Molecular Analysis of Anthocyanin Biosynthetic Pathway Genes in *Cymbidium* Orchids

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Abstract

Anthocyanin biosynthesis was examined in cymbidium orchid (*Cymbidium* Swartz). Cymbidium orchids lack true red and purple/blue flowers, because they accumulate cyanidin and peonidin (pink) anthocyanins, but not pelargonidin (red) or delphinidin (blue). Transient gene expression studies showed that the use of a heterologous flavonoid biosynthetic gene, dihydroflavonol reductase (*DFR*) from *Anthurium*, enhanced production of pelargonidin in cymbidium floral tissues. Similarly, delphinidin was produced when a pansy flavonoid 3′, 5′-hydroxylase (*F3′5′H*) was introduced. The maize *Lc*/*C1* transcription factors in combination with *DFR* or *F3′5′H* was necessary in order to observe upregulation of the anthocyanin pathway and changes in anthocyanin pigment accumulation. The presence of the new anthocyanins was confirmed by TLC and HPLC.

cDNA clones of the flavonoid biosynthetic genes chalcone synthase (*CHS*), *DFR* and flavonoid 3′ hydroxylase (*F3′H*) were isolated using PCR. The full length cymbidium *CHS* (1173 bp) was ~85% identical at DNA level with *CHS* from *Oncidium* 'Gower Ramsey', a *Phalaenopsis* hybrid cultivar, as well as bibenzyl synthase from *Phalaenopsis* sp. The 792 bp partial-length *F3′H* sequence was ~ 66% identical with *F3′H* from *Pelargonidium*×*hortorum*, *Verbena hybrida* and *Sorghum bicolor*, while the *DFR* sequence was highly homologous with the published cymbidium *DFR*. The deduced protein sequences contained domains or conserved residues typical of CHS and F3′H.

Southern analysis showed both cymbidium *CHS* and *F3′H* are represented by small gene families, with *CHS* consisting of at least three members and *F3′H* up to three genes. By contrast, *DFR* is likely to be presented as a single gene. Using different coloured cymbidium cultivars, it was shown that *DFR* expression correlated with cyanidin production in the flower. The *CHS* clone was most highly expressed in leaf tissues and in late developmental stages in floral tissues of Vanguard Mas Beauty (a green cultivar). This expression pattern did not correlate with pigment production, and hence this gene is unlikely to be involved in anthocyanin production in flowers. *F3′H*

expression was not detected in leaf and floral tissues at any developmental stages examined.

DFR and *CHS* promoters were isolated by genome walking, in an attempt to identify organ specific promoters suitable for use in cymbidium. A 1544 bp *DFR* promoter and a 1561 bp *CHS* promoter were cloned upstream of a *GFP* reporter gene and transient gene expression studies showed that *CHS* promoter had the ability to drive GFP production in white and pink petal tissues and in leaf tissues. However, the *DFR* promoter activated *GFP* expression only in the white petal tissues. These transient gene expression studies also demonstrated that maize *Lc*/*C1* transcription factors greatly enhanced the activities of both *CHS* and *DFR* promoters. The success of this transient expression system indicates that MYB and bHLH transcription factor are likely to be involved in anthocyanin production in cymbidium.

Together, the results of this study confirm that a suite of molecular strategies to modify flower colour in cymbidium are feasible, as well as providing essential information on flavonoid and anthocyanin genes that expand our knowledge and understanding of this complex flowering plant.

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List of Abbreviations

A	absorbance, at the wavelength indicated by the numerical value
Amp	ampicillin
ANS	anthocyanidin synthase
AUS	aurone synthase
BSA	bovine serum albumin
bp	base pairs
CH_3CN	acetonitrile
CHS	chalcone synthase
CTAB	cetyltrimethyl ammonium bromide
CaMV35S	cauliflower mosaic virus 35S promoter
C1	COLOURLESS; MYB transcription factor from maize
cDNA	complementary deoxyribonucleic acid
DFR	dihydroflavonol 4-reductase
dATP	deoxyadenosine triphosphate
dCTP	deoxycytidine triphosphate
dGTP	deoxyguanosine triphosphate
dTTP	deoxythymidine triphosphate
dNTP	deoxyribonucleotide triphosphate
EDTA	ethylenediamine-tetraacetate
F3H	flavanone 3-hydroxylase
F3′H	flavonoid 3′-hydroxylase
F3′5′H	flavonoid 3′5′-hydroxylase
FNS	flavone synthase
FLS	flavanol synthase
FW	fresh weight
GFP	green fluorescent protein, oringinally from *Aequorea victoria*
HOAc	acetic acid
HPLC	high performance liquid chromatography
gDNA	genomic DNA

h	hour
IAA	indole-3-acetic acid
IPTG	isopropyl-beta-D-thiogalactopyranoside
JFDP	Jung Frau dos Pueblos
kb	kilobases
kPa	kiloPascal
LB	Luria-Bertani; bacterial growth media
LRF	Lisa Rose Flammingo
Lc	LEAF COLOUR; bHLH transcription factor from maize
M	molar; moles per litre
MOPS	3-(N-morpholino) propanesulfonic acid
MS	Murashige and Skoog: tissue culture media
MT	methyltransferase
min	minute
milliQ	water which has been purified using Milli-Q Ultrapure system
N	normal
NJG	Narella Jennifer Gail
NaOAC	sodium acetate
ORF	open reading frame
PCR	polymerase chain reaction
PVP	polyvinyl pyrrolidone
psi	pounds per square inch
RACE	rapid amplification of cDNA ends
RE	restriction endonuclease
RF	relative to the front
RT	reverse transcription
rpm	revolutions per minute
rRNA	ribosomal ribonucleic acid
SDS	sodium dodecyl sulphate
SSC	sodium chloride-sodium citrate
TBE	tris borate EDTA buffer
TE	tris-EDTA
TLC	thin-layer chromatography
Tris	tris(hydroxymethyl) aminomethane

U	enzyme unit
UF3GT	UDP-glucose:flavonoid 3-*O*-glucosyltransferase
UV	ultra violet
v/v	volume/volume
w/v	weight/volume
X-Gal	5-bromo-4-chloro-3-indolyl- beta-D-galactopyranoside
%	percentage

Chapter 1

Introduction

1.1 Cymbidium orchids in New Zealand

Cymbidium orchids are the major export cut flower crop for New Zealand. Cymbidium flowers are available in green, white, pink and yellow colours, but lack true red and purple/blue colours. Novel flower colour is a major driving force in the floriculture industry, with new colour cultivars demanded by the market. Genetic limitations mean that many commercially important flower species are unable to be bred for certain colours, especially the highly desired blue-range colours. Genetic engineering provides the method of inserting specific colour-contributing gene(s) into elite commercial lines and modifying colour production, overcoming some barriers encounted by traditional breeding. Carnation and rose, for example, are both commercially important flower crops where blue-hue flower colours have been developed using molecular breeding. Therefore, molecular breeding does offer options for generating orchids with novel flower colours. This project is about the use of molecular technologies to study genes involved in pigment production in cymbidium. Developing new orchid cultivars by modifying flower colours and patterns will help to grow export markets and will benefit the floriculture industry in New Zealand.

1.2 Orchids- a worldwide flower crop

Orchids are monocotyledons and make up the largest family of flowering plants, the orchidaceae, which comprises more than 800 genera and 25,000 species. They can be found in most parts of the world, but mainly grow in tropical, sub-tropical regions, and some in more temperate climates (Bechtel et al., 1981; Leigh, 1990; James, 1993;

Banks, 2005). Orchids have flowers with diverse colour, shape, sizes, fragrance and texture. Many orchid genera also flower persistently, and their flowers have a long shelf life as both cut flowers and pot plants.

Orchids make up to 8% of the global floriculture trade (Martin and Madassery, 2006). The common commercially grown orchids come from the genera *Cymbidium, Dendrobium, Phalaenopsis, Oncidium* and *Cattleya* (John and Molloy, 1983; Arditti, 1992). Asian countries have the largest tropical and sub-tropical orchid production, with Taiwan and Thailand being the largest phalaenopsis and dendrobium exporter countries, respectively. New Zealand has a cooler climate, and is an ideal environment for temperate orchids such as cymbidium. Cymbidiums account for 50% of exported cut flowers from New Zealand (Heyes and Johnston, 1998). In 2006, New Zealand exported NZ$20.2m worth of cymbidium orchids, mainly to the US and Japan, and provide 19% of the total Japanese orchid consumption (Wang, 2004).

1.3 The genus *Cymbidium*

The genus *Cymbidium* belongs to the tribe Cymbidieae, comprises approximately fifty species and originates in Asia. *Cymbidium* (Sw. 1799) species have a wide distribution from the Himalayas throughout Southeast Asia and northern Australia (Bechtel et al., 1981; Leigh, 1990; James, 1993; Banks, 2005). Cymbidium, in general, can be divided into two groups: one from the high altitudes requiring substantial changes in temperature at night, while the other group is more tropical and requires warmer conditions (Bechtel et al., 1981; Du Puy and Gribb, 1998; Choi et al., 2006). Cymbidium has a sympodial growth pattern and various habitats. Most species from the mountains are terrestrial (grow in the ground), while low land species are epiphytic (grow on other plants) and some are lithophytic (grow on rocks) (Leigh, 1990; James, 1993; Banks, 2005).

1.3.1 Cymbidium cultivars

Cymbidiums have been cultivated for thousands of years in China, but became popular in Europe during Victorian times. The modern cymbidium cultivars are hybrids, with a range of flower colors and sizes, including white, green, yellow, pink and maroon (Figure 1.1). Those colour cultivars were developed from a base of six main species. The species *C. hiijerianum (grandiflorum)* and *C. lowianum* were important for breeding the green colour flowers and *C. tracyanum* was used to breed for yellow flowers. The white to rose pink flowers were bred from species of South Asia origin, *C. parishii, C. insignei* and *C. erythrostylum*, and dark pink-red colours were bred from *C. iansonii* (Tomlinson, 1985). Thousands of hybrids have been developed from these species and the commercial cymbidium cultivars are mostly hybrids. Most commercial cultivars are triploid although diploid and tetraploid cultures do exist, whereas the wild species are diploid (Tomlinson, 1985; Zhu et al., 2006).

1.3.2 The cymbidium plant

Cymbidium has a horizontal growth pattern. Each year new growth is initiated from vegetative buds, developing from a pesudobulb. The bud forms a lead, which grows horizontally, becoming a horizontal stem, or rhizome. Roots also develop, emerging from the base of the rhizome from the previous year. The new rhizomes then grow upwards and thicken to form a secondary stem, or pseudobulb (Skelsey, 1978; Leigh, 1990; James, 1993). The leaves, which emerge from the pseudoblub, are long and narrow with parallel veins. The leaves are arranged alternately on opposite sides of the stem. The leaves are conduplicated, which means they are folded at the middle when juvenile. As the leaf extends, it opens up and forms a V-shape in cross section. Inflorescences develop from the axils at the base of the pseudobulb. An inflorescence can have between five and thirty flowers, which open for up to ten weeks.

1.3.3 The cymbidium flower

All orchid flowers including cymbidium are zygomorphic (one plane of symmetry) with their floral organs arranged in threes (Sheehan, 2002). The floral organs are in whorls,

with sepals in the first whorl, petals in the second whorl, and the column (gynostemium) in the centre. The column consists of both the male parts (the anthers and stamens) and the female parts (the style and stigma), which are fused (John and Molloy, 1983; Arditti, 1992). The median petal located opposite to the column is named the lip or labellum and has a very different appearance compared with the other two petals. The lip is further developed into three lobes, and where the middle lobe curves backwards (Figure 1.2). The lip has two pubescent medium ridges and is usually highly pigmented in combinations of spots, stripes or patches, to direct pollinators into the centre of the flower (Bechtel et al., 1981). Cymbidium belongs to the Liliflorae super order. Plants belonging to this order have highly petalised sepals, which means there is no clear distinction between sepals and petals. The size, shape and colour of the sepals are all very similar to the petals (Dressler, 1981). Orchid flowers are highly evolved in terms of colour and texture in order to select the specific pollinator. The main pollinators for cymbidium are bees while some species pollinated by wasps (Davies et. al., 2006a).

1.4 Plant pigments

Plant pigments are secondary metabolites responsible for the colours in plant tissues. Different plant pigments absorb the visible light spectrum differently, thereby providing a range of different colours. Plant pigments serve multiple functions in plants, including protection against UV light, defense against pathogens and herbivores, plant development (auxin metabolism), stress response, and seed and fruit dispersal (Dooner et al., 1991; Koes et al., 1994; Winkel-Shirley, 1996; 2001). The main function of pigments in reproductive tissues is attracting pollinators or as seed dispersal agents.

Plant pigments can be classified into four major groups based on their distinctive chemical structures: chlorophylls, carotenoids, betalains and anthocyanins. While chlorophylls and carotenoids are present in all photosynthetic cells of vegetative tissue, carotenoids, betalains or anthocyanins are the predominant pigments in flowers. Carotenoids are synthesized in the plastids and are lipid-soluble pigments that absorb light at the wavelength of 450 nm (Hirschberg, 2001). They are responsible for many of the yellow and orange colour in plants. Carotenoids are also involved in photosynthesis

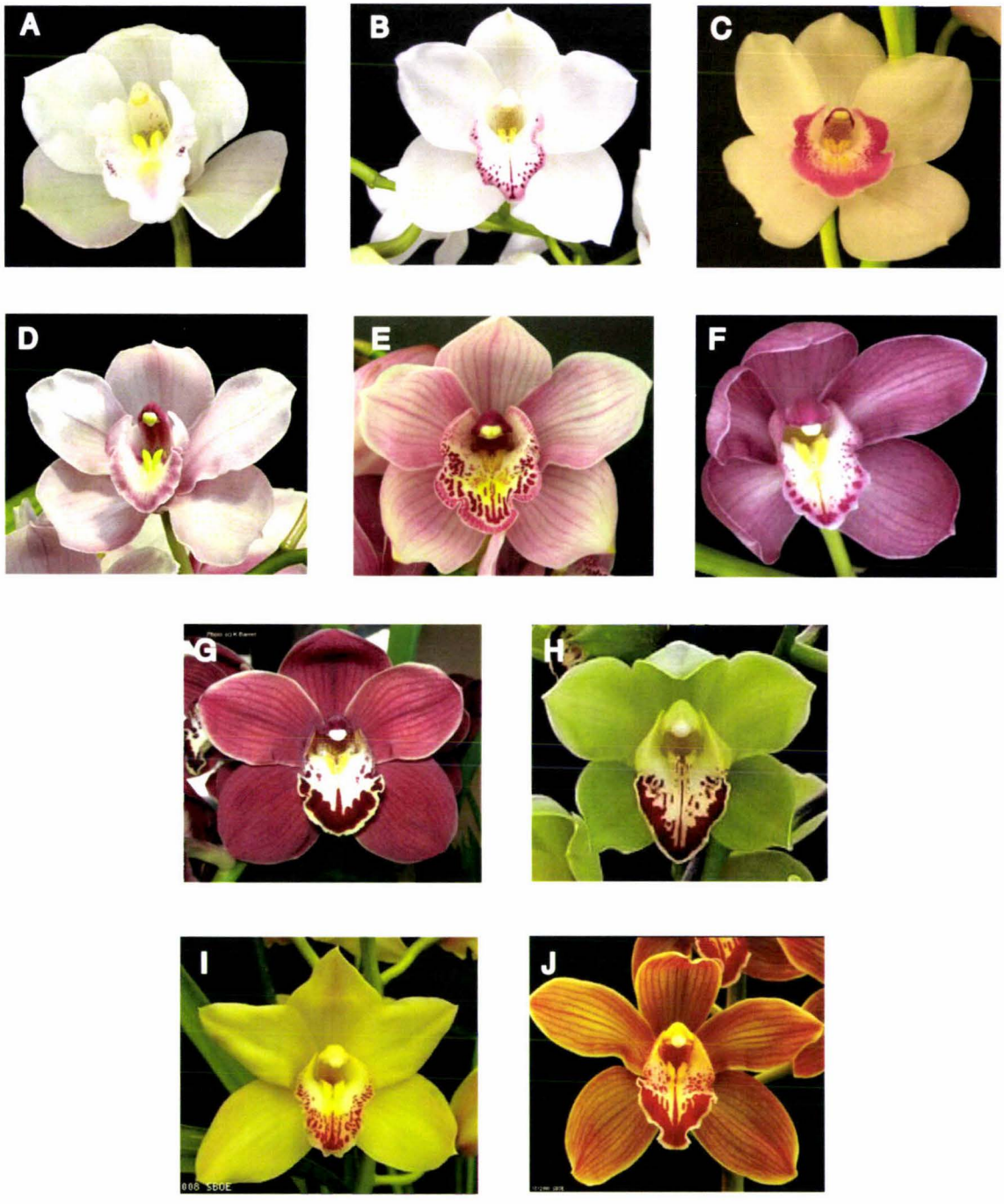

Figure 1.1: A range of cymbidium cultivars, **A-J** showing individual cultivars used in this study, and illustrating the flower colour range. **A-C**, white cultivars: **A**, Jung Frau dos Pueblos, JFDP; **B**, Winter Bride Peter's Choice; **C**, Virgin. **D-F**, pink cultivars: **D**, Narella Jennifer Gail, NJG; **E**, Lisa Rose Flamingo; LRF; **F**, Fantasy. **G**, Maroon cultivar: Clarissie Austin South Pacific, CASP. **H**, green cultivar: Vangard Mas Beauty, VMB. **I**, Yellow cultivar: Marissa Golden Wedding. **J**, Orange red cultivar: Tethys 'Butterscotch'. Photo **D** is taken from http://home.alphalink.com.au; **G** is taken from www.aospacificcentral.org; photo **H-J** is taken from www.santabarbaraorchidestate.com.

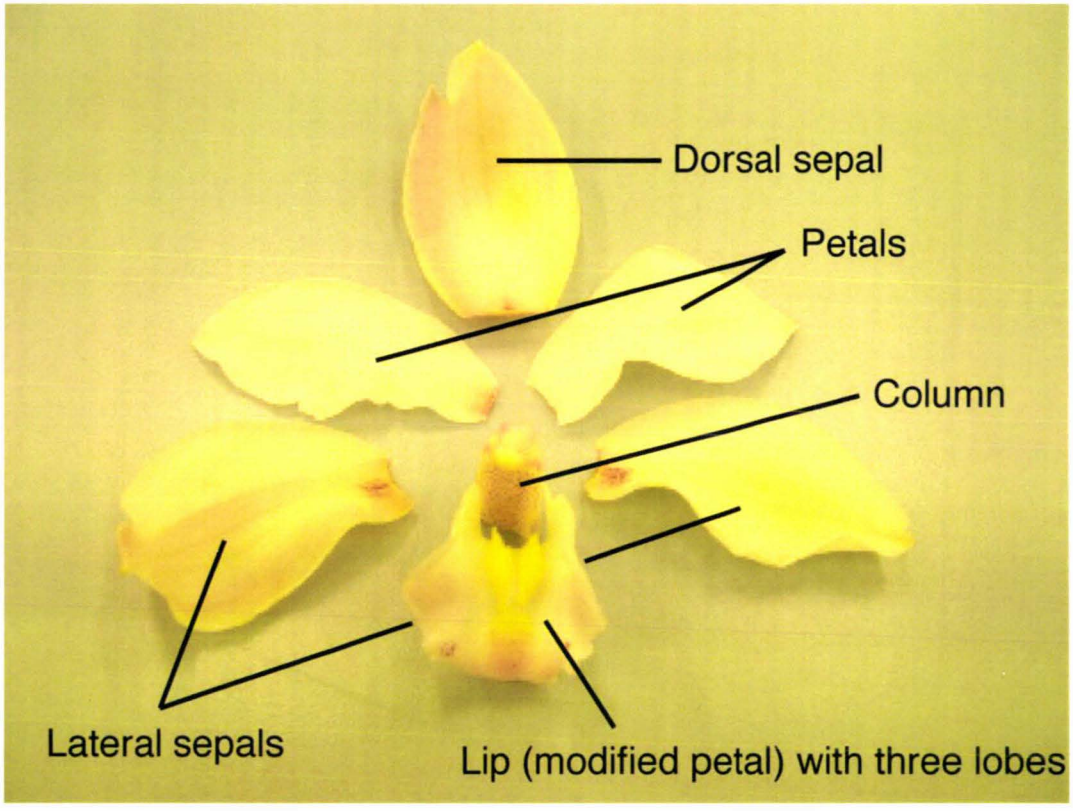

Figure 1.2: Structure of cymbidium flower.

as accessory pigments and may play a role in light energy dissipation. Carotenoids have some similar properties to chlorophylls, and these two pigment groups can co-exist in flowers. Carotenoids can also be produced along with red or purple anthocyanins to give brown or orange colours in the same plant tissues (Forkmann, 1991).

Betalains are water-soluble pigments, and have two major classes, betaxanthins and the betacyanins, showing yellow and red-violet with absorption wavelengths at 480 nm and 540 nm respectively. Betalains are synthesized from 3-(3,4-dihydroxyphenyl) alanine (DOPA) via condensation of betalamic acid (Grotewold, 2006). Betalains have only been found in the order Caryophyllales and some fungi, and have not been found to co-exist with anthocyanins (Stafford, 1994).

Flavonoids are a diverse and widespread group of plant secondary metabolites. Anthocyanins are a major class of flavonoids and give colours from orange to red, purple and blue. Some classes of flavonoids do not absorb visible light, and are therefore "colourless", but can still influence the colour of plant tissue. These are the flavones, flavonones and flavonols, and they are important in contributing the "depth" of white and cream colours in flowers. They can also co-exist with anthocyanin and influence the flower colour (Goto, 1987; Forkmann, 1991). There are some other coloured flavonoids that may act as pigments or as key precursors in the production of anthoycanin, specifically the yellow coloured chalcones and aurones (Kuhn et al., 1978; Forkmann, 1991; Davies et al., 2006b).

Flavonoids are derived from the phenylpropanoid pathway. The water-soluble flavonoids are synthesised in the cytosol and stored in the vacuole. The various flavonoids share the same basic structure (Figure 1.3). The primary flavonoid structure has a C6-C3-C6 frame, which consists of two aromatic rings (A- and B- rings) that are connected by a heterocyclic ring (C-ring) (Schwinn and Davies, 2004; Yu et al., 2006). There are six major anthocyanidin groups: pelargonidin, cyanidin/peonidin, delphinidin and malvidin/petunidin, representing orange/red, pink/mauve and blue/purple colours. These anthocyanidins are different in the level of hydroxylation and methyl groups at the 3′(R1) and 5′(R2) positions of the B-ring (Table 1.1). An increase in the number of hydroxyl groups means the pigment absorbs at longer wavelengths, therefore pushing the spectrum from the orange to the blue end (Schwinn and Davies, 2004). Side

branches of the flavonoid pathway produce compounds that are involved in plant defense, UV protection, and the synthesis of lignins and tannins (Koes et al., 1994). The main focus of this thesis, however, is on the anthocyanins.

1.5 Pigment composition in cymbidium orchid

An understanding of pigment composition and gene regulation in cymbidium is the first step to manipulation of floral colour. Surveys on the pigment composition of flowers from several cymbidium cultivars have shown that three pigment groups are involved. Carotenoids are the main pigment in yellow cultivars and are also present in small amounts in the green cultivars. The main carotenoids in yellow cultivars are antherxanthin and β-carotene, whereas in green cultivars they are lutein and β-carotene (Lewis, 2001; Mudalige and Kuehnle, 2004). Chlorophylls are the main pigments in the petals of green cultivars, whereas there was no chlorophyll detected in other coloured cultivars (Lewis, 2001). White cultivars do not contain any coloured pigments, but do have colourless flavonoids.

Pigment profiling of flower tissues from various maroon and pink cymbidium cultivars and the red lip from non-pink cultivars, have shown similar anthocyanins present in all cultivars. Three major anthocyanins were identified as cyanidin 3-rutinoside, cyanidin 3-glucoside and cyanidin 3-(6″-malonylglucoside), and three minor anthocyanins as peonidin 3-glucoside, peonidin 3-rutinoside, and peonidin 3-malonylglucoside (Sugiyama et al., 1977; Tatsuzawa et al., 1996; Lewis, 2001). White cultivars have no anthocyanin accumulation in sepals and petals, but the red lip contains cyanidin- and peonidin-based anthocyanins.

Petal tissues from all the cymbidium cultivars analysed also contain kaempferol and quercetin glycosides. These flavonols can act as co-pigments with the coloured anthocyanins, thus their presence contributes to the colour of cymbidium. These results show that the pink/maroon cymbidium cultivars only synthesise dihydroxylated anthocyanins, and there is a lack of tri- and mono-oxylated anthocyanins, which explains the lack of blue/purple and brick-red colour cymbidium.

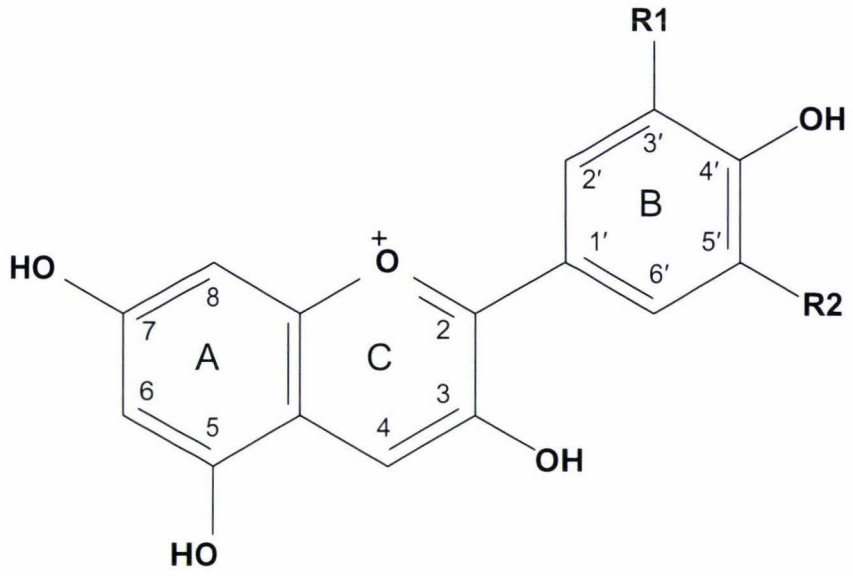

Figure 1.3: The basic flavonoid structure. The A, B and C rings are labelled, the numbers indicate the carbons in the aromatic rings. Addition of an oxygen atom at C4 position sets apart flavonols from anthocyanidins. The flavonol backbone has a carboxyl group, but the anthocyanidin backbone lacks oxygen at the C4 position. The various flavonols are differentiated by hydroxylation at the R1 and R2 positions and the level of hydroxylation and methylation at R1 and R2 positions determine various anthocyanidins as shown in Table 1.1. Figure is taken from Albert (2006).

Table 1.1: Substitution on the B-ring of anthocyanins.

Anthocyanins	R1	R2
pelargonidin	H	H
cyanidin	OH	H
delphinidin	OH	OH
peonidin	OCH_3	H
petunidin	OH	OCH_3
malvidin	OCH_3	OCH_3

1.6 Flavonoid biosynthesis

The flavonoid pathway is well characterized in many plant species such as petunia, *Antirrhinum* and maize (Dooner et al., 1991; Holton and Cornish, 1995; Mol et al., 1998). The first committed step in the flavonoid pathway is the formation of the chalcones (Figure 1.4). In most plant species, chalcone synthase (CHS) most commonly converts three acetate residues derived from three molecules of malonyl CoA with one molecule of *p*-coumaryl-CoA to form the C_{15} naringenin chalcone, although it may also catalyse the condensation of three caffeoyl CoA molecules with 4-coumaroyl-CoA to form eriodictyol chalcone (Heller and Hahlbrock, 1980; Yu et al., 2006). Naringenin chalcone is the first key step for flavonoid production (Grotewold, 2006). It is rapidly isomerised by chalcone isomerase (CHI) to form the colourless flavanone, naringenin (Jez and Noel, 2002).

The formation of the dihydroflavonols: dihydrokaempferol (DHK), dihydroquercetin (DHQ) and dihydromyricetin (DHM), begins with hydroxylation of naringenin, catalysed by flavone 3-hydroxylase (F3H) (Forkmann, 1991; Yu et al., 2006; Schwinn and Davies, 2004). F3H converts naringenin to DHK. At this point, hydroxyl groups may be added on to the 3′ position of DHK by the enzymatic reaction catalysed by flavonoid 3′ hydroxylase (F3′H) to form DHQ. Alternatively, the OH group can be added on to both 3′ and 5′ positions by flavonoid 3′5′-hydroxylase (F3′5′H) to form DHM (Figure 1.4).

The formation of flavonols such as kaempferol and quercetin comes from the dihydroflavonol substrates, DHK and DHQ, respectively. This reaction is catalysed by flavonol synthase (FLS), which is a 2-oxoglutarate-dependent dioxygenase (Yu et al., 2006). Flavonols play important roles in co-pigmentation in flower and fruit colouration, pollen germinantion, disease resistance and cell signaling (van der Meer et al., 1992; Yu et al., 2006).

DHK, DHQ and DHM are the precursors of pelargonidin, cyanidin, and delphinidin, respectively. A series of enzymatic reactions lead to the conversion of the colourless

dihydroflavonols to colourful anthocyanidins. First, dihydroflavonol 4-reductase (DFR) reduces the dihydroflavonol (DHFs) to a leucoanthocyanidin (Heller et al., 1985a, b). Then anthocyanidin synthase (ANS) converts the leucoanthocyanidin to their respective anthocyanidin. F3′H and F3′5′H activity may still be present in this stage, converting leucopelargonidin to form either leucocyanidin or leucodelphinidin. The anthocyanidins are separated as pelargonidin, cyanidin or delphinidin depending on the hydroxylation of the B-ring and give red/orange, pink/magenta and purple/blue colours respectively (Schwinn and Davies, 2004). The greater the number of hydroxyl groups, the deeper blue colour (Holton et al., 1993; Harborne, 1976).

Anthocyanidin molecules are generally water-soluble and easily broken down. Therefore, the addition of a side group is required for stability (Grotewold, 2006). The most common stable anthocyanin is glycosylated at position 3 of the C-ring. Glycosylation occurs when a sugar side group is attached to the flavonoid molecule and the most common sugar attached to anthocyanin is glucose. This process does not change the flower colour, but in some cases can affect intensity or co-pigmentation (Forkmann, 1991). The position of glycosylation often affects acylation, which is the addition of an acid group to the sugar residues of anthocyanins (Schwinn and Davies, 2004). Acylation is important for stabilizing the tertiary structure of the anthocyanin. Glycosylation is catalysed by UDP-glucose:flavonoid 3-*O*-glucosyltransferase (UF3GT), resulting in anthocyanin 3-glucoside (Nakajima et al., 2001). The glycosylation means the water solubility of the anthocyanin increases, and anthocyanins become more stable at cellular pH (Yu et al., 2006). At this point three basic classes of anthocyanins are formed. The anthocyanin can be further modified with a number of other side groups through methylation, glycosylation and acylation in a species-specific manner. Methylation of the B-ring hydroxyl groups is catalysed by *O*-methyltransferases (MT). Petunia for example, has four MT genes involved the transfer of a methyl group from *S*-adenosyl methionine to the 3′ or 3′, 5′ positions of the B-ring (Forkmann, 1991; Yu et al., 2006). Cyanidin can be methylated to form peonidin 3-glycoside, and delphinidin can be methylated to generate petunidin or malvidin (Forkmann, 1991).

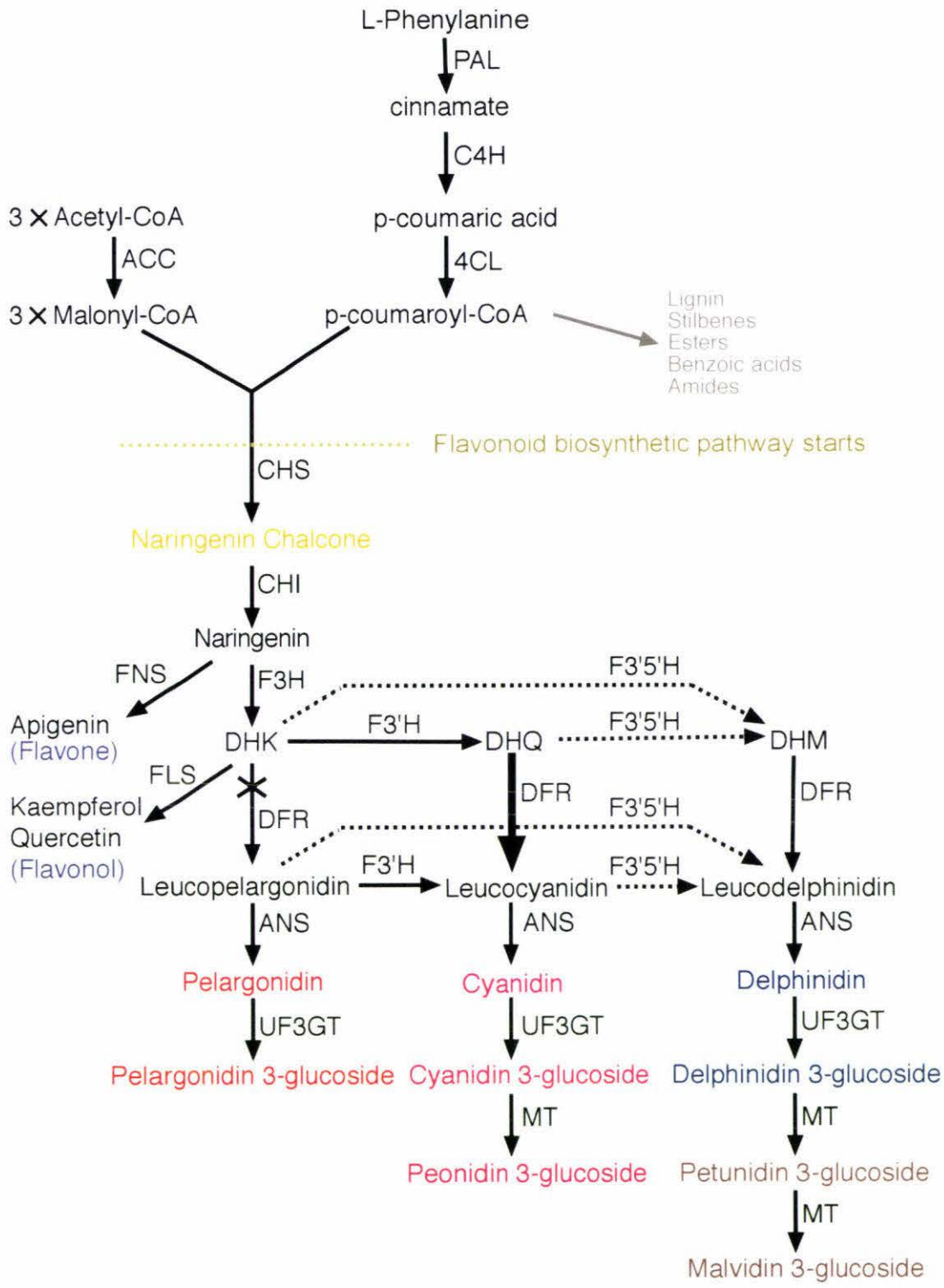

Figure 1.4: Schematic presentation of the flavonoid biosynthetic pathway. Classes of flavonoids are indicated in brackets. Crosses indicate known limitations of pigment biosynthesis in cymbidium. Dotted arrows represent flavonoids not produced appreciably in cymbidium. Bold arrow represent flavonoids produced appreciably in cymbidium. The full names of the enzymes are: AUS, aurone synthase; FNS, flavone synthase; FLS, flavonol synthase; CHS, chalcone synthase; CHI, chalcone isomerase; DFR, dihydroflavonol reductase; F3H, flavanone 3-hydroxylase; F3′H, flavonoid 3′-hydroxylase; F3′5′H, flavonoid 3′5′-hydroxylase; ANS, anthocyanidin synthase; UF3GT, UDP-glucose:flavonoid 3-*O*-glucosyltransferase and MT, methyltransferase.

1.7 Key anthocyanin biosynthetic genes

Many flavonoid/ anthocyanin genes (both structural and regulatory types) have been identified. Only a few anthocyanin biosynthetic genes have been cloned from orchids, and the control of the pigmentation pattern is not well understood. General information on the key flavonoid biosynthetic genes identified from a range of plants is outlined below.

1.7.1 Chalcone synthase (*CHS*)

CHS is the first enzyme in the flavonoid biosynthesis pathway and is responsible for chalcone formation (Heller and Hahlbrock, 1980). Chalcone leads to synthesis of all class of flavonoids, thus the activity of CHS is important for anthoycanins and flavonol formation in cymbidium. CHS activity was originally identified in parsley (Kreuzaler and Hahlbrock, 1972). CHS is encoded by a multi-gene family in most plants, with the exception of parsley, *Antirrhinum* and *Arabidopsis*, where only a single *CHS* gene is present (Kreuzaler and Hahlbrock, 1972; Sommer and Saedler, 1986; Feinbaum and Aussubel, 1988). The evolution of CHS has resulted in divergent forms, with each potentially regulated by different developmental or environmental signals (Chappell and Hahlbrock, 1984). For example, *CHS D* and *E* in *Ipomea* both catalyse the condensation reaction to form naringenin chalcone but *CHS E* is expressed in the flower tube, whereas *CHS D* is expressed in flower limb. Thus *CHS D* is responsible for anthocyanin biosynthesis in the limb (Habu et al., 1998; Durbin et al., 2000; Shiokawa et al. 2000).

CHS genes have been isolated from a number of monocotyledonous ornamental plants, including *Anthurium* (Collette, 2004), *Asiatic* hybrid lily (Nakatuska et al., 2003) and some members of the orchidaceae, such as *Bromheadia, Dendrobium* and *Phalaenopsis* orchids (Liew et al., 1998a; Mudalige-Jayawickrama et al., 2005; Han et al., 2005, 2006a). It has been shown that at least three *CHS* members are present in *Bromheadia finlaysoniana* and *Phalaenopsis* hybrid orchids (Liew et al., 1998a; Han et al., 2005; Han et al., 2006a), although only one *CHS* gene from *Phalaenopsis* orchid has been functionally confirmed as a flower-specific, anthocyanin accumulation-related *CHS* (Han et al., 2006a and b).

CHS is a member of the type III polyketide synthase (PKS) superfamily. Many secondary metabolite enzymes such as bibenzyl synthase, stilbene synthase (STS) and acridone synthase also belong to the PKS family (Helariutta et al., 1995; Liew et al., 1998a). These synthases, such as STS, have similar sequences to CHS and use the same substrates as CHS to making anti-fungal phytoalexins (Austin and Noel, 2003). Thus it is difficult to distinguish *CHS* genes from other *PKS* genes (Helariutta et al., 1995; Liew et al., 1998a; Durbin et al., 2000). Careful confirmation of putative *CHS* genes for their function is required.

1.7.2 Dihydroflavonol 4-reductase (*DFR*)

DFR is an important enzyme in anthocyanin production in plants, because it catalyses the reduction of specific dihydroflavonols to their prospective leucoanthocyanidins, and this will subsequently determine the type of anthocyanidins that accumulate in flower tissue. Substrate specificity of the DFR found in several plant species had a large effect on flower colour. DFR in *Antirrhinum* and *Zea mays*, for example, uses DHK and DHQ, and therefore pelargonidin and cyanidin accumulate in these plants (Reddy et al., 1987; Beld et al., 1989). By contrast, petunia DFR showed higher affinity for DHM than DHQ, and did not use DHK. This specificity results in the accumulation of delphinidins and cyanidins, but a lack of pelargonidin in petunia flowers (Gerats et al., 1982; Forkmann and Ruhnau, 1987). The pigment analysis of cymbidium and dendrobium floral tissues showed there is an absence of pelargonidin-type anthocyanins (orange to brick red) (Sugiyama et al., 1977; Tatsuzawa et al., 1996; Lewis, 2001; Mudalige and Kuhnle, 2004). This is consistent with data showing that the cymbidium DFR does not reduce dihydrokaempferol (DHK) efficiently, thereby limiting pelargonidin production (Johnson et al., 1999). It has been suggested that all DFRs accepting DHK as substrate have a conserved four-amino acid domain near the active site (Johnson et al., 2001). However, both the cymbidium and dendrobium DFR have this domain, but do not bind to DHK, suggesting that this domain is not the only factor determining DFR substrate specificity (Mudalige-Jayawickrama et al., 2005). Studies on DFR genes in different orchid suggest that they are less likely to have multigenes in the genome. For example, the *Cymbidium* cultivar `Rosannagirl Mild' and *Bromheadia finaysoniana* contain a single *DFR* gene in the genome, although three *DFR* genes have been found in

Oncidium orchid (Liew et al., 1998b; Johnson et al., 1999; Mudalige-Jayawickrama et al., 2005; Hieber et al., 2006).

1.7.3 Cytochrome P450 gene family

F3′H and F3′5′H belong to the microsomal cytochrome P450 gene family (Tanaka et al., 1998) and catalyse the hydroxylation reaction at the 3′ and 3′, 5′ of the B-ring in the flavonoid molecule. These enzymes have a wide substrate range, at multiple steps within the flavonoid pathway, including conversion of naringenin (a flavanone) to eriodictyol; DHK (a dihydroflavonol) to DHQ or DHM; leucopelargonidin (a leucoanthocyanin) to leucocyanidin or leucodelphinidin; and apegenin (a flavone) to luteolinidin. The F3′H and F3′5′H enzymes are known as the "red" and "blue" genes, and therefore have been targeted to modify flower colour. Examples of altering F3′H and/or F3′5′H activity to create novel flower colour in carnation, rose and torenia have been reviewed in Tanaka et al. (2005).

Cytochrome P450 monooxygenases contain a heme domain and require NADPH for the oxygenation reaction (Schuler and Werck-Reichhart, 2003; Seitz et al., 2006). F3′5′H belongs to CYP A and F3′H belongs to CYP B cluster. The amino acid sequences of the two genes in plants are very diverse, but the architecture remains conserved (Hasemann et al., 1995). F3′H and F3′5′H genes are well characterized among the dicotyledons. These genes were first studied in petunia showed that F3′H is corresponding to the *Ht1* locus, whereas F3′5′H activity is controlled by *Hf1* and *Hf2* loci, and the F3′5′H is enhanced by Cytochrome b_5 (encoded by *difF*), (Holton et al., 1993; Brugliera et al., 1999). The available monocotyledon F3′H and F3′5′H sequences are limited. Only sequences from *Allium cepa* (AAS48419), sorghum bicolor (ABG54319, ABG54321, AAV74195, AAV74194), *Oryza sativa* (AAM00948) and *Phalaenopsis* hybrid (AAZ79451) are published in databases, and many researches have not yet published. Recently, the F3′5′H from phalaenopsis has been isolated, and the sequence has all the characteristic-conserved motifs (Su and Hsu, 2003; Wang et al., 2006). *PhF3′5′H* is expressed at high levels at stages when flowers have just opened, correlating with the accumulation of delphinidin in the flower and expression is stronger in a purple cultivar compared with a yellow cultivar.

1.8 Factors determining the final flower colour

Flower colours are basically determined by the pigments formed in floral tissues. The final colour shade, intensity, and texture are also influenced by other chemical and physical factors in addition to anthocyanins. Some key factors that impact on final colour are described below.

1.8.1 Co-pigmentation

Flavones and flavonols commonly form complexes with anthocyanins (Goto, 1987; Forkmann, 1991); co-pigmentation usually pushes the visible light absorption to longer wavelengths (bathochromic shift), resulting in a bluer colour (Schwinn and Davies, 2004; Yu, et al., 2006). Increasing the ratio of flavanol and anthocyanin in *Ceanothus papllosus*, for example, changes the maximum absorption from 580 nm to 680 nm (Bloor, 1997). Bluer torenia flower colour was developed by transformation of an antisense *DFR* into torenia. A block in anthocyanidin production meant that naringenin was re-directed into flavone synthesis instead of anthocyanindin synthesis, causing increased flavone levels, and a bluer colour (Aida et al., 2000a). Metal ions including Fe^{3+}, Cu^{2+}, Ca^{2+}, Al^{3+}, Mg^{2+}, and Mo^{2+} are also found to co-exist with anthocyanins in co-pigment complexes in many plant species (Shiono et al., 2005; Yu, et al., 2006). The presence of the metal ions can alter flower colour by shifting the absorption wavelength towards blue and stabilising the anthocyanin complex (Kando et al., 1992). Cymbidium accumulates the colourless flavonoids, kampferol and quercetin, that may act as co-pigments (Lewis, 2001).

1.8.2 Vacuolar pH

Anthocyanins accumulate in the vacuole, and the pH in the vacuole is important for colour shifts displayed by anthocyanins (Brouillard, 1988; Mol et al., 1998; Grotewold, 2006). In a less acidic pH (pH 5-6), the anthocyanins tend to show blue colour, but red colours are shown in more acidic conditions (pH 3-4) (Forkmann, 1991). Plant vacuoles usually have pH recorded around 5.5 (Forkmann, 1991).

The colour changes in *Ipomea* from reddish-purple at the bud stage to blue when the flower is fully opened correlates with the change of vacuolar pH from 6.6 to 7.7 (Yoshida et al., 1995). One of the key genes affecting vacuolar pH, *Purple* (*Pr*), has been isolated. *Pr* encodes for a vacuolar Na^+/H^+ antiporter ion exchanger (*InNHX1*) (Yamaguchi et al., 2001). In the future, genes responsible for controlling vacuolar pH can potentially be used for engineering flower colours. There is no information on cymbidium vacuolar pH so far.

1.8.3 Pigment distribution and cell shape

Distribution of anthocyanins in cell layers and cell shape affect colour intensity. A study of colour intensity and epidermal cell shape of dendrobium showed that in the low colour intensity petals, anthocyanins are only localised in epidermal cells or subepidermal cells, whereas the more intense colour is the result of localization of pigments in several layers of mesophyll as well as epidermal cells (Mudalige et al., 2003). A striped pattern forms when pigments are concentrated in cells around vascular bundles (Mudalige et al., 2003). Moreover, the shape of epidermal cells and how cells are packed in mesophyll cells affects the texture of petals and sepals. The flat and tightly packed epidermal cells give a shining surface; in contrast, the conical and tightly packed epidermal cells give a rough texture (Mudalige et al., 2003). No studies in cymbidium pigment distribution and cell shape have been published. A MYB transcription factor, *Mixta*, have been isolated from *Antirrhinum* (Noda et al., 1994). It has been shown that *Mixta* is necessary for conical cell formation. Petals of *Antirrhinum mixta* mutant have flat cells instead of conical cells, and this physical difference caused changes in the way that ultraviolet light was reflected or absorbed by the cells, resulting in lack of petal pigmentation (Glover and Martin, 1998).

1.9 Genetic regulation of anthocyanin biosynthesis

The expression of flavonoid structural genes in the anthocyanin biosynthetic pathway is usually controlled by floral developmental and environmental signals. It is well demonstrated that the structural genes are regulated at the transcriptional level by transcription factors (Dooner, 1983; Mol et al., 1989). Transcription factors can enhance

or repress the transcription rate of the structural genes through controlling the rate of mRNA synthesis initiation via RNA polymerase II (Ranish and Hahn, 1996; Endt et al., 2002; Jaakola et al., 2002). Through regulating the overall activity of the structural genes, transcription factors are involved in controlling pigmentation intensity and patterning in a tissue-specific manner. Some transcription factors also influence the physiological properties of petal cells such as vacuolar pH and cell shape, in addition to pigmentation (Noda et al., 1994; Quattrocchio et al., 2006). The regulatory genes themselves respond to both developmental signal such as seed development and trichome development, and environmental changes such as lighting and the circadian clock (Sablowski et al., 1994; Spelt et al., 2002; Schwinn et al., 2006; Quattrocchio et al., 2006).

Many anthocyanin regulatory genes have been identified in several model plant species such as maize, petunia, *Antirrhinum* and *Arabidopsis*. Two transcription factor families commonly linked with the regulation of flavonoid biosynthesis are the MYB and the bHLH transcription factors (Mol et al., 1998). The anthocyanin-regulating MYB protein has two helix-turn-helix motifs, the R2 and R3 repeats, which have the ability to bind to the target DNA sequence. The bHLH is similar to the MYC protein in animal systems, and has a basic helix-loop-helix domain (Mol et al., 1998). The basic mechanism of regulatory action by transcription factors is likely to be that bHLH protein forms a complex with MYB protein and subsequently the complex binds to the *cis* element of a target gene to activate the transcription. Usually the binding site is at a specific sequence in promoter regions (Sainz et al., 1997; Grotewold et al., 2000). Another transcription factor involved is a leucine-rich repeat protein, the WD40. The WD40 gene family does not interact with target genes, but functions by stabilizing protein-protein interactions between MYBs and bHLH complex (Quattricchio et al., 1998). The formation of transcription factors complex is being seen as critical for controlling secondary metabolites during plant development (Davies and Schwinn, 2003).

1.9.1 Maize transcription factors

Some of the earliest transcription factors regulatory genes identified and characterized have been from maize. The MYB factor *C1* gene family includes *Colourless* (*C1*) (Cone

et al., 1986; Paz-Ares et al., 1986) and *Purple leaf* (*pl*) (Cone et al., 1993). The bHLH protein is encoded by the *R/B* gene family, includes *Leaf colour* (*Lc*) (Ludwig, 1989), *Red* (*R*) (Chandler et al, 1989), *Booster* (*b*) (Chandler et al., 1989) and *Sienna* (*sn*) (Tonelli et al., 1991). Different member of *R* and *C1* transcription factor families encode proteins with similar function, but each member has particular temporal and spatial expression patterns, depending on their response to environmental and developmental cues (Davies and Schwinn, 2003). The regulation of the anthocyanin pathway genes in maize is regulated by the direct interaction between MYB and bHLH genes. Combined expression of *R* and *C1* in unpigmented maize cell culture is necessary and sufficient to activate the entire anthocyanin biosynthetic pathway genes, and induce anthocyanin production (Goff et al., 1990; Ludwig, 1990; Grotewold et al., 1998; Bruce et al., 2000).

Ectopic expression of members of the *R* and MYB gene families, driven by the *CaMV35S* promoter, is sufficient to up-regulate pigmentation in some dicotyledon species too. Maize *Lc* and *C1* regulatory genes have been used successfully in some species to enhance anthocyanin production. Co-expression of *Lc* and *C1* in tobacco, *Arabidopsis* and white clover, for example, has resulted in intensified pigmentation in the flower (Lloyd et al., 1992; Quattrocchio et al., 1993; de Majnik et al., 2000). In *Arabidopsis*, induction of flavonols and trichrome formation was also observed (Lloyd et al., 1992). These results showed that *Lc* and *Cl* can activate the biosynthetic pathway not only in maize but also in other plant species and potentially can be used to test and modify anthocyanin production in target species.

1.9.2 MYB and bHLH transcription factors in dicotyledons

Homologous genes to the MYB and bHLH transcription factors from maize have also been isolated from a number of dicotyledonous plants. The regulation of anthocyanin biosynthesis, however, appears to be more complicated in dicotyledons, compared with maize. It appears that the anthocyanin biosynthetic genes in the dicotyledonous plant are regulated in groups. They can be classified into early biosynthetic genes (EBGs) and late biosynthetic genes (LBGs), with the division point are either at F3H or DFR, depending on whether flavones or flavonols are the predominant co-pigments with

anthocyanins (Martin and Great, 1993; Davies and Schwinn, 2003). The MYB and bHLH protein complex system that is seen in maize, is primarily targeting expression of LBGs rather than EBGs in petals of *Antirrhinum* and petunia (Martin et al., 1991; Quattrocchio et al., 1998; 1999). Late biosynthetic genes (LBGs) such as *DFR* in dicotyledonous flowering plants are regulated separately by MYB- and bHLH-type regulatory proteins, while early biosynthetic genes (EBGs) such as *CHS* are regulated by different regulatory proteins. MYB and bHLH paralogous genes isolated from petals of *Antirrhinum* and petunia have different activities, allowing for variation of pigmentation in the same tissue. The regulation of anthocyanin biosynthesis genes in flowers is much better characterized in petunia and *Antirrhinum* (reviewed in Davies and Schwinn, 2003) but is not well understood in the flowers of monocotyledon species.

1.9.3 Anthocyanin biosynthesis regulation in orchid

The regulation of anthocyanin biosynthesis in orchid is not well understood yet, but a few studies have indicated that pigment production in orchid is likely to be regulated by the MYB- and bHLH-type transcription factors. The up-regulation of anthocyanin production using both MYB and bHLH type regulatory genes offers a useful tool to monitor anthocyanin production. The maize *C1* and *B* genes have been tested transiently for up-regulating pigmentation in petals of two mutants, the alba mutant (white coloured) and the albescent mutant (white with light purple lip), of *Doritis Pulcherrima* orchid (Griesbach and Klein, 1993). The result showed that maize *C1* and *B* genes are able to complement pigmentation in the albescent mutant, but not alba mutant. This is consistent with albescent being a regulatory mutant, while alba is a structural gene mutant (Griesbach and Klein, 1993). Recent studies in phalaenopsis and oncidium suggest that pink pigmented spots or patches in petals are the result of up-regulation of anthocyanin biosynthetic genes by MYB-type transcription factors (Ma et al., 2008; Chiou and Yeh, 2008). A MYB-type transcription factor, *OgMYB1*, has been isolated from *Oncidium* 'Gower Ramsey'. *OgMYB1* is active during red sepal and petal tissues during floral development, but does not express in the yellow coloured lip tissue. Over-expression of *OgMYB1* in *Oncidium* Gower Ramsey resulted in up-regulation of pigmentation in lip tissue via activation of *OgCHI* and *OgDFR* transcription (Chiou and Yeh, 2008).

1.10 Genetic engineering for novel colours

An obstacle to breeding novel flower colours for the ornamental industry is when the genes resource for a desired colour are lacking in a particular species. Conventional breeding techniques normally have great difficulty to introduce a desired trait into a species that does not have the necessary genes. Genetic engineering has the ability of "cutting" and "inserting" genes from a source species to a target species, breaking the limitation of the gene complement in a certain species (Tanaka, 2005).

Strategies for employing genetic modification to obtain novel flower colours include:
1) Down regulation or silencing of targeted enzyme activities to block the formation of a specific flavonoid.
2) Introducing gene(s) that are absent in the target plant to open the pathway to the synthesis of flavonoid compounds that would not be normally formed.
3) Expression of a suitable gene from another species or cultivar to overcome the rate-limiting steps of the target plant.

Some examples of how these strategies have been used in ornamental plants to modify flower colour are given below.

1.10.1 Generating white flower colour

CHS is a key enzyme at the start of the flavonoid pathway, controlling the formation of all flavonoids. Silencing *CHS* to create white colour flowers was first demonstrated in petunia, using antisense *CHS* (van der Krol et al., 1988). Since then silencing of *CHS* has been carried out in various plant species (Elomaa, 1993; Courtney-Gutterson et al., 1994; Gutterson, 1995; Deroles et al., 1998; Napoli et al., 1999; Aida et al., 2000b; Suzuki et al., 2000), using different silencing constructs. The transgenic plants have white flowers as the synthesis of all flavonoids, including anthocyanins, is blocked (van der Krol et al., 1988). Since flavonoids also play important roles in response to environmental stress, plant defence and signaling, this blocking of the whole flavonoid pathway can result in pleiotropic effects, such as male sterility and sensitivity to environmental stress (Davies and Schwinn, 2003). Some transgenic lines also exhibited

flowers with complex patterning. This variation in patterning was shown when sense or antisense silencing of *CHS* was used in petunia (van der Krol et al., 1990), lisianthus (Deroles et al., 1998) and torenia (Aida et al., 2000b). These variations in flower pattern represent the instability of the silencing in the plant, which is a disadvantage for commercialization (Bradley et al., 2000). Therefore *CHS* silencing can help achieve white colour but may also have colour instability problems in some species.

An alternative target is down-regulation of key enzymes in the flavonoid pathway such as DFR or F3H (Figure 1.4). Zuker and colleagues have down-regulated *F3H* in carnation, and they found not only a reduction in anthocyanin levels in flowers, but also an increase in methyl benzoate, and thus more fragrance (Zuker et al., 2002). On the other hand, suppression of *DFR* in petunia does not have a clear colour phenotypic effect, but did induce female infertility (Jorgensen et al., 2002). Thus altering the activities of anthocyanin biosynthesis pathway gene may have other consequences and this should be assessed as part of the approach.

1.10.2 Generating blue flower colour

Rose, carnation and chrysanthemum are all commercially important cut flowers, where a lot of effort has been put in to create blue colour flowers. The strategy is to enable these plant species to produce delphinidin-type anthocyanins, pigments responsible for blue colour in many species but not produced in these species (Holton and Tanaka, 1994). Transgenic roses accumulating over 95% of delphinidin have been developed. The success in accumulating high levels of delphinidin was the result of over-expressing the viola *F3′5′H* gene and down-regulating in a selected cultivar the endogenous *DFR* to minimize its competition for the DHK substrate (Katsumoto et al., 2007). The transgenic line produces flowers with a dusky purple colour.

Mauve carnation flowers are another example of creating purple/blue coloured flowers. During early experiments, overexpression of the petunia *F3′5′H* in carnation did not result in high levels of delphinidin and this was due to competition between the petunia F3′5′H and endogenous carnation F3′H and DFR enzymes (Tanaka et al., 2005). This was solved by co-expressing both the petunia *F3′5′H* and cytochrome b_5 in the same

carnation line that has weak endogenous *F3'H* and *DFR* expression, resulting in a range of pink to violet coloured carnations (de Vetten et al., 1999; Tanaka et al., 2005). Two companies, Florigene and Suntory have developed a range of violet colour carnations by introducing a petunia *F3'5'H* and petunia *DFR* into a carnation *dfr* and *f3'h* double mutant background (Tanaka et al., 2005; Chandler and Tanaka, 2007). The petal also contained apigenin, which is considered to have a strong co-pigment effect and the vacuolar pH is around 5.5 (Fukui et al., 2003).

The two strategies described above showed that manipulation of anthocyanin biosynthetic genes is not enough to change flower colour to true blue, despite delphinidin being the predominant anthocyanin. Modification of secondary factors that contribute to flower colours, such as metal ions, co-pigments and higher cellular pH are necessary. Current understanding of genes involved in pH regulation and metal ion uptake, is however, not well characterized. Attempts to engineer genes involved in flavone production have also been carried out. Over expression of torenia *FNSII* gene in petunia resulted in increased flavones but decreased anthocyanin due to reduced supply of substrate, and a change in flower colour from deep purple to pale purple (Tsuda et al., 2004). Similarly, down regulation of *FNSII* gene in blue torenia also resulted in a decrease of anthocyanin. It is thought that flavones may stablise anthocyanins in torenia flower tissue, and decreased flavone levels led to increased anthocyanin degradation (Ueyama et al., 2002). Although a true blue colour is yet to be achieved, the accumulation of delphinidin-based anthocyanins in both carnation and rose has demonstrated the power of modern genetic engineering technology.

1.10.3 Generating red and orange flower colours

Many commercially important ornamental plants like petunia, gentian and cymbidium have a DFR which cannot reduce DHK efficiently (Forkmann and Ruhaun, 1987; Tanaka et al., 1996; Johnson et al., 1999; Johnson et al., 2001). Thus, even if F3′H and F3′5′H activity is silenced in these plants, they still cannot produce pelargonidin. The strategy used to create orange-red flowers has been to introduce a DFR that is able to reduce DHK to pelargonindin in order to overcome the substrate specificity issue of the endogenous DFR. Brick-red petunia has been created using this strategy by introducing

a foreign DFR from maize (Meyer et al., 1987), gerbera (Meyer et al., 1987) and rose (Tanaka et al., 1995) into a petunia mutant which is deficient in F3′H (*hf1*) and F3′5′H (*hf1* and *hf2*) enzyme activity. The reason for using a mutant background is that the petunia F3′H and F3′5′H will normally compete for the same leucopelargonidin substrate, which makes formation of pelargonidin less efficient. This strategy cannot be applied in plants without the triple mutation. Yet, most commercial plants are bred from multiple hybrid lines, and therefore a new strategy was needed. A chimeric RNAi construct that silences both *FLS* and *F3′H* and expresses the gerbera *DFR* gene was used to overcome this problem and produce pelargonidin in tobacco (Nakatsuka et al., 2007).

1.11 Thesis aims

This project focuses specifically on the flavonoid pathway and anthocyanin pigments in cymbidium orchids: a flower crop that is missing two colour groups- red and blue. Limited research has been conducted on the subject of flavonoid biosynthetic genes in orchids. To date, the only anthocyanin biosynthetic gene that has been isolated from cymbidium is *DFR*. Research on the molecular aspects of pigment production in cymbidium will allow us to better understand anthocyanin formation in cymbidium, and contribute to possible strategies for manipulating flower colour.

The project also focuses on obtaining the molecular tools for manipulating pigment production in a tissue-specific manner. The *CaMV35S* promoter activates the expression of the gene of interest throughout the plant, but it does not create the most desirable outcomes. Genes expressed in a tissue specific manner can provide novel colours in specific organs such as leaf or petals and sepals. Thus the project aims are:

1. To assess the possibility of novel pigment production in cymbidium using transient gene expression studies.
2. To isolate and characterize the key flavonoid biosynthetic genes *F3′H*, *DFR* and *CHS* from cymbidium orchids.
3. To investigate and identify tissue specific gene promoters in cymbidium orchid.