is 10-11% among siblings while it is less than 1% among the somatic mutants of the same clone (somaclonal variation). Moreover, subtle phenotypic differences of flower color can be directly linked to some of the polymorphic bands in the AFLP pattern. Therefore, AFLP can be used to identify flower color in segregating progenies in the seedling stage.

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CHAPTER 2

PIGMENT DISTRIBUTION AND EPIDERMAL CELL SHAPE OF SOME *DENDROBIUM* SPECIES AND HYBRIDS

2.1 INTRODUCTION

Key components of color in flowers and fruits of vast majority of higher plants are flavonoids (including anthocyanins), located in vacuoles, and carotenoids and chlorophylls, located in plastids (Mol et al., 1998; Strack and Wray, 1993). Flowers acquire their characteristic hue due to these pigments in combination with other chemical and physical factors. The physical factors encompass spatial localization of pigments and the optical properties of petal epidermal cells (Kay et al., 1981; Mol et al., 1998). The most extensive study to date on pigment distribution and epidermal cell shape in petals was done by Kay et al. (1981) wherein 201 species in 61 families were surveyed. The majority (85 out of 97 species) of flowers surveyed have anthocyanins confined to the epidermis, and the majority of epidermal cells have their otherwise flat outer walls raised into a dome or a conical shape (Christensen and Hansen, 1988; Kay et al., 1981). These physical factors influence the role of the plant epidermis in pollinator attraction and in our perception of flower color and visual texture (Glover, 2000; Glover and Martin, 2000; Gorton and Vogelmann 1996; Noda et al., 1994). An understanding of the latter is valuable to flower breeding programs.

In Orchidaceae, data on pigment distribution and epidermal cell shape are limited to a few species. Matsui (1990 & 1992) showed in *Cattleya* Lindl. and allied

genera that the spatial localization of anthocyanins and carotenoids affected perception of color intensity and quality, while the height/width ratio of epidermal cells determined the visual texture. In *Dendrobium,* little is known regarding the histology of flower pigmentation. Flower epidermal cell shapes are reported only for the labella of *D. phalaenopsis* Fitzg., *D. superbiens* Fitzg., and *D. discolor* Lindl. (Vajrabhaya and Vajrabhaya, 1984). The objectives of this study were to describe distribution patterns of pigments in the *Dendrobium* perianth tissues, to identify epidermal cell shapes in *Dendrobium* flowers, and to relate these factors to the color and texture perceived.

2.2 MATERIALS AND METHODS

2.2.1 Plant Material

Inflorescences of 34 *Dendrobium* species and hybrids were selected to represent a range of flower colors and two visual textures. Six different color groups were examined: whites, pale/pastel colors, lavender/purple, striped, blue, and purple/yellow combinations. Two visual texture groups were selected: glossy and velvety. Detailed Royal Horticulture Society (RHS) color descriptions of most flowers can be found in Kamemoto et al. (1987) and Kuehnle et al. (1997). Inflorescences were harvested from plants maintained at the orchid greenhouse facility of the University of Hawaii at Manoa and submerged in tap water for 10-15 minutes. Stems were recut under water, bases were left immersed in water, and flowers were analyzed on the same day whenever possible or within two days.

2.2.2 Preparation of Tissue Sections and Analysis

A simple new method was used to make transverse sections. Perianth parts were separated and immersed in a series of polyethylene glycol (PEG) solutions (M.W. 8000, Sigma Chemical Co., St Louis, Mo.) ranging from 0.1% to 0.5% (w/v) prior to sectioning. The solution with an osmotic potential closest to that of the perianth cells was determined by observing the amount of plasmolysis in the intact cells of the sections. Our observations indicated that 0.25% PEG solution has an osmotic potential close to that of perianth cells. For flowers with large and abundant intercellular air spaces in the mesophyll, perianth parts were first infiltrated with 0.25% PEG under house vacuum (~600 Hg mm). Perianth parts sank to the bottom of the solution after successful infiltration. Vacuum infiltration was required for all flowers with loosely packed mesophyll, i.e., with large and numerous air spaces. It improved the sectioning substantially since replacement of air with PEG solution resulted in firmer tissues (hydrostatic pressure) and eliminated air bubbles trapped inside sections that otherwise hindered analysis. In contrast, *Dendrobium* petals with glossy texture were easier to section and did not need vacuum infiltration. Transverse sections (0.1-0.2 mm, 2-3 cell layers thick) from the middle of the petal, sepal, and the labellum of each flower were then taken using a sharp razor blade, mounted in 0.25% PEG solution, and examined using a Nikon Microphot SA microscope with Nikon SX 35A camera attachment. Pigment location (vacuole or plastid), color, distribution within the different cell layers, and adaxial epidermal cell shapes were noted for each sample. Sections found to have both anthocyanins and

carotenoids were sealed by applying a layer of nail varnish around the coverslip, and kept overnight until the purple color disappeared, to visualize the distribution of carotenoids without interference from anthocyanins.

2.3 RESULTS

2.3.1 Spatial Localization of Anthocyanins

As expected, white flowers lacked any colored cells in their tissues (Fig. 2.1A and B). Anthocyanins were confined to a single layer of cells, either to the epidermal or to the subepidermal layer, in petals and sepals of all light colored flowers grouped under the pale/pastel and white/purple mixed categories (Table 2.1; Fig. 2.1C-F). In contrast, anthocyanins were present either in two cell layers (epidermal and subepidermal layer), or in many cell layers of epidermis and mesophyll, in all more intensely colored flowers as grouped in the lavender/purple (Table 2.1; Fig. 2.1G-J) and the blue categories. Multiple layering of the colored cells likely increased the pathlength of light and thereby increased light absorption thus darkening the flower color. Intensely colored spherical pigment bodies similar to anthocyanoplasts described by Pecket and Small (1980), were seen within the pigmented cells of some examples in the blue category (*D.* Betty Goto). These authors also found anthocyanoplasts in the petals of the orchid *Laelia anceps* Lindl

Color intensity also was determined by distribution of pigment within a single tissue. For example, very light colored *D.* Jaquelyn Thomas cultivars 'Uniwai Blush', Y166-1 (Fig. 2.1C and D), and *D.* Neo-Hawaii had only a few colored cells in the cell layer beneath the adaxial epidermis (subepidermal layer).

Table 2.1. Pigment distribution in the epidermal, subepidermal, and mesophyll cells of the perianth of *Dendrobium.*

Color grouping Species or Hybrid (Univ. of Hawaii identification)	Pigment in perianth[a]								
	Petal			Sepal			Labellum		
	epi	sub	mes	epi	sub	mes	epi	sub	mes
White									
D. Jaquelyn Thomas 'Uniwai Mist' (UH800)	--	--	--	--	--	--	--	--	--
D. Nanae [white form, (UH1041)]	--	--	--	--	--	--	--	--	--
Pale/Pastel									
Blush									
D. Jaquelyn Thomas (Y166-1)	--	a	--	--	a	--	a	a	--
D. Jaquelyn Thomas 'Uniwai Blush' (UH44)	--	a	--	--	a	--	a	a	--
D. Neo-Hawaii (D452)	--	a	--	--	a	--	a	a	--
Light pink									
D. Icy Pink 'Sakura'	--	a	--	--	a	--	a	a	--
Lavender/purple									
Light lavender / two tone									
D. Jaquelyn Thomas (O580-4N)	a	a	a	a	a	a	a	a	a
Purple									
D. bigibbum Lindl. (K388)	a	a	a	a	a	a	a	a	a
D. Evie Nakasato	a	a	a	a	a	a	a	a	a
D. Jaquelyn Thomas 'Uniwai Prince' (UH 503)	a	a	a	a	a	a	a	a	a
D. Manoa Beauty (K404-2)	a	a	a	a	a	a	a	a	a
D. phalaenopsis var. *compactum* Fitzg. (D356-1)	--	a	a	--	a	a	a	a	--
Purple, dark									
D. Et-Roi x *D.* Takami Kodama (D499)	a	a	a g	a	a	a g	a	a	a
Reddish purple									
D. Ekapol 'Red' (D439 clone)	a	a	a	a	a	a	a	a	a
D. Jaquelyn Thomas (D168-12)	a c	a c	a c	a c	a c	a c	a c	a c	a c
D. Manoa Sunrise (K1520-26)	a c	a c	a c	a c	a c	a c	a c	a c	a c
D. Sabin (D430)	a	a	a	a	a	a	a	a	a
D. Sonia 'Red' (D438 clone)	a	a	a	a	a	a	a	a	a
Reddish purple, dark									
D. Jaquelyn Concert (D239)	a c	a c	a c	a c	a c	a c	a c	a c	a c
White/purple mixed									
D. Hiang Beauty [purple form]	--	a	--	--	a	--	a	a	--
D. Nanae (UH1041-42)	--	a	--	--	a	--	a	a	--

Table 2.1. (Continued) Pigment distribution in the epidermal, subepidermal, and mesophyll cells of the perianth of *Dendrobium.*

Color grouping Species or Hybrid (Univ. of Hawaii identification)	Pigment in perianth[a]								
	Petal			Sepal			Labellum		
	epi	sub	mes	epi	sub	mes	epi	sub	mes
Purple/yellow combinations									
Bronze									
D. Imelda Romualdez (D216)	a c	a c	c	a c	a c	c	a c	a c	c
Bronze with purple labellum									
D. Autumn Lace (K432)	c g	a c g	a c g	c g	a c g	a c g	a c g	a c g	c g
D. canaliculatum x *D. taurinum* (D428-13)	c g	a c g	c g	c g	a c g	c g	a c	a c	c
Brown									
D. lasianthera J.J. Sm. (K1007)	c	a c	a c	c	a c	a c	a c	a c	c
D. gouldii Rchb. F. (K1250-10)	c	a c	a c	c	a c	a c	a c	a c	c
Yellow with brown/red marking									
D. moschatum Sw.	c	c	c	c	c	c	a c	c	c
D. spectabile (Blume) Miq.	a c	a c	c	a c	a c	c	a c	a c	c
Yellow with purple labellum									
D. Sri Siam (D326-1)	c	c	c	c	c	c	a c	a c	c
(*D.* Jaquelyn Thomas x *D.* Field King) x *D.* May Neal 'Srisopon' (K527-24)	c	c	c	c	c	c	a c	a c	c
Blue									
D. Betty Goto (D500)	a	a	a	a	a	--	a	a	--
D. Pompadour 'Blue' x *D. gouldii* 'Blue' (K1164-7 seedling selection)	a	a	--	a	a	--	a	a	--
(K1164 – 18 seedling selection)	a	a	--	a	a	--	a	a	--
(K1164 – 22 seedling selection)	a	a	--	a	a	--	a	a	--
White with blue									
D. gouldii Rchb. f. (K280-6)	--	a	a	--	a	a	a	a	--
Striped									
Purple, striped									
D. Hirota x *D.* Candy Stripes (D465-2)	--	a	a	--	a	a	a	a	--
Orange with red stripes									
D. bullenianum Rchb. f.	c	c	a c	c	c	a c	a c	a c	a c

[a] epi = epidermal layer, sub = subepidermal layer, mes = mesophyll; a = anthocyanin, c = carotenoids, g = chlorophyll, -- = lack of pigment.

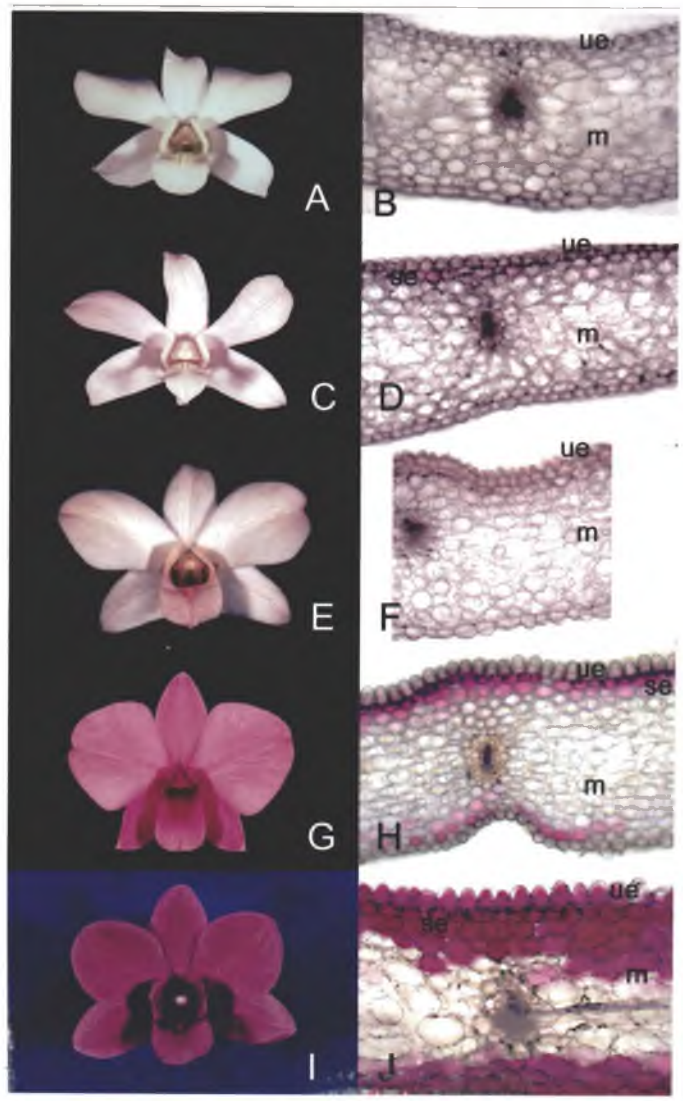

Figure 2.1. Front view and transverse sections (t.s.) of the perianths of *Dendrobium* flowers showing the location of pigmented cells. (A) and (B) *D.* Nanae (white form) with t.s. of petal x100. (C) and (D) *D.* Jaquelyn Thomas Y166-1 with t.s. of petal x100. (E) and (F) *D.* Icy Pink 'Sakura' with t. s. of labellum x100. (G) and (H) *D. phalaenopsis* var. *compactum* with t.s. of petal x100. (I) and (J) *D.* Et-Roi × *D.* Takami Kodama with t.s. of petal x100. ue = epidermis, se = subepidermal layer, m = mesophyll.

Whereas, the 'darker' pastel *D.* Icy Pink 'Sakura' had most of the subepidermal layer consisting of colored cells.

Labella of pale/pastel and white/purple flowers had darker coloration than the corresponding petals and sepals and contained anthocyanin in both the epidermal and subepidermal cells (Fig. 2.1E and F). A majority of the flowers (19/34) across all phenotypic classes shared a similar pigment distribution pattern in petals and sepals that differed from the labella (Table 2.1).

2.3.2 Flowers with Striped Patterns

Flowers with striped patterns generally had pigmentation confined to the epidermal or mesophyll cells close to the vascular bundles (Fig. 2.2). Anthocyanin-containing cells were restricted to the mesophyll surrounding the vascular bundles in *D.* Hirota x *D.* Candy Stripes (Fig. 2.2A and B) whereas they were confined to parts of the adaxial epidermis directly above vascular bundles in the labellum of *D. canaliculatum* x *D. taurinum* (Fig. 2.2C and D). In addition, carotenoids in the striped *D. bullenianum* (Fig. 2.2E and F) were found in the cytoplasm in unique reticulate structures, which are presently being investigated for detailed ultrastructure. One exception to stripes mimicking the venation pattern of the perianth is *D. spectabile*, where the striped pattern of the labellum was independent of venation.

2.3.3 Distribution of Pigments in Purple/Yellow Combinations

Flowers in this category were bronze, bronze with purple labellum, brown, yellow with brown markings, and yellow with purple labellum.

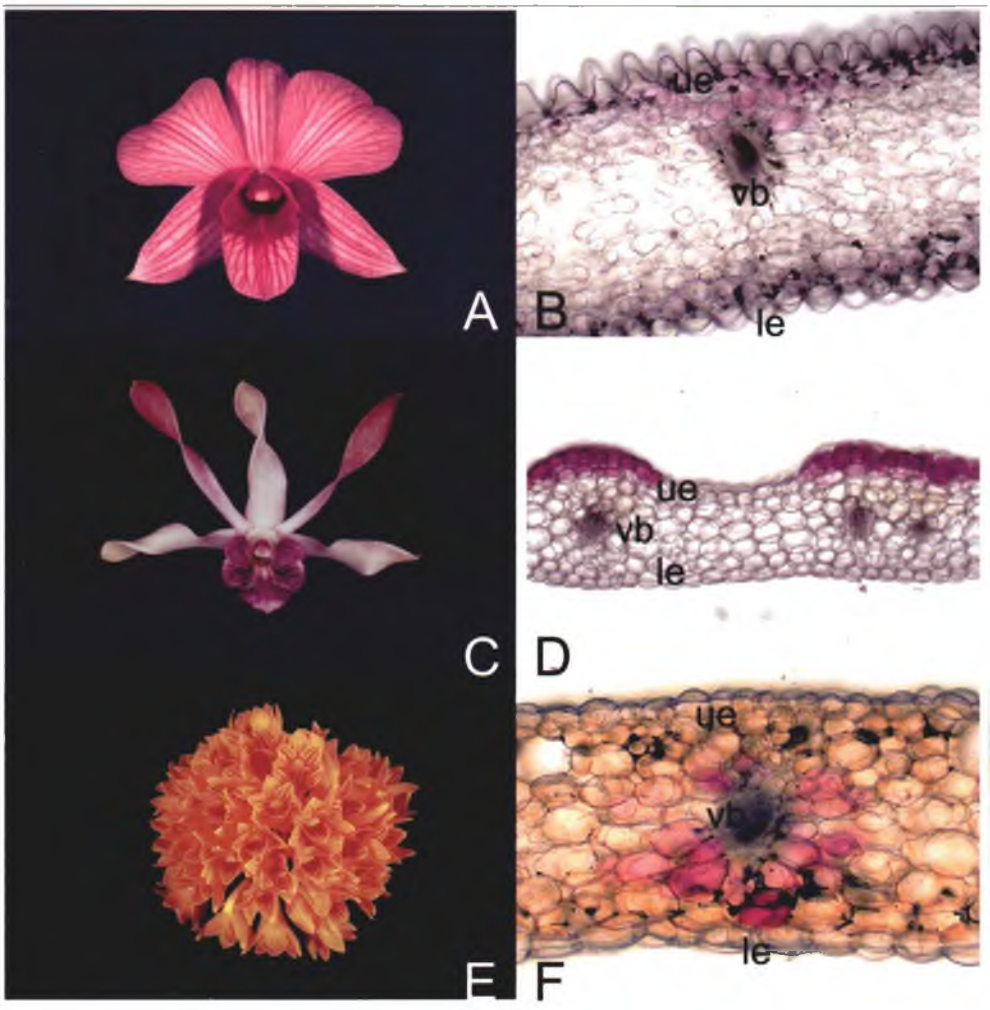

Figure 2.2. Localization of pigmented cells above and around the vascular bundle in *Dendrobium* flowers with striped patterns. (A) and (B) *D.* Hirota × *D.* Candy Stripe, with petal t.s. x100. (C) and (D) *D. canaliculatum* x *D. taurinum*, with labellar t.s. x100. (E) and (F) *D. bullenianum*, with petal t.s. x100. ue = upper epidermis, vb = vascular bundle, m = mesophyll.

Carotenoids were present throughout the epidermal, subepidermal, and mesophyll layers in all color groups. In contrast, anthocyanin localization varied considerably within the purple/yellow category: only in the epidermis, as in labellum of *D.* Sri Siam and (*D.* Jaquelyn Thomas x *D.* Field King) x *D.* May Neal 'Srisopon' (Table 2.1; Fig. 2.3A and B) or to the mesophyll as in petals and sepals of *D. gouldii* (Table 2.1; Fig. 2.3C and D). Anthocyanins were also located in the epidermis and subepidermal layer in bronze colored *D.* Imelda Romualdez, (Fig. 2.3E and F) or across all cell layers (Fig. 2.3G and H) in reddish purple *D.* Jaquelyn Concert.

2.3.4. Cell Shapes of the Upper Epidermis

Four types of epidermal cell shapes were found in *Dendrobium* flowers: flat, dome (height/width <1.2), elongated dome (height/width > 1.2) and papillate (Table 2.2; Fig. 2.4). Of all the flowers we examined, those belonging to section *Spatulata* and their hybrids (*D. gouldii*, *D. lasianthera*, *D. canaliculatum* x *D. taurinum*, *D.* Autumn Lace and *D.* Betty Goto) had flat epidermal cells (Table 2.2; Fig. 2.4A) in the petals and sepals. Cuticles of glossy textured flowers were twice as thick as those of velvety textured flowers (Fig. 2.4A). All *D.* Jaquelyn Thomas-type hybrids (section *Spatulata* x section *Phalaenanthe*) such as 'Uniwai Blush', 'Uniwai Prince', O580-4N, D168-12,'Uniwai Mist', *D.* Neo-Hawaii, *D.* Manoa Sunrise, and *D.* Jaquelyn Concert had dome-shaped epidermal cells (Table 2.2; Fig. 2.4B) in their petals and sepals. In addition, *D.* Hiang Beauty, *D.* Icy Pink 'Sakura', *D.* Nanae (white and purple-white forms), and *D.* Evie Nakasato also had dome-shaped cells in the petals and sepals (Table 2.2).

Table 2.2. Shapes of the adaxial epidermal cells of the perianth of *Dendrobium.*

Species or Hybrid (Univ. of Hawaii identification)	Adaxial epidermal cell shape[a] Petal	Sepal	Labellum
Glossy texture			
D. Autumn Lace (K432)	f	f	f
D. Betty Goto (D500)	f	f	p
D. canaliculatum x *D. taurinum* (K428-13)	f	f	f
D. *gouldii* blue phenotype (K280-6)	f	f	f
D. gouldii brown phenotype (K1250-10)	f	f	f
D. Imelda Romualdez (D216)	f	f	p
(*D.* Jaquelyn Thomas x *D.* Field King) x *D.* May Neal 'Srisopon' (K527-24)	f	f	f, p
D. lasianthera (K1007)	f	f	f
D. moschatum	f	f	f, p, h[b]
D. Sri Siam (D326-1)	f	f	f, p
Velvety texture			
D. bigibbum (K388)	e	e	e, p
D. bullenianum	f, d	f, d	f, d
D. Ekapol 'Red' (D439)	e	e	p, h
D. Evie Nakasato	d	d	p
D. Et-Roi x *D.* Takami Kodama (D499)	e	d	e, p, h
D. Hiang Beauty	d	d	d, p
D. Hirota x *D.* Candy Stripes (D465-2)	e	e, d	e, p
D. Icy Pink 'Sakura' (K 1224)	d	d	d, p
D. Jaquelyn Concert (D239)	d	d	d, p
D. Jaquelyn Thomas (O580-4N)	d	d	f, d, p
D. Jaquelyn Thomas (Y166-1)	d	d	f, d, p
D. Jaquelyn Thomas (D168-12)	d	d	f, d, p
D. Jaquelyn Thomas 'Uniwai Blush' (UH44)	d	d	f, p
D. Jaquelyn Thomas 'Uniwai Mist' (UH800)	d	d	d, p
D. Jaquelyn Thomas 'Uniwai Prince' (UH503)	d	d	f, d, p
D. Manoa Beauty (K404-2)	e	e	e, p, h
D. Manoa Sunrise (K1520-26)	d	d	f, d, p
D. Nanae purple & white phenotypes (UH1041)	d	d	d, p
D. Neo-Hawaii (D452)	d	d	f, p
D. phalaenopsis var. *compactum* (D356-1)	e	e, d	e, p
D. Pompadour 'Blue' x *D. gouldii* 'Blue' (K 1164-7 seedling selection)	f, d	f, d	f, d
(K 1164 – 18 seedling selection)	d	d	f, d
(K 1164 – 22.seedling selection)	d	f, d	f, d
D. Sabin (D430)	e	e	e, p, h
D. Sonia 'Red' (D438)	e, d	d	e, p, h
D. spectabile	d, e	f, d	d,e

[a] f = flat, d = dome, e = elongated dome, p = papillate, h = epidermal hair

[b] multicellular hair

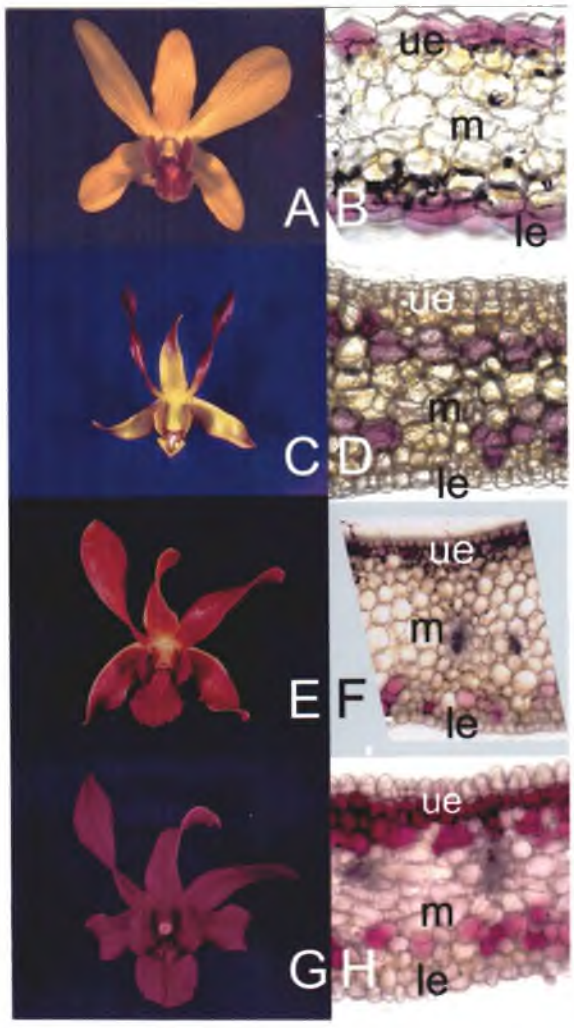

Figure 2.3. Effect of relative location of anthocyanin in combination with yellow carotenoids on perception of flower color in *Dendrobium*. Front view of flowers and t.s. of the perianth showing the perceived color and the relative location of yellow carotenoids (in plastids) and purple anthocyanins (in vacuoles) in perianth tissues. (A) and (B) yellow flower of (*D.* Jaquelyn Thomas x *D.* Field King) x *D.* May Neal 'Srisopon' showing purple labellum and its section x160. (C) and (D) brown flower of *D. gouldii*, with petal section x100. (E) and (F) bronze colored *D.* Imelda Romualdez, with petal section x160. (G) and (H) reddish purple *D.* Jaquelyn Concert, with sepal section x100. ue = upper epidermis, m = mesophyll, le = lower epidermis.

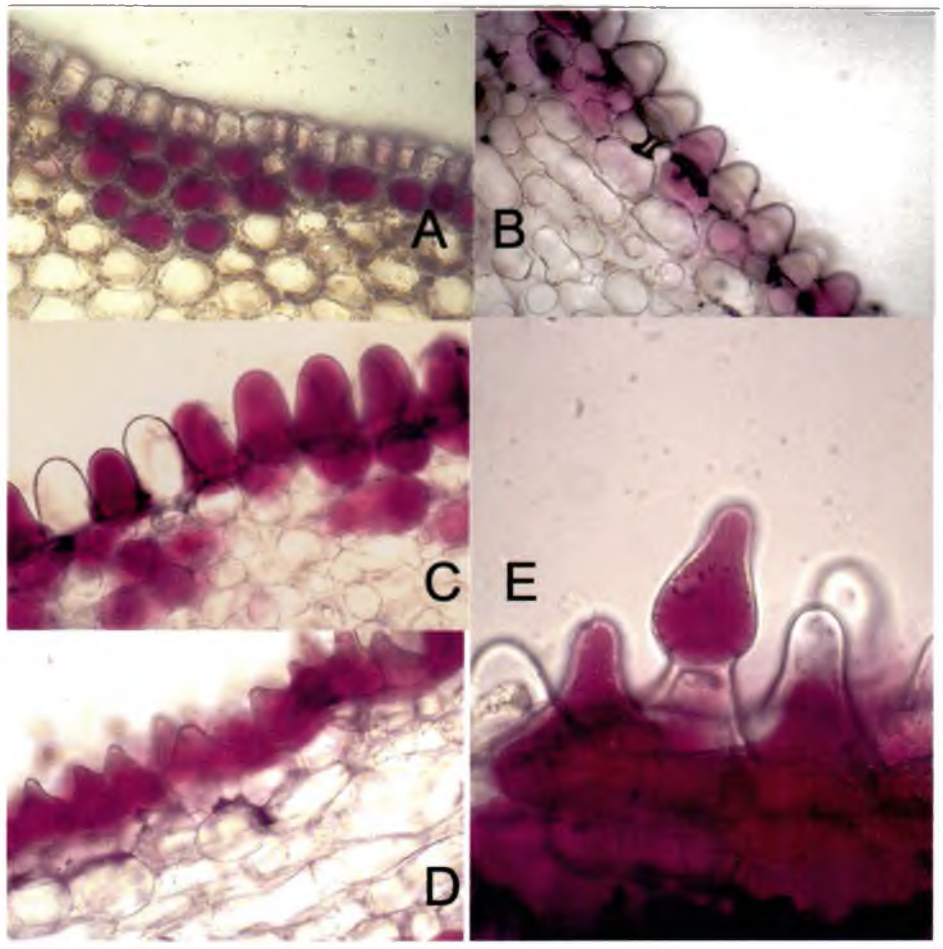

Figure 2.4. Various shapes of adaxial epidermal cells found in the perianth of *Dendrobium*. (A) flat epidermal cells of glossy textured *D. lasianthera* (Section *Spatulata*) x400. (B) domed cells of velvety textured *D.* Jaquelyn Thomas O580-4N (Section *Spatulata* x Section *Phalaenanthe* x200. (C) elongated dome shape of velvety textured *D. bigibbum* (Section *Phalaenanthe*) x200. (D) papillate cells of *D.* Betty Goto (labellum section) x200. (E) epidermal hair on the labellum of *D.* Sabin x400.

Dendrobium phalaenopsis var. *compactum* and *D. bigibbum* (section *Phalaenanthe*) had elongated dome-shaped epidermal cells (Table 2.2; Fig. 2.4C)

Height of epidermal cells appears to be more uniform in petals compared to sepals. Height/width ratios of the sepal epidermal cells varied considerably (0.8 to1.2); that made it difficult to differentiate between dome and the elongated dome shapes in some flowers. An epidermal hair comprised of two cells, a cylindrical base and a conical head, was seen in the labellar sections of the following: *D.* Sonia 'Red', *D.* Ekapol 'Red', *D.* Sabin (Fig 2.4D), *D.* Et-Roi x *D.* Takami Kodama and *D.* Manoa Beauty (Table 2.2), while *D. moschatum* was the only exception with multicellular hairs on its labellum.

Labella in *Dendrobium* appear more complex than petals and sepals in terms of epidermal cell shape. Various shapes of epidermal cells can be observed in a single labellum. Epidermal cells towards the distal end of the labellum were often similar to those found on the petals (Table 2.2). When sectioned across the ridges or keels, papillate cells were the most common cell type found in most flowers examined (Table 2.2; Fig. 2.4E). Papillate cells of labella also had visible surface striations on their cuticles similar to those observed by Vajrabhaya and Vajrabhaya (1984).

2.4 DISCUSSION

Distribution of anthocyanins appeared to be much more variable in *Dendrobium* compared to other plant genera. Kay et al. (1981) found that the anthocyanins were confined to the epidermis in the petals of a majority of species surveyed (85 out of 97) while they were confined to the mesophyll only in a few

species of Boraginaceae. A few species (7/97) had anthocyanins in epidermis as well as mesophyll. In *Dendrobium*, anthocyanin distribution varied considerably within the genus. It can be confined to the epidermis or mesophyll in some species/hybrids while it is distributed throughout all cell layers in others. However, pigment distribution pattern in *Dendrobium* is consistent with some of the genera previously examined in the Orchidaceae. In most *Cattleya* species, anthocyanin was confined to the mesopyhyll, while *Cattleya* with splashed petals, and some species of *Laelia* with intensely colored flowers, had anthocyanin in the epidermis as well as mesophyll (Matsui, 1990). Anthocyanin is confined to the epidermis in *Sophronitis* and *Sophronitella* species (Matsui, 1990). This unusual variation of pigment distribution patterns in orchids might be a result of an extremely large number of man-made interspecific and intergeneric hybrids.

Combinations of yellow carotenoids and purple anthocyanins result in perceived flower colors of brown, bronze, red, or wine (Griesbach, 1984; Vogelpoel, 1990). Griesbach (1984) attributed the differences in color perception to the differences in relative concentrations of yellow and purple pigments. According to this explanation, high ratios of yellow carotenoid/ purple anthocyanin give a brown color and the opposite gives red colors, while equal concentration of the two give a bronze color to the flowers. Our observations indicate that this explanation might be too simplistic for *Dendrobium*, for which the accumulated purple pigment is predominantly cyanidin glycosides (Kuehnle et al., 1997). The relative location of yellow and purple pigments may be as important as relative concentration in determining flower color. A good example of this is found in *D. bullenianum* where red striping resulted from the combination of orange carotenoids with mesophyll-restricted purple anthocyanins. This phenomenon has been observed in other orchids

such as *Laelia milleri* Blumensch. ex Pabst, *Sophronitis coccinea* (Lindl.) Rchb. f., and *Broughtonia sanguinea* (Sw.) R. Br., in which red color was also ascribed to the coexistence of carotenoids and anthocyanins (Matsui, 1990). This emphasizes the fact that the relative location of the two pigments is critically important in determining the shade of color.

A survey of epidermal patterns in the Angiosperms revealed two main types: tabular (flat) and papillose (Christensen and Hansen, 1998). In papillose type, the outer epidermal cell wall is raised above the epidermis into a conical, dome or papillate shape. Papillose petal surfaces are predominant among insect pollinated flowers (Christensen and Hansen, 1998). Shape and size of the epidermal cells, especially the ratio of height/width, is known to affect flower texture (Matsui, 1990). In *Cattleya,* glossy flowers have square-shaped epidermal cells, whereas velvety textured petals have 'deltate' epidermal cells with greater height/width ratios. Apparently in *Dendrobium,* both the epidermal cell shape and the nature of the aerenchymatous layer (mesophyll layer) influence the visual texture of flowers. Square cells with a thicker smooth cuticle and tightly packed mesophyll with few air spaces gave a glossy texture to the perianth, while domed cells with a thin cuticle and loosely packed mesophyll with large and numerous air spaces produced a velvety texture. Papillate cells seem to be common in a number of orchids and occur in *Anacamptis pyramidalis* (L.), *Dactylorhiza fuchsii* (Druce) Soó, (Kay et al., 1981), and many species of *Cattleya* and *Laelia* (Matsui, 1990). The preponderance of *Dendrobium* hybrids containing velvety textured flowers suggests that their dome-shaped cells are perhaps more attractive to the human eye due to the enhanced absorption of light by dome shaped cells.

Both papillate and dome-shaped cells absorb light over a greater part of their surfaces when compared to flat outer surfaces of square epidermal cells. In addition, surface striations perhaps function as an additional light-trapping device by reducing the surface reflection while smooth un-striated cuticles contributed towards strong surface reflections as suggested by Kay et al. (1981). Endress (1994) stated that intercellular spaces in the mesophyll influence the light reflection in addition to organ surface structures. Our observation on the influence of epidermal cell shape and mesophyll packing upon visual texture is confirmed by these explanations. Christensen and Hansen (1998) also revealed that the shape of papillae in a single petal can vary, with more distinct papillae towards the distal end and less pronounced papillose cells towards the proximal end. The authors speculate such zonation can act as guides to the insect. Our data on labella showed such differences in epidermal cell shape and reinforced the presumed function of the labellum as a specialized petal for insect landing and guidance. Quantitative data on floral flavonoids in *Dendrobium* showed that the labella contained two to four times more anthocyanins than the sepals on a fresh weight basis (Kuehnle et al., 1997). Presence of papillate cells, surface striations, the different pigment distribution patterns and higher pigment quantities, contribute to the darker color of labella seen in many flowers when compared to the color of other perianth parts.

Many chemical factors such as type of pigment, their combinations, vacuolar pH, and co-pigments affect flower color. The types of flavonoids and carotenoids found in *Dendrobium* species and hybrids are already documented (Kuehnle et al., 1997; Thammasiri et al., 1986). This study complements the earlier work by revealing the physical factors (spatial localization of pigments, epidermal cell shape) and their effects on color perception. Since methodology for successful genetic

transformation of *Dendrobium* is available (Nan and Kuehnle, 1995), understanding of the detailed spatial distribution of pigments will facilitate directed engineering of flower colors by permitting more sophisticated approaches in selection of appropriate target phenotypes and tissues for the expression of pigment-modifying genes. For example, a petal specific epidermal promoter will be appropriate in color manipulation of pastel/pale flowers while a constitutive promoter is more appropriate for the dark purple phenotypes. Isolation of color-modifying genes from *Dendrobium* flowers and the study of spatial and temporal regulation of these genes will bring important breakthroughs for development of novel phenotypes, while further research on inheritance of distribution pattern and epidermal cell shapes will benefit classical breeding programs.

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CHAPTER 3

CLONING AND CHARACTERIZATON OF ANTHOCYANIN BIOSYNTHETIC GENES FROM *DENDROBIUM*

3.1 INTRODUCTION

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 (reviewed in 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 the anthocyanin biosynthesis are well characterized in crops such as petunia, maize and snapdragon (reviewed by Davies and Schwinn, 1997; Dooner et al., 1991; Forkmann, 1993; Martin and Gerats, 1993; Mol et al., 1998).

3.1.1 Flavonoid Pathway

Flavonoids are synthesized via a complex biochemical pathway known as the phenylpropanoid pathway (Fig. 3.1). The first committed step of flavonoid biosynthesis the condensation of 3 molecules of malonyl-CoA with a single molecule of 4-coumaroyl-CoA to form chalcone, catalyzed by the enzyme chalcone synthase (CHS). Chalcone is readily isomerized to naringenin, a colorless flavonone, by chalcone isomerase (CHI). Naringenin is subsequently hydroxylated by flavanone 3-hydroxylase (F3H) to form the key intermediate dihydrokaempferol (*DHK*). *DHK* can be hydroxylated at the 3' position of the B ring to form dihydroquercetin (*DHQ*) or at both the 3' and 5' positions to form dihydromyricetin (*DHM*).

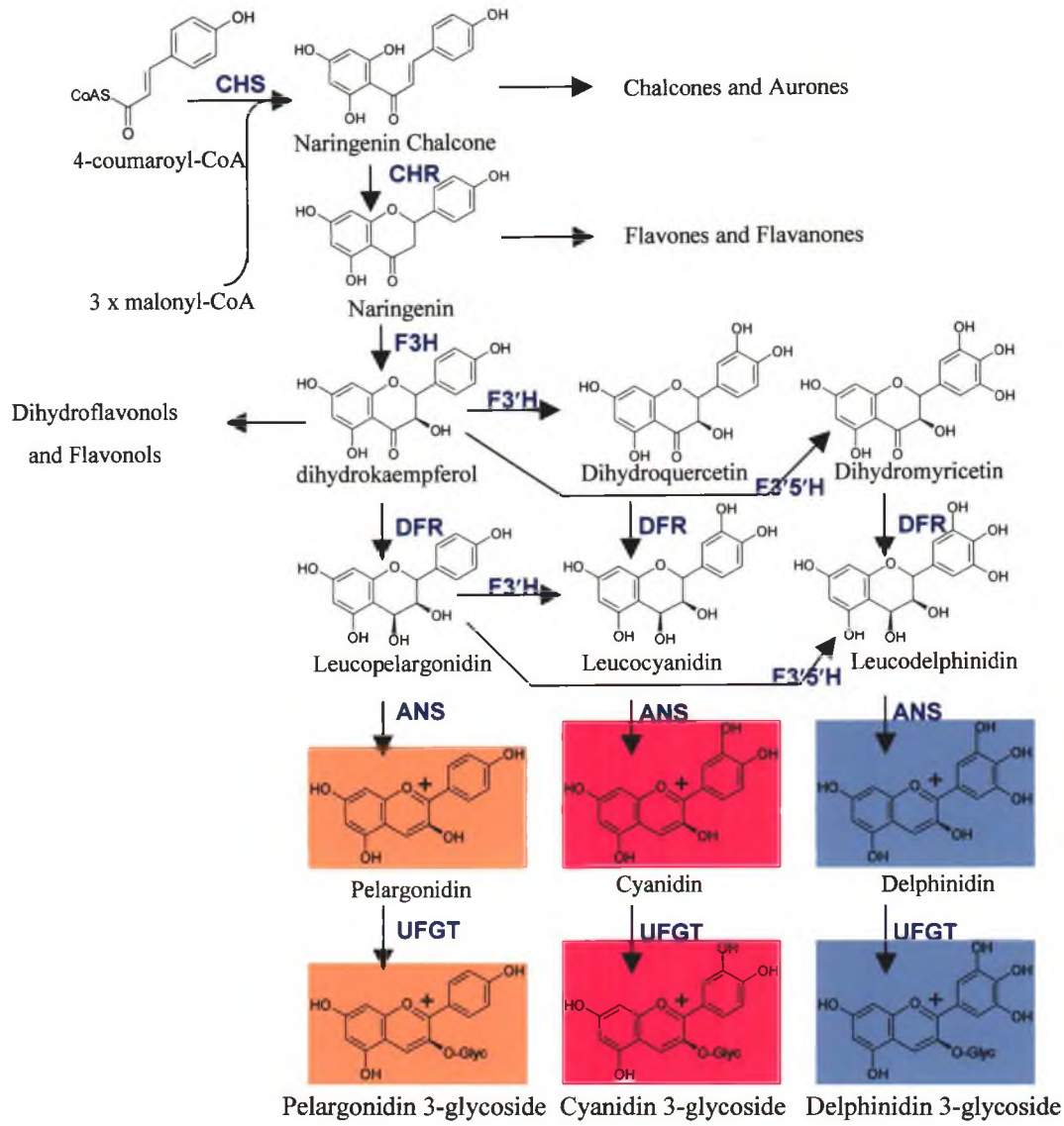

Figure 3.1. A diagrammatic representation of the flavonoid biosynthetic pathway. Enzyme catalyzing each reaction is shown in uppercase letter codes. Colored anthocyanins are depicted in corresponding colored boxes. Enzyme abbreviations are as follows: CHS=chalcone synthase, CHI=Chlacone isomerase, F3H=flavanone 3-hydroxylase, DFR=dihydroflavonol 4-reductase, F3'H=flavonoid 3'-hydroxylase, F3'5'H=flavonoid 3', 5'-hydroxylase, ANS=anthocyanidin synthase, UFGT= UDPG:flavonoid-3-*o*-glucosyl transferase.

The first reaction is catalyzed by flavonoid 3'-hydroxylase (F3'H) while the latter is catalyzed by flavonoid 3', 5'-hydroxylase (F3'5'H). These 3 dihydroflavonols are the precursors of anthocyanins. The first committed step of anthocyanin synthesis is the reduction of dihydroflavonols to their corresponding leucoanthocyanidin, catalyzed by the enzyme dihydroflavonol 4-reductase (DFR), which is subsequently converted to anthocyanidins by the enzyme/enzymes anthocyanidin synthase (ANS). Finally, these unstable anthocyanidins are glycosylated to form the anthocyanins by the enzyme UDPG:flavonoid-3-*o*-glucosyl transferase (UFGT).

3.1.2 Anthocyanin Skeleton

The basic skeleton of an anthocyanin molecule consists of two aromatic rings (A&B) with a heterocycle (C) in the middle (Fig. 3.2). The number of OH groups attached to the B ring and their methylation status influence the color directly (Stafford, 1990). The hydroxyl group on position 3 is glycosylated by rhamnose or glucose in the stable anthocyanin molecule. Acylation and methylation of the basic skeleton provide an enormous array of colors found in nature (Koes et al., 1994)

3.1.3 Chalcone Synthase

The enzyme CHS, a member of the polyketide synthase family, has been the focal point of much research since it catalyzes the production of chalcone, the C_{15} intermediate of all flavonoids (Heller and Forkmann, 1988). It is also used to study the regulation of gene expression in plants since *chs* genes and their regulatory factors make an attractive system due to easy to track anthocyanin production. No cofactors are required for the catalysis of this enzyme.

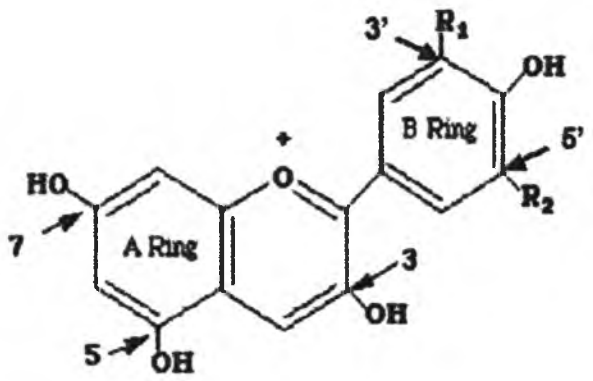

Figure 3.2. Basic carbon skeleton of the flavonoid molecule. Two aromatic rings are named as A and B ring. A hetero-cycle with an oxygen atom is found in the middle. In anthocyanins OH group at position 3 is glycosyated. Color of the anthocyanin molecule directly depends on the chemical nature of the R_1 and R_2 groups attached at the 3' and 5' positions of the B ring.

In addition to 4-coumaroyl-CoA, CHS from some plants such as *Dianthus caryophyllus*, *Verbena hybrida* and *Callistephus chinensis* can accept caffeoyl-CoA as a substrate (Spribille and Forkmann 1982b; Stotz et al., 1984). In *Verbena hybrida*, cyanidin derivatives are found despite the lack of measurable F3'H activity due to the use of caffeoyl-CoA as a substrate by its CHS (Stotz et al., 1984).

There are regulatory genes that directly control the CHS activity in many plants (reviewed by Heller and Forkmann, 1988). The *c2* gene of *Zea mays* (Dooner, 1983) *f* gene from *Matthiola*, and *niv* gene from *Antirrhinum*, (Spribille and Forkmann, 1981 & 1982a) genetically control the CHS activity of these plants. Recessive conditions of each allele completely block the CHS activity in the respective plant. In many plants, several *chs* genes constitute a small multi-gene family. In *Ipomoea* as many as 13 *chs* genes were found in the genome (Durbin et al., 1995), while 3 and 10 were found in *Gerbera* and *Petunia*, respectively (Helariutta et al., 1995; Koes et al., 1989). Promoter regions of these genes recognize, and are activated by, different environmental stimuli (Martin, 1993).

3.1.4 Dihydroflavonol 4-Reductase

Genes encoding DFR, namely the *a1* locus of *Zea mays* (O'Reilly et al., 1985) and the *pallida* locus of *Antirrhinum majus* (Coen et al., 1986), were cloned using transposable elements as tags. The enzyme requires NADPH as a cofactor for the reduction. However, the DFR enzyme from some plant species such as *Matthiola incana* and *Hordeum vulgare* can accept NADH as well as NADPH (Heller et al., 1985; Kristiansen, 1986). One interesting aspect of the DFR enzyme is

its substrate specificity. DFR from *Petunia* and *Cymbidium* (an orchid), cannot reduce *DHK* efficiently, explaining the lack of pelargonidin-accumulating orange flowers, even in the absence of competing enzymes F3'H and F3'5'H (Forkmann and Ruhnau, 1987; Gerats et al., 1982; Johnson et al., 1999). A hypothesis regarding the 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) identified 4 amino acid residues that determine the substrate specificity of *Petunia* DFR. However, these 4 amino acids are not conserved between *Cymbidium* and *Petunia*, ruling out the possibility of these amino acid residues as the region of substrate specificity in *Cymbidium*.

In *Dendrobium*, analysis of floral flavonoids found in species and hybrids identified 3' hydroxylated cyanidin as the major pigment while pelargonidin was found to be rare (Kuehnle et al., 1997). We intend to isolate the gene(s) encoding DFR from a pelargonidin-accumulating clone *Dendrobium* Icy Pink 'Sakura' (K1224) and from the cyanidin-accumulating hybrid *Dendrobium* Jaquelyn Thomas 'Uniwai Prince' (UH503) to determine nucleotide sequences, expression pattern and copy number.

3.1.5 Flavonoid Hydroxylases

Hydroxylase enzymes play a very important role in determining flower color. F3'H catalyzes the addition of a OH group at the 3' position of the B ring, while F3'5'H adds two OH groups at 3' and 5' positions (Fig. 3.2). The actions of the two enzymes produce *DHQ* and *DHM*, which lead to the production of purple cyanidin

and blue delphinidin, respectively. Both enzymes are cytochrome P450 dependent monooxygenases that require NADPH as a cofactor and oxygen as a substrate (Forkmann, 1991). The Cytochrome P450 enzymes constitute a superfamily of enzymes belonging to heme-thiolate proteins, involved in oxidation and metabolism of many substrates. Activity of F3'H has been first demonstrated in microsomal fractions of *Haplopappus* cell cultures (Fritsch and Griesbach, 1975). In some plants DFR can efficiently reduce all 3 substrates *DHK*, *DHQ* and *DHM*, giving the possibility of a full array of flower colors (Helariutta et al., 1993; Heller et al., 1985; Meyer et al., 1987; Stich et al., 1992; Tanaka et al., 1995). In such cases, the flower color is primarily determined by the activity of the two hydroxylases. In some ornamentals, such as chrysanthemum, carnation, and roses, blue color is absent due to the lack of F3'5'H activity. In *Dendrobium* blue delphinidin is absent although 3'5' hydroxylated flavonols were found in some flowers as a minor constituent (Kuehnle et al., 1997). We intend to isolate cDNA clones of *f3'h* and *f3'5'h* using Reverse Transcription-Polymerase Chain Reaction (RT-PCR) from flower bud mRNA. Expression and the characterization of these two genes in *Dendrobium* will give insight into the predominance of purple and lack of blue colors in *Dendrobium*.

3.2 OBJECTIVE

In *Dendrobium*, there has been no nucleotide sequences of any flower color genes published in any of the accessible databases (*chs* was reported as cloned in abstract only by Yong and Chua, 1990). Genes encoding three enzymes of the flavonoid pathway, CHS, DFR and F3H, were isolated from other orchids (see

chapter 1, Table 1.1). Our objective was to isolate and characterize the genes encoding key anthocyanin biosynthetic enzymes, namely CHS, DFR, F3'H and F3'5'H from *Dendrobium* and to understand the basis of flower color at the molecular level. This information will enable the identification of the most appropriate genetic background for flower color manipulation, and find insight into the most probable cause for the absence of blue color in *Dendrobium* and scarcity of pelargonidin accumulation.

3.3 MATERIALS AND METHODS

3.3.1 RNA Extraction and cDNA Synthesis

Inflorescences of *Dendrobium* Jaquelyn Thomas 'Uniwai Prince' (UH 503) were harvested from the University of Hawaii greenhouse grown plants. Total RNA was extracted from unopened buds according to the method of Champagne and Kuehnle (2000).

cDNA was synthesized from 5μg of total RNA using 200 units of SuperScript™ II RNase H⁻ reverse transcriptase (Invitrogen, Carlsbad, CA) in 1X first strand synthesis buffer (50 mM Tris-HCl, 75 mM KCl, 3mM $MgCl_2$, pH 8.3), supplemented with 0.01M DTT and 0.5mM dNTPs, by incubating the reaction mixture at 42°C for 50 minutes. Oligo dT (dT_{16} or dT_{20}-T7) primers were used for first strand cDNA synthesis. 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, WI) at 37°C for 20 minutes.

RNAse H was inactivated by incubation at 60°C for 10 min. The samples were stored at –20°C for PCR amplifications.

3.3.2 PCR with Degenerate Primers to Amplify Flavonoid Genes

Degenerate oligonucleotide primers were designed by Dr. M.M. Champagne (Table 3.1) and synthesized (IDT, Coralville, IA), 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 µls using RedTaq (Sigma, St. Louis, Mo) in a 1X amplification buffer [0.01M Tris (pH 8.3), 0.05M KCl, 0.01% gelatin, 0.2mM dNTPs, 1.0mM $MgCl_2$] in iCycler thermal cycler (Bio-Rad, Hercules, CA). Flower bud cDNA made from 250 ng of total RNA was used with 200 nM primer concentrations for each reaction. Sequences of each primer and PCR conditions used are listed in Table 3.1.

For *f3'h/f3'5'h* genes, PCR was carried out in 1X HotStar Taq buffer (Qiagen, Valencia, CA) supplemented with 0.2mM dNTP, 0.5 µM degenerate primers (Table 3.1) and 2.5 Units of Hotstar Taq DNA polymerase. A touchdown PCR was performed with an annealing temperature of 62°C-42°C (Table 3.1), with a reduction of 2°C per 3 cycles. Final amplification was carried out with the annealing temperature of 45°C in iCycler thermal cycler (Bio-Rad, Hercules, CA).

PCR products were separated on agarose gels in 1X TAE buffer (40mM Tris-acetate, 1mM Na_2EDTA). Amplified DNA fragments with expected molecular weight were excised and purified using GENECLEAN II kit (BIO 101, Carlsbad, CA) and ligated to pCR II-TOPO (Invitrogen, Carlsbad, CA) TA vector.

Table 3.1. Degenerate and oligo dT primers, reverse transcription conditions, and PCR conditions used for amplification of flavonoid genes from *Dendrobium*.

Designation	Primer combinations[a]	PCR/transcription reaction conditions
dT_{16}	ttttttttttttttt-3'	Reverse transcription at 42°C for 50 min.
T_{20}-T7	taatacgactcactatagggtttttttttttttttt-3'	
CHS-L	cggaattca(c/t)ca(a/g)ca(a/g)ggitg(c/t)tt(c/t)g-3'	95°C-3 min (95°C-1 min, 40°C-1 min, 72°C-1 min) x 30 72°C-5 min. 1.0 mM $MgCl_2$ concentration, Red Taq (Sigma)
CHS-R	cgggatccc(a/g)aaia(a/g)nacicccca(c/t)t-3'	
DFR-L	Cggaattcgggnccigtngtigtn-3'	95°C-3 min (95°C-1 min, 40°C-1 min, 72°C-1 min) x 30 72°C-5 min. 1.0 mM $MgCl_2$ concentration, Red Taq (Sigma)
DFR-R	cgggatcctacatccanccngtcat(c/t)tt-3'	
Helix 1L.2	gc(a/g/t)ggiac(a/g/t)ga(c/t)acitc-3'	95°C-3 min (95°C-1 min, 62-42°C -1 min, 72°C-1 min) x 30 72°C-1min (94°C-30 sec, 45°C-30sec, 72°C-30sec) x 30 1.5 mM $MgCl_2$ concentration, Red Taq (Sigma)
Helix KR	a(a/g)iggigtig(a/g)(a/t)gg(a/g)tg-3'	

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

Ligation and transformation was carried out according to the manufacturer's instructions using chemically competent *Escherichia coli* TOP 10F cells provided with the kit. PCR product inserts were fully sequenced (plus and minus strands) at the University of Hawaii Biotechnology Core Facility.

3.3.3 3'RACE and 5'RACE to Obtain the Full/Partial Sequences

UH503 flower bud cDNA was synthesized according to the reverse transcription protocol of SuperScript™ II as described above, using dT_{20}-T7 primer (Table 3.1). A Gene specific primer and the T7 adapter primer were used in subsequent 3' RACE (Rapid Amplification of cDNA Ends) reactions. Primers and the PCR conditions used in each reaction are listed in Table 3.2. A skewed ratio of 10:1 gene-specific primer to general primer (T7) was used in all 3'RACE reactions (Bespalova et al., 1998), which was crucial for the successful amplification of the gene. PCR products were isolated on an agarose gel and purified using a GENECLEAN II kit. Products were ligated into a TA cloning vector [pCRII-TOPO cloning kit (Invitrogen, Carlsbad, CA)] or pGEM-Teasy (Promega, Madison, WI)] according to the manufacturers' protocols. Plasmid inserts were fully sequenced at the UH Biotechnology Core Facility.

For 5'RACE reaction, synthesized cDNA was cleaned in order to remove the extra dNTPs and other impurities using GENECLEAN II kit. Cleaned cDNA was boiled for 5 minutes and quickly chilled on ice. A poly-C tail was added to the single-stranded cDNA using Terminal deoxyribonucleotide Transferase (TdT) (Promega, Madison, WI).

Table 3.2. Primers and PCR conditions used in 3' RACE and 5' RACE reactions to amplify flavonoid genes.

Designation	Primer combinations	PCR conditions
Den-CHS-6-L T7	gtcccctgttccaactcgta-3' taatacgactcactata-3'	95°C-5 min (95°C-1 min, 55°C-1 min, 68°C-1 min) x 35 68°C-7 min. 2.0 mM $MgCl_2$ concentration, Pfx Taq polymerase (Invitrogen)
Den-DRF-L T7	gggttatgtggtcagggcta-3' taatacgactcactata-3'	94°C-5 min (94°C-5 min, 55°C-2 min, 72°C-30 min) x 1 (94°C-1 min, 55°C-1 min, 72°C-2 min) x 30 72°C-7 min. 1.5 mM $MgCl_2$ concentration, Red Taq (Sigma)
Poly G Den-DFR-R	ggccacgcgtcgactagtacgg gggggggggggggg-3' agtcaaggtcactccagcag-3'	95°C-5 min (94°C-5 min, 55°C-2 min, 72°C-30 min) x 1 (94°C-1 min, 55°C -1 min, 72°C-2 min) x 30 72°C-7min. re-amplification: 95°C-5 min (94°C-1 min, 55°C -1 min, 72°C-2 min) x 30 72°C-7min. 1.5 mM $MgCl_2$ concentration, RedTaq (Sigma)
Den-F3'H-L T7	gcggggactgacacgtcctcag c-3' taatacgactcactata-3'	95°C-15 min (95°C-1 min, 65-53°C -1 min, 72°C-2 min) x 30 72°C-7min. touch down with 0.4°C decrease in temperature every cycle final amplification: (95°C-1 min, 55°C -1 min, 72°C-1 min) x 30 72°C-7min. 1.7 mM $MgCl_2$ concentration, Hot Star Taq (Qiagen)
Den-F3'5'H-L T7	gcggggacagacacgtccgcc-3' taatacgactcactata-3'	95°C-5 min (95°C-1 min, 55-59°C-1 min, 72°-1 min) x 30 72°C-7 min Temperature gradient was used 1.5 mM $MgCl_2$ concentration, Red Taq (Sigma)

TdT enzyme reaction was carried out in 1X tailing buffer (10mM Tris-HCL, pH 8.4, 25mM KCl, 1.5mM $MgCl_2$) supplemented with 0.8 mM dCTP at 37°C for 40 min. Enzyme was inactivated by incubation at 65°C for 10 minutes. A polyG primer with an adaptor, and a gene specific primer (Table 3.2) were used in amplification of the 5' end of the gene. Primer sequences and PCR conditions are listed in Table 3.2. A single microliter (μl) of 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.

3.3.4 Radioactive Probe Synthesis for Northern and Southern Hybridization

The cDNA inserts of the flavonoid genes were isolated by restriction digestion of the clones with *Eco*RI enzyme and separation on 1% agarose gel. DNA fragments were purified (GENECLEAN II kit) and 20ng was labeled with α^{32}P-ATP using random primer labeling (Prime a Gene Kit, Promega, Madison, WI). Labeled product was cleaned using Elutip®-D column (Schleicher and Shuell, Keene, NH). Cleaned probe was added to the hybridization buffer at a concentration of 1 million cpm/1ml of buffer.

3.3.5 Northern Blot Hybridization

Floral buds and flowers from harvested inflorescences of UH503 and K1224 were divided into 10 different developmental stages, 1 being the most immature (Table 3.3). RNA was extracted from bud/flower stages and mature leaves according to Champagne and Kuehnle (2000) extraction protocol. Ten μg of total RNA from different bud/flower stages were size fractionated on a standard 2%

formaldehyde agarose (0.9%) gel electrophoresis (1X MOPS buffer). RNA was transferred overnight onto Nytran Supercharge™ nylon membrane (Schleicher & Schuell, Keene, NH) by downward capillary transfer using alkaline transfer buffer (3MNaCl, 0.01N NaOH).

Table 3.3. Description of the bud and flower stages of *Dendrobium* 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 perianth.
2	1.5-1.7	Small buds, most of the adaxial surface turned purple, abaxial surface still green.
3	1.7-1.9	Medium-small buds, adaxial surface of perianth turning dark purple, abaxial surface turning purple.
4	1.9-2.1	Medium size buds, dark purple perianth.
5	2.1-2.3	Medium-large unopened buds, dark purple.
6	2.4-2.8	Most mature buds, unopened, dark purple on adaxial and abaxial sides of perianth.
7	Not measured	Flowers just opened (half open).
8	Not measured	Flowers fully opened, dark purple perianth, 1 position below stage 7 flower.
9	Not measured	Open flower, 2 flowers below stage 7 on the raceme, dark purple.
10	Not measured	Older flower, 3 flowers below stage 7, dark purple

Hybridization and washing was done according to Church and Gilbert (1984) protocol with modifications of molality and pH of $Na_2HPO_4 \cdot 7H_2O$ in hybridization buffer from 0.5M to 0.25M and pH from 7.2 to 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, St. Louis, MO), 1mM $Na_2EDTA \cdot 2H_2O$, 0.25M $Na_2HPO_4 \cdot 7H_2O$ (pH 7.4)]. Blots were washed twice at 60°C for 10 min, using phosphate wash buffer with BSA [0.5% BSA, 1mM $Na_2EDTA \cdot 2H_2O$, 5% SDS, 40mM Na_2HPO_4. $7H_2O$ (pH 7.4)]. Final two washes were done at 60°C for 20 min. using phosphate buffer without BSA [1mM $Na_2EDTA \cdot 2H_2O$, 1% SDS, 40mM $Na_2HPO_4 \cdot 7H_2O$ (pH 7.4)]. Signals were detected by autoradiography or Bio-Rad Personal Molecular Imager® FX (Bio-Rad, Hercules, CA). The density tool in Quantity one, vesion 4.2.1 (Bio Rad, Hercules, CA) software program was used to detect the relative strengths of the signal.

3.3.4 Genomic DNA Extraction and Southern Hybridization

Young leaves from greenhouse-grown plants or tissue-cultured plants were pulverized into a fine powder using liquid nitrogen in chilled mortar and pestles. Powdered tissue was incubated in 20 mLs of CTAB [cetyltriammonium bromide (Sigma, St. Louis, MO)] extraction buffer [100mM Tris-HCL (pH 9.5), 2% CTAB (w/v), 1.4 M NaCl, 1% (w/v) PEG-8000 (polyethylene glycol, MW8000, Sigma)] at 65°C for 1 hr with occasional mixing. DNA was cleaned with an equal volume of chloroform:isoamylalcohol (24:1) and precipitated according to the protocol of Carlson et al. (1991) protocol. RNA was removed by treatment with RNAse A for 1

hr at 37°C. Each digestion mixture was extracted twice with an equal volume of phenol (pH 6.7):chloroform:isoamyl alcohol (25:24:1) followed by a single extraction of chloroform:isoamyl alcohol (24:1). DNA was precipitated by addition of an equal volume of 2-propanol. Pelleted DNA was washed twice with 70% ethanol and dissolved in sterile water. Isolated DNA (15 μg) was digested with restriction enzymes in 0.2-0.3 mL volume. Digested DNA was salt precipitated and redissolved in 50 μLs of water and size fractionated on a 0.8% agarose gel electrophoresis in 1X TAE buffer. Separated DNA was transferred onto Nytran Supercharge™ (Schleicher & Schuell, Keene, NH) nylon membrane by downward capillary transfer using a 20X SSC buffer according to manufacturer's instructions (neutral transfer method). Membranes were hybridized with ^{32}P labeled denatured DNA probes at 60°C overnight and washed as in northern blot analysis. Signals were visualized by autoradiography or Bio-Rad Personal Molecular Imager® FX (Bio-Rad, Hercules, CA).

3.4 RESULTS

3.4.1 Cloning and Sequence Analysis of Flavonoid Biosynthetic Genes

Dihydroflavonol 4-reductase. Two partial cDNA clones were isolated from UH503 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. A full-length clone of 1320 bp (Fig. 3.3), *Den-dfr,* was obtained by ligation of 5' and 3' ends of the gene using a unique restriction site, *Pst*I, in the overlapping region of two partial clones.

```
                                M  E  N  E  K  K  G  P  V  V
aactggcgttgaggagagagaaaaaaagaaatggagaatgagaagaagggaccagtagtg  60
 V  T  G  A  S  G  Y  V  G  S  W  L  V  M  K  L  L  K  K  G
gtgactggagccagtggctacgtgggttcatggttggtgatgaagcttcttaaaaagggt  120
 Y  V  V  R  A  T  V  R  D  P  T  N  L  T  K  V  K  P  L  L
tatgtggtaagggctacagtgagagatccaacaaatcttacgaaagtgaagccattgttg  180
 D  L  P  R  S  N  E  L  L  S  I  W  K  A  D  L  D  D  V  E
gatctgccgcgctccaatgaactgctcagcatttggaaagcagacctagatgacgtcgaa  240
 G  S  F  D  E  V  I  R  G  S  I  G  V  F  H  V  A  T  P  M
ggtagcttcgatgaggtgatacgtggcagcattggagtgttccacgtcgctactcccatg  300
 N  F  Q  S  K  D  P  E  N  E  V  I  K  P  A  I  N  G  L  L
aattttcaatccaaagaccctgagaatgaagtgataaaaccggcaatcaacggtctgctg  360
 G  I  L  R  S  C  K  K  A  G  S  V  Q  R  V  I  F  T  S  S
ggcatcttgaggtcttgcaaaaaggccggcagcgttcagcgagtgatattcacgtcttct  420
 A  G  T  V  N  V  E  E  H  Q  A  A  V  Y  D  E  S  C  W  S
gcaggaacagtaaatgtggaggaacaccaagcagcagtgtatgacgagagctgctggagt  480
 D  L  D  F  V  N  R  V  K  M  T  G  W  M  Y  F  L  S  K  T
gaccttgacttcgtcaaccgagtcaagatgaccggttggatgtacttcctgtcaaaaaca  540
 L  A  E  K  A  A  W  E  F  V  K  D  N  D  I  H  L  I  T  I
cttgctgagaaggctgcttgggagtttgtgaaggacaatgacattcatttaataaccatt  600
 I  P  T  L  V  V  G  S  F  I  T  S  E  M  P  P  S  M  I  T
attccgactttggtggtggggtccttcataacatctgaaatgccaccaagcatgatcact  660
 A  L  S  L  I  T  G  N  D  A  H  Y  S  I  L  K  Q  V  Q  F
gcactatcattaattacaggaaatgatgcccattactcaattttaaagcaagttcaattt  720
 V  H  L  D  D  V  C  D  A  H  I  F  L  F  E  H  P  K  A  N
gttcatttggatgacgtatgtgatgctcacattttccttttcgagcatcccaaagcaaat  780
 G  R  Y  I  C  S  S  Y  D  S  T  I  Y  G  L  A  E  M  L  K
ggtagatacatttgctcttcctatgactccacaatttatggcttagcagaaatgctgaag  840
 N  R  Y  P  T  Y  V  I  P  Q  K  F  K  E  I  D  P  D  I  K
aacagatatcccacatatgtcattcctcagaagtttaaggaaattgatccagatattaag  900
 C  V  S  F  S  Y  K  K  L  L  E  L  G  F  K  Y  K  Y  S  M
tgtgtaagcttctcttataagaaattgctggagcttggctttaagtataagtatagtatg  960
 E  E  M  F  D  D  A  I  N  T  C  R  D  K  K  L  I  P  L  N
gaggagatgtttgatgatgctatcaatacctgtagggataagaagcttatcccactcaac  1020
 T  D  Q  E  I  V  L  A  A  E  K  F  E  E  V  K  E  Q  I  A
actgatcaggaaatagtcttagctgctgagaaatttgaggaagttaaagagcagattgct  1080
 V  K  *
gttaagtgaaaaatgaatgagaaaggagaagctaatgttgttgttttaattttctgtgca  1140

ctgtcctattgattttctaagtgctttacactatcattggatgtatctttacttattata  1200

gtgggcttttgattatggcttttgcaatggacatgtaatagcatctgtaataaattttaa  1260

ttctatgtggtaaatttgaatcataatcatatatgctttaaaaaaaaaaaaaaaaaaaaa  1320
```

Figure 3.3. Nucleotide sequence and the deduced amino acid sequence of *Den-dfr* cDNA clone isolated from the flower bud cDNA of *Dendrobium* Jaquelyn Thomas 'Uniwai Prince' (UH503). Amino acid sequence is shown above the nucleotide sequence in single-letter codes. Stop codon is represented as an asterisk. Polyadenylation signal is represented in bold letters.

The first ATG found 30 nucletide downstream from the 5' end of the clone was the most probable start codon since it was immediately preceded by AAAGAAATGG which resembles the plant consensus sequence TAAACAATGG (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). The nucleotide sequence of the *dfr* clone isolated from *Dendrobium* Icy Pink 'Sakura' (K1224) was identical to the clone isolated from UH503.

The nucleotide sequence of *Den-dfr* revealed a similarity ranging from 65-87% compared to 19 other *dfr* sequences from plants available in GenBank. Multiple alignment of *Den-dfr* with other *dfr* sequences revealed a high similarity to other orchids (87% to *Bromheadia*; 84% to *Cymbidium*) and to *Liliaceae* (74% to *Lilium hybrida*). Phylogenetic tree based on CLUSTAL W alignment program show all monocotyledonous sequences to cluster into a single branch showing their common ancestry, while dicotyledonous sequences clustered into two distinct groups (Fig. 3.4).

Chalcone synthase. PCR with degenerate primers resulted in the isolation of two *chs* clones, *chs-6* and *chs-9*, with significant similarity to a *Phalaenopsis* sp. 'True Lady' *chs* homolog (Champagne and Kuehnle, unpublished data). We were able to obtain the 3' end of a chalcone synthase gene (*Den-chs-11*) consisting of 704 bp with the longest ORF encoding 159 amino acids (Fig. 3.5) using gene-specific primers (Table 3.2) designed with the *chs-6* clone.

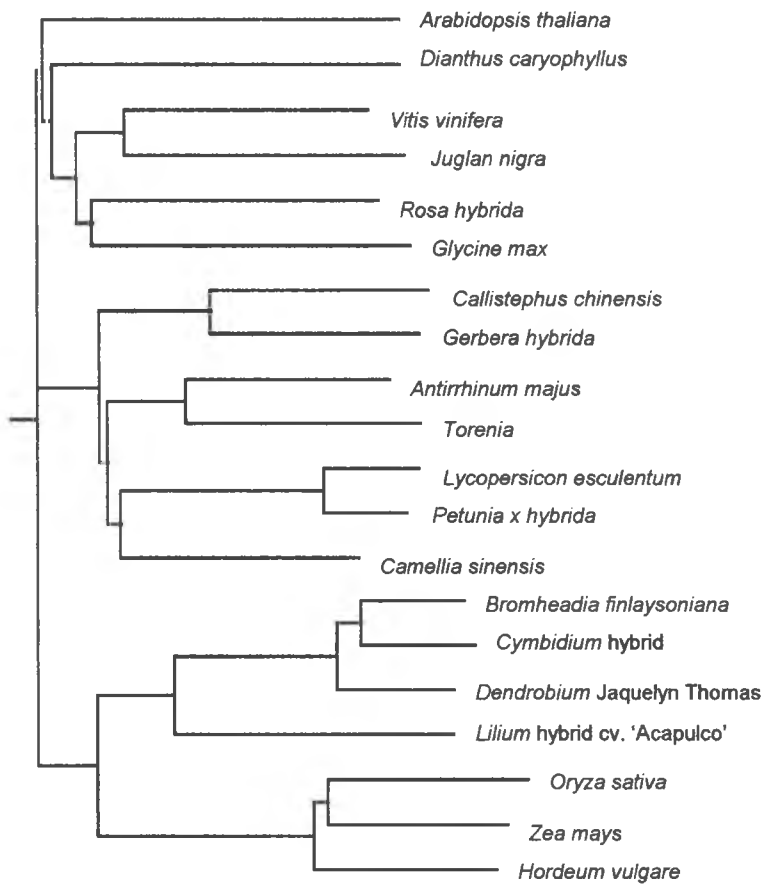

Figure 3.4. Phylogenetic tree drawn from Phylogenetic Inference Package (PHYLIP version 3.5c; Felsenstein, 1993.) showing relationship among the nucleotide sequences of *dfr* genes from different plant species. Sequence alignment was performed with CLUSTAL W version 3.2 with a gap penalty of 3. GenBank accession numbers for each species are listed below: *A. thaliana*-M86359; *D. caryophyllus*-Z67983; *V. vinifera* X75964; *J. nigra*-AJ278459; *Rosa hybrida*-D85102; *C. Chinensis*-Z67981; *G. hybrida*-Z17221; *A. majus*- X15536; *T. hybrida*-AB012924; *L. esculentum*-Z18277; *P. x hybrida*-AF233639; *C. sinensis*-AB018686; *G. max*-AF167556; *B. filaysoniana*-AF007096; *Cymbidium hybrida*-AF017451; *Lilium* hybrid-AF169801; *O. sativa*-AB003496; *Z. mays*-X05068; *H. vulgare*-S69616.

```
 P  L  F  Q  L  V  S  A  S  Q  T  I  L  P  E  S  E  G  A  I
ccccctgttccaactcgtatcggcttcccagaccatccttccggagtccgagggcgccatt  62
 D  G  H  L  R  E  M  G  L  T  F  H  L  L  K  D  V  P  G  L
gatggccatctacgcgagatgggactaaccttccacctactgaaagacgtcccaggcttg  122
 I  S  K  N  I  Q  K  S  L  V  E  A  F  K  P  L  G  I  H  D
atctctaaaaacattcaaaagagtctcgtagaggcattcaagccacttggtattcacgac  182
 W  N  S  I  F  W  I  A  H  P  G  G  P  A  I  L  D  Q  V  E
tggaattcgatcttctggattgcgcatccgggcggtccggcaatactcgaccaagtagaa  242
 V  K  L  G  L  K  A  E  K  L  A  A  S  R  N  V  L  A  E  Y
gttaagcttggacttaaagctgagaagctcgcggccagtagaaacgtgcttgcggagtat  302
 G  N  M  S  S  A  C  V  L  F  I  L  D  E  M  R  R  R  S  A
gggaatatgtccagcgcttgtgtgcttttcatacttgatgaaatgaggcggaggtcggcg  362
 E  A  G  Q  A  T  T  G  E  G  L  E  W  G  A  L  F  G  F  G
gaggctgggcaagctaccaccggagaggggttggagtggggagcattgttcggattcggt  422
 P  G  L  T  V  E  T  V  V  L  R  S  V  P  I  A  G  A  V  *
ccggggcttacagttgaaaccgttgtgttacgcagcgttccgattgctggtgcggtgtga  482

tggatcgaccagcttgtttagattattggtatttgatctgttgtactgttctttttatta  542

taattggcttgtttctgcttgctctaaatggctagtgctgggttgggtggagctataaag  602

gctggtggggaaaggaatgcaccatactgttattttatgttatctgtggccatggttcta  662

ttttt**aataaa**gaagtcttatgaaaaaaaaaaaaaaaaaaa  704
```

Figure 3.5. Nucleotide sequence and the deduced amino acid sequence of *Den-chs-11* cDNA clone isolated from the flower bud cDNA of *Dendrobium* Jaquelyn Thomas 'Uniwai Prince' (UH503). Amino acid sequence is shown above the nucleotide sequence in single-letter codes. Stop codon is represented as an asterisk. Polyadenylation signal is represented in bold letters.

Surprisingly, *Den-chs-11* is only 70% similar to the *chs-6* clone. The 3'RACE performed with primers designed against the *chs-9* clone resulted in a 500 bp fragment (Appendix A), which showed high similarity to bibenzyl synthase, a close relative of CHS enzyme since both enzymes catalyze similar chemical reactions. Bibenzyl synthase is an important enzyme 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* homolgue from *Phalaenopsis* sp. 'True Lady' (Hsu et al., 1997, GenBank No. U88077, protein ID AAB650941) with BLAST search revealed this *Phalaenopsis* cDNA clone is indeed more similar to bibenzyl synthase clones of *Bromheadia* and other *Phalaenopsis* hybrids than to *chs*. This explains the apparent discrepancy in our results.

The closest sequence, from the orchid *Bromheadia finlaysoniana,* is 84% identical to the *Den-chs-11* at the nucleotide level and 94% identical (97% similar) to the deduced amino acid sequence (Table 3.4). The closest non-orchid sequence is *Sorghum bicolor* with 83% identity (94% similarity) at the amino acid level. Amino acid sequences of *chs* from 25 plant species have shown 76%-94% identity to the deduced amino acid sequence of *Den-chs-11* (Table 3.4).

Flavonoid hydroxylase. Degenerate primers to obtain both flavonoid hydroxylases were designed based on the conserved regions of cytochrome p450 sequences available in the GenBank (Champagne and Kuehnle, unpublished). A 200 bp band amplified by PCR contained sequences similar to both *f3'h* and *f3'5'h.*

Table 3.4. Comparison of partial clone *Den-chs-11* with chalcone synthase from other plants at the amino acid level.

Plant species	Common name	GenBank accession no.	Length of the sequence	% identity[a]
Dendrobium (UH503)	Dendrobium	----------	159	100
Bromheadia finlaysoniana	Seraman	AF007099	394	94
Matthiola incana		AJ427537	394	82
Sorghum bicolor	Sorghum	AF152551	401	83
Petunia x hybrida	Petunia	X14599	319	83
Brassica napus		AF07633	374	82
Zea mays	Corn	X60205	400	81
Oryza sativa	Rice	X89859	398	81
Lilium hybrida	Lily	AF169800	393	81
Gerbera hybrida	Gerbera	Z38096	398	81
Arabidopsis thaliana		AY090376	395	81
Vitis vinifera	Grape vine	AB066275	393	81
Nicotiana tobaccum	Tobacco	AF11783	389	81
Camellia sinensis	Tea	D26593	389	80
Malus x domestica	Apple	AB074485	391	80
Catheranthus roseus	Periwinkle	AJ131813	389	80
Callistephus chinensis	China Aster	Z67988	398	79
Petroselinum crispum	Parsley	V01538	398	79
Daucus carota	Carrot	AJ006780	397	79
Solanum tuberosum	Potato	U47738	389	79
Antirrhinum majus	Snapdragon	X03710	390	79
Lycopersicon esculentum	Tomato	X55195	389	79
Hydrangea macrophylla	Hydrangea	AB011467	389	79
Glycine max	Soybean	L07647	388	79
Hordeum vulgare	Barley	X58339	389	78
Allium cepa	Onion	AF268382	253	76

[a] Sequences are aligned with CLUSTAL W (Thompson et al. 1994) program in SDSC Biology Workbench.

Gene specific primers designed from these sequences were used to perform 3'RACEs to obtain the 3' end of the two genes. Two partial cDNA clones, *Den-f3'h-6* and *Den-f3'5'h-8* (Fig. 3.6 and Fig. 3.7), with the signature heme-binding domains were obtained. Deduced amino acid sequences of the two clones were aligned with the available flavonoid hydroxylases (Fig. 3.8 and Fig. 3.9). Sequence alignments of *f3'h* sequences with *Den-f3'h-6* revealed 57 of 219 residues are conserved across all 10 species (Fig. 3.8). *Den-f3'h-6* clone has shown 43% identity (62% similarity) to *Petunia* and 47% identity to *Glycine max f3'h* amino acid sequences, respectively. A search with BLAST program revealed many other cytochrome P450 genes with even higher similarity to *Den-f3'h-6* from monocots such as *Asparagus* and *Musa.* However, the functions of these cytochrome p450 enzymes are not known yet. Lack of known *f3'h* sequences from monocotyledonous plants makes it difficult to confirm the identity of the gene by sequence similarity alone. The *Den-f3'5'h-8* sequence has shown 63% identity (76% similarity) to the corresponding *Petunia* sequence. A high proportion of amino acid residues (73 of 219) are fully conserved among all 9 sequences compared (Fig. 3.9). Thirty eight of the others are conservative substitutions.

3.4.2 Gene Expression by Northern Analyses

Temporal expression of *dfr* in the developing inflorescences of UH503 and K1224 showed that *dfr* transcripts are most abundant in the flower buds and are reduced to nearly undetectable levels in the open flowers (Fig. 3.10). However, RT-PCR analysis has shown that *dfr* transcripts were present in low levels in flowers.

```
 A  G  T  D  T  S  S  A  T  V  I  W  T  M  T  E  L  M  K  N
gcggggactgacacgtcctcagcaacagttatatggacgatgacagagctgatgaagaat  60
 P  R  V  M  T  K  L  Q  Q  E  L  Q  E  A  I  F  N  K  T  K
ccaagggtaatgaccaaattgcaacaagagctacaagaggccattttcaacaaaaccaag  120
 V  E  E  G  E  L  Q  Q  L  K  Y  L  K  L  V  I  K  E  S  L
gttgaagaaggcgagctccaacaactaaaatacctcaagctcgtcatcaaagagtcgctt  180
 R  L  H  P  P  A  P  L  L  V  P  R  E  T  L  E  S  C  N  I
agattacaccctccagcaccgcttctagttcctcgcgaaaccctagaatcatgcaatatt  240
 E  G  Y  D  I  P  A  K  T  R  V  F  I  N  A  V  A  I  A  T
gaaggttatgacattccagccaagacacgagttttcattaacgccgtcgccattgccaca  300
 D  P  K  T  W  K  N  P  N  E  F  W  P  E  R  F  T  S  T  S
gatccaaaaacttggaagaaccctaatgagttttggcctgagagattcacttcgaccagt  360
 I  D  L  K  G  Q  D  F  N  F  V  P  F  G  I  G  R  R  S  C
atagatctgaaagggcaggacttcaattttgtgccttttggtattggtcgcaggagttgc  420
 P  G  V  D  F  A  T  V  L  V  E  L  V  L  A  N  L  L  H  C
ccaggtgttgattttgctacagttcttgtggagcttgttttagctaatcttttgcattgt  480
 F  E  W  S  L  P  D  G  M  K  P  E  D  I  D  M  G  E  A  C
tttgaatggagtttacctgatggaatgaaacctgaggatattgatatgggagaggcgtgt  540
 G  L  T  T  H  K  K  V  P  L  C  M  V  A  K  P  K  M  H  *
ggccttacaacgcataaaaaagtgcctctttgcatggttgctaagccaaagatgcattga  600

attgttgtcactggaagtgattgtgggggatgataatgttctttgtgcttgtaagtgaga  660

ttgattgctatttgggacatttataatgtggctgaaatttttataaaattgaaaacttat  720

gttaatctgtttttttttttctttataattgggggttaaataaccaaaaaaaaaaaaaaa  780

aaaaaaccctatagggggggggcggta  806
```

Figure 3.6. Nucleotide sequence and the deduced amino acid sequence of *Den-f3'h-6* partial cDNA clone isolated from the flower bud cDNA of *Dendrobium* Jaquelyn Thomas 'Uniwai Prince' (UH503). Amino acid sequence is shown above the nucleotide sequence in single-letter codes. Stop codon is represented as an asterisk.

```
 A  G  T  D  T  S  A  I  V  I  E  W  A  M  A  E  M  L  K  N
gcggggacagacacgtccgccatagtcatcgagtgggcgatggccgaaatgcttaaaaac  60
 P  S  I  L  Q  R  A  Q  Q  E  T  D  R  V  V  G  R  H  R  L
ccatcaatcctccaacgagcacaacaggaaaccgatcgcgtcgtcggccgccaccgtctt  120
 L  D  E  S  D  I  P  K  L  P  Y  L  Q  A  I  C  K  E  A  L
ctcgacgaatccgacataccaaagctcccctacctccaagccatctgcaaggaagctctc  180
 R  K  H  P  P  T  P  L  S  I  P  H  Y  A  S  E  P  C  E  V
cgaaaacaccctccaacacctctcagcatacctcactacgcctccgaaccctgcgaggta  240
 E  G  Y  H  I  P  G  K  T  W  L  L  V  N  I  W  A  I  G  R
gaaggctaccacattcccgggaagacttggctgctcgtcaacatatgggccatcgggcgg  300
 D  P  D  V  W  E  N  P  L  L  F  D  P  E  R  F  L  Q  G  K
gacccggacgtgtgggagaacccgttgctgttcgacccggagaggtttctgcaagggaag  360
 M  A  R  I  D  P  M  G  N  D  F  E  L  I  P  F  G  A  G  R
atggcgagaatcgatccgatgggaaacgacttcgagctcataccgtttggagccgggagg  420
 R  I  C  A  G  K  L  A  G  M  L  M  V  Q  Y  Y  L  G  T  L
aggatttgcgccgggaagttagcggggatgctgatggtgcagtattatttgggaacgctg  480
 V  H  A  F  D  W  S  L  P  E  G  R  W  G  A  G  H  G  G  R
gtgcatgcctttgactggagtttgccggaaggacgttggggagctggacatggaggaagg  540
 A  G  V  G  V  A  E  A  V  P  L  S  V  M  A  R  P  R  L  A
gccggggttggtgttgccgaagctgtgccgctctcggtgatggcgaggccgaggctggcg  600
 P  A  L  Y  G  L  L  *
ccggcgctttatggccttctttaagggagaaagattgcctaacgaatttatgacataaat  660

tacgtttgaatatttttatatgatttttttttttgnttgttgttaagattaaaagagatt  720

ttttctcttatgttcgaattaagttataaaatatnaataaatgaataatctctttcnaaa  780
```

Figure 3.7. Nucleotide sequence and the deduced amino acid sequence of *Den-f3'5'h-8* partial cDNA clone isolated from the flower bud cDNA of *Dendrobium* Jaquelyn Thomas 'Uniwai Prince' (UH503). Amino acid sequence is shown above the nucleotide sequence in single-letter codes. Stop codon is represented as an asterisk.

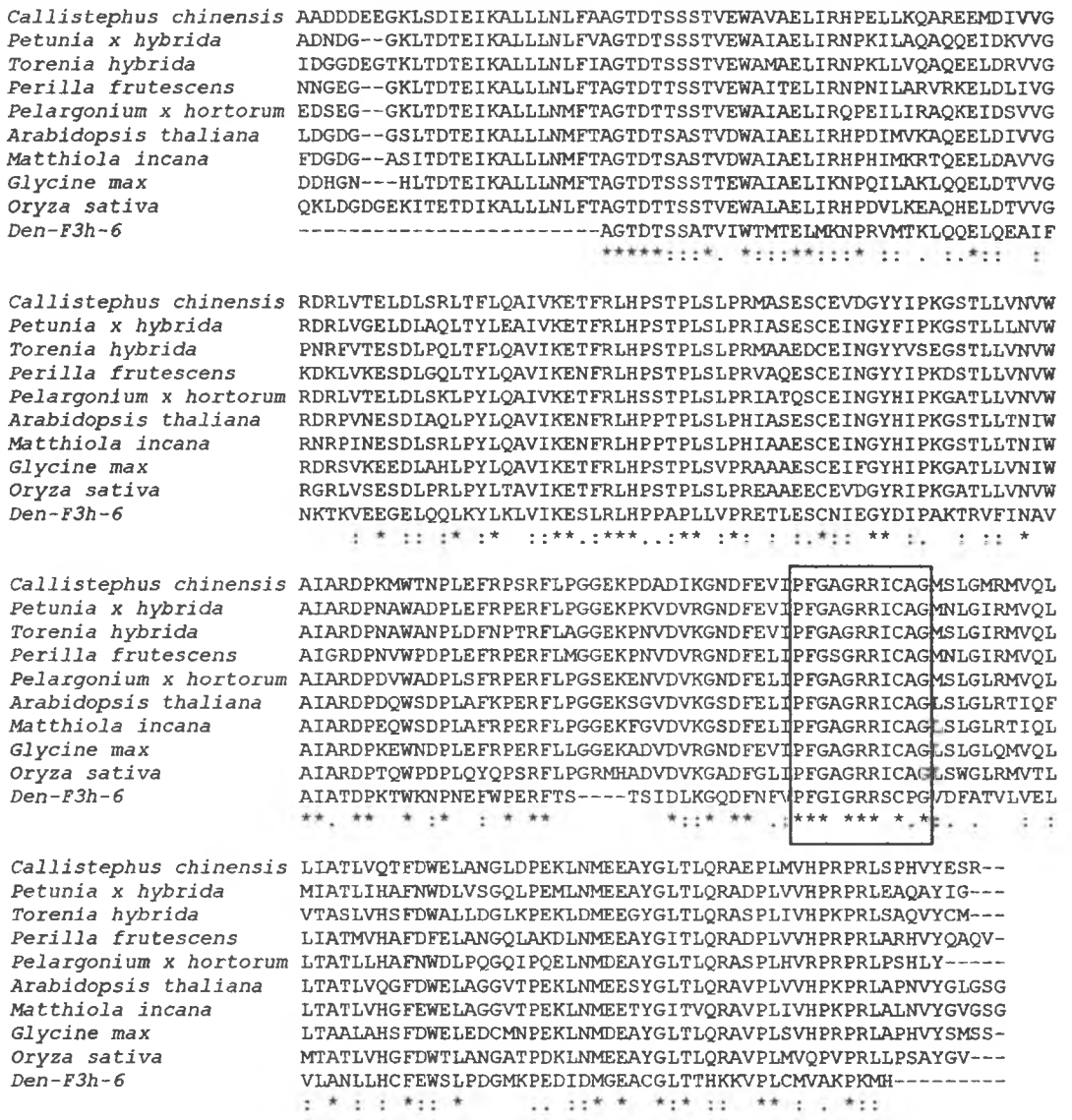

Figure 3.8. Alignment of amino acid sequences of F3'H enzymes showing the conserved regions and sequence similarities. Heme binding domain is included in the box. *=amino acid residues conserved among all 9 sequences. :=amino acids with similar functional groups (highly conserved substitutions). .=weakly conserved substitutions.

Figure 3.9. Alignment of amino acid sequences of F3'5'H enzymes showing the conserved regions and sequence similarities. Heme binding domain is included in the box. *=amino acid residues conserved completely among all 9 sequences. :=amino acids with similar functional groups (highly conserved substitutions). .=weakly conserved substitutions.

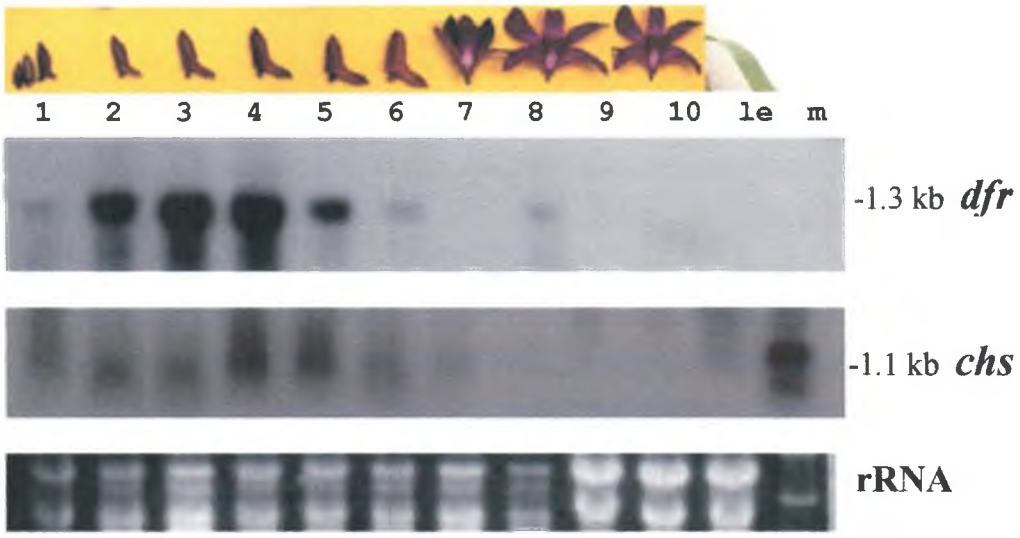

Figure 3.10. Northern blots showing temporal expression of *dfr* and *chs* mRNA in the developing inflorescence, and young leaf of *Dendrobium* Jaquelyn Thomas 'Uniwai Prince' (UH503). Upper panel shows the unopened buds divided according to size, and flowers sorted according to the position on raceme (stage 9 not shown) used in RNA extraction. Total RNA was extracted from these tissues and size fractionated (10 μg/lane) on a 2% formaldehyde agarose gel electrophoresis. RNA was blotted onto a charged nylon membrane and was hybridized with ^{32}P-labeled denatured cDNA probes of *Den-dfr* or *Den-chs-11*. Autoradiographs were exposed to the signal for 3 days for *dfr* and 2 weeks for *chs*. Lower panel indicate the loading level of RNA in ethidium bromide stained gel. *dfr* transcript is 1.3 kb in size. *chs* transcript is 1.1 kb in size. le=leaf; m=molecular weight marker.

The *dfr* transcripts can be amplified from the total RNA of open flowers in both genotypes (Notebook No. 4, page 91). No *dfr* transcripts were detected in the leaves through northern or RT-PCR analysis in either genotype. Similar expression data was observed with the *dfr* of *Bromheadia finlaysoniana* (Liew et al., 1998b).

Chalcone synthase transcripts follow a similar temporal expression pattern to *dfr* in the developing inflorescences of K1224 (Fig. 3.11). Expression of *chs* in UH503 is highest in stage 4 floral buds and decline as the buds become mature and fully open. However, unlike *dfr* expression, *chs* expression is still detectable in stage 7 open flowers and in young leaves of UH 503 (Fig. 3.11)

We were unable to detect expression of *f3'h* in buds, flowers or leaves of UH503, and K1224, using 10μg of total RNA. When the total RNA load was increased to 20μg, a faint signal was visible in flowers, buds and leaves of UH503 (Fig. 3.12A). It was still non-detectable in the buds of K1224 and a very faint signal is present in flowers of K1224. Densitometry reading revealed a higher signal of *f3'h* in UH503 compared to K1224 in all 3 tissues (Density tool of Quantity One 4.2.1 software program, Bio-Rad molecular imager system).

Presence of the *f3'5'h* mRNA in floral buds of UH503 was evident by RT-PCR and 3'RACE experiments (Notebook No. 5, page 34 & 58). However, the expression level is very minimal and almost undetectable in Northern even when loaded with 20 μg of total RNA (3.12B). The broad band appearing in K1224 for leaf tissue suggests that *f3'5'h* is present more in leaves than in buds or the probe cross hybridizes with other closely related cytochrome 450 transcripts.

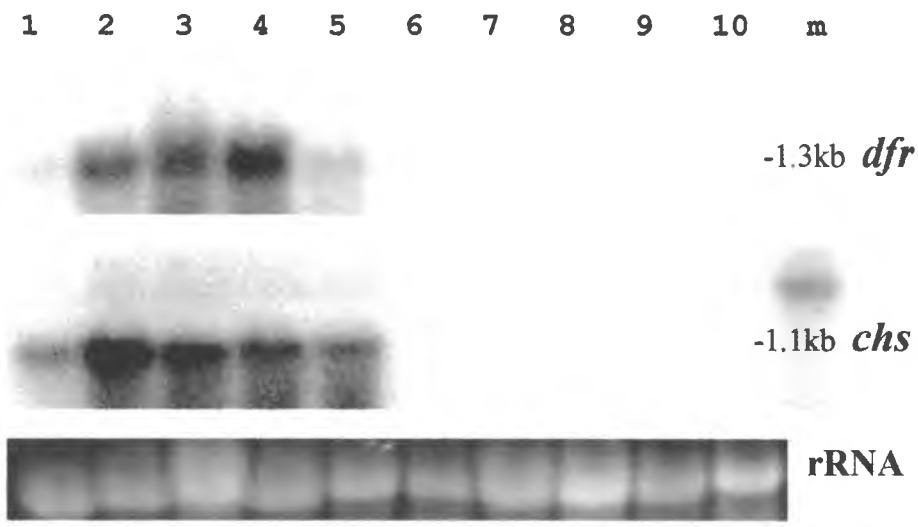

Figure 3.11. Northern blots showing temporal expression of *dfr* and *chs* in the developing inflorescence of *Dendrobium* Icy Pink 'Sakura' (K1224). RNA extracted from 10 bud and flower stages were size fractionated on a 2% formaldehyde agarose gel electrophoresis and blotted on to a charged nylon membrane. Total RNA (10 μg/lane) was hybridized with ^{32}P-labeled denatured cDNA probes of *Den-dfr* or *Den-chs-11*. Imager screen K-HD (Bio-Rad) was exposed to the signal for approximately 3 days. Lower panel indicate the loading levels of RNA in ethidium bromide stained 28S rRNA band. 1-6=flower buds from youngest to most mature; 7-10=open flowers; m=molecular weight marker.

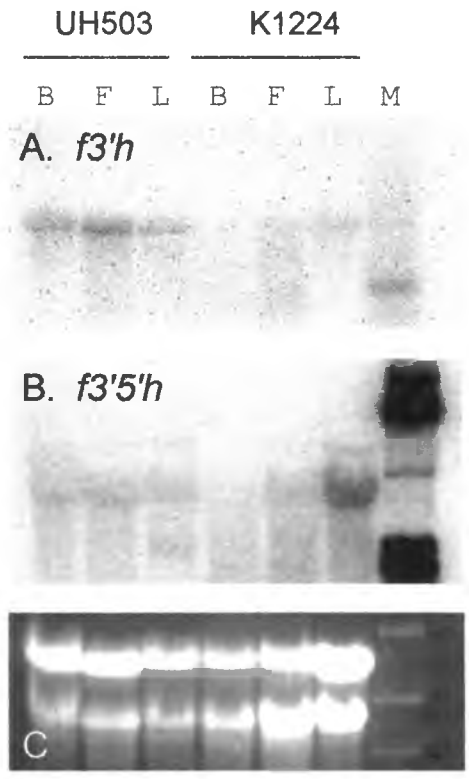

Figure 3.12. RNA blot analyses of buds, open flowers, and mature leaves of *Dendrobium* Jaquelyn Thomas 'Uniwai Prince' (UH 503) and *Dendrobium* Icy Pink 'Sakura' (K1224) showing expression of flvonoid hydroxylase genes. (A) RNA blot hybridized with *Den-f3'h-6* clone. (B) RNA blot hybridized with *Den-f3'5'h-8* clone. (C) Ethidium bromide stained rRNA bands as an indication of RNA loading levels. Both genotypes have very low level of expression in all 3 tissue types. Imager screen K-HD (Bio-Rad) was exposed to the signal for 3 days. Open flowers and buds of UH503 have higher expression levels compared to K1224. B=buds; F=flower; L=leaf; M=molecular weight marker.

3.4.3 Genomic Southern Analysis of *Dendrobium* (UH503 & K1224) DNA

Dendrobium genomic DNA was difficult to digest and 24-36 hours of digestion was needed for complete digestion, even at a concentration of 10 units of enzyme/μg of DNA. For detection of the *dfr* gene, DNA was digested with *Eco*RI, *Pst*I, *Bam*HI, or *Kpn*I sites. The *dfr* probe used for hybridization contains a single *Pst*I site. The *Bam*HI digest indicates 2 bands while *Eco*RI digest contain 6 bands (Fig. 3.13A). The *Pst*I digestion also results in 3 bands contrary to the expected number of bands (2) from a single copy of a *dfr* gene. The number of *dfr* gene copies cannot be accurately inferred from the available data. In other orchids, *Cymbidium* (Johnson et al., 1999) and *Bromheadia* (Liew et al., 1998b), *dfr* was represented by a single copy. Since UH503 is a hybrid of two amphidiploid (4N) parents (Kamemoto et al., 1999) presence of more than one copy of the *dfr* gene is possible. Six bands appearing in the *Eco*RI digest might be due to the presence of restriction sites in the introns.

A similar result was found for K1224 *dfr* gene also. A single band was detected in *Bam*HI and *Pst*I digests (Fig. 3.13B). Digestions with *Hind*III, *Xba*I and *Xho*I resulted in 4, 5 and 2 bands, respectively. However, the hybridization signals of all bands are not equal in intensity in K1224. Surprisingly, there is only a single band resulting from *Pst*I digestion (Fig. 3.13B). This might be due to a disruption of the restriction site by an intron or partial digestion. There is also a possibility of cross-hybridization with closely related gene sequences. It is difficult to infer the gene copy number with the available data.

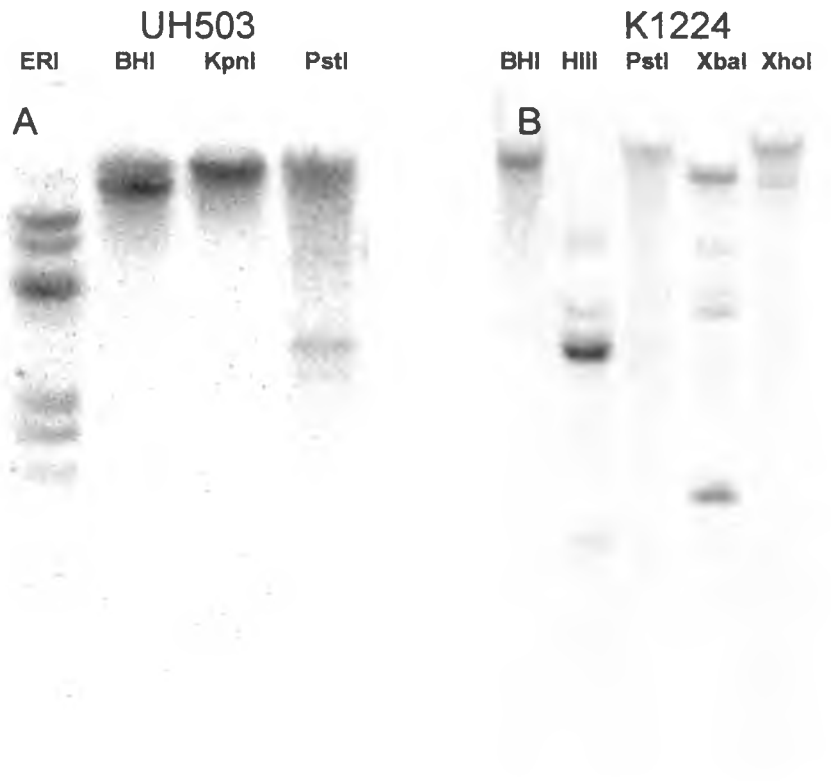

Figure 3.13. Genomic southern blots of *Dendrobium* DNA hybridized with ^{32}P labeled *Den-dfr* cDNA probe. Each lane represents different restriction digests of 15 μg of DNA. Number of bands hybridized with the probe varied in each digestion. Imager screen K-HD (Bio-Rad) was exposed to the signal for 4 days. (A) UH503 genomic DNA digested with *Eco*RI (ERI), *Bam*HI (BHI), *Kpn*I and *Pst*I. (B) K1224 genomic DNA digested with *Bam*HI, *Hind*III (HIII), *Xba*I and *Xho*I.

Southern analyses of UH503 and K1224 for chalcone synthase clearly indicate multiple bands in *Xba*I digests with strong hybridization signal (Fig. 3.14A & B). Both *Hind*III and *Eco*RI digests of K1224 give multiple bands with *Den-chs-11* probe (Fig. 3.14B). *Bam*HI digest of UH503 also resulted in 2 bands (Fig. 3.14A), while a single band is found in *Xho*I and *Kpn*I digests. This might be due to the large fragment sizes (≅ 20-30 kb) that result from XhoI and KpnI restriction enzymes. High molecular weight bands cannot be resolved on a 0.8% agarose gel thus giving a single hybridizing band from multiple fragments containing the gene. These results indicate that *chs* might be represented by several copies in UH503 and K1224 genomes. However, a southern blot of the parental genome along with UH503 and K1224 might give a better indication of gene copy number.

Restriction enzymes with no cutting sites in the cDNA clone was used for southern analyses of *f3'h* and *f3'5'h*. Southern blots of UH503 (Fig. 3.15A) hybridized with *f3'h* clearly indicate the presence of 2 bands in the *Hind*III, *Pst*I and *Xba*I digests. Three bands found in *EcoR*I digest might be due to a restriction sites in the intron region of *f3'h*. Therefore, UH503 genome most likely contain 2 copies of *f3'h* gene according to the available data of Southern analysis. There are 2 bands of *f3'5'h* also in UH503 shown in *Hind*III and *Bam*HI digests (Fig. 3.15B). The single band in *Xba*I can be due to the high molecular weight of the fragments containing the two copies of the *f3'5'h* gene. Therefore, the best deduction for the number of copies of *f3'5'h* is also 2 for UH503 genome.

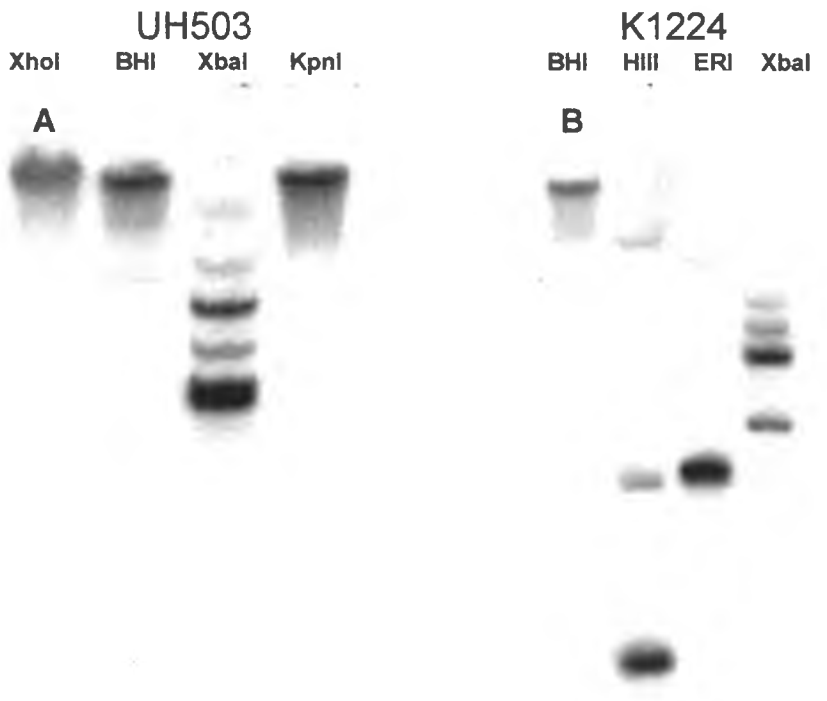

Figure 3.14. Genomic southern blots of *Dendrobium* DNA hybridized with ^{32}P labeled *Den-chs-11* cDNA probe. Each lane represents different restriction digests of 15 μg of DNA. Imager screen K-HD (Bio Rad) was exposed to the signal for 4 days. (A) UH503 genomic DNA digested with *Xho*I, *Bam*HI (BHI), *Xba*I and *Kpn*I. (B) K1224 genomic DNA digested with *Bam*HI (BHI), *Hind*III (HIII), *Eco*RI (ERI) and *Xba*I.

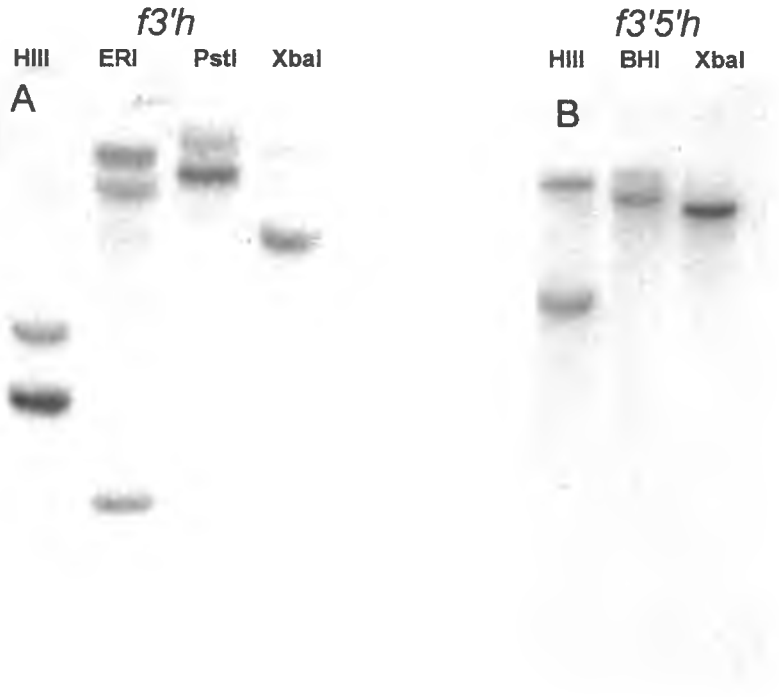

Figure 3.15. Genomic southern blots of *Dendrobium* Jaquelyn Thomas 'Uniwai Prince' (UH503) DNA to determine the copy number of *f3'h* and *f3'5'h* genes. Each lane represents different restriction digests of 15 μg of DNA. Imager screen K-HD was exposed to the signal for 4 days. (A) *Dendrobium* UH 503 DNA hybridized with ^{32}P labeled *Den-f3'h-6* cDNA. (B) *Dendrobium* UH503 genomic DNA hybridized with ^{32}P labeled *Den-f3'5'h-8* cDNA. ERI=*Eco*RI; BHI=*Bam*HI; HIII=*Hind*III.

3.5 DISCUSSION

We have successfully isolated a full-length cDNA clone encoding DFR from *Dendrobium.* Sequence analysis revealed it is 87% identical to the *Cymbidium* DFR which does not efficiently reduce *DHK* to form orange pelargonidin (Johnson et al., 1999). The region of substrate specificity of *Petunia* DFR was determined to be between 131-167 amino acids in the polypeptide. A comparison of this region of *Petunia* DFR with known *DHK* accepting enzymes revealed that 4 amino acid residues (underlined in Fig. 3.16) were conserved among all species, except in *Petunia.* However, these 4 residues are not shared by *Petunia* and *Cymbidium* DFR ruling out the possibility of common substrate specificity region in the two species. Our comparison of *Dendrobium* and *Cymbidium* DFR has shown that the 4 amino acids unique to *Petunia* DFR are not shared by *Dendrobium* also (Fig. 3.16). In fact these 4 amino acids are conserved among all *DHK* accepting enzymes as well as *Cymbidium* and *Dendrobium*. Therefore, the substrate specificity of orchids must be determined by another region of the enzyme.

Most *Dendrobium* hybrids contain cyanindin and peonidin as their major pigment aglycone (Kuehnle et al., 1997). Two unique lines, *D.* Icy Pink 'Sakura' and *D.* Waianae Blush, have pelargonidin as their major anthocyanidin with the amount of cyanidin limited to only 2%. The pedigree of K1224 shows that this rare unique colored phenotype occurred in the progeny of a cross of white x two-tone lavender parents (Kamemoto et al., 1999). Such a color change can occur due to two reasons as follows: a mutation of DFR enzyme changing its substrate specificity

from *DHQ* to *DHM*; or a mutation of F3'H enzyme reducing the amount of *DHQ* making *DHK* the most abundant substrate available for DFR. Sequence comparison of DFR from a typical purple phenotype, UH503, with that of K1224 (pelargonidin accumulating line) has shown that the 2 sequences are identical in the coding region of the gene. This is not surprising since the pedigree analysis of UH503 and K1224 clearly indicates the presence of *Dendrobium* Jaquelyn Thomas (O580-4N) as a common genome in the background of the two hybrids (Kamemoto et al., 1999). Therefore, the most probable reason for the pelargonidin accumulation in K1224 is a mutation leading to a reduction of F3'H enzyme activity.

Rosa hybrida	SVNVEETQKPVYNESNWSDVEFCRRVKMTGWMYFAS
Dianthus caryophyllus	TVNVEATQKPVYDETCWSDLDFIRSVKMTGWMYFVS
Gerbera hybrida	TVNGQEKQLHVYDESHWSDLDFIYSKKMTAWMYFVS
Antirrhinum majus	TVNVEEHQKPVYDETDSSDMDFINSKKMTGWMYFVS
Zea mays	TVNLEERQRPVYDEESWTDVDFCRRVKMTGWMYFVS
Petunia x hybrida	TLDVQEQQKLFYDQTSWSDLDFIYAKKMTGWMYFAS
Cymbidium	TVNVEEHQATVYDESSWSDLDFVTRVKMTGWMYFVS
Den-DFR	TVNVEEHQAAVYDESCWSDLDFVNRVKMTGWMYFLS

Figure 3.16. Alignment of part of amino acid sequences of 5 DFR enzymes accepting *DHK* as a substrate, along with *Petunia*, *Cymbidium*, and *Dendrobium* DFR. Fully conserved residues are in bold letters. Amino acids that are unique to *Petunia* and are important in determining substrate specificity of DFR are underlined. *Cymbidium* and *Dendrobium* DFR contain the same amino acid residues in these positions as other *DHK* accepting enzymes and differ from the DFR of *Petunia*.

Our northern analysis indicates that the *f3'h* expression is non-detectable in K1224 flower buds, while UH503 has detectable amounts of *f3'h* mRNA. Expression of *f3'5'h* is barely detectable even in 20 μg of total RNA in both phenotypes. These evidence points towards a reduction of *f3'h* activity in K1224 as the reason for its pelargonidin production.

Niesbach-Klosgen et al. (1987) used chalcone synthase as a tool to study the evolutionary relationships among plants. Our data for the amino acid sequence of chalcone synthase gene shows that amino acid sequence is highly conserved across 25 plant species. The *Bromheadia* CHS, the closest relative of *Dendrobium* has 97% similarity (94% identity) at the amino acid level. Amino acid sequence alignment showed 76-94% identity between the *Dendrobium* CHS and other plant sequences. However, the presence of other enzymes 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., 1998a). Northern analysis with the *Den-chs-1 1* revealed expression in buds flowers and leaves with the highest expression in stage 2 to 5 (small-medium) buds. This is consistent with the results of *Bromheadia* (Liew et al., 1998a). Southern analysis indicates that multiple genes of chalcone synthase are found in *Dendrobium*. However, there is a possibility of cross-reaction of the probe with related genes such as bibenzyl synthase and stilbene synthase as explaned in Liew et al., (1998a). 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;

reviewed in 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). The clone we have isolated seemed to be strongly expressed in floral buds. This gene will be a useful tool in the future for shutting down the anthocyanin biosynthesis in *Dendrobium* for breeding white phenotypes.

Isolation of cytochrome P450 genes involved in flower color is fairly recent compared to *dfr* and *chs* genes. The first report was of *f3'5'h,* isolated from *Petunia hybrida* (Holton et al., 1993), and *f3'h* clone was isolated only in 1999 (Brugliera et al., 1999). Although highly conserved heme-binding domains are present in both genes, these sequences have high degeneracy making it difficult to design effective primers (Shimada et al., 1999). We were able to isolate a partial clone (40% of the ORF) of *f3'5'h* with very high similarity to other proven *f3'5'h* sequences. Expression of this gene is extremely low in *Dendrobium* flower buds possibly explaining the lack 3', 5' hydroxylated anthocyanidins (delphinidin) in *Dendrobium.* Introduction of a *f3'5'h* gene from another plant species is a possible path of color manipulation towards blue in *Dendrobium.*

3.6 CONCLUSION

Isolation of flavonoid genes from *Dendrobium* is an extremely important step towards the understanding flower color of *Dendrobium.* Information on anthocyanins present in flowers (Kuehnle et al., 1997) along with data on expression of flavonoid genes will enhance the classical breeding program. This will allow the plant breeder to understand the molecular basis of flower color in parent plants

enabling better prediction of flower color in the progeny. In addition, pinpointing the genetic block of the pathway and substrate specificity of the enzymes will enable the scientists to identify the best phenotypes for genetic engineering to modify flower color.

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CHAPTER 4

METABOLIC ENGINEERING OF *DENDROBIUM* ORCHIDS TO MODIFY FLOWER COLOR

4.1 INTRODUCTION

Flavonoids not only provide an attractive array of colors for flowers and fruits but also play an important role in human health and nutrition due to their antioxidant, estrogenic, and anticancer properties (Adlercreutz and Mazur, 1997; Dixon, 1999, Rice-Evans et al., 1997). Therefore, considerable interest in metabolic engineering of the phenylpropanoid pathway to produce new flavonoids and isoflavonoids is found in recent research (reviewed by Dixon and Steele, 1999). For flower color manipulations, the flavonoid synthetic pathway is the main focus in metabolic engineering since it is the most studied and the easiest to manipulate without much adverse effect on the whole plant (Davies et al., in Press).

There are three main approaches in flower color manipulation as follows: 1) Introduction of biosynthetic genes from other species to produce novel colors; 2) Sense or antisense suppression of the biosynthetic enzymes; 3) Introduction of regulatory genes in order to activate the native biosynthetic gene(s).

4.1.1 New Colors through Introduction of Biosynthetic Genes

The first report on flower color modification using biotechnology was found in *Petunia* where a *dfr* gene (*a1*) from *Zea mays* was introduced into a *Petunia* mutant that accumulates dihydrokaempferol (*DHK*) to produce brick-red colored

flowers (Meyer et al., 1987). It was used to overcome the substrate specificity of *Petunia* Dihydroflavonol 4-reductase enzyme (DFR), allowing the flowers to accumulate orange pelargonidin-based anthocyanins in a mutant that lack flavonoid hydroxylase activity. Later, traditional breeding of these transgenic plants provided attractive orange-colored *Petunia* flowers (Griesbach, 1993; Johan et al., 1995). Introduction of *Rosa* and *Gerbera dfr* into *Petunia* also resulted in similar colors (Helariutta et al., 1993; Tanaka et al., 1995). Comparison of all three transgenics revealed that *Gerbera* DFR gave a more consistent and stronger expression in *Petunia,* emphasizing the importance of the source of the transgene in the success of color manipulation (Elomaa et al., 1995). Substrate specificity of DFR was also shown to be the reason of lack of orange colors in *Cymbidium* (Johnson et al., 1999).

Some major ornamental plant species such as rose, chrysanthemum, and carnation have little or no delphinidin in their flowers and lack blue flowers. Successful introduction of the flavonoid 3', 5'-hydroxylase (F3'5'H) enzyme that produces delphinidin has already been accomplished in carnation (by International Flower Developments, a joint venture between Florigene Ltd., Australia and Suntory Ltd., Japan). Introduction of *Petunia f3'5'h* and *dfr* genes into a DFR-deficient white carnation has enabled the production of mauve colored carnation flowers (reviewed in Tanaka et al., 1998; described in International Patent Application PCT/AU96/00296). Commercial varieties named Moondust™ and Moonshadow™ are marketed in Australia, Japan and USA (Davies et al., in Press; Tanaka et al., 1998). However, introduction of the F3'5'H enzyme alone might be insufficient to

produce a true blue flower in ornamentals. Full activity of F3'5'H also requires a cytochrome b_5 protein in *Petunia* (de Vetten et al., 1999). Introduction of a *Petunia* cytochrome b_5 together with a *Petunia* F3'5'H increased the activity of the introduced F3'5'H in transgenic carnation (Brugliera et al., 2000). Other factors such as vacuolar pH, copigmentation, and intramolecular interactions are also important in getting a true blue color (Brouillard and Dangles, 1993). An important step towards controlling the vacuolar pH of the petals is already made with the isolation of genes controlling this trait in morning glory flowers (Fukuda-Tanaka et al., 2000; Yamaguchi et al., 2001).

Another approach to generate novel colors is through production and accumulation of colored flavonoids such as chalcones and aurones, which are yellow in color (Davies et al., in Press). Chalcones are unstable intermediates involved in flavonoid biosynthesis and appear colorless or light yellow. Several important ornamentals such as pelargonium, cyclamen, lisianthus and impatiens do not have yellow colored flowers. Transgenic plants that accumulate yellow colored flavonoids were produced by introducing a chalcone reductase (CHR) cDNA from *Medicago sativa* into a white-flowered *Petunia* mutant (Davies et al., 1998). Transgenic plants accumulated a new flavonoid, 6'-deoxychalcones, instead of more common 6'-hydroxychalcone. Deoxychalcone is not accepted as a substrate by chalcone isomerase, thereby reducing the flow of intermediates towards anthocyanins and allowing light yellow chalcones to be accumulated in flowers.

Another type of anthocyanidins known as 3-deoxyanthocyanidins confers bright red, orange and scarlet in some species of Gesneriaceae (Harborne, 1966). These anthocyanidins differ from the more common type since they do not have a hydroxyl group at the C-3 position, which is usually glycosylated. A cDNA encoding a key enzyme in this pathway, flavonone reductase (FNR) isolated from *Sinningia cardinalis,* was introduced into *Arabidopsis* and *Petunia* mutants under the control of CaMV 35S promoter (Davies et al., in Press). However, the authors did not detect any production of 3-deoxyanthocyanins.

4.1.2 Sense or Antisense Suppression of the Flavonoid Biosynthetic Genes

Expression of a gene can be inhibited by introducing a gene of interest or a close homologue of it, in sense (cosuppression) or antisense direction (Mol et al., 1990). Although it seems to be a simple task, suppression of a gene is more difficult to achieve than expression (Tanaka et al., 1998). Efficiency of the suppression was shown to be dependent on the transgene promoter strength and homology between the transgene and the native gene (Que et al., 1997). Several distinct theories are put forward that involve DNA-DNA, DNA-RNA, and RNA-RNA interactions in order to explain the cosuppression phenomena (reviewed in Depicker and van Montagu, 1997; Fagard and Vaucheret, 2000; Gallie, 1998; Joseph et al., 1997; Jorgensen, 1995). One theory that is increasingly gaining more evidence suggests it to be mediated by double stranded RNA (dsRNA) (Montgomery and Fire, 1998).

Despite the difficulty, inhibition of pigment synthesis by introduction of sense and antisense *chs* or *dfr* was achieved in *Petunia* (Napoli et al., 1990; van

Blokland et al., 1994; van der Krol et al., 1988 & 1990), *Chrysanthemum, Dianthus caryophyllus, Eustoma grandiflorum, Gerbera hybrida, Torenia hybrida,* and *Rosa hybrida* (reviewed in Ben-Meir et al., 2002; Davies and Schwinn, 1997; Elomaa and Holton, 1994; Tanaka et al., 1998). Uniform white flowers were obtained in *Chrysanthemum* and *Dianthus,* while white-patterned phenotypes resulted in *Petunia, Eustoma* and *Torenia.* Some transformants of *Gerbera hybrida, Rosa hybrida, Dianthus caryophyllus* and *Torenia hybrida* gave pale colored phenotypes due to reduction of anthocyanin synthesis throughout the flower (reviewed in Tanaka et al., 1998).

Another interesting approach through suppression is to redirect intermediate substrates into a different branch of the flavonoid pathway for production of new colors. Intermediates such as dihydroflavonols are the precursors of colored anthocyanins as well as colorless flavonols. Introduction of flavonol synthase *(fls)* gene in antisense direction has resulted in higher anthocyanin content in petunia (Davies et al., 2003), tobacco (Holton et al., 1993a) and lisianthus (Nielsen et al., 2003). A similar result was reported by sense suppression of *fls* in a petunia cv. Surfinia Pink where flower color changed from pink to red purple (reviewed in Tanaka et al., 1998).

4.1.3 Introduction of Regulatory Genes to Activate the Native Biosynthetic Gene(s)

There are various regulatory genes belonging to Myb, and bHLH (basic helix-loop-helix) families of transcription factors, which activate distinct branches of

the anthocyanin biosynthesis isolated from maize (e.g. *c1*- Myb type, *r*-bHLH type), snapdragon (*delila*-bHLH type) and petunia (*an2*-Myb) (reviewed in Holton and Cornish, 1995; Mol et al., 1998). These regulatory genes were used to modify plant and flower color in *Arabidopsis, Nicotiana,* and *Petunia* (reviewed in Martin et al., 2001). *Petunia* transformants containing a maize regulatory gene, *leaf color* (*Lc,* belonging to the Myb family), under the control of the CaMV 35S promoter contained very high levels of anthocyanin in the foliage and floral tube (Bradley et al., 1998). However, the same construct did not give any difference in phenotype in lisianthus, pelargonium, or chrysanthemum (Bradley et al., 1999) but has shown a color change from pink to intense red in tobacco (Lloyd et al., 1992). Introduction of *Delila* (a bHLH type homologue of r gene from snapdragon) into tobacco and tomato also resulted in increased anthocyanin content in the flowers and foliage (Mooney et al., 1995). A Myc type transcription factor, *c1* from maize, driven by the CaMV 35S promoter had no effect on flower color of tobacco (Lloyd et al., 1992). Another important regulatory gene belonging to Myb family was isolated from *Antirrhinum,* which is responsible for the venation pattern in flowers (Schwinn et al., 2001). This might be a useful gene to create genetically stable colored venation patterns as we have seen in some *Dendrobium* flowers.

4.2 OBJECTIVE

Our objective is to produce new colors of orange-red and blue shades in *Dendrobium* through metabolic engineering. We have chosen a pelargonidin-accumulating hybrid *Dendrobium* Icy Pink 'Sakura' (K1224) as the prime candidate

for genetic manipulation. Chemical analysis (Kuehnle et al., 1997) and molecular analysis (Chapter 3) together with classical breeding data suggest that substrate preference of DFR and predominance of the F3'H activity as the most probable reason for the rarity of orange pelargonidin in *Dendrobium.* In order to produce orange shades in *Dendrobium*, we have introduced an *Antirrhinum dfr* gene (courtesy of Dr. Kathie Martin via Food and Crop Research Ltd. New Zealand), which encodes a DFR that readily accepts *DHK* as a substrate. It is also important to use a genotype that does not have F3′H and F3′5′H activity since these two enzymes compete with DFR for the same substrate. Chemical analysis of K1224 revealed the absence of detectable levels of 3′-hydroxylated, and 3′, 5′-hydroxylated intermediates (Kuehnle et al., 1997) and molecular analysis revealed undetectable levels of transcription of these 2 genes in K1224 (Chapter 3). Production of anthocyanin in K1224 suggests that, the downstream enzymes, ANS and UFGT are active in K1224, making it a suitable candidate to produce orange shades.

Dendrobium Jaquelyn Thomas 'Uniwai Prince' (UH503), a purple cyanidin accumulating flower, has shown the presence of both *f3'h* and *f3'5'h* cDNA in their flower buds despite the lack of 3', 5'-hydroxylated anthocyanins in *Dendrobium.* This can be due to low activity of the enzyme, low transcription of the enzyme as shown by no detectable amounts even in 20 μg of total RNA (Chapter 3), competition of the two enzymes for the same substrate (F3′H and F3′5′H), or a combination of both. In order to avoid substrate competition, it is important to choose a genotype, which is deficient in F3′H activity. Therefore, K1224, which

contain only 2% 3′-hydroxylated anthocyanins, is a suitable candidate for insertion of *f3′5′h* gene. We have chosen a *f3′5′h* gene from a non-orchid plant (proprietary information, obtained from Florigene Ltd., Melbourne, Australia under a limited license agreement with University of Hawaii) to engineer blue flower color into *Dendrobium.*

4.3 MATERIALS AND METHODS

4.3.1 Plant Material

Apical and axillary buds of *Dendrobium* Icy Pink 'Sakura' (K1224) was surface sterilized in 10% Clorox solution and placed in modified VW (Vacin and Went, 1949; Sagawa and Kunisaki, 1984) liquid growth medium supplemented with 2% sucrose (w/v) and 15% (v/v) coconut water, for generation of protocorm-like-bodies (PLB). PLBs were multiplied and maintained in VW medium at 100 rpm, 16-hour photoperiod of 19.0 ± 5 μmol m^{-2} sec^{-1} photon flux density provided by cool white and Gro-lux Sylvania fluorescent lamps (GTE Corp., Danvers, MA). Thirty five to forty PLBs were placed on ½ strength solidified MS (Murashige and Skoog, 1962) media, supplemented with 2% sucrose (w/v), and 0.7% granulated agar (w/v) (Fisher Scientific, Fair Lawn, NJ) in 6.0 x 1.5 cm disposable Petri plates for bombardment.

4.3.2 Color Genes, Plasmid DNA and Particle Bombardment

We have used the *dfr* gene from *Antirrhinum majus* (*Snp-dfr*), the *f3′5′h* and *f3′h* genes from *Petunia x hybrida* (*Pet-f3′5′h*, *Pet-f3′h*) (Holton et al., 1993b; Brugliera et al., 1999) obtained from Florigene Ltd., Melbourne, Australia, and

another *f3'5'h* gene from a non-orchid plant (*NP-f3'5'h*) for color modification experiments. The *dfr* gene and the two genes from *Petunia* were sub-cloned into the plasmid vectors of pBI525 and pSAN150 (courtesy of Sanford Scientific Inc.) in both sense and anti-sense directions (Table 4.1). The *NP-f3'5'h* gene was subcloned into pSAN150 in sense direction only. The two plasmids, pBI525 and pSAN150, have double Cauliflower Mosaic Virus (CaMV) 35S promoter (35S-35S) and *Arabidopsis* ubiquitin3 (UBQ3) promoter (courtesy of Sanford Scientific with permission from Dr. J. Callis), respectively. Two selectable marker genes were used for co-bombardment: The *uidA* (neomycin phosphotransferase) under the control of 35S-35S promoter in plasmid pBI426 (gift of W. Crosby, Plant Biotechnology Institute, Saskatoon, Canada), and *hpt* (hygromycin phosphotransferase) under the control of UBQ3 promoter in plasmid pSAN154 (Table 4.1). All constructed plasmids were transformed into *Escherichia coli* DH5α strain and plasmid DNA was extracted using plasmid DNA isolation miniprep or midiprep kits (Qiagen, Valencia, CA).

Plasmids containing color gene and the selectable marker were coated onto 1.6 μm gold particles (Bio-Rad, Hercules, CA) in a 1:1 ratio, using the procedure used by Nan (1994). Microprojectile bombardments were carried out using Bio-Rad model PDS-1000/He Biolistic® particle delivery system (Bio-Rad, Hercules, CA) at a vacuum pressure of 26-28 Hg mm, rupture disc pressure of 1100 psi, and target tissue placed on the 4th stage from the top using the standard laboratory protocol (Nan, 1994). Plates with shot PLBs were kept in dark for 2 days. All plant tissues

were placed on growth media (½ strength MS solid or VW liquid) for approximately two weeks without selection and subsequently transferred into selection media.

Table 4.1. Different plasmid constructs of color genes, selectable markers, and promoters used for particle bombardment into orchid tissue.

Plasmid ID	Gene[a]	Plant source	Promoter	Purpose
pSAN150-SNP-DFR	*dfr*	*Antirrhinum majus*	UBQ3	Increase the orange-red shades
pSAN150-ANTI-DFR	Antisense *dfr*	*A. majus*	UBQ3	Reduce the activity of *Dendrobium* DFR
pBI525-SNP-DFR	*dfr*	*A. majus*	35S-35S	Increase the orange- red shades
pBI525-ANTI-DFR	Antisense *dfr*	*A. majus*	35S-35S	Reduce the activity of *Dendrobium* DFR
pSAN150-NP-F3'5'H	*f3'5'h*	(Proprietary information)	UBQ3	Induce the production of blue colored anthocyanins
pBI525-PET-F3'5'H	*f3'5'h*	*Petunia x hybrida*	35S-35S	Induce the production of blue colored anthocyanins
PBI525-ANTI-F3'5'H	Antisense *f3'5'h*	*P. x hybrida*	35S-35S	Shut down the production of cyanidin
pSAN150-ANTI-F3'5'H	Antisense *f3'5'h*	*P. x hybrida*	UBQ3	Shut down the production of cyanidin
pSAN150-PET-F3'H	*F3'h*	*P. x hybrida*	UBQ3	Suppress or increase the production of cyanidin
pBI525-PET-F3'H	*F3'h*	*P. x hybrida*	35S-35S	Suppress or increase the production of cyanidin
pSAN150-ANTI-F3'H	*F3'h*	*P. x hybrida*	UBQ3	Suppress the production of cyanidin
pBI525-ANTI-F3'H	*F3'h*	*P. x hybrida*	35S-35S	Suppress the production of cyanidin
pSAN154	*hpt*		UBQ3	Selectable marker for screening transformed plants
pBI426	*uidA*		35S-35S	Selectable marker for screening transformed plants

[a] GenBank accession numbers for genes are as follows: *Snp-dfr*=X15536, *Pet-f3'h*=Z22544, *Pet-f3'5'h*=AF155322.

All bombardments done with *Dendrobium* Icy Pink 'Sakura' (K1224) are listed in Table 4.2. All other bombardments we have done with different *Dendrobium* hybrids are listed in Appendices B-D.

4.3.3 Selection and Regeneration of Transformants

Different levels of geneticin (Sigma, St. Louis, MO) ranging from 25-50 $mg \cdot L^{-1}$ were used for selection of PLBs co-bombarded with pBI525 while hygromycin B (Sigma, St. Louis, MO) levels of 10-30 $mg \cdot L^{-1}$ were used for those co-bombarded with pSAN154. Selection regime used for each bombardment is listed with details in Table 4.2 and appendices B-D. Plantlets were regenerated from PLBs on ½ strength MS or banana medium (VW medium supplemented with 75 g of blended unripe banana flesh and solidified with 15 $g \cdot L^{-1}$ agar) in 10 cm x 2.5 cm disposable Petri plates. Growing plantlets were transferred into Magenta boxes (G12) for further growth while keeping track of the plate number and the PLB number of each plantlet.

4.3.4 PCR and RT-PCR Analyses

Two methods of sampling were used in DNA extraction of regenerated plantlets. For those with a few surviving plants, each individual plant within a magenta box was numbered and sampled separately for DNA extraction. When the numbers were too large for individual sampling, one leaf from each plant in a single magenta box was cut and all leaves were extracted together (pooled samples). Genomic DNA was extracted from collected leaves using DNeasy® Plant minikit (Qiagen, Valencia, CA) following manufacturer's instruction

Table 4.2. Co-bombardment of *Dendrobium* Icy Pink 'Sakura' (K1224) PLBs.

Constructs and Date of Bombardment	# of[a] Plates	Selection Regime	Transfer to Regeneration Medium	% Survival of PLBs[b]	Status of the experiment
pBI426 pBI525-PET-F3'5'H 12/22/97	3	17 days in liquid, no selection 14 days in liquid with Geneticin 50 mg·L^{-1}	1/21/98	4.7% (5/105) 7/30/98	Two compots were potted on 4/28/99, 1 plant PCR+, flowered in July, 2000, no color change relative to controls. Flowers normal, transformation and regeneration protocol non-mutagenic.
None 12/22/97	2	17 days in liquid, no selection 14 days in liquid with Geneticin 50 mg·L^{-1}	1/21/98	1% (1/70) 7/30/98	
pSAN154 pSAN150-SNP-DFR 9/17/98	6	11 days in liquid, no selection 1) 15 days liquid with Hygromycin 12.5 mg·L^{-1} 2) 15 weeks on solid with Hyg. 12.5 mg·L^{-1}	10/14/98 2nd selection on 2/10/98 re: 3/25/99	24% (50/210) 5/24/99	85 pooled samples tested for PCR, 50% positive for color gene, potted out all positive plants in growth chamber or green house, first flowering in Nov. 2002. No visual color change compared to non-bombarded plants.
pSAN154 pSAN150-SNP-DFR 9/17/98	2	11 days liquid, no selection 1) 15 days liquid with Hygromycin 12.5 mg·L^{-1} 2) 15 weeks on solid with Hyg. 12.5 mg·L^{-1}	10/14/98 2nd selection[c] on 12/10/98 re: 3/25/99	37% (26/70) 5/24/99	
None 9/17/98	1	11 days liquid, no selection 1) 15 days liquid with Hygromycin 12.5 mg·L^{-1} 2) 15 weeks on solid with Hyg. 12.5 mg·L^{-1}	10/14/98 2nd selection[c] on 12/10/98 re: 3/25/99	15% (6/40) 5/24/99	

[a] 35-40 PLBs per plate

[b] Number of PLBs producing plantlets after antibiotic selection out of total number bombarded

[c] PLBs were transferred onto regeneration medium and transferred back to selection medium on 12/10/98 for a 2nd round of selection Final regeneration on 3/25/99

Table 4.2. (Continued) Co-bombardment of *Dendrobium* Icy Pink 'Sakura' (K1224) PLBs.

Constructs and Date of Bombardment	# of[a] Plates	Selection Regime	Transfer to Regeneration Medium	% Survival of PLBs[b]	Status of the experiment
pSAN154 pSAN150-NP-F3'5'H 4/5/99	7	17 days on solid, no selection 3 months on solid Hygromycin 10 mg·L^{-1}	7/26/99	35% (82/233) 11/2/99	11/54 pooled samples and 26/53 individuals positive for color gene. Most potted out.
None 4/5/99	1	17 days on solid, no selection 3 months on solid Hygromycin 10 mg·L^{-1}	7/26/99	10% (4/40) 11/2/99	
pSAN154 pSAN150-ANTI-DFR pSAN150-NP-F3'5'H 6/7/99	5	14 days on solid, no selection 4.5 months on solid with Hygromycin 12.5 mg·L^{-1}	11/2/99	19% (37/191) 11/2/99	In G12 Magenta boxes, PCR testing is not completed yet.
None 6/7/99	1	14 days on solid, no selection 4.5 months on solid with Hygromycin 12.5 mg·L^{-1}	11/2/99	32% (12/37) 11/2/99	
pSAN154 pSAN150-NP-F3'5'H 1:3 ratio 12/17/99	10	14 days in liquid, no selection 4 weeks in liquid, Hygromycin 30 mg·L^{-1}	1/31/00	10% (35/350)	In 40 G12 Magenta boxes, PCR sampling is not completed yet. Low survival rate due to high selection pressure.
None 12/17/99	4	14 days in liquid, no selection 4 weeks in liquid, Hygromycin 30 mg·L^{-1}	1/31/00	8.5% (3/35)	

[a] 35-40 PLBs per plate

[b] Number of PLBs producing plantlets after antibiotic selection out of total number bombarded

Primer sequences and PCR conditions used for detection of different genes are summarized in Table 4.3. All PCR reactions were carried out with 200-250 ng of genomic DNA, RedTaq DNA polymerase (Sigma, St. Louis, MO), 0.4 μM primer concentration and 0.2mM dNTP concentration in an iCycler thermal cycler (Bio-Rad, Hercules, CA).

For RT-PCR analyses, RNA from leaf tissues of transformed plants that tested positive for the inserted color gene by PCR, were extracted using extraction method of Champagne and Kuehnle (2000). An aliquot containing 1 μg of total RNA was treated with 1 unit of DNase I (Ambion Inc., Austin, TX) in 1X DNase I buffer in a 10 μL volume at 37°C for 20 minutes, according to the manufacturer's protocol. The activity of DNase was stopped by adding 1.1 μL of DNase inactivation reagent provided with the enzyme. cDNA was synthesized from 1μg of total RNA using 100 units of SuperScript® II RNase H^- reverse transcriptase (Invitrogen, Carlsbad, CA) in 1X first strand synthesis buffer (50 mM Tris-HCl, 75 mM KCl, 3mM $MgCl_2$, pH 8.3), supplemented with 0.01M DTT and 0.5mM dNTPs, by incubating the reaction mixture at 42°C for 50 minutes. Oligo dT (T_{20}-T7) primer was used to prime the first strand synthesis. Reaction was stopped by incubation of the mixture at 70°C for 15 min. RNA template was removed by incubating the reaction mixture with 2 units of RNase H (Promega, Madison, WI) at 37°C for 20 minutes. The RNase H enzyme was inactivated by incubation at 60°C for 10 min. Sample was stored at –20°C for PCR amplifications. PCR conditions are same as listed in Table 4.3.

Table 4.3. Primer sequences and PCR conditions used in amplification of the inserted gene from transgenic plants.

Primer ID	sequence	Gene amplified	PCR conditions
antDFR-L	ggctacaccgttcgtg-3'	*Snp-dfr* gene	94°C-4 min (94°C-45 sec, 54°C-30 sec, 72°C-1 min) x 30 72°C-8 min. 2.9 mM $MgCl_2$ concentration, RedTaq (Sigma)
antDFR-R	atttcttgacggtttttgcttgc-3'		
FL-NP-2L	cgaagatgatgaaggagcacag-3'	*NP-f3'5'h* gene	95°C-4 min (95°C-45 sec, 55°C-45 sec, 72°C-45 sec) x 35 72°C-7 min. 1.0 mM $MgCl_2$ concentration, RedTaq (Sigma)
FL-NP-2R	caatcgtggactgaccgtag-3'		
HYG-5'	aagttcgacagcgtctccgac-3'	*hpt*	95°C-4 min (95°C-1 min, 61°C-1 min, 72°C-2 min) x 40 72°C-8 min. 2.3 mM $MgCl_2$ concentration, RedTaq (Sigma)
HYG-3'	ttctacacagccatcggtcca-3'		
F3'5'H-5'	gtggcggagatgttgac-3'	*Pet-f3'5'h*	94°C-4 min (94°C-1 min, 58°C-1 min, 72°C-2 min) x 40 72°C-8 min. 1.7 mM $MgCl_2$ concentration, RedTaq (Sigma)
F3'5'H-3'	attcttcgtccagcacc-3'		
NEO-5'	aggctattcggctatgactgg-3'	*uidA* (*nptII*)	95°C-5 min (95°C-1 min, 58°C-1 min, 72°C-2 min) x 30 72°C-7 min. 1.7 mM $MgCl_2$ concentration, RedTaq (Sigma)
NEO-3'	agaaggcgatagaaggcgatg-3'		

RT-PCR products were separated on a 1.0% agarose gel in 1X TAE buffer for Southern blotting. The DNA was transferred overnight onto Biodyne B membrane (Pierce, Rockford, IL) by downward capillary transfer using alkaline transfer buffer (0.4M NaOH). Membrane was neutralized by immersing in neutralization buffer [0.5M Tris-HCl (pH 7.2), 1M NaCl] for 15 minutes.

4.3.5 Northern Blot Analysis

Leaf samples were harvested from plants that were positive for RT-PCR testing. Total RNA was extracted as described previously. Different amounts of total RNA (8, 5, or 3 μg) from different plants was size fractionated on a 2% formaldehyde agarose (0.9%, w/v) gel electrophoresis. The RNA was transferred overnight onto Nytran Supercharge™ nylon membrane (Schleicher & Schuell, Keene, NH) by downward capillary transfer using alkaline transfer buffer (3MNaCl, 0.01N NaOH). Hybridization and washing was done according to modified Church and Gilbert (1984) procedure as described previously in Chapter 3.

4.3.6 Probe synthesis for Northern and Southern Hybridization

Gene constructs used in bombardment was cut with appropriate restriction enzymes to release the color gene inserts. The cDNA inserts of the color genes were separated from the plasmid backbone by size fractionation on a 1% agarose gel in 1X TAE buffer. DNA fragments were cut out from the gel and purified (GENECLEAN II kit, Bio101, Carlsbad, CA). A 20 ng of the purified cDNA was labeled with α^{32}P-ATP using random primer labeling (Prime a Gene kit, Promega, Madison, WI). Labeled product was cleaned using purification column (Schleicher and Shuell,

Keene, NH). Cleaned probe was added to the hybridization buffer at a concentration of 1 million cpm/1ml of buffer. Blots were hybridized and washed using modified Church and Gilbert (1984) procedure as described in Chapter 3.

4.3.7 Southern Hybridization and Development

Southern hybridization was done with a non-radioactive North2South® direct HRP labeling and detection kit (Pierce, Rockford, IL). The labeling was done using 100ng of purified *NP-f3'5'h* insert in a 30μL volume using direct HRP (Horse Radish Peroxidase) label in reaction buffer provided with the kit at 45°C for 15 minutes. Reaction was stopped by adding 30 μLs of enzyme stabilization solution.

The membrane was pre hybridized with 10 mLs of prehybridization buffer provided with the kit at 55°C. The labeled probe (100 ng) was added to the prehybridization buffer and incubated for 2 hours at 55°C. The membrane was moved to a new tube and washed three times with 40 mLs of wash buffer (2X SSC, 0.1% SDS) at 55°C for 15 minutes each. Three more washes were done with 2X SSC at room temperature for 15 minutes each. Excess fluid was drained off the membrane and was placed on a plastic tray. Chemiluminiscent substrate was prepared by mixing 5mLs of luminol/enhancer solution with 5 mLs of stable peroxide solution. The hybridized membrane was covered with the substrate solution and incubated for 5 minutes at room temperature. Substrate was drained and the developed blot was placed in between two plastic sheets. An X ray film was exposed to the signal for 1 minute and developed according to the manufacturer's instruction.

4.3.8 Potting, Acclimatization and Maintenance of PCR Positive Plants

All PCR positive plants were planted in shredded hapuu (tree fern) media or Sphagnum moss (Gold Moss brand, Puerto Varas, Chile) in 3" pots. When there are several plants per magenta box, they were planted together in 6" community pots. All plants were kept on the lab bench for 1-2 weeks to acclimatize to the low humidity and low light. Plants were later moved into the growth chamber and placed on humidi-trays at 130±5 μmol $m^{-2}s^{-1}$ light intensity. Once the plant got firmly established with new roots and shoots, they were moved to screened insect-proof boxes with a light intensity of 57±5 μmol $m^{-2}s^{-1}$. After 4 months of growth, plants were moved into a white colored larger box, which allowed more light (102±5 μmol $m^{-2}s^{-1}$) to penetrate for better growth.

4.4 RESULTS

4.4.1 Selection and Regeneration

We have used different selection regimes (Table 4.2) in both liquid and solid growth media for selecting the transformed plants from non-transformed plants. There was a high variation in tolerance of antibiotics among different *Dendrobium* hybrids (Table 4.2; Appendices B-D). High levels of antibiotic (geneticin 50 $mg{\cdot}L^{-1}$) for two weeks gave only a 5% recovery rate in PLBs of K1224 (Fig. 4.1A). This is an insufficient number of plants to obtain 20-40 individual transformants. Second selection regime (hygromycin 12.5 $mg{\cdot}L^{-1}$) for 15 days was not sufficient enough to kill all the non-transformed plants. However, selection in liquid media could not be continued due to bacterial contamination. We observed a more than 50% recovery in PLBs on

regeneration medium. A second round of selection with hygromycin (10 mg·L^{-1}) was done on solid media up to 15 weeks. A percentage survival rate of 24% and 37% was obtained after the second selection (Table 4.2; Fig. 4.1B). The number of plantlets that is regenerated from a single surviving PLB ranged from 1 to 26. We have used a selection on solid media supplemented with 10 mg·L^{-1} for 3 months for the next experiment. A 35% survival rate was obtained from this selection protocol.

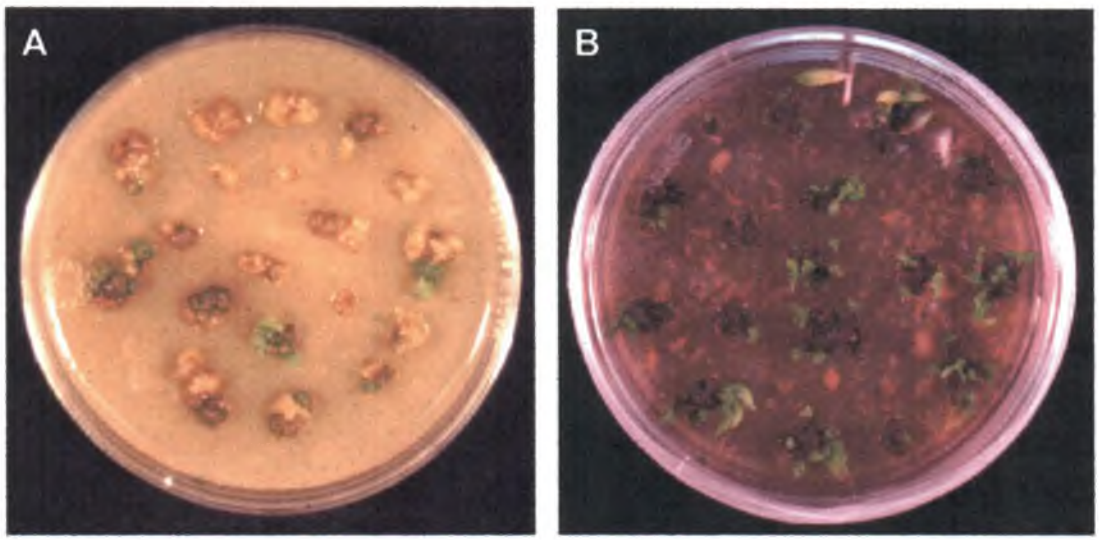

Figure 4.1. *Dendrobium* Icy Pink 'Sakura' (K1224) protocorm-like-bodies regenerating on banana media after antibiotic selection. (A) geneticin 50 mg·L-1 in liquid VW media for 2 weeks. (B) hygromycin 12.5 mg·L-1 for 2 weeks in liquid VW followed by hygromycin 10 mg·L-1 for 15 weeks in solid banana media.

One of the major problems we faced in liquid selection was contamination with bacteria. Bacterial contamination is easy to spread in liquid selection media and results in loss of all PLBs from a single plate. In solid selection, contamination was easier to control. Discarding the contaminated PLBs when transferring onto fresh media can minimize the loss. However, the solid selection requires a considerably longer time than liquid selection.

4.4.2 PCR Analyses

We have analyzed two sets of transgenic plants for the inserted color gene. Plants that are 1-2" tall (Fig. 4.2) were used for DNA extraction. The first set bombarded with pSAN154 and pSAN150-SNP-DFR (9/17/98) was analyzed for the color gene (*Snp-dfr*) as well as the selectable marker (*hpt*). An example of an ethidium bromide stained gel photograph of PCR product of the *Snp-dfr* gene is depicted in Fig. 4.3. Genomic DNA isolated from a non-transformed plant and sterile water without DNA was used as negative controls. The plasmid DNA (pSAN150-SNP-DFR) and/or genomic DNA from a known PCR positive plant were used as positive controls. From 83 samples tested, 18 samples were positive for both genes while 24 samples did not contain either of the genes (Table 4.4). There were 29 samples positive for the color gene only, while 12 were positive only for the selectable marker gene (Table 4.4). In summary, 48/85 samples were indicated to contain the color gene. Plants that are positive only for the color gene are very valuable since the antibiotic resistant gene is considered as an environmental concern in producing transgenic plants.

Table 4.4. Results of PCR experiments to detect the presence of *Antirrhinum dfr* gene and *hpt* gene from bombarded and selected plants of *Dendrobium* Icy Pink 'Sakura'.

DNA sample	µls[a]	DFR[b]	Hyg[c]	Pot date and status
1-1 A1	5.1	strong+	weak+	Potted 2/20/01, died
1-1 A2	6.1	strong+	weak+	Potted 2/20/01, moved to greenhouse 5/02/02
1-2 A1	3.6	weak+	strong+	Potted 2/20/01, moved to greenhouse 5/02/02
1-2 A2	3.9	strong+	weak+	Potted 2/20/01, moved to greenhouse 5/02/02
1-3 A1	6.4	-	weak+	Discarded
1-3 A2	7.1	-	-	Discarded
1-4 A1	6.7	-	-	Discarded
1-4 A2	6.7	-	-	Discarded
1-5 A1	6.9	-	-	Discarded
1-5 A2	5.1	-	-	Discarded
1-6 A1	6	medium+	weak+	Potted 2/20/01, moved to greenhouse 5/02/02
1-6 A2	5.7	strong+	weak+	Potted 2/20/01, moved to greenhouse 5/02/02
1-7 A1	5.6	strong+	-	Potted 01/22/02, died
1-7 A2	4.2	strong+	-	Potted 11/20/02
2-1 A1	7.3	-	-	Discarded
2-1 A2	7.8	-	-	Discarded
2-2 A1	8	-	-	Discarded
2-2 A2	8.9		-	Discarded
2-4 A1	7.8	-	weak+	Died before potting
2-4 A2	7.1	strong+	weak+	Potted 1/22/2, in growth chamber
2-5 A1	8.1	strong+	-	Potted 11/09/02, in growth Chamber
2-5 A2	10.9	strong+	-	Potted 11/09/02, in growth chamber
4-1 A1	8.9	-	-	Discarded
4-1 A2	10	-	weak+	Discarded
4-3 A1	8	-	-	Discarded
4-3 A2	12.5	-	-	Discarded
5-1 A1	6	-	weak+	Discarded
5-1 A2	9.2	-	-	Discarded
5-2 A1	11.7	weak+	strong+	Potted 10/24/01, died
5-2 A2	13	strong+	-	Potted 01/22/02, in growth chamber
5-3 A1	7	weak+	strong+	Potted 01/22/02, in growth chamber
5-3 A2	7	strong+	-	Potted 01/22/02, died
5-4 A1	6	-	-	Discarded
5-4 A2	6	-	-	Discarded
5-5 A1	5.7	Weak+	strong+	Discarded
5-5 A2	6.3	strong+	-	Discarded
5-6 A1	3.8	-	-	Discarded
5-6 A2	5.1	Medium+	-	Potted 06/24/02, in growth chamber.

Table 4.4. (Continued) Results of PCR experiments to detect the presence of *Antirrhinum dfr* gene and *hpt* gene from bombarded and selected plants of *Dendrobium* Icy Pink 'Sakura'.

DNA sample	µls[a]	DFR[b]	Hyg[c]	Pot date and status
5-7 A1	6.2	weak+	-	Discarded
5-7 A2	6.7	Medium+	-	Potted 06/24/02, in growth chamber.
6-1 A1	8.6	-	-	Discarded
6-1 A2	5.5	-	n.d.	Discarded
6-2/3 A1	5.1	-	weak+	Discarded
6-2/3 A2	4.6	-	-	Discarded
6-5 A1	5	-	strong+	Discarded
6-5 A2	5	Medium+	weak+	Potted 01/22/02, died
6-7 A1	5.6	-	-	Discarded
6-7 A2	4.4	-	-	Discarded
6-8 A1	5	strong+	strong+	Potted 2/20/01, moved to greenhouse 5/02 /02
6-8 A2	4.7	strong+	strong+	Potted 2/20/01, moved to greenhouse 5/02/02
6-9 A1	4	-	strong+	Discarded
6-9 A2	4.4	strong+	-	Potted 01/22/02, died
6-10 A1	6.6	-	weak+	Potted 6/24/02, in growth chamber
6-10 A2	5.1	-	-	Discarded
7-1+2 A1	4.4	weak+	-	Potted 01/22/02, in growth chamber
7-1+2 A2	4.2	weak+	-	Potted 01/22/02, in growth chamber
7-3 A1	6.1	medium+	-	Discarded
7-4 A1	5.7	strong+	n.d.	Discarded
7-4 A2	7.1	strong+	-	Potted 01/22/02, died
7-5 A1	4.4	weak+	-	Potted 01/22/02, in growth chamber
7-5 A2	4.7	-	-	Discarded
7-7 A1	6.6	-	weak+	Discarded
7-8 A1	5.4	medium+	-	Potted 8/17/01, moved to greenhouse 5/2/02
7-8 A2	8.9	strong+	weak+	Potted 1/22/02, died
7-9 A1	7.9	strong+	-	Discarded
7-9 A2	7.1	strong+	-	Potted 1/22/02 &11/9/02, in growth chamber
7-10 A1	7.6	strong+	strong+	Potted 8/17/01, moved to greenhouse 5/2/02
7-10 A2	8.1	strong+	-	Potted 10/20/01, moved to greenhouse 5/2/02
7-11 A1	5.3	weak+	-	Potted 10/24/01, moved to greenhouse 5/2/02
7-11 A2	5.3	-	strong+	Discarded
7-12 A1	6.9	weak+	-	Discarded
7-12 A2	6.9	medium+	weak+	Potted 11/09/02, in growth chamber
7-13 A1	4.3	medium+	weak+	Discarded
7-13 A2	5.9	medium+	weak+	Potted 01/22/02, in growth chamber
8-1 A1	7.1	medium+	-	Discarded
8-1 A2	6.6	-	n.d.	Discarded
8-6 A1	5.5	-	-	Discarded

Table 4.4. (Continued) Results of PCR experiments to detect the presence of *Antirrhinum dfr* gene and *hpt* gene from bombarded and selected plants of *Dendrobium* Icy Pink 'Sakura'.

DNA sample	µls[a]	DFR[b]	Hyg[c]	Pot date and status
8-7 A1	6.3	strong+	strong+	Potted 3/16/01, moved to greenhouse 5/2/02
8-7 A2	5.8	-	medium+	Discarded
8-8 A1	5.1	medium+	-	Potted 8/17/01, moved to greenhouse
8-8 A2	6.4	weak+	-	Potted 10/24/01,died
8-9 A1	6	strong+	strong+	Potted 1/22/02, in growth chamber
8-9 A2	4.8	strong+	-	Potted 10/24/01, in growth chamber
8-11 A1	6.1	strong+	-	Potted 01/22/02, in growth chamber
8-11 A2	7.6	-	-	Discarded

[a] Number of µLs containing 200 ng of DNA used for a PCR reaction.
[b] PCR result for the *Antirrhinum dfr* gene, strong, medium and weak represent the brightness of the DNA band. +=positive PCR result; -=negative PCR result; n.d.=not done.
[c] PCR result for the *hpt* gene, strong, medium and weak represent the brightness of the DNA band. +=positive PCR result; -=negative PCR result; n.d.=not done.

Figure 4.2. Regenerated plantlets of *Dendrobium* Icy Pink 'Sakura' (K1224) growing on ½ strength Murashige and Skoog medium after hygromycin selection and regeneration on banana medium. Leaves of regenerated plantlets were sampled to test for the presence of the inserted genes using PCR. All the plants in a magenta box (pooled sample) or individual plants were used for DNA extraction.

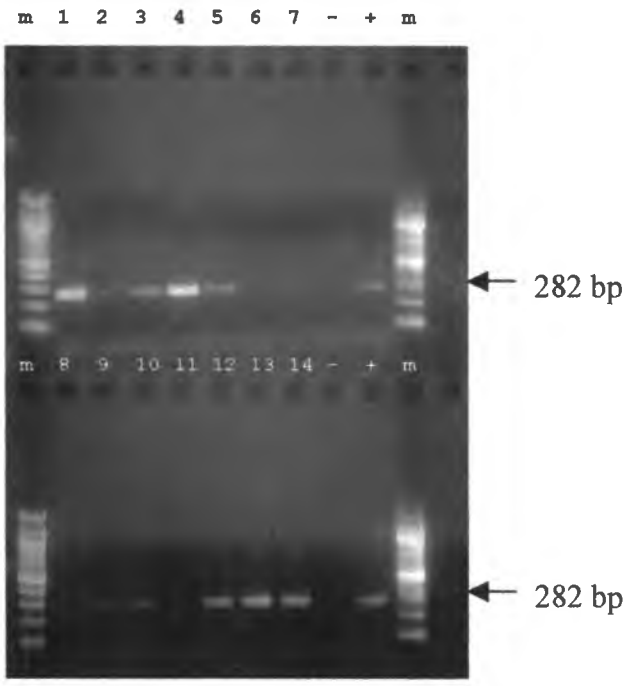

Figure 4.3. Agarose gel electrophoresis of PCR amplified products of *Antirrhinum dfr* (*Snp-dfr*) gene from bombarded *Dendrobium* K1224 plants. Primers specific for *Snp-dfr* gene were used to amplify a 282 bp fragment from genomic DNA of *Dendrobium* K1224 plants regenerated from PLBs co-bombarded with pSAN150-SNP-DFR and pSAN154. Lanes m=molecular weight markers; lanes 1-14=genomic DNA from pooled samples 1-1 A1, 1-2 A1, 1-6 A1, 1-7 A1, 2-4 A2, 2-5 A1, 5-2 A1, 5-3 A1, 5-5 A1, 5-6 A2, 5-7 A1, 6-5 A2, 6-8 A1 and 6-9 A2, respectively; lane -=genomic DNA from a control plant that was not bombarded with *Antirrhinum dfr*; lane +=genomic DNA from a known PCR positive plant.

A second set of K1224 plants bombarded with pSAN154 and pSAN150-NP-F3'5'H (4/5/99) were also analyzed through PCR for the presence of the color gene. Genomic DNA extracted from pooled samples or from individual plants was used for PCR analysis. A very low rate of positive transformants (11/54) was found among the pooled samples. But, half of the individual plants (26/53) were positive for the *NP-f3'5'h* gene (Table 4.5; Fig. 4.4). All the samples that were indicated to be positive were potted up for growth and flower color observations.

It is noteworthy that all the different plantlets growing from a single PLB did not show the same PCR result. Therefore, insertion and integration of the transgene might be different for each individual plant arising from a single PLB.

4.4.3 RT-PCR and Northern Analyses

We have used three individual plants and three pooled samples to test the expression of the transgene (*Snp-dfr)* from the first set of PCR positive plants. RT-PCR analysis indicated that all three individual plants of the pooled sample (6-8 A2) were positive for the expression of the inserted *Snp-dfr* gene (Fig. 4.5). In addition, two pooled samples (1-6 A1 and 1-1 A1) were also proven to be positive. Southern hybridization with HRP labeled cDNA probe of *Snp-dfr* show strong hybridization with the RT-PCR product confirming that the amplified fragment is *Antirrhinum dfr.*

Ten RNA samples from the second set of transformants were tested for the expression of the inserted color gene. Three individual plants 8-2-1, 8-2-4 and 8-2-6 were positive for the expression of *NP-f3'5'h* gene (Fig. 4.6).

Table 4.5. Results of PCR experiments to detect the presence of *NP f3'5'h* gene from bombarded and selected plants of *Dendrobium* Icy Pink 'Sakura'.

Pooled sample	PCR[a] *NP-f3'5'h*	Pot date & Status	Individual plant	PCR[a] *NP-f3'5'h*	Pot date & status
3-4A-1	-	Discarded	2A+2B-12	-	Discarded
3-4A-2	Strong+	11/20/02	2A+2B-13	-	Discarded
3-10A-1	Weak+	11/20/02	2A+2B-15	-	Discarded
3-10A-2	-	Discarded	2A+2B-16	-	Discarded
4-1 A-1	-	Discarded	2A+2B-24	-	Discarded
4-1 A-2	-	Discarded	2A+2B-25	-	Discarded
4-2 A-1	-	Discarded	2A+2B-29	-	Discarded
4-2 A-2	-	Discarded	3A-1-1	Strong+	7/23/01, greenhouse
4-3 A-1	-	Discarded	3A-1-2	Strong+	7/23/01, greenhouse
4-3 A-2	-	Discarded	3A-1-3	Weak+	7/23/01, greenhouse
4-4 A-1	-	Discarded	3A-1-4	-	Discarded
4-4 A-2	-	Discarded	3A-1-5	-	Discarded
4-5 A-1	-	Discarded	3A-2-1	-	Discarded
4-5 A-2	-	Discarded	3A-2-2	Weak+	10/30/2, growth chamber
4-6 A-1/A-2	-	Discarded	3A-2-3	-	Discarded
4-7 A-1	-	Discarded	3B-1-1	-	Discarded
4-8 A-1	Strong+	8/7/01, greenhouse	3B-1-2	-	Discarded
4-8 A-2	-	Discarded	3B-1-3	-	Discarded
4-9 A-1	-	Discarded	3B-1-4	-	Discarded
4-9 A-2	-	Discarded	3B-1-5	-	Discarded
4-11 A-1	Weak+	In G12 Magenta	3B-2-1	-	Discarded
4-11 A-2	-	Discarded	3B-2-2	-	Discarded
4-12 A-1	-	Discarded	3B-2-3	-	Discarded
4-12 A-2	-	Discarded	3B-2-4	-	Discarded
4-13 A-1	-	Discarded	3B-2-5	-	Discarded
4-13 A-2	-	Discarded	3B-2-6	-	Discarded
4-14 A-1	-	Discarded	3B-2-7	-	Discarded
4-14 A-2	-	Discarded	3B-2-8	-	Discarded
4-16 A-1	-	Discarded	3B-3-2	-	Discarded
4-16 A-2	-	Discarded	8-1-2	Weak+	7/20/01, died[b]
4-18 A-1	-	Discarded	8-1-3	Strong+	7/20/01, died[b]
4-18 A-2	-	Discarded	8-1-4	Strong+	7/20/01, died[b]
4-19 A-1	-	Discarded	8-1-5	Strong+	7/20/01, died[b]

Table 4.5. (Continued) Results of PCR experiments to detect the presence of *NP f3'5'h* gene from bombarded and selected plants of *Dendrobium* Icy Pink 'Sakura'.

Pooled sample	PCR[a] *NP-f3'5'h*	Pot date & Status	Individual plant	PCR[a] *NP-f3'5'h*	Pot date & status
4-19 A-2	-	Discarded	8-1-6	Strong+	7/20/01, died[b]
5-4 A-1	Weak+	In Magenta boxe	8-2-1	Strong+	11/20/02[c]
5-4 A-2	Strong+	In Magenta box	8-2-2	Strong+	11/20/02
5-5 A-2	-	Discarded	8-2-3	Strong+	11/20/02
5-6 A-1	-	Discarded	8-2-4	Weak+	10/30/02
5-7 A-1	-	Discarded	8-2-5	-	Discarded
5-7 A-2	-	Discarded	8-2-6	Strong +	11/20/02[c]
5-9 A-1	-	10/30/2, growth chamber	8-3-1	Weak+	7/20/01, greenhouse.
5-9 A-2	-	Discarded	8-3-2	Weak+	7/20/01, greenhouse
5-10 A-1	-	Discarded	8-3-3	Weak+	7/20/01, greenhouse
5-10 A-2	-	Discarded	8-3-4	Weak+	7/20/01, greenhouse
5-11 A-1	Weak+	In Magenta box	8-3-5	Strong+	7/20/01, greenhouse
5-11 A-2	Weak+	In Magenta box	8-3-6	Strong+	7/20/01, greenhouse
9-2 A-1	Weak+	10/30/02, died	8-3-7	Strong+	7/20/01, greenhouse
9-2 A-2	Strong+	10/30/02, died	8-3-8/9	Strong+	7/20/01, greenhouse
9-5 A-1	-	Discarded	8-4-1	Strong+	10/30/02
9-5 A-2	-	Discarded	8-4-2	-	Discarded
9-10 A-1	-	Discarded	8-4-3/4	Strong+	10/30/02
9-10 A-2	-	Discarded	8-4-5/6	Strong+	10/30/02
9-12 A-1	-	10/30/02	8-4-7/8	Weak+	10/30/02
9-12 A-2	Weak+	10/30/02			

[a] PCR result for the *NP-f3'5'h* gene, strong medium and weak represents the brightness of the amplified DNA band on ethidium bromide stained agarose gel. +=positive PCR result; -=negative PCR result.

[b] died due to bacterial contamination.

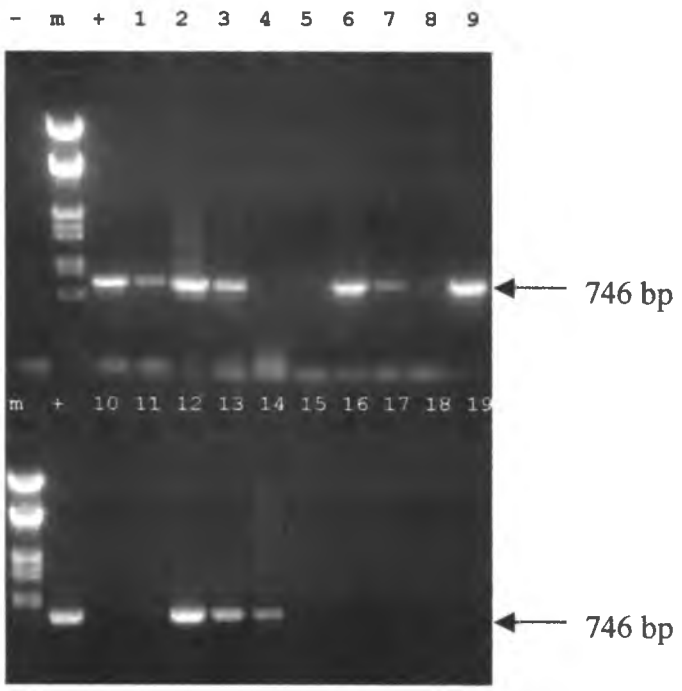

Figure 4.4. Agarose gel electrophoresis of PCR amplified products of *NP-f3'5'h* gene from bombarded and selected *Dendrobium* Icy Pink 'Sakura' (K1224) plants. Primers specific for *NP-f3'5'h* gene was used to amplify a 746 bp fragment from genomic DNA of K1224 plants regenerated from PLBs co-bombarded with pSAN150-NP-F3'5'H and pSAN154. Lanes 1-19=genomic DNA from individual samples 3A-1-1, 8-3-8/9, 8-3-7, 3B-1-1, 8-4-7/8, 8-4-5/6, 8-4-3/4, 8-1-2, 8-4-1, 8-2-5, 8-2-4, 8-2-3, 8-2-2, 8-2-1, 3A-2-3, 3B-2-8, 3B-2-7, 3B-2-6, and 3B-2-5, respectively; lane m=molecular weight marker; lane +=genomic DNA from a known PCR + plant (8-3-5); lane -=negative control without DNA (sterile water).

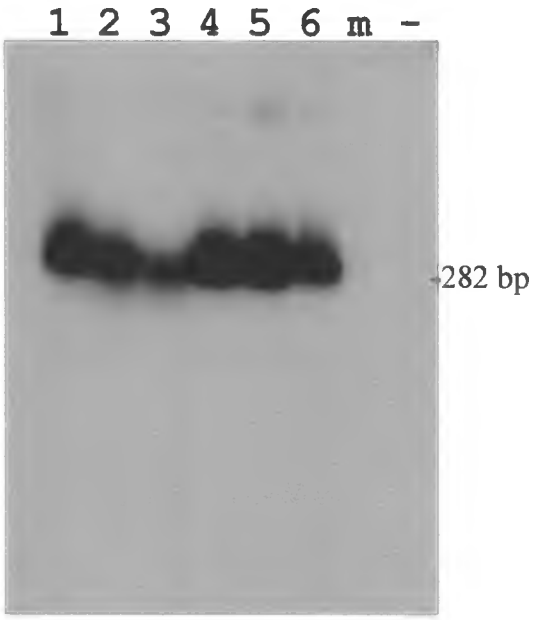

Figure 4.5. Southern blot of RT-PCR products of amplified cDNA of *Dendrobium* K1224 leaves from plants that were positive for the presence of *Antirrhinum dfr* gene by PCR, hybridized with *Antirrhinum dfr* cDNA probe directly labeled with Horse Radish Peroxidase (HRP) enzyme using North2South detection kit (Pierce). After hybridization membrane was washed 3 times with 2X SSC, 0.1% SDS buffer at 55°C for 15 minutes each, followed by 3 washes with 0.2X SSC, 0.1% SDS buffer at room temperature. Membrane was incubated with fluorescent substrate, luminol, and hydrogen peroxide for 5 minutes. An X ray film was exposed to the fluorescent signal for 1 minute. Lanes 1-2 = cDNA from pooled samples of 1-1 A2, 1-6 A1; lane 3 = *Snp-dfr* cDNA as a positive control; lanes 4-6 = cDNA from 1-3 individual plants of 6-8 A2 pooled sample; lane m=molecular weight marker; lane -= negative control without DNA (sterile water).

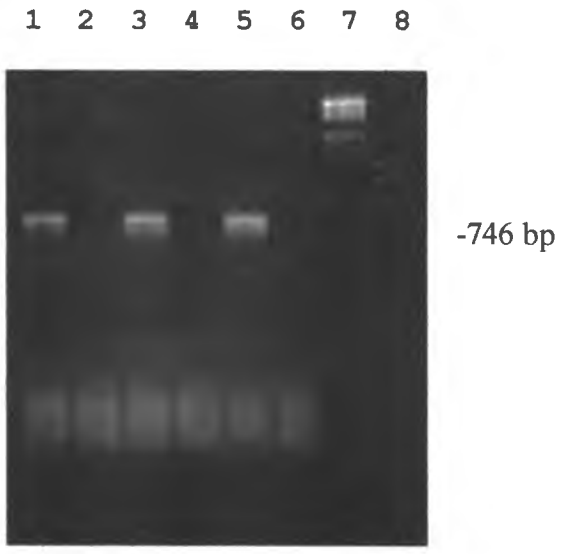

Figure 4.6. Agarose gel electrophoresis of RT-PCR products of amplified cDNA from transgenic *Dendrobium* Icy Pink 'Sakura' (K1224) plants indicated to contain the color gene (*NP-f3'5'h*) by PCR. Primers used for amplification were specific to a 746 bp fragment of *NP-f3'5'h* gene. Lanes 1-5=cDNA from individual plants of 8-2-1, 8-2-2, 8-2-4, 8-2-5 and 8-2-6, respectively; lane 6=negative control without cDNA; lanes 7 & 8=molecular weight markers.

All other pooled samples and individual plants tested were negative for the expression of *NP-f3'5'h* (Table 4.6). RNA samples were digested with DNase I in order to remove all residual DNA, which might give a false positive on PCR. Although we were unable to confirm the integration of the gene through Southern anaysis, RT-PCR results reveal that the gene is being expressed.

Two samples, 8-2-1 and 8-2-6 show faint bands of 1.8 kb on northern blot (Fig. 4.7) that corresponds to the molecular weight of *NP-f3'5'h*. This indicates the expression of the transgene in these two samples. However, other samples cannot be concluded to be negative since we have loaded only 3-5 micrograms of RNA from some samples due to inadequate amounts available for equal loading. These plants are potted out and tagged for further analysis when more leaf tissues are available for sampling (Fig. 4.8). Northern analysis might be an easier way to prove the integration and expression of the transgene since repeated efforts on Southern analysis did not yield any positive results despite the indication of the presence or the transgene via PCR and RT-PCR. However, transcription does not necessarily indicate the activity of the enzyme since post-transcriptional gene silencing can still shut down the activity of the transgene. It is important to do enzyme activity studies and flavonoid profiles on flower buds, once potential transgenic plants begin flowering.

Table 4.6. Results of RT-PCR experiments indicating the expression of *NP-f3'5'h* gene from bombarded PCR positive plants of *Dendrobium* Icy Pink 'Sakura' (K1224)

Sample ID	RT-PCR[a] result
control	-
3-4A-2	-
3-10A-1	-
4-8A-1	-
8-2-1	strong+
8-2-2	-
8-2-4	strong+
8-2-5	-
8-2-6	strong+
8-4-1	-
8-4-2	-

[a] RT-PCR result for *NP-f3'5'h* gene, strong represents the brightness of the amplified DNA band on ethidium bromide stained agarose gel; +=positive PCR result; -=negative PCR result.

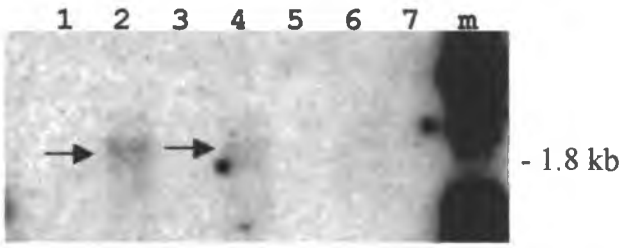

Figure 4.7. RNA blot showing expression of *NP-f3'5'h* in transgenic *Dendrobium* K1224 plants indicated to contain the color gene (*NP-f3'5'h*) by PCR. Total RNA from leaves were hybridized with ^{32}P-labeled denatured cDNA probes of *NP-f3'5'h* according to the modified Church and Gilbert (1984) procedure. Autoradiograph was exposed to the signal for two weeks. Lanes 1-3=8 μg of total RNA from 8-2-2, 8-2-6 and 8-4-1 plants respectively; lanes 4-5= 5 μg of total RNA from 8-2-1 and 8-2-5 plants; lane 6=3 μg of total RNA from 8-2-4 plant; lane 7=flower bud RNA from *Dendrobium* Icy Pink as a negative control; lane m=molecular weight marker.

Figure 4.8. Transformed *Dendrobium* Icy Pink 'Sakura (K1224) plants indicated to contain the inserted color gene by PCR. Plants are potted out in shredded hapuu and acclimatized to the outside environment in the growth chamber.

4.5 DISCUSSION

4.5.1 PCR Results and Selection of Transformed Plants

Two different color genes, *Snp-dfr* and *NP-f3'5'h,* under the constitutive promoter UBQ3 were inserted into *Dendrobium* Icy Pink 'Sakura' with the intention of creating new colors of orange-red and blue shades which are absent in commercial *Dendrobium.* Presence of the transgene in two sets of transformants was confirmed by PCR. Expression of the transgene from a few plants was indicated by RT-PCR and northern analysis. We were not able to determine the copy number and the DNA integration events since Southern analysis did not give a good signal despite repeated efforts. Most of the tested plants are potted out for flower color observations and follow up experiments will be done to identify plants with high activity of the transgene.

Selection is the most crucial step in plant transformation since this is the key to separate the transformants from the non-transformed plants. Our experience suggests that a 15-20% survival rate on non-bombarded control plates will allow a good selection pressure to kill most of the non-transformed plants and not toxic enough to kill the transformants. But, this selection does not eliminate all non-transformed plants. High levels of antibiotic for short period of time might be too toxic to the plants killing the transformed plants along with the non-transformed ones. It is difficult to directly compare two different selection protocols since there is high variation in particle delivery from one experiment to the other. Our selection

protocols have to be changed to eliminate the problem of bacterial contamination encountered during the experiment.

Although it is difficult to recommend a single selection procedure for K1224, our percentage survival data and PCR results can be used as a guideline to avoid antibiotic concentrations that are too low or too high. For K1224, 3-4 weeks in a liquid media containing Hygromycin B concentration of 12.5-15 $mg \cdot L^{-1}$ might be a good selection regime for PLBs bombarded with pSAN154. A regeneration efficiency of 19%-37% was obtained with hygromycin concentration of 10-12.5 $mg \cdot L^{-1}$ in liquid and solid selection for the shot PLBs (Table 4.2). Although liquid selection is faster, it increases the losses due to bacterial contamination, as we have encountered for the shooting experiment on 9/17/98.

4.5.2 Transgene Expression and Silencing

There are many examples of successful introduction and expression of different *dfr* genes into *Petunia.* The *dfr* genes from *Gerbera* (Helariutta et al., 1993), *Rosa* (Tanaka et al., 1995), *Zea mays* (Meyer et al., 1987) and *Cymbidium* (Johnson et al., 1999) have been successfully introduced and expressed in *Petunia. Antirrinum dfr* gene has been introduced into *Forsythia x intermedia* (Rosati et al., 1999) with the intention of producing anthocyanins in acyanic petals and was also used in antisense orientation in order to reduce condensed tannin accumulation in *Lotus corniculatus,* a forage plant for livestock (Robbins et al., 1998). These examples indicate that expression of active DFR is possible among even distantly

related plant species. Our RT-PCR and northern analysis data confirm the expression of *Antirrhinum dfr* in a few transgenic plants (Table 4.6; Fig. 4.5).

A few PCR positive plants, 1-1 A2, 1-2 A1, 8-7 A1 and 8-8 A1, have flowered in late November, 2002 (Fig. 4.9). No obvious enhancement of color was observed in these flowers. However, RT-PCR analysis has proven these plants to be negatve for the expression of *Antirrhinum-dfr* gene. A detailed analysis on flavonoid profile and gene expression is needed before any conclusion can be drawn on the success of genetic manipulation with *Antirrhinum dfr*.in *Dendrobium*. One important observation is that the flower color of the PCR positive plants did not reduce suggesting that the native *dfr* is not silenced by cosuppression.

Many examples of research on transgenetic plants revealed that the inserted gene is detected and silenced by various mechanisms unless the transgene is integrated into the "correct place" (Kumpatla et al., 1998; Matzke and Matzke, 1998; Gallie, 1998). Since there are no techniques available to target the integration into the "correct position" of the genome, it is necessary to create many transgenic plants in order to select a desirable, stable phenotype (Tanaka et al., 1998). For the first set of K1224 transformants with pSAN150-SNP-DFR, we have got more than 50% (48/85 pooled samples) samples indicated to contain the inserted color gene. Although the survival rate is high enough to give a fairly high number of transformed plants, there is still a considerable room for improvement of the selection procedure to obtain a higher probability for the presence of the inserted gene.

Figure 4.9. Acclimatized and transformed *Dendrobium* Icy Pink 'Sakura' (K1224) plants growing in screened insect proof boxes in University of Hawaii greenhouse facility. No adverse effects were noted on the vegetative growth or flowering after bombardment and selection. (A) plants at vegetative stage and (B) a few flowering plants that were PCR positive but RT-PCR negative for *Antirrhinum dfr* gene.

The biggest obstacle in producing a transgenic plant that expresses the transgene is gene silencing. Gene silencing is a phenomenon that is fortuitously discovered by analyzing transgenic plants (Stam et al., 1997). Fagard and Vaucheret (2000) have made a comprehensive review on all the theories regarding (trans)gene silencing in plants. A transgene can be silenced at the transcriptional level, preventing the transcription before the production of mRNA (transcriptional gene silencing or TGS) or after the transcription by degradation of mRNA (post transcriptional gene silencing PTGS). TGS is usually not dependent on sequence homology and is triggered by the immediate environment of the transgene locus and the organization of the transgene. PTGS and cosuppression requires high homology between the silenced genes. Our recent results show that *Antirrhinum dfr* is only 65% similar to *Dendrobium dfr* gene and cosuppression is highly unlikely in transformed plants. However, integration of multiple copies and tandem repeats can silence the transgene itself without affecting the native gene. This cannot be ruled out for the *Dendrobium* transgenics since we were not able to get any information about transgene copy number or arrangement by Southern analysis. However, active transcription of *Antirrhinum dfr* and *NP-f3'5'h* gene even in a few transgenic plants show that the promoter and the termination cassette we have used is working as expected. Only the flower color changes and/or changes in flavonoid profile and enzyme studies will reveal the activity of the transgene in orchids.

4.5.3 Other Bombardment Experiments and Their Status

First set of experiments conducted with K1224 plants bombarded with *Pet-f3'5'h* gene did not give any color changes (Table 4.2) and was not expected since all plants except one was PCR negative. Lack of any aberrations in plant and flower morphology proved that bombardment, selection, and regeneration protocols are not detrimental to the plant. Plants were eventually discarded. The number of PLBs recovered after selection was not high enough to give a stable transformant with a color change.

We have done several bombardment experiments with *Dendrobium* Samarai PLBs with the intention of shutting down the anthocyanin production in the lip (Appendices B-D). All the plants were planted in the greenhouse during August-October 1999 without PCR analysis, since the numbers were too large to do individual analysis. No color changes of the lips were observed with 35S-35S:: antisense/sense *Snp-dfr* or 35S-35S:: antisense *Pet-f3'h* treatments. Plants were eventually discarded. We have done most of these bombardments before isolation of any *Dendrobium* color genes. We did not observe any morphological aberrations due to the bombardment and antibiotic selection.

Our recent results on sequence similarity of *Dendrobium f3'h* and *Petunia f3'h* indicated that they are only 45% similar at the amino acid level. The *Dendrobium dfr* gene is only 62% similar to that of *Antirrhinum* at the amino acid level and 45% at the nucleotide level. These observations indicate that suppression of orchid genes by inserting these two dicotyledonous sequences into *Dendrobium*

might not be possible since cosuppression as well as antisense suppresson needs high sequence similarity. Therefore, we did not pursue most of the experiments done with the intention of shutting down the enzyme activity. The *dfr* gene isolated from *Dendrobium* itself (chapter 3) is a better candidate for shutting down the anthocyanin synthesis in *Dendrobium.*

APPENDIX A

Nucleotide and putative amino acid sequences of a *Den-chs-9 clone*

```
 P  S  V  E  H  Q  D  D  L  V  T  Q  A  L  F  A  D  G  A  S
ccttctgtggagcaccaggatgatcttgttactcaagctttatttgctgatggtgcatcc  -61
 A  V  I  V  G  A  D  P  D  E  A  A  D  E  H  A  S  F  V  I
gcggttatagtaggtgccgatccagatgaggcggccgatgagcacgccagcttcgtcata  -121
 V  S  T  S  Q  V  L  L  P  E  S  A  G  A  I  G  G  H  V  S
gtctctacatctcaagtcttactaccagagtcagcaggtgccatcggaggccatgtaagt  -181
 E  G  G  L  L  A  T  L  H  R  D  V  P  Q  I  V  S  K  N  V
gaggggggcctcttagccacgcttcatagagatgtcccgcaaattgtttccaaaaatgtt  -241
 G  K  C  L  E  D  A  F  T  P  L  G  I  S  D  W  N  S  I  F
ggaaagtgtttggaagatgcattcaccccacttggtatttcggactggaactctatcttc  -301
 W  V  P  H  P  G  G  R  A  I  X  D  Q  V  E  E  R  V  G  L
tgggtgccgcatccaggcggtcgagccattntagaccaggtggaggagagggtggggctg  -361
 K  P  E  K  L  L  I  S  R  H  V  L  A  E  Y  G  N  M  S  S
aagccagagaagctgcttatttcaaggcatgtgcttgcagagtatggtaatatgtcgagt  -421
 V  C  V  H  F  A  L  D  E  M  R  K  R  S  A  K  E  G  K  A
gtctgcgtgcactttgctcttgatgaaatgcgcaaaaggtctgcaaaagaaggtaaggct  -481
 T  T  G  E  G  L
acaaccggcgaaggccttg  -500
```

Nucleotide sequence and putative amino acid sequence of *Den-chs-6* clone. Amino acid sequence is represented by single letter codes above the nucleotide seqeuence. This clone has shown significant similarity to bibenzyl synthase clones from *Phalaenopsis* and *Bromheadia*

APPENDIX B

Co-bombardment of *Dendrobium* Samarai (UH988) seed-derived tissues (splb).

Constructs and Date of Bombardment	# of Plates[a]	Selection Regime	Antibiotic Mg L^{-1}	Transfer to Regen. Medium	% Survival of PLBs	# of magentas or compots	Potted date
pBI426 pBI525-SNP-DFR 3/19/98	5	22 days liquid, then 10 days liquid	no selection Geneticin up to 50 mg/L	4/20/98	11% (20/177) 6/30/98	26 compots	7/19/99 potted and move to greenhouse, plants were too numerous to be analyzed by PCR. Plants flowered in September 2000, No visual change in lip color, discarded
None 3/19/98	2[b]	22 days liquid, then 10 days liquid	no selection Geneticin up to 50 mg/L	4/20/98	7% (6/80) 6/30/98	0	
pBI426 pBI525-ANTI-F3'H 4/13/98	7	20 days liquid, then 19 days liquid	no selection Geneticin up to 35 mg/L	5/22/98	19% (47/245) 7/30/98	32 compots	8/16 through 10/7/99 potted and move to greenhouse, plants were too numerous to be analyzed by PCR. Plants flowered in September 2000, No visual change in lip color, discarded
None 4/13/98	1	20 days liquid, then 19 days liquid	no selection Geneticin up to 35 mg/L	5/22/98	0% (0/35) 7/30/98	0	
pBI426 pBI525-ANTI-DFR 7/15/98	5	18 days liquid, then 14 days liquid	no selection Geneticin up to 35 mg/L	8/16/98	1% (2/175) 12/23/98	5 magenta	8/16 through 10/7/99 potted and move to greenhouse, plants were too numerous to be analyzed by PCR. Plants flowered in September 2000, No visual change in lip color, discarded
None 7/15/98	1	18 days liquid, then 14 days liquid	no selection Geneticin up to 35 mg/L	8/16/98	0% (0/35) 12/23/98		

[a] 35-37 PLBs/plate, 5-8 mm in diameter
[b] 40 PLBs/plate, 4-6 mm in diameter

APPENDIX C

Co-bombardment of *Dendrobium* Jacqueline Thomas 'Uniwai Princess (UH507) protocorms

Constructs[a] and Date of Bombardment	# of Plates[b]	Selection Regime	Antibiotic mg L^{-1}	Transfer to Regen. Medium	% Survival of PLBs	# of magentas or compots	Status
pSAN154 pSAN150-SNP-DFR pSAN150-PET-F3'H 6/14/99	5	24 days solid 3 months solid on antibiotics	no selection 7/9 Hygromycin 12.5 7/30 Hygromycin 15 8/31 Hygromycin 15[c]	10/8/99	5% (39/779) 11/2/99	In 10 G12 Magenta boxes	To be analyzed by PCR
None 6/14/99	1	24 days solid 3 months solid on antibiotics	no selection 7/9 Hygromycin 12.5 7/30 Hygromycin 15 8/31 Hygromycin 15	10/8/99	11% (19/170) 11/2/99	In 4 G12 Magenta boxes	

[a] Hypothesis 1: To increase pigment production using sense DFR, Hypothesis 2: Shut down cyanidin production with antisense *f3'h* to shift the pathway towards pelargonidin.

[b] 35-37 PLBs/plate, 5-8 mm in diameter.

[c] Notes: Used Hyg 15mg/L even though the kill curve for this line suggests it can go up to 20mg/L, because of delayed, residual killing by Hygromycin.

APPENDIX D

Co-bombardment of *Dendrobium* Susan Takahashi (UH999) plbs.

Constructs[a] and Date of Bombardment	# of Plates[b]	Selection Regime	Antibiotic mg L^{-1}	Transfer to Regen. Medium	% Survival of PLBs	# of magentas or compots	Status of experiment
pSAN154 pSAN150-SNP-DFR 10/22/98	5	19 days liquid 64 days solid	no selection Hygromycin 10	1/14/99	28% (40/140) 1/27/99	33 magentas	All plants are discarded since sense suppression will not work with *Antirrhinum dfr.*
None 10/22/98	1	contamin. Discarded					
pSAN154 pSAN150-PET-F3'H 4/13/99	4	9 days solid 4.5 months solid	no selection Hygromycin 12.5	9/3/99	58% (62/106) 11/2/99		All plants are discarded since sense suppression will not work with *Petunia f3'h.*
None 4/13/99	1	9 days solid 4.5 months solid	no selection Hygromycin 12.5	9/3/99	58% (21/36) 11/2/99		
pSAN154 pSAN150-PET-F3'H 4/24/99	5	9 days solid 4.25 months solid	no selection Hygromycin 12.5	9/3/99	60% (111/184) 11/2/99		All plants are discarded since sense suppression will not work with *Petunia f3'h*

[a] Hypothesis 1: To decrease color production using antisense *Snp-dfr*, Hypothesis 2: Shut down cyanidin production via *f3'h* antisense to shift the pathway towards pelargonidin.

[b] 35 plbs/plate 5-15 mm in diameter

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