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

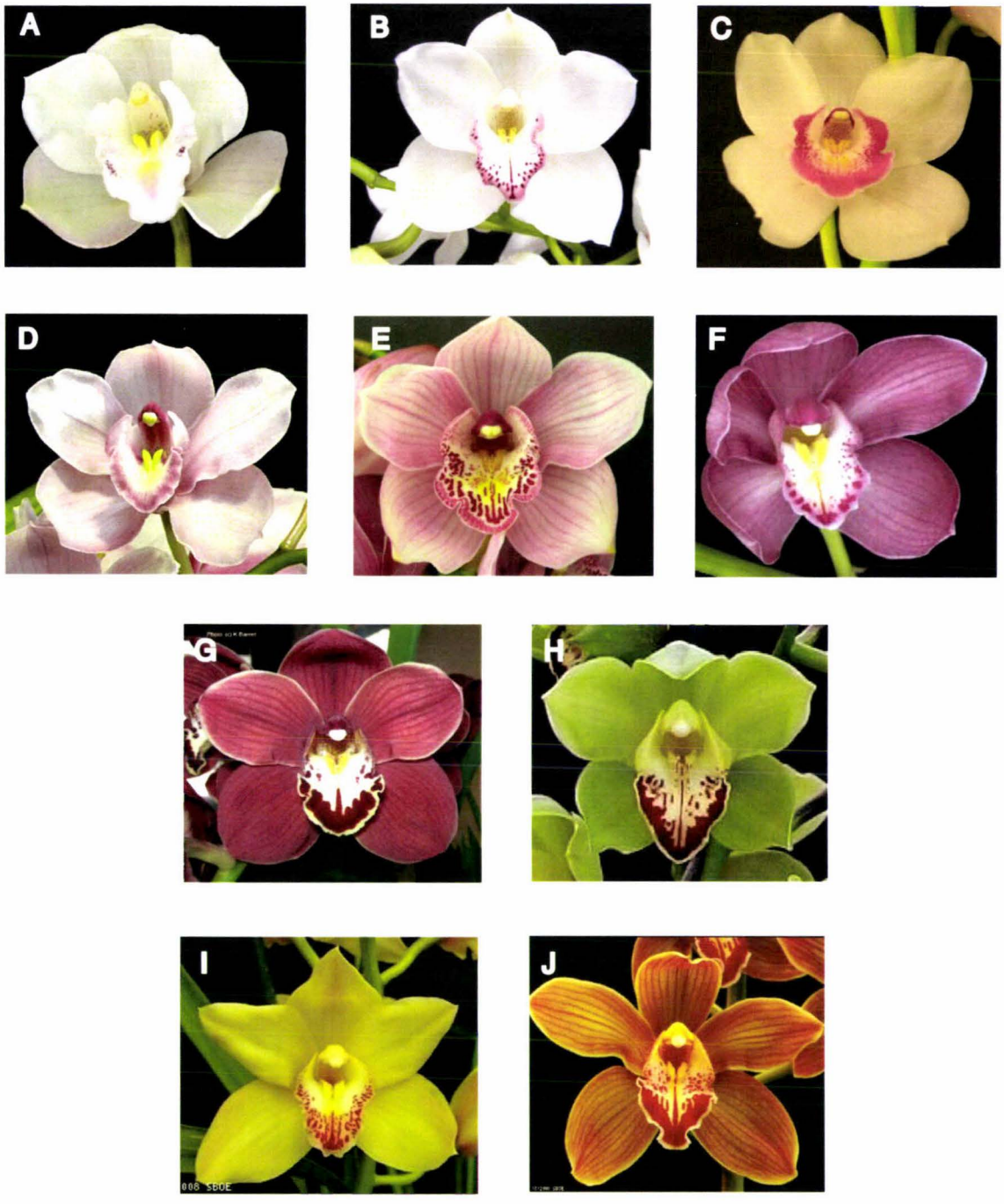


Figure 1.1: A range of cymbidium cultivars, **A-J** showing individual cultivars used in this study, and illustrating the flower colour range. **A-C**, white cultivars: **A**, Jung Frau dos Pueblos, JFDP; **B**, Winter Bride Peter's Choice; **C**, Virgin. **D-F**, pink cultivars: **D**, Narella Jennifer 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.

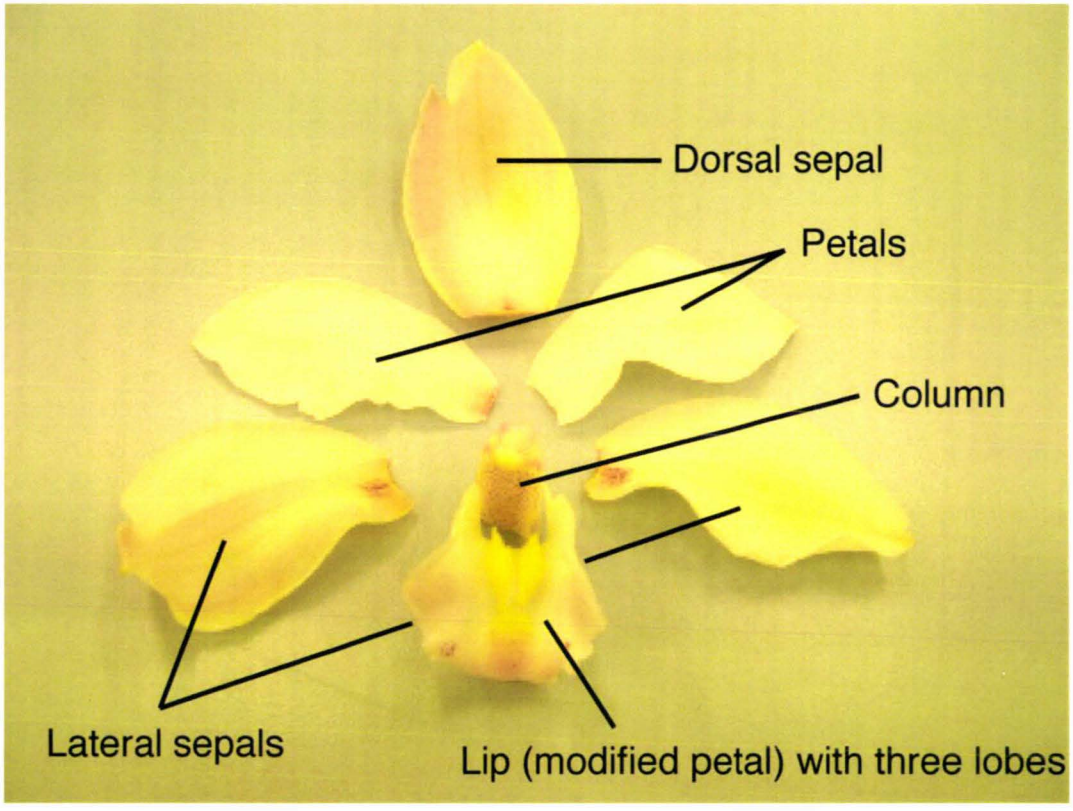


Figure 1.2: Structure of cymbidium flower.

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.

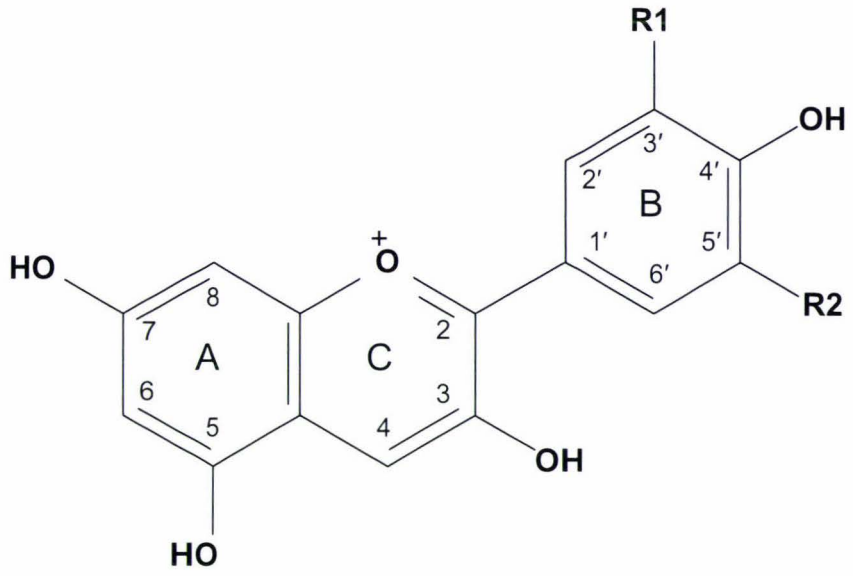


Figure 1.3: The basic flavonoid structure. The A, B and C rings are labelled, the numbers indicate the carbons in the aromatic rings. Addition of an oxygen atom at C4 position sets apart flavonols from anthocyanidins. The flavonol backbone has a carboxyl group, but the anthocyanidin backbone lacks oxygen 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).

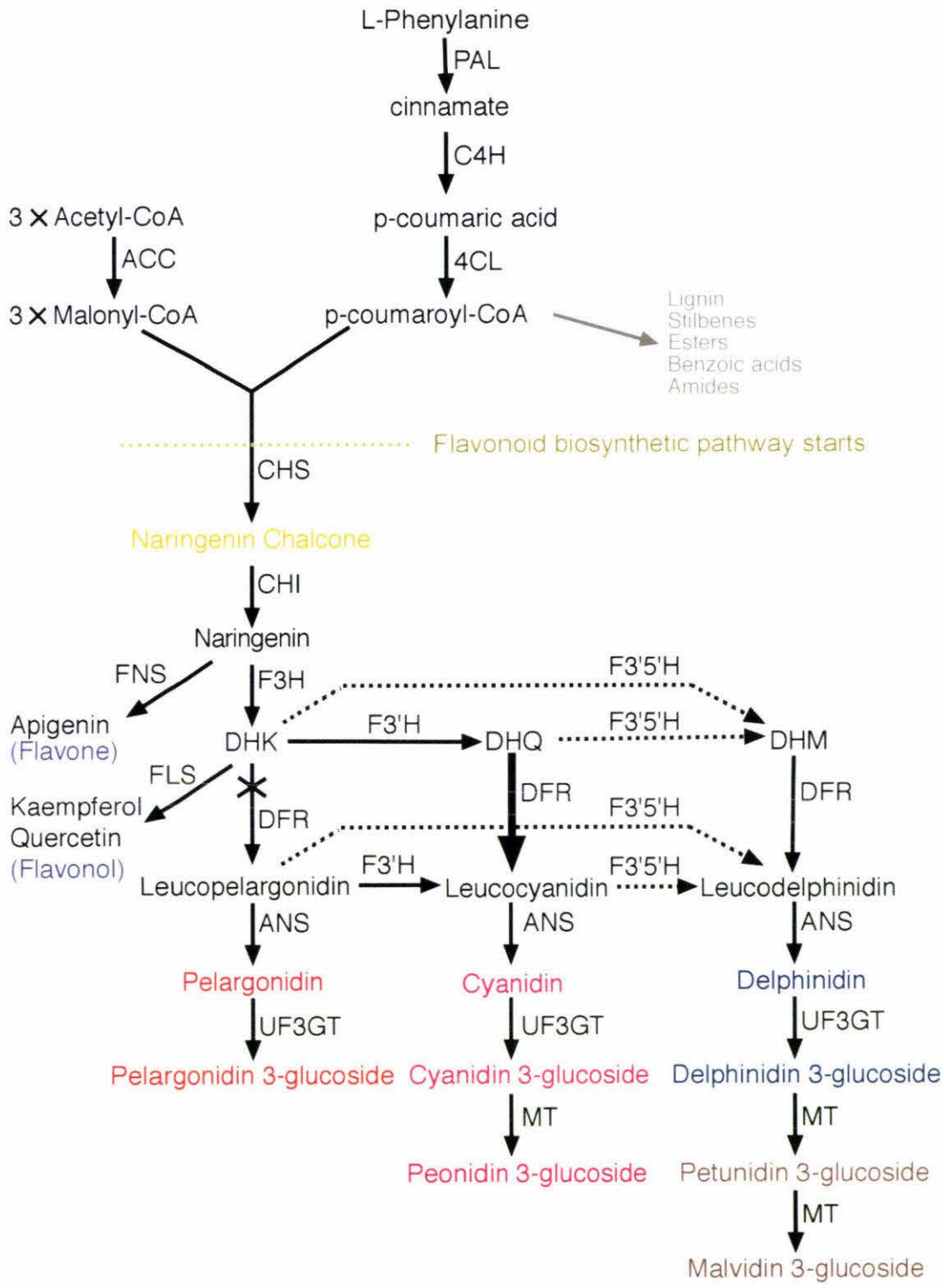


Figure 1.4: Schematic presentation of the flavonoid biosynthetic pathway. Classes of flavonoids are indicated in brackets. Crosses indicate known limitations of pigment biosynthesis in cymbidium. Dotted arrows represent flavonoids not produced appreciably in cymbidium. Bold arrow represent flavonoids 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.

Chapter 2

General materials and methods

2.1 Chemicals

Unless otherwise stated, all chemicals, reagents and solvents were supplied by Merck, BDH, Sigma or Roche.

2.2 Media, buffers and solutions

MilliQ water was used for all culture media and other solutions. LB-ampicillin (100 μg mL^{-1}) plates for *E. coli* culture and Media 2 (Appendix 1.6, used for tissue culture of plant material post transformation for transient gene expression studies) were sterilized by autoclaving at 121°C, 15 psi for 20 min. Agar solutions for solid media were cooled to hand temperature before the addition of antibiotics. Solutions that could not be autoclaved before use, such as those containing SDS, used autoclaved MilliQ water. Recipes for media and stock solutions are given in Appendix 1.

2.3 Plant materials

Cymbidium cut flower and leaf materials used in this study were obtained from three commercial cut flower growers (Dave and Nancy Beck, West Coast Orchids, Auckland; Peter Moffatt, Kiwi Orchids, Nelson; Ian Floyd, Airborne Cymbidiums, Auckland). Cut flowers were harvested when one flower was open on the stem, and were transported by overnight courier to the research lab at Crop and Food Research, Palmerston North. On arrival, cut stems and cut leaves were held in tubes of tap water in a vase-life room

maintained at a constant temperature of 20°C and illuminated with a 12 h photoperiod by cool fluorescent lights (20-25 μmol m^{-2} s^{-1}). Floral tissues were collected from the cymbidium cultivars shown in Table 2.1.

Table 2.1: Cymbidium cultivars used for the collection of floral tissues.

Cultivar	Abbreviation
Clarissie Austin South Pacific	CASP
Jung Frau dos Pueblos	JFDP
Narella Jennifer Gail	NJG
Vanguard Mas Beauty	VMB
Virgin	-

Photos of the cultivars used are shown as Figure 1.1. Cymbidium inflorescences consist of buds or flowers at different developmental stages. Flowers continued to develop sequentially from the base of the inflorescence under the conditions of the vase-life room. A sequence of six developmental stages was defined to ensure buds and flowers were harvested in a consistent manner, shown in Figure 2.1. Sepal, petal and lip tissues for each stage were detached, ground up in liquid nitrogen and stored at -80°C. Leaf material was collected from the cymbidium cultivars shown in Table 2.2.

Table 2.2: Cymbidium cultivars used for the collection of leaf tissues.

Cultivar	Abbreviation
Forty Niner Alice Anderson	AA
Big Chief Kirawee	BCK
Lymonese	Ly
Vanguard Mas Beauty	VMB
Narella Jennifer Gail	NJG
Piedmont	Pd

Young leaves were taken from the same plants as those used for inflorescence harvests. Leaf tissues were also ground up in liquid nitrogen and stored at -80°C.

2.4 PCR amplification of DNA fragments

cDNA fragments of cymbidium *CHS*, *DFR* and *F3'H* were amplified by PCR, using the general procedures are stated below. The specific template, primer, enzyme and annealing temperature used for each experiment are stated in the methods section of each chapter.

2.4.1 First-strand cDNA synthesis

Five μg of total RNA was used for cDNA synthesis. The first strand cDNA synthesis was carried out using Superscript III reverse transcriptase (Invitrogen), following the manufacturer's protocol. The poly(T) oligonucleotide primer possessed an anchor region that was used as a primer in subsequent PCR amplification (described in each chapter). The cDNA reaction solutions were diluted to 1 mL with TE buffer, and 1 μL used as template for subsequent polymerase chain reaction (PCR) amplifications.

2.4.2 Polymerase Chain Reaction (PCR)

All PCR reactions were carried out in a total volume of 50 μL, using either an Eppendorf or Biorad PCR thermocycler. The components of PCR reaction mixes are shown in Appendix 1.2. Amplifications were carried out using conditions as described in the relevant chapter.

2.4.2.1 Analysis of PCR products

The PCR products were analysed by electrophoresis on agarose gels (Section 2.5.6). A total of 5 μL of loading dye was added to 50 μL of PCR reaction solution prior to loading samples on to the gel. The resolved bands on the gels were then examined under

Figure 2.1: Developmental stages defined for cymbidium flowers.
Stage 1: Immature small, green buds.
Stage 2: Tightly closed small buds with pink pigment starting to form on the abaxial surface and reduced chlorophyll.
Stage 3: Medium-sized buds, the colour has turn creamy yellow with a dash of pink on the sepal.
Stage 4: Large buds with crinkly sepal, flower is ready to open.
Stage 5: The bud has just opened to the degree that the column can be seen from the opening of the flower.
Stage 6: Fully opened flower, sepals are white on adaxial surface with stripes of pink on the abaxial surface. Petals are white on both sides. Lip is mainly white with light pink patches scattered on the lip and column pink pigmentation on all surfaces.

UV light, and the desirable bands of DNA were subsequently excised with a blade. The DNA fragment from gel pieces were recovered according to the procedures described in Section 2.5.1.

2.4.2.2 Adding overhanging adenines to PCR fragments

In order to use TA cloning to insert DNA fragments into pGEM-T Easy (Promega, Madison, WI, USA), the DNA fragment has to possess an overhanging adenine on its ends. When the PCR reaction was carried out using a proofreading DNA polymerase such as *Pwo* polymerase, the resultant DNA fragments are blunt-ended. Therefore, adding A-overhangs to the blunt-ended DNA fragments is necessary before carrying out TA cloning. The reaction mix contained 7 μL of PCR fragment, 1 μL of 10× *Taq* polymerase buffer (Invitrogen), 1 μL of *Taq* polymerase and 1 μL of dATP (2 mM). The mix was incubated at 70°C for 30 min. The DNA fragments with A-overhangs were retrieved using the High Pure PCR Purification kit (Roche) before being used for ligations.

2.5 General cloning procedures

2.5.1 Purification of DNA fragments and PCR products

PCR fragments or restriction-digested plasmid backbones were isolated by either the freeze-thaw method or by using a High Pure PCR Purification kit (Roche). In the freeze-thaw method, the agarose gel piece containing the DNA fragments was frozen in liquid nitrogen and centrifuged at 14 000 rpm at room temperature for 5 min. The supernatants were transferred to fresh microfuge tubes. This freeze-thaw procedure was repeated three times until no more liquid came out of the gel. The collected liquid was mixed with one-tenth of final volume of 3 M NaOAc and 2 volumes of 100% ethanol, incubated at -80°C for 1 h then centrifuged at 14 000 rpm for 20 min. DNA pellets were washed with 70% (v/v) ethanol, air dried and then dissolved in 10 μL of sterile water. PCR fragments isolated using the High Pure PCR Purification kit (Roche) followed the manufacturer's instructions.

2.5.2 Cloning PCR fragments into pGEM-T Easy

All PCR fragments with adenine-overhanging ends were ligated into the pGEM-T Easy vector system according to the manufacturer's instructions, and then transformed into bacteria as below.

2.5.3 Transformation into *E. coli* and growing bacterial cultures

Ligation mixtures were used to transform *E. coli* competent cells (strain XL1-Blue) to multiply ligated plasmids. Competent cells (in 50 μL aliquots) were taken out from the -80°C freezers and thawed on ice. Ligation reactions (5 μL) were added to the competent cells, mixed briefly and incubated on ice for 20 min. The cells were then heat-shocked at 42°C in a water bath for 45 s, and then incubated on ice for a further 5 min. The cells were spread onto LB-agar plates containing ampicillin (100 mg L^{-1}, Appendix 1.1), spread with 4 μL IPTG (200 mg mL^{-1}, Appendix 1.1) and 40 μL X-Gal (20 mg mL^{-1}, Appendix 1.1). Inclusion of IPTG and X-Gal allows blue/white selection to be used to identify insert-positive clones.

All transformed cells were grown overnight at 37°C in an incubator. Individual white colonies were picked and grown overnight in 2 ml LB media with ampicillin selection (100 mg L^{-1}) in a 37°C shaking incubator.

2.5.4 Plasmid DNA preparations using alkaline lysis

Bacterial cultures were transferred to microfuge tubes and centrifuged at 14 000 rpm for 1 min to pellet cells. The supernatants were discarded, and cells resuspended in 100 μL of ice-cold solution I (see Appendix 1.3). The cells were lysed by adding 200 μL of freshly prepared solution II, inverting the tubes a few times to allow complete lysis. To neutralize the solutions, 150 μL of solution III was added and mixed well. At this stage, the solution appeared as a viscous bacterial lysate. The tube was centrifuged at 14 000 rpm for 5 min to pellet protein, carbohydrate and bacterial genomic DNA. The clear supernatant was transferred to a new set of tubes containing ice-cold 100% ethanol. The DNA plasmids were pelleted by centrifugation at 14 000 rpm for 20 min, then washed

with 80% (v/v) ethanol and air dried for 15 min. DNA pellets were dissolved in 50 μL of sterile water.

2.5.5 Restriction-endonuclease (RE) digestion of plasmid DNA

This method was used for routine diagnosis of positive clones or isolated DNA fragments from a plasmid for either downstream cloning or making probes for northern and Southern analysis.

Plasmid DNA (usually 1 μg) was digested with 10 units of restriction enzyme or enzyme combinations (Roche) in 1× restriction enzyme buffer, using the buffer recommended by the manufacturer which provides the best conditions for enzymatic activity. The pGEM-T Easy clones were always diagnosed using *Eco*RI enzyme to release the inserts. The digestion reaction mixes were incubated at 37°C (or 30°C, for certain restriction enzymes) in a water bath, for a minimum of 1.5 h. The digested DNAs were separated by agarose-gel electrophoresis.

2.5.6 Agarose-gel electrophoresis

Agarose-gel electrophoresis was routinely used for plasmid DNA or gDNA analysis. A 1% (w/v) gel was made by adding the appropriate amount of agarose to 1× TBE buffer (Appendix 1.4), and heated until the agarose had completely melted. A total of 5 μL of ethidium bromide (10 mg mL^{-1}) was added to 250 mL of agarose solution before pouring into the gel apparatus. The gel was left to set at room temperature for 1 h. A one-tenth volume of 10 × sample loading dye (Appendix 1.4) was added to each sample before loading onto the gel, which was run in 1 × TBE buffer at 100 – 120 V for 1 - 1.5 h. DNA was visualized by ethidium bromide fluorescence using a short wavelength UV transilluminator. Photographs were obtained using an Alphaimager 2000 Documentation and Analysis System (Alpha Innotech). Alternatively, the gel images were scanned by the FLA-5100 imaging system (Fujifilm). DNA size was estimated by comparing DNA fragments with a 1 kb + DNA ladder (Invitrogen) on each gel.

2.5.7 Plasmid DNA and DNA fragment purification

Plasmid DNA was purified for sequencing purposes using the High Pure PCR Purification kit (Roche), following the manufacturer's instructions. For every 100 μL of DNA miniprep sample, 300 μL of binding buffer (which contains high salt) was added and well-mixed. Then the mixture was added to the spin column, centrifuged at 14 000 rpm for the DNA to bind to the silica column and the solution to pass through. DNA bound to the column was then washed to remove salt with the wash buffer supplied. The DNA was eluted from the column using 50 μL of elution buffer supplied.

2.5.8 DNA sequencing

DNA sequencing was undertaken by either the Allan Wilson Centre Genome Service, Massey University (Palmerston North) or by the Waikato University DNA Sequencing Facility (Hamilton).

2.6 RNA and gDNA extraction

The following procedures were used to extract total RNA from the flower and leaf materials and genomic DNA from young leaves. Isolated RNAs were used for northern analysis and RT-PCR, gDNAs were used for Southern analysis and genome walking experiments.

2.6.1 RNA extraction by the Hot Borate method

Obtaining good quality RNA from flowers can be difficult, due to the presence of phenolics and carbohydrates and very high levels of RNases. Preliminary experiments with Trizol (Invitrogen) and a CTAB method found large amounts of carbohydrate remaining with the RNA. However, a modified Hot Borate method (Hunter et al., 2002) was found to give the best quality and quantity of RNA.

The extraction buffer was made by adding 50 µL of 1 M DTT (final conc. of 10 mM), 50 µL of Nonidet P-40 (1%, v/v) and 0.1 g of PVP (2%, w/v) to every 5 mL aliquot of XT buffer (see Appendix 1.5) and then was heating to 80°C just before use.

Approximately 0.8 g fresh weight of frozen sample was added to 5 mL of hot extraction buffer in an Oakridge tube, the mixture briefly vortexed, and 37.5 µL of proteinase K (0.53 mg) added. The sample was incubated in a rocker at 42°C for 90 min. At the end of incubation, 400 µL of 2 M KCl (final conc. of 145 mM) was added, the sample gently mixed and then incubated on ice for 30 min. The sample was centrifuged at 12 000 rpm for 20 min at 4°C, the supernatant transferred to a new tube, and an equal volume of 4 M LiCl added (final conc. of 2 M), and after mixing, the solution was left at 4°C overnight.

To collect the RNA, the sample was centrifuged at 12 000 rpm for 30 min at 4°C, the supernatants was removed and the RNA pellet resuspended in 500 µL of sterile water plus 50 µL of 3 M sodium acetate. The RNA was purified by adding 500 µL of chloroform/isoamyl alcohol (24:1), vortexed and centrifuged at 14 000 rpm for 5 min at 4°C. The upper aqueous phase was transferred to a new tube. A total of 400 µL of water was added to the remaining chloroform, the phases vortexed and centrifuged again. The supernatant was removed and combined with the supernatant collected from the previous step. A total of 30 µL of sodium acetate and 800 µL of isopropanol were then added to the combined aqueous extracts and the precipitated RNA was pelleted by centrifugation at 14 000 rpm for 30 min at 4°C. The pellets were washed with 80% (v/v) ethanol and resuspended in 50 µL milliQ water. RNA preparations were stored at -80°C.

2.6.1.1 RNA quantification

RNA samples were quantified by spectrometry using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA), measuring absorbance at 280, 260 and 230 nm. The purity of RNA against protien contamination is indicated by the A_{260}/A_{280} ratio, while the A_{260}/A_{230} ratio gives an indication of organic contamination such as phenolics and polysaccharides. A value close to two indicates

that RNA is pure. RNA concentration was calculated using the absorbance at 260 nm and the extinction coefficient for RNA of 40 (i.e., 40 μg mL^{-1} of RNA has an $A_{260} = 1.0$). Therefore:

Absorbance at 260 nm x 40 = RNA concentration in μg mL^{-1}

2.6.2 Genomic DNA extraction by the Urea method

Flower tissue (~2.3 g FW) was added to 16 mL of urea buffer (Appendix 1.5) in a 50 mL Falcon tube and incubated on a rocker at room temperature for 10 min. Phenol/chloroform/IAA (12 mL, Appendix 1.5) was then added, the samples mixed by vortexing and then rocked for 10 min at room temperature. The sample was centrifuged at 3 000 rpm for 10 min and the supernatant was transferred to a new tube. The gDNA was precipitated by adding 1.2 mL of 3 M NaOAc (pH 6) and an equal volume of isopropanol. The precipitated gDNA was then hooked out with a bent Pasteur pipette into a 15 mL Falcon tube, washed with 70% (v/v) ethanol and then transferred to a new tube containing 4 mL of TE buffer (Appendix 1.5) for dissolving the gDNA. The gDNA was further purified by adding 0.4 mL of NaOAc and 0.5 volumes of phenol/chloroform, vortexing and centrifuging at 3 000 rpm for 10 min. The supernatant was transferred to a new tube and re-precipitated with 2 volumes of 100% ethanol. The gDNA was hooked out again, washed with 70% (v/v) ethanol and left to air dry. The final gDNA pellet was dissolved in 500 μL of TE buffer. The quality of gDNA was assessed on an agarose gel to ensure minimal degradation, and stored at 4°C (to avoid shear damage caused by freezing).

2.6.2.1 gDNA quantification

All gDNA and plasmid DNA samples were quantified using a Nanodrop spectrophotometer, as described in Section 2.6.1.1, except that the extinction coefficient for double-stranded DNA is 50 μg mL^{-1} (i.e., 50 μg mL^{-1} of double-stranded DNA has an $A_{260} = 1.0$). Therefore:

Absorbance at 260 nm × 50 = DNA concentration in μg mL^{-1}

2.7 Northern and Southern analysis

2.7.1 Northern Analysis

2.7.1.1 RNA denaturing gels

The apparatus used to run RNA gels was treated with 2% (w/v) Absolve (PerkinElmer Life Science) overnight, rinsed in de-ionised water, and left to dry in the fume hood. The gel was made by melting agarose in water in a microwave, and after the gel solution had cooled to around 55°C, 5% of formaldehyde was added and one-tenth of final volume of 10 × MOPS buffer (Appendix 1.4) to give a final agarose concentration of 1.2% (w/v). The gel mix was poured into the gel tray, the comb inserted and left to set.

2.7.1.2 Checking for RNA degradation

Before running the gel for the northern blot, the integrity of the RNA and the accuracy of quantification were examined on a denaturing gel. A total of 3 μg of RNA sample was treated as described in Section 2.7.1.3 below, then separated on a denaturing gel as described above at 70 V for 4 h. The gel was examined under UV light to assess any degradation and the equality of loading between the samples.

2.7.1.3 RNA sample treatment

A total of 20 μg of RNA dissolved in 10 μL of water was loaded for northern analysis. The following chemicals were also added to each RNA sample to denature the RNA and prevent degradation: 1.5 μL of 10 × MOPS, 12.5 μL of formamide, 4 μL of 37% formaldehyde, 1 μL each of ethidium bromide (1 μg mL^{-1}) and bromophenol blue (0.4% w/v) to give a final volume of 25 μL. All RNA samples were kept on ice during preparation. The samples were then incubated at 65°C for 10 min to denature the RNA, immediately quenched on ice for 5 min, then centrifuged briefly to ensure samples were all at the bottom of the tubes. Samples were then placed back on ice prior to loading. A

10 µL aliquot of RNA ladder (0.75 kb - 9.74 kb) (Invitrogen) was denatured in the same way as the samples and then loaded on the gel for sizing of the RNA fragments.

2.7.1.4 Electrophoresis of RNA samples

After sample treatment, RNA samples were loaded onto a denaturing gel and separated by electrophoresis at 15 V overnight using 1 × MOPS as running buffer. The RNA gel was examined under UV light to check RNA separation. A fluorescent ruler was photographed next to the gel.

2.7.1.5 Blotting of RNA

The gel was blotted on to Hybond N+ Membrane (Amersham Pharmacia Biotech, Cardiff, UK) following the procedure as described in Brown (1999). The transfer buffer used was 10 × sodium chloride-sodium citrate (SSC) buffer (Appendix 1.5). After transfer overnight the RNA was fixed to the membrane using a UV-C 500 crosslinker (Hoefer Pharmacia Biotech, San Franscisco, CA, USA) for 1 min at 70 000 µJ cm^{-2}. The membrane was then stored in a plastic bag at room temperature until needed.

2.7.2 Southern analysis

2.7.2.1 Restriction digestion of gDNA

A total of 60 µg of gDNA was digested with 3000 U of the appropriate restriction enzyme (Roche) in a total volume of 1 mL including the recommended buffer at 37°C overnight. To ensure the digest was complete, 5 µL of each digestion reaction was examined on an agarose gel (see Section 2.5.6).

The DNA in the remaining digestion reactions was precipitated by adding one-tenth volume of 3 M NaOAc and two volumes of 100% ethanol, and incubating at 4°C for 1 h. DNA was pelleted by centrifugation at 14 000 rpm for 10 min. The supernatants were discarded, and the pellets were washed with 70% (v/v) ethanol. A second centrifugation step at 14 000 rpm for 2 min was included to collect the pellets, and supernatants were

discarded. The microfuge tubes was left open to allow pellets to air dry, then the pellets were dissolved in 50 μL of TE buffer.

2.7.2.2 Electrophoretic separation of digested gDNA

To each DNA sample 0.3 volume of 10 × loading dye was loaded, the samples mixed and then loaded onto a "dry" gel (a gel in but not submerged in its running buffer) to prevent samples floating out. A high molecular weight DNA ladder was also loaded for sizing the hybridized fragment. The gel was run at 30 V until all samples had entered the agarose gel, and then 1×TBE buffer was added to cover the gel. The samples were then separated at 80 V until the dye front had migrated about two-third of the length of the gel.

2.7.2.3 Blotting gDNA

The blotting apparatus for gDNA was the same set up for blotting RNA (see Section 2.7.1.5), except that the transfer buffer used was 0.4 M NaOH.

2.7.3 Probing northern and Southern blots

2.7.3.1 Probe labelling

Double-stranded DNA template was labelled by random priming using the method of Feinberg and Vogelstein (1983). A total of 100 ng of DNA fragment (the probe template) was denatured at 90°C for 15 min then chilled on ice for 5 min. The following solutions were added to the probe: 2.5 μL of 10 × RP buffer (Appendix 1.5), 2 μL of random hexamers (Roche), 5 μL of [α- ^{32}P]-dATP (110 TBq mmol^{-1}, Amersham Biosciences) and 1 μL of the Klenow fragment of DNA polymerase I (3′-5′ exo$^-$) (Roche). The mixture was then incubated at 37°C for 3 h and then 25 μL of water was added to gave a total volume of 50 μL. The labelled DNA was purified from unincorporated radioactive nucleotides using a ProbeQuant^{TM} G-50 Micro Column (Amersham Biociences), and denatured at 90°C for 10 min before adding to the hybridisation solution.

2.7.3.2 Hybridisation

The membrane was pre-hybridized in 50 mL of Church and Gilbert buffer (Church and Gilbert, 1984, Appendix 1.5) at 60°C on a rocking platform for 2 h. This reduces non-specific binding of the probe to the membrane. The pre-hybridization buffer was discarded, and replaced with sufficient fresh Church and Gilbert buffer to cover the membrane, prior to adding the denatured probe. Membranes were incubated overnight on a shaking platform at 60°C to allow hybridisation.

2.7.3.3 Stringency washes

Unbound probe was removed by washing the membranes with a series of solutions of increasing stringency (decreasing salt) at constant temperature. The hybridisation solution was discarded and membranes were washed with different stringency SSC buffers (all containing 0.1% SDS), with each wash step at 1 h at 60°C. The washes started with 2 × SSC, followed by 1 × SSC, and then a final wash of 0.5 × SSC. This stringency was sufficient to remove all background and non-specifically bound probe, but not the desired radioactive signal.

2.7.3.4 Autoradiography

After completion of washes, the membrane was sealed in plastic and then exposed to a phosphorimager plate overnight. The image was scanned by an FLA-5100 phosphorimager imaging system (Fujifilm, Tokyo, Japan). After the image was scanned, the membrane was exposed to X-ray film (Kodak) to obtain a better quality image. The film cassette was stored at -80°C for two weeks before the film was developed.

2.7.3.5 Stripping membranes

Membranes were stripped in hot 1% (w/v) SDS (near 100°C) and left on the rocker to cool to room temperature. Membranes were washed briefly in 2 × SSC and the efficiency of stripping assessed with a Geiger counter or by exposure to a phosphorimager plate overnight.

2.8 Transient gene expression assay using biolistic particle bombardment

Transient gene expression was carried out using biolistic particle bombardment. In this technique, DNA is introduced into tissues by adsorbing it onto gold particles, which are shot into the tissue using high-pressure gas. Transient expression of the introduced genes in the cells around the site of the bombardment can be visually examined.

2.8.1 Gold particle preparation

Gold particles of 1 μm and 1.6 μm diameter were prepared by weighing out 50 mg of gold particles (Bio-Rad, Hercules, USA) and suspending them in 500 μL of isopropanol in a 1.5 mL microfuge tube. The gold particles were vortexed for 10 min, then pelleted by centrifugation and the supernatant discarded. This step was followed by washing the gold particles three times with 500 μL of H_2O, then 500 μL of H_2O was added to the gold pellet. The pellet was resuspended evenly, then aliquoted out in 50 μL fractions. The aliquots were stored at -20°C.

2.8.2 Sterilization of plant materials

Plant materials were sterilized for 10 min in 10% bleach (containing 4.2% w/v sodium hypochlorite) containing a few drops of Tween 20. The sterilizing solution was stirred gently throughout this time. The plant material was rinsed three times in sterile water and left to dry in a laminar flow hood before use.

2.8.3 Preparation of DNA/gold suspension

A total of 10 μL of combined plasmid DNA was added to 50 μL of gold particles. The amount of each DNA construct used is stated in the methods section of each chapter. Following the addition of DNA, 20 μL of spermidine (100 mM) and 50 μL of 2.5M $CaCl_2$ was also added to the gold particles. The mixture was vortexed for 3 min,

centrifuged briefly and then 85 μL of supernatant was discarded. The gold particles were resuspended in the remaining solution, and 5 μL used for each shot.

2.8.4 Particle bombardment conditions

Particle bombardment experiments were performed in a laminar flow hood using a modified helium particle inflow gun based on Finer et al. (1992), but with a high-speed direct current solenoid valve. Petal/sepal tissues were placed on media 2 (Appendix 1.6) at 120-160 mm from the nozzle, while leaf tissues were placed on media 2 at 40-50 mm from the nozzle. An aliquot of 5 μL of the experimental DNA adsorbed to the gold particle suspension was placed onto the filter for each bombardment, and when the trigger button was pressed, the gold particles were accelerated in a helium stream onto the tissue (helium pressures were set at 300 kPa for petal/sepal tissues and at 600 kPa for leaf tissues, both in a partial vaccum of -14 psi). The standard solenoid opening time was 30 milliseconds. Each sample was bombarded twice (unless otherwise stated).

2.8.5 Post-bombardment handling of plant materials

After shooting, all tissues were subsequently implanted into media 2 and kept in sealed tubs in a tissue culture room at 25°C with a 16 h photoperiod under cool white fluorescent lights (25 μmol m^{-2} s^{-1}).

2.8.6 Assessment of shooting results

The time of assessment post-shooting varied depending on the tissue and the constructs being tested (see Chapter 3, 4 and 5). Anthocyanin production in plant tissues was assessed using an Olympus SZX12 light microscope. GFP production in plant tissues was examined by using a blue light source with a GFP filter set attached on the microscope. All images were taken by a Leica Microsystems DC500 digital camera.

Chapter 3

Transient gene expression and anthocyanin accumulation in floral tissues of cymbidium

3.1 Introduction

Production of different anthocyanidins depends on the availability of the substrates DHK, DHQ and DHM, and the enzymatic activity of F3′H, DFR and F3′5′H (Figure 3.1). The lack of pelargonidin in cymbidium is because the endogenous DFR can only use DHQ as a substrate for cyanidin synthesis, and not DHK as a substrate for pelargonidin synthesis (Johnson et al., 1999). It is not clear why delphinidin is absent in cymbidium, but it may due to a mutation or lack of a functional F3′5′H in the flavonoid biosynthetic pathway.

Modern biotechnology has provided useful tools to manipulate the activities of key enzymes in the flavonoid pathway, potentially making new pigments in plants that would not be possible by traditional breeding methods. The production of stable transgenic plants with a new phenotype is, however, an expensive and time-consuming process. Some plant species do not have a transformation systems established. Although a transformation system is established for cymbidium (Yang et al., 1999; Chin et al., 2007), it usually takes three to four years for transgenic cymbidium to develop from tissue-cultured plantlets to mature flowering plants. A careful assessment of the gene of interest in the target plant is therefore important, before stable genetic modifications are attempted. Detailed characterization of both molecular and biochemical aspects of anthocyanin production prior to initiating stable transformation experiments, will improve the likelihood of success in generating a new cymbidium flower colour.

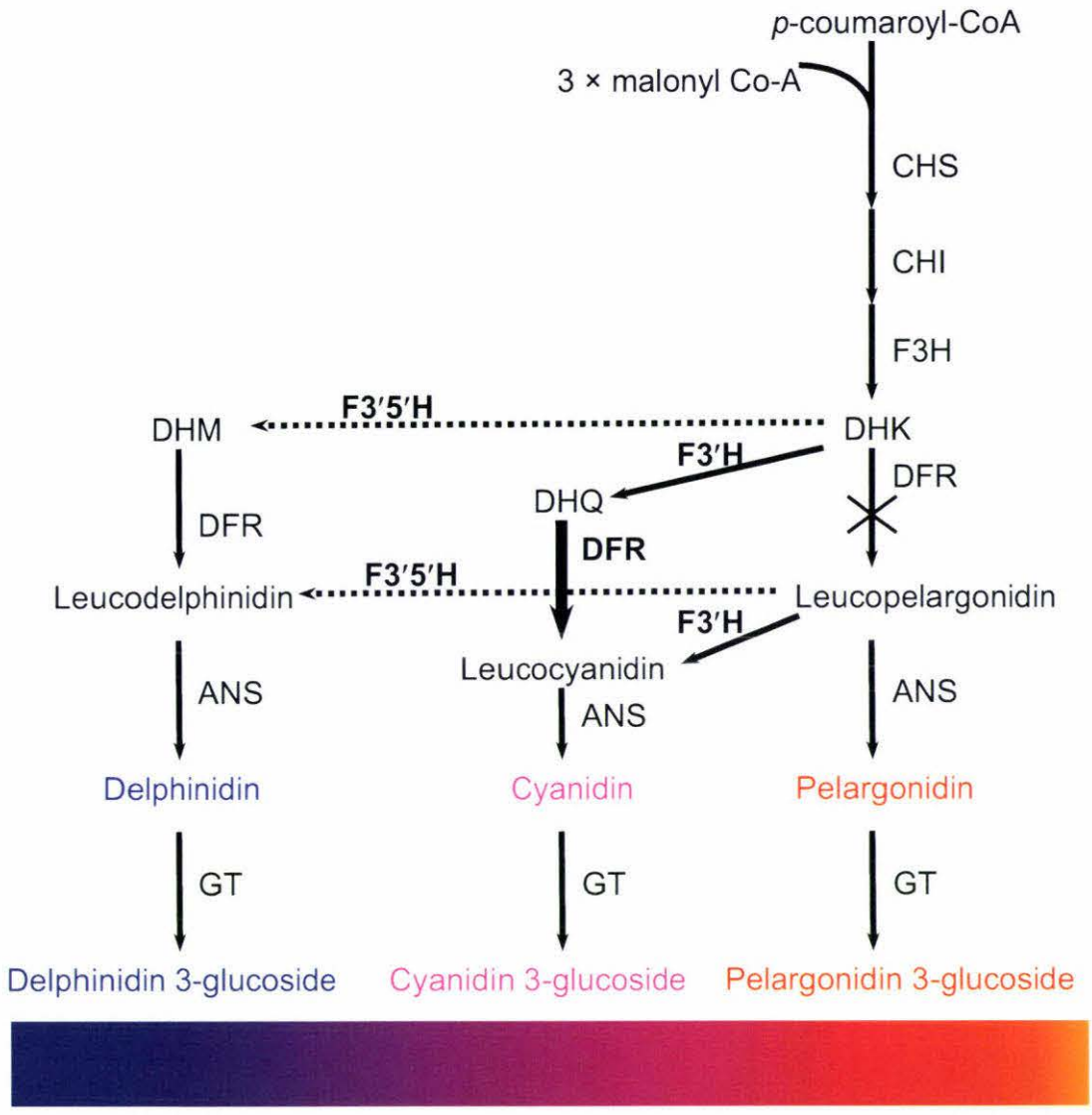


Figure 3.1: Schematic representation of the flavonoid biosynthetic pathway. The cross represents known limitations of pigment biosynthesis in cymbidium. Dotted arrows represent flavonoid not produced appreciably in cymbidium. Bold arrows represent flavonoids produced appreciably in cymbidium. CHS, chalcone synthase; CHI, chalcone isomerase; F3H, flavone 3-hydroxylase; F3′5′H, flavonoid 3′5′-hydroxylase; F3′H, flavonoid 3′-hydroxylase; DFR, dihydroflavonol reductase; ANS, anthocyanidin synthase; GT, glucosyltransferase; DHM, dihydromyricetin; DHQ, dihydroquercetin; and DHK, dihydrokaempferol.

One way to characterize the anthocyanin production pathway is to feed plant tissues with various soluble flavonoid precursors to determine where blocks in the pathway may exist. If anthocyanins are synthesized, the phenotype can be assessed easily by visual colour change, and the pigments can be further analyzed using photometric and chromatographic methods (Martens et al., 2003). Some characterization of the anthocyanin biosynthetic pathway has already been carried out in cymbidium. Feeding leucopelargonidin to cymbidium petals did not result in anthocyanin production (D. Lewis, pers. comm.). This is consistent with findings by Johnson et al. (2001), who concluded that pelargonidin production would require a DFR that could use leucopelargonidin as substrate. When leucodelphinidin was supplied to cymbidium petals, delphinidin accumulated. Therefore, this result indicated that the endogenous cymbidium DFR is able to process delphinidin precursors, but the necessary F3′5′H activity to produce those precursors was not present. The introduction of a functional *F3′5′H* gene from another plant into a cymbidium plant system may overcome the limitation in delphinidin production.

A second approach to study pigmentation *in planta* is to use a transient gene expression system. This approach allows the visualization of what colour might be formed in the petal and sepal when specific transgene or transgene combinations are introduced into the plant system. The pigments formed can be further analysed by various chromatography techniques. Previous studies have used the transient expression system to demonstrate that overexpression of maize anthocyanin regulatory genes (*MYB/C1* and *MYC/R* families) can complement the albescent mutation of *Doritis pulcherrima* and white *Phalaenopsis* species, resulting in up-regulation of anthocyanin production in white cultivars of orchid (Griesbach and Klein, 1993; Ma et al., 2008). Crop and Food Research have also tested the regulatory ability of maize *Lc/C1* transcription factors in cymbidium by transiently expressing maize transcription factor genes, and shown that introduction of *Lc/C1* to JFDP petals/sepals leads to pigmentation in the petals/sepals (D Lewis, pers. comm.). This indicates maize transcription factors can up-regulate endogenous biosynthetic gene expression in cymbidium, as has been reported in other plants (Lloyd et al., 1992; Quattrocchio et al., 1993; Goldsbrough et al., 1996; Bradley et al., 1998; Bovy et al., 2002). This also suggests that the white phenotype is due to a lack of regulatory gene expression rather than to a defective biosynthetic gene (Griesbach and Klein, 1993).

These observations led to the first objective of this chapter: to determine if up-regulation of pigmentation caused by overexpression of maize *Lc/C1* transcription factors is uniform in different cymbidium cultivars. A transient expression system has been established for flower petal tissue (Shang et al., 2007), and transient overexpression of maize *Lc/C1* in cymbidium has been used previously as a tool to select the cymbidium cultivar with the best potential for colour strategies to be assessed.

The second objective was to determine if novel pigments could be generated in cymbidium floral tissue. The strategy was to re-channel parts of the flavonoid pathway by introduction of heterologous anthocyanin biosynthetic genes to overcome the lack of certain anthocyanin biosynthetic enzymes in cymbidium. One of the candidate gene sources is from *Anthurium,* an ornamental monocot plant whose DFR can convert DHK to pelargonidin (Collette, 2002). *Anthurium DFR* was tested for its ability to induce pelargonidin production in cymbidium petals/sepals. An *F3'5'H* gene from pansy (*Viola tricolor*) was introduced into cymbidium to enhance delphinidin production. This follows the work of Katsumoto et al. who used this strategy to accumulate delphinidin pigment in rose (Katsumoto et al., 2007). Both genes will be tested for the synthesis of novel colours in cymbidium using a transient gene expression approach.

The third objective was to determine whether RNAi technology can be used to silence heterologous or homologous gene expression in cymbidium petal/sepal tissues using the transient expression system. The effects of RNAi technology were tested by co-introducing pansy *F3'5'H* driven by the *CaMV35S* promoter with a pansy *F3'5'H* RNAi construct into cymbidium petals/sepals via biolistic bombardment.

3.2 Materials and methods

3.2.1 Plant materials

Fresh flower stems of two white cultivars, Jung Frau dos Pueblos (JFDP) and Virgin, and two pink cultivars, Narella Jennifer Gail (NJG) and Lisa Rose Flammingo (LRF), were obtained from a commercial grower. All stems had water tubes attached to the cut

ends and were kept in a controlled temperature room prior to experiments as per Section 2.3. Flowers were graded into six developmental stages as shown in Figure 2.1. Stage six petals and sepals of all cultivars were used for biolistic transformation experiments.

3.2.2 Transient gene expression using a gene gun

All transient gene expression experiments were carried out as described in Section 2.8. Stage six petals and sepals were detached from the flower and sterilized before biolistic transformation. Gold particles used for biolistic bombardment transformation of petals and sepals were 1 μm diameter and aliquoted as 10 mg per 50 μL of water fractions. Each petal or sepal was bombarded twice on the adaxial side and was kept in a plastic tub on Media 2 (Appendix 1.6) in a culture room at 20°C under a 16-hour light and 8-hour dark photoperiod.

3.2.2.1 Assessment

In all transient expression experiments, assessment of GFP expression in floral tissues started 24 h after the introduction of the DNA into the sepals and petals. GFP expression was recorded as present or absent. Pigment production was monitored from 48 h to 14 days post transformation. General assessment of anthocyanin induction in petal/sepal tissue was made by scoring the foci number. Petals/sepals were grouped as having of ~10, ~100, or >100 colour foci.

3.2.3 Transient gene expression experiments using heterologous transcription factors

Two constructs containing the ORF sequences of two maize transcription factors were obtained from Crop and Food Research (Bradley et al., 1998). Construct pLc349 contains the maize *Lc* gene, and pLN44 contains the maize *C1* gene, and these were used for transient expression experiments to up-regulate pigment production in cymbidium petals/sepals (Appendix 2.1). Both constructs are pART7-based expression vectors, meaning both the *Lc* or *C1* genes were under the control of the *CaMV35S* promoter. A mixture of 3 μg of each pLc349 and pLN44 plasmid DNA was coated onto

the gold particles for transient gene expression studies. Stage six petals/sepals from the JFDP, Virgin, NAG and LRF cultivars were tested for up-regulation of pigmentation.

Construct pPEP-GFP is also a pART7-based expression construct, in which a *GFP5* coding sequence is driven by the *CaMV35S* promoter (Siemering et al., 1996). Two control experiments using this *GFP* construct were included: biolistic transient expression of pPEP-GFP alone, and pPEP-GFP co-introduced into petals/sepals together with pLc349 and pLN44. The *GFP*-only control was to test if pigment formation in cymbidium petals/sepals was induced by a wounding response caused by shooting damage. Similarly, co-introduction of *GFP* and maize transcription factors was carried out in order to be able to detect the expression of the transgenes in petals/sepals where increased pigment was observed, to confirm the transformation event. In each case, 3 μg of pPEP-GFP was used. The control experiments were carried out in the petals/sepals of the JFDP and Virgin cultivars.

3.2.4 Transient expression of anthocyanin biosynthetic genes

Transient gene expression of heterologous biosynthetic and regulatory genes were used to test the feasibility of producing novel pigments in the petal/sepal of cymbidium.

Two constructs containing biosynthetic genes were used to generate synthesis of the novel pigments in the epidermal cells of cymbidium petals/sepals. Construct pAa*DFR* contains *Anthurium DFR* (AY232492) and construct pPansyF3′5′H40 contains a pansy *F3′5′H* gene (AB332097). Both constructs were in a pART7-based vector with a *CaMV35S* promoter to drive the gene expression (Appendix 2.1). Two μg of plasmid DNA was used in each case to coat the gold particles and then introduced into the petals/sepals of the JFDP cultivar.

In order to boost the anthocyanin level when the biosynthetic genes were being tested to produce different pigments, the pLc349 and pPN44 constructs were mixed with pAaDFR, or pPansyF3′5′H#40. The respective DNA mixtures containing both transcription factors and one structural gene were coated onto gold particles. The effect of each construct combination on pigmentation was observed after they were introduced

into the petals/sepals of JFDP. Two μg of each pLc349, pPN44, pAaDFR and pPansyF3′5′H#40 DNA were used for all the experiments

3.2.5 Silencing *F3′5′H* activity of pansy

A pansy *F3′5′H* RNAi construct was tested to confirm that the pigments apparently induced by transient expression of a pansy *F3′5′H* transgene in cymbidium petals/sepals and sepals were caused by the introduced enzyme activity, as well as testing RNAi technology in cymbidium floral tissues.

3.2.5.1 Assembly of a pansy *F3′5′H* RNAi construct

Two pansy *F3′5′H* gene-specific primers were designed: pansy*F3′5′H* RNAi-F (ATATCTAGAGAGATGGATCGAGTCATCG) and pansy*F3′5′H* RNAi-R (ATAGGATCCGATTGATCTTCCCATTCTCTT). The forward primer has an *Xba*I restriction site and the reverse primer contains a *Bam*HI site (restriction sites are underlined). Both primers have three additional nucleotides added on the 5′-end to ensure efficient cleavage during the enzyme digestion. A Pansy cDNA was gifted by H. Zhang and used as the template for PCR amplification. The PCRs were performed to amplify fragments: either *Pwo* polymerase (Roche) or *Taq* polymerase (Qiagen) were used. The reactions were carried out at annealing temperature of 60°C or at 65°C, extension at 72°C for 1 min (detailed PCR reactions are described in Appendix 1.2). At the end of the PCR reactions, the PCR product was separated on an agarose gel to determine the size of products. The PCR product was excised from the gel and DNA was recovered using a Roche High Pure PCR purification kit, following the manufacturer's instructions (Section 2.5.7). The extracted DNA was then digested with 20 U each of *Xba*I and *Bam*HI as described in Section 2.5.5. The digested DNA was purified from the reaction using a Roche High Pure PCR purification kit and then used for cloning.

The pansy *F3′5′H* fragment with *Xba*I site and *Bam*HI sites was first cloned into the pDAH2 RNAi base vector between *Xba*1 and *Bgl*II. After sequencing confirmation of the positive clone for the first arm inserted into pDAH2 (Appendix 2.2), the second arm

was cloned into the construct between the *Bam*HI and *Nhe*I sites. The positive clone was digested with *Eco*RI to linearise the vector, before sequencing of the final construct.

3.2.5.2 Testing the pansy *F3′5′H* RNAi construct by particle bombardment

Control experiments used 3 μg of DNA of each of pLN44, pLc349 and pPansyF3′5′H#40 plasmids mixed together and bombarded into JFDP petals/sepals. The silencing of the endogenous *F3′5′H* by the RNAi construct was tested using a total of 5 μg of pPansyF3′5′HRNAi mixed with all the constructs mentioned above. All constructs were bombarded into JFDP and Virgin petals/sepals and pigment production/ reduction was assessed during the following days.

3.2.6 Pigment analysis

Anthocyanins formed in the transformed cells during transient gene expression were analysed to reveal changes in anthocyanins. Areas of cymbidium sepals and petals containing coloured foci were excised with a scalpel blade and ground into powder in liquid nitrogen. Anthocyanins were extracted by a hydrolysis method for TLC analysis and an aqueous methanol method for HPLC analysis. Approximately 300 mg FW of ground tissues weas collected and was divided into two to accommodate the two different analyses.

3.2.6.1 Thin-layer chromatography (TLC) analysis of anthocyanidins

Hydrolysis extraction was carried out to release the anthocyanidins from transformed tissues. Six mL of 3 M HCl was added to 150 mg of tissue, the samples mixed well and incubated in a 100°C oil bath for 45 min. The sample was centrifuged for 15 min at 3000 rpm and the supernatants transferred to a new tube. A total of 1 mL of ethyl acetate was added to the collected supernatant mixture, vortexed and centrifuged at 3000 rpm for 6 min. The aqueous phase (bottom layer) was transferred to new tube containing 600 μL of isoamyl alcohol, before vortexing and then standing for separation. The coloured amyl alcohol fraction on the top layer, was collected by a glass pipette and transferred to a PCR tube. Anthocyanidins were purified through a C18-E column

(55 μm, 70A, 100 mg/mL, Phenomenex). The column was prepared by washing with 1 mL of methanol twice, followed by three rinses with 1 mL of water for each rinse. The collected coloured amyl alcohol fractions were dried, resuspended in methanol, and loaded onto the washed a C18-E column. The column was then washed three times with 1 mL of water, and the flavonoids were eluted with 0.5 mL of 0.1 N HCl in methanol. The eluted fraction was then concentrated down by evaporation under nitrogen and resuspended in 10 μL of methanol. The collected anthocyanidins were used for TLC analysis.

Anthocyanidins were separated using a POLYGRAM® CELL 400, 20×20 cm TLC plate coated with a 0.1 mm layer of microcrystalline cellulose. A total of 2 μL of each pelargonidin, cyanidin and delphinidin standard was loaded onto the marked spots on the TLC plate. The hydrolysed flavonoid extraction from *Lc/C1*, *Lc/C1+* anthurium *DFR* and *Lc/C1+* pansy *F3'5'H* tissues were also loaded onto the plate. The plate was placed in a sealed glass tank, containing 30 mL of formic acid: hydrochloric acid: water (8:2:3). The TLC was run for approximately 90 min, and once the solvent front was 1 cm from the top, the TLC plate was left to dry in the fume hood and an image of the plate was obtained by scanning using a Epson Perfection 4990 photo scanner.

3.2.6.2 High performance liquid chromatography (HPLC) analysis of anthocyanin

Anthocyanin extraction for HPLC analysis was carried out using a methanol extraction method. Approximately 2 mL of 70% (v/v) methanol with 3% (v/v) acetic acid was added to 300 mg sample tissues to cover the samples, which were then mixed well and soaked in the solvent at 4°C for 7 h. The sample was then centrifuged at 3000 rpm for 10 min, and the supernatant transferred to another tube and left at 4°C. The pellet was used for the second extraction with 90% (v/v) methanol with 3% (v/v) acetic acid overnight at 4°C. Next day, the second extraction was centrifuged at 3000 rpm for 15 min, the supernatant was collected and combined with the supernatant from the first extraction. The combined supernatant fraction was then dried down to 500 μL by vacuum centrifugation, and the extract was centrifuged at 14 000 rpm for 1 min to pellet the solid matter. The concentrated supernatant fraction was then analysed by HPLC.

HPLC analysis was carried out using a Waters 600 solvent delivery system with a Phenomenex Prodigy (5 μm, 250 × 4.6 mm) RP-18 end-capped column (column temperature 30°C) and a Waters 996 PDA detector. Elution (0.8 mL min^{-1}) was performed using a solvent system comprising solvent A [HOAc:CH_3CN:H_3PO_4:H_2O (20:24:1.5:54.5)] and 1.5% H_3PO_4 (solvent B) and a linear gradient starting with 35% A, then increasing to 67% A at 20 min, 90% A at 23 min and finally 100% A at 29.3 min, remaining at 100% A for a further 10 min. Anthocyanins were detected at 530 nm, using the Waters Millenium software system. Peaks were identified using the spectral maxima and comparison against known standards.

3.3 Results

3.3.1 Maize *Lc* and *C1* up-regulate the anthocyanin biosynthesis pathway in petals and sepals of cymbidium

Transient expression of maize *Lc*/*C1* transcription factors in all four cymbidium cultivars JFDP, Virgin, NAG and LRF led to strong pigmentation in the petals/sepals (Figure 3.2). Strong zones of colour foci were observed within seven days for all the cultivars tested. These zones of foci could be seen clearly by the naked eye. A more detailed close-up view displaying individual pigmented cells is shown in Figure 3.2B, D, F, H. A control experiment was carried out to ensure that the up-regulation of pigmentation was not simply due to a wounding response by biolistic bombardment. A *CaMV35S::GFP* construct (pPEP-GFP) was transiently expressed in JFDP petals/sepals by bombardment, and the result showed that no coloured foci were formed (Figure 3.3A, B). Co-introduction of *GFP* (pPEP-GFP) with *Lc* (pLc3489) and *C1* (pPN44) produced identical patterns on the petal/sepal between the GFP fluorescence zone and the anthocyanin zone (Figure 3.3C-F). Close-up images show clearly that the GFP and anthocyanins are produced in the same cells (Figure 3.3E, F), indicating that GFP can be used as a marker to trace the cells that have been transformed with DNA-coated gold particles. The co-expression also showed that the GFP fluorescence appeared

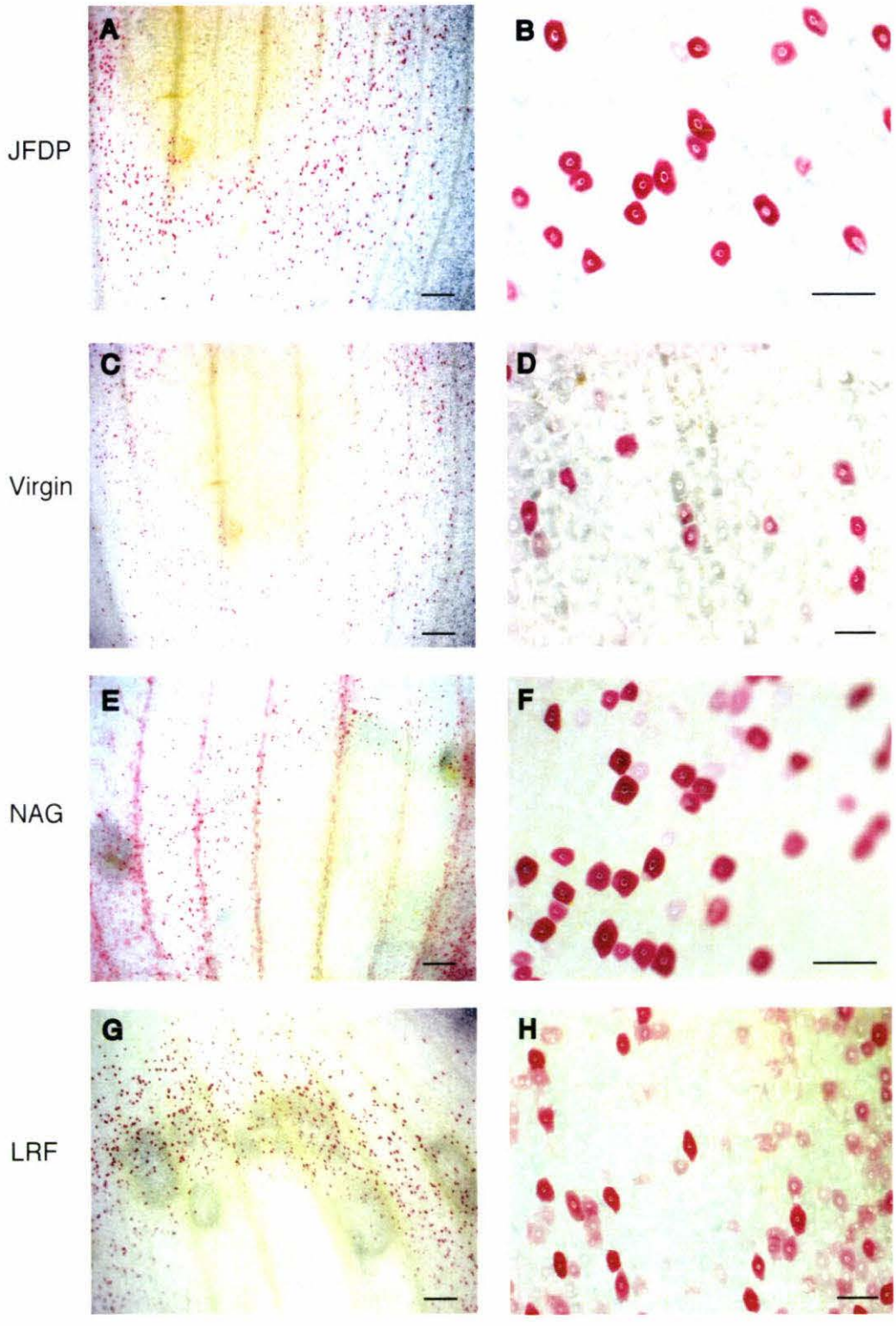
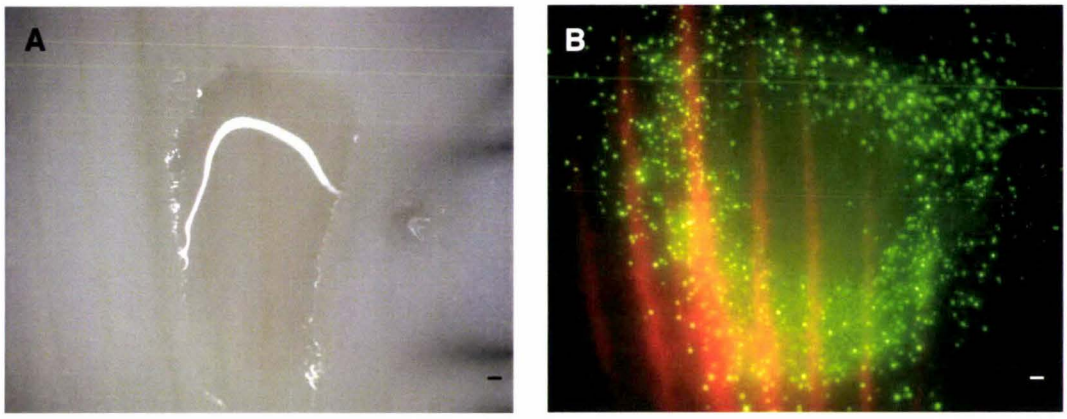


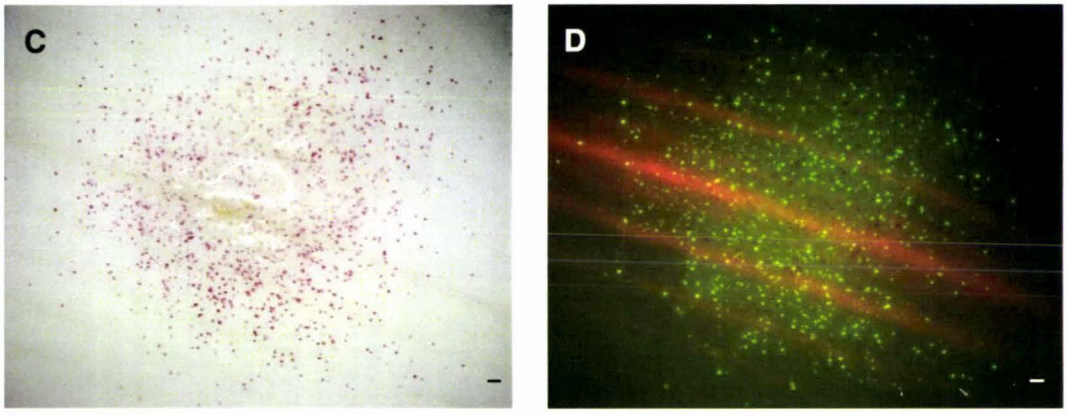
Figure 3.2: Induction of anthocyanin biosynthesis by maize *Lc* (pLc349) and *C1* (pPN44) transcription factors in cymbidium petal/sepal tissues. Microscope images show the biolistic blast (**A**, **C**, **E**, **G**) and individual cells (**B**, **D**, **F**, **H**) that have accumulated anthocyanin following transient expression of *Lc*/*C1*. Bars on panel **A**, **C**, **E** and **G** represent 1 mm. Bars on panel **B**, **D**, **F** and **H** represent 100 µm. Photos were taken 1 week post shooting. JFDP, Jung Frau dos Pueblos; Virgin; NAG, Narella Jennifer Gail; LRF, Lisa Rose Flamingo.

GFP control in JFDP



Lc /C1+GFP

JFDP



Virgin

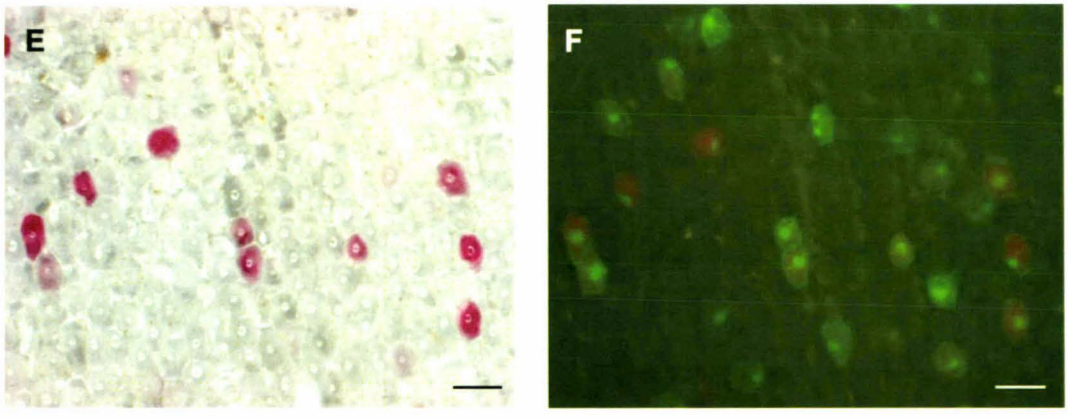


Figure 3.3: Controls for particle bombardment experiments using cymbidium petal/sepal tissues. **A** and **B**, expression of *CaMV35S*::*GFP* (pPEP-GFP) only; **C**-**F**, co-expression of *Lc* (pLc349), *C1* (pPN44) and *GFP* in JFDP and Virgin as indicated. Left panel is under white light and the right panel is under blue light. **A**-**D**, scale bar = 1 mm; **E** and **F,** scale bar = 100 μm.

earlier than anthocyanins (Figure 3.3E, F). In fact, the GFP could be observed less than 24 h post-bombardment.

Pigmentation was initially observed around 48 h after shooting. Sparse pale pink foci were observed in JFDP petals/sepals, but none were observed in Virgin or in the pink cultivars. It was very hard to distinguish coloured foci in pink cultivars within 48 h. The number and intensity of colour foci, however, increased rapidly in the following days. Virgin had visible foci by day three. A ring of pigmented foci matching the shot zone could be seen by the naked eye four days post-shooting. The colour at the shot zone intensified with time, with the coloured foci dark red in colour at ten days post-shooting.

Visual observations indicated pigmentation induced by *Lc/C1* was different amongst the cymbidium cultivars tested. Virgin gave the weakest pigmentation, and the pigmentation appeared latest among the tested cultivars. Approximately 36 h post-shooting, the foci in Virgin were only pale pink but a dark pink colour was seen in the other cultivars. Strong pigmentation was observed in the JFDP, NAG and LRF cultivars. Out of the four cultivars tested, JFDP was found to be the best cultivar in terms of responding to *Lc/C1* up-regulation of pigmentation in the petal and sepal. Although pink cultivars did show strong up-regulation of pigmentation in response to *Lc/C1*, they were not ideal for testing heterologous anthocyanin biosynthetic gene function. This is because it is hard to distinguish colour foci that are induced by heterologous biosynthetic gene activity from its natural pink background. Therefore, JFDP appears to be a good candidate for further experiments, as it has a white background, which allowed new pigments to be easily observed, and the anthocyanin biosynthetic genes were strongly up-regulated by the maize transcription factors.

The anthocyanins induced by *Lc/C1* were examined by TLC and HPLC. The anthocyanidin extract from the pigment foci in the petal/sepal tissue induced by *Lc/C1* showed a major band that migrated similarly with the cyanidin standard, and a second band with a slightly slower migration than pelargonidin (Figure 3.4). This is likely to be peonidin, since the RF value is approximately that reported for peonidin and the colour is different to that of pelargonidin.

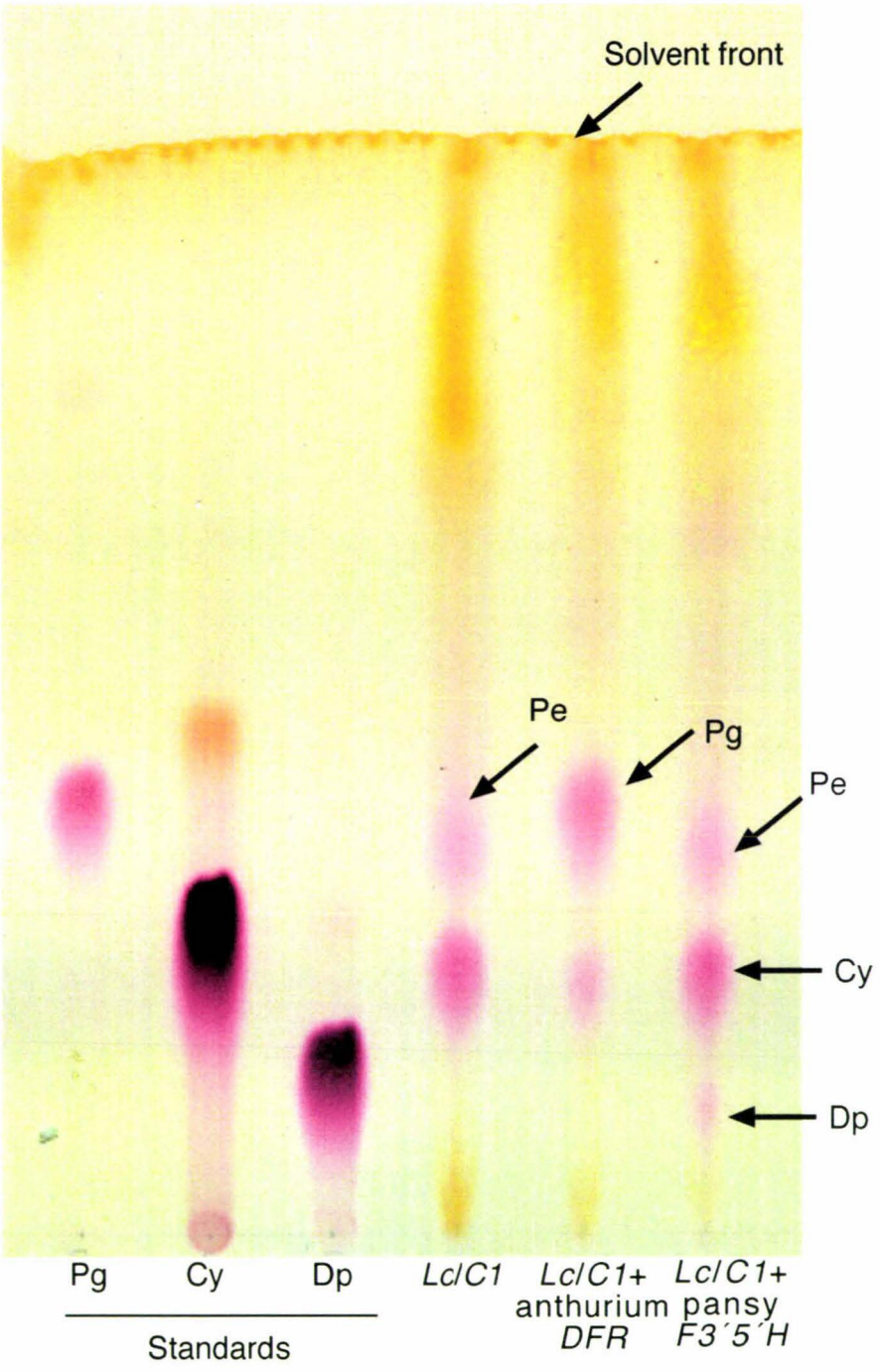


Figure 3.4: Thin layer chromatography of hydrolysed fraction of anthocyanins extracted from petals/sepals biolistically transformed with heterologus flavonoid biosynthetic genes. Classes of anthocyanin was indicated; Pg, pelargonidin; Cy, cyanidin; Dp, Delphinidin. Pe, peonidin. Peonidin is labelled using its known position relative to pelargonidin.

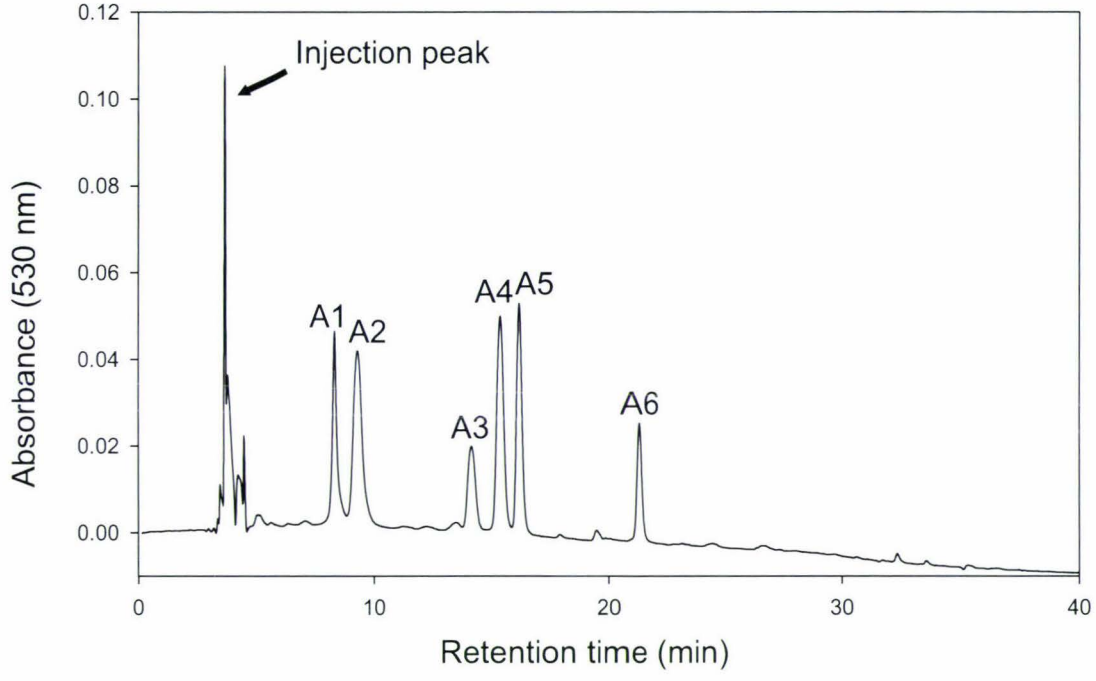


Figure 3.5: HPLC chromatogram of the anthocyanins extracted from cymbidium petal/sepal tissues transformed by *Lc* (pLc349) and *C1* (pPN44).

Further HPLC analysis of the methanol extract from the same foci showed that six different anthocyanins were induced by *Lc/C1* transient expression (Figure 3.5). The anthocyanidins were putatively identified as A1, cyanidin 3-glucoside; A2, cyanidin 3-rutinoside; A3, peonidin 3-glucoside; A4, peonidin 3-rutinoside; A5, cyanidin 3-malonylglucoside; A6, peonidin 3-malonylglucoside. All these anthocyanins are cyanidin/peonidin-based, which is consistent with the TLC results.

3.3.2 Induction of anthocyanin pigment in cymbidium by anthurium *DFR*

Cymbidium does not have the ability to produce pelargonidin-based anthocyanins naturally due to the lack of DFR capable of using DHK as a substrate. The possibility of creating pelargonidin-based anthocyanins in the petals/sepals of cymbidium was tested in JFDP using a transient expression system and the anthurium *DFR*, driven by the *CaMV35S* promoter. Over-expression of anthurium *DFR* alone was able to up-regulate pigment synthesis in a small number of cells only (Figure 3.6 C). The locations of the coloured foci appear to be around petal/sepal veins of the shot zone. The colour of the cells was faint pink. An extended exposure time (208 ms) was needed to be able to show the pigmentation using the Leica camera. The number of coloured foci induced by anthurium *DFR* was very low, and only around 30-40 foci per petal/sepal were observed during the experiments, with the majority of the foci being pink and occasionally dark red.

Since the effect of *DFR* overexpression alone was inconclusive in the petals/sepals, *Lc/C1* was co-induced with anthurium *DFR* to maximize pigment accumulation. The bombardment results showed that, along with *Lc/C1*, anthurium *DFR* expression gave more coloured foci and they appeared to be redder (Figure 3.6 D) in comparison with the pink foci produced by *Lc/C1* without anthurium *DFR* (Figure 3.6 C). Hundreds of coloured foci could be observed in each petal/sepal, and the outline of the shot zone can be seen clearly.

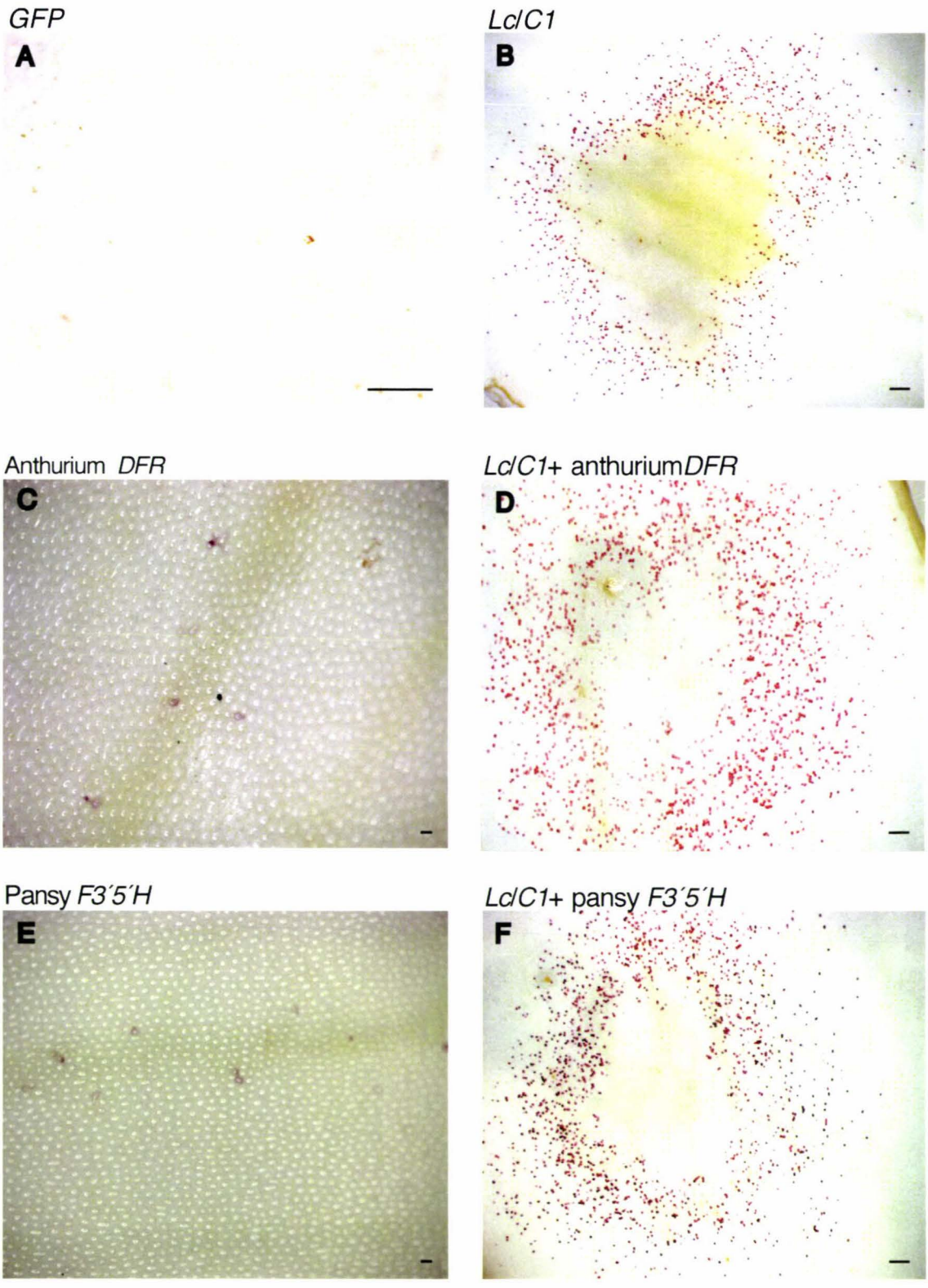


Figure 3.6: Transient expression of anthurium *DFR* and pansy *F3′5′H* with and without *Lc/C1* in petal/sepal tissues of JFDP cultivar. **A**, negative control of *GFP* (pPEP-GFP) under normal light **B**, co-expression of *Lc/C1*; **C** and **E** are petal/sepal transformed with anthurium *DFR* (pAaDFR) and pansy *F3′5′H* (pPansyF3′5′H#40), respectively. **D** and **F** are co-expression of *Lc/C1* with anthurium *DFR* and pansy *F3′5′H*, respectively in petal/sepal tissues. **A**, **B**, **C** and **E**, scale bar = 1000 μm; **B**, **D**, **F**, scale bar = 1 mm.

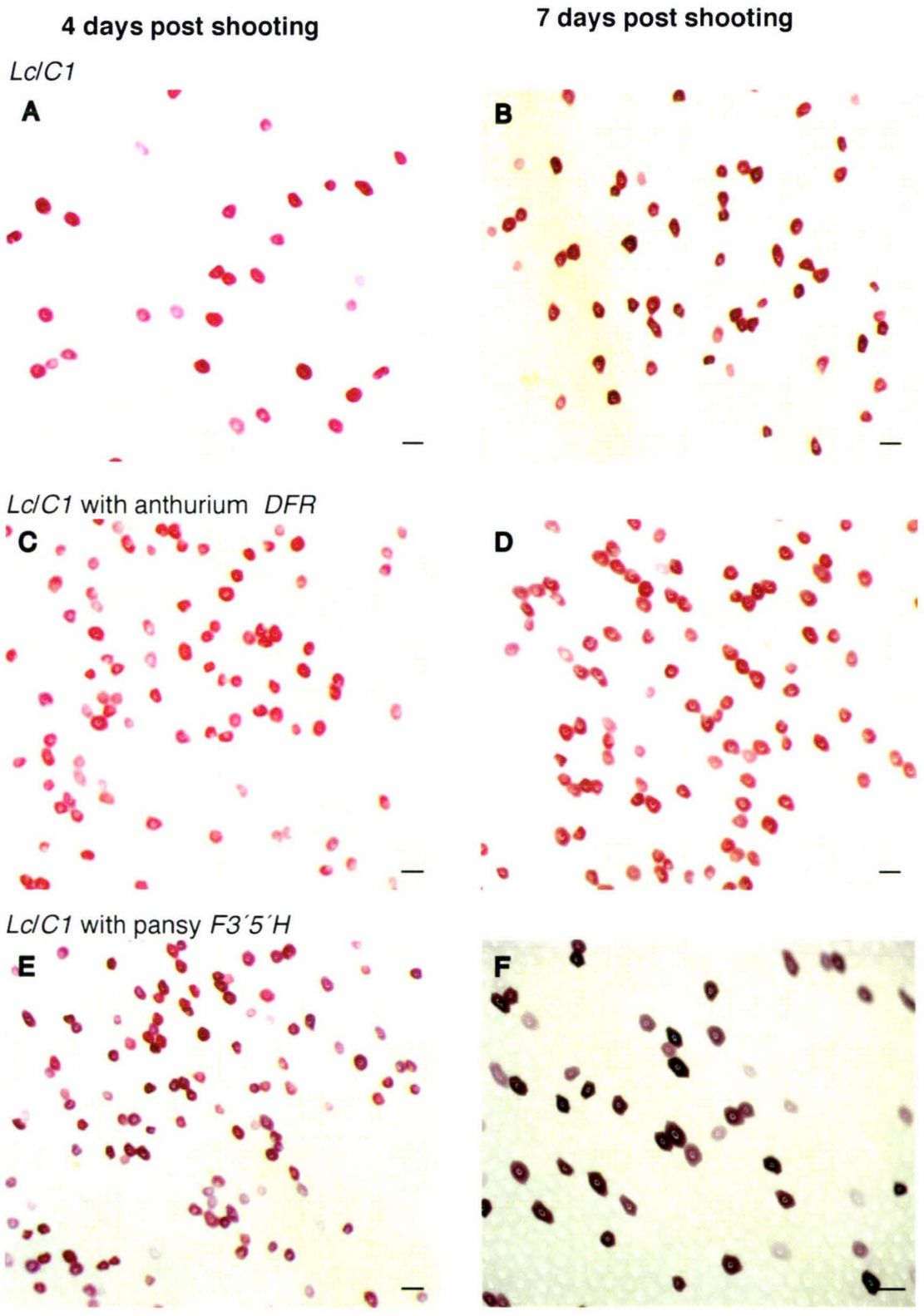


Figure 3.7: Changes of colour in transformed cymbidium petal/sepal cells over time. **A** and **B** are petal/sepal transformed with *Lc/C1* (pLc349 and pPN44); **C** and **D** are petal/sepal transformed with anthurium *DFR* (pAaDFR) and *Lc/C1*; **E** and **F** are petal/sepal transformed with pansy *F3′5′H* (pPansyF3′5′H) and *Lc/C1*. **A**, **C** and **E** are images taken at four days post shooting; **B, D** and **F** are images taken at seven days post shooting. Scale bars represent 100 μm.

Cells transformed with a mixture of plasmids containing anthurium *DFR*, *Lc*/*C1* are shown at higher magnification (Figure 3.7C), displaying a mixture of bright red cells and pale pink cells. The colour of all transformed cells intensified over a period of two weeks, in a similar manner to that observed in the *Lc*/*C1* experiment. The first appearance of coloured foci was observed 48 h post-shooting under the light microscope, and only a very few, very lightly coloured cells were observed. At three to four days post-shooting, "colour rings" can be seen easily by the naked eye. The colour of the foci was very dark by the seventh day. A slight change in colour was observed with time for cells transformed with anthurium *DFR* (Figure 3.7C and D). The petals/sepals would normally survive on the media in a reasonably healthy state for eight to ten days post-transformation. Foci were still visible at 14 days, even though petal/sepal senescence was well advanced.

TLC pigment analysis was carried out to confirm that the observed colour change was the result of pelargonidin-based anthocyanin production in petals/sepals after the introduction of anthurium *DFR*. The anthocyanidin extract from petal/sepal tissues transformed with *Lc*/*C1*+anthurium *DFR* showed the presence of a large amount of pelargonidin (a major band) as well as a reduction in the amount of cyanidin compared with the *Lc*/*C1* sample (Figure 3.4, fifth lane). The TLC analysis indicated that different pigments had been induced by the introduction of a heterologous anthocyanin biosynthetic gene.

HPLC analysis was also carried out to further identify the pigments produced. Apart from the six peaks identical to those in *Lc*/*C1* transformed cells, three new peaks with spectral absorption maxima at 501, 506 and 504 nm were detected in *Lc*/*C1*+ anthurium *DFR*-transformed cells (Figure 3.8). These spectral maxima correspond with the presence of monohydroxylated anthocyanins. Although the original six cyanidin-based anthocyanins were still present, they were reduced in comparison to *Lc*/*C1* transformants. The three new peaks represent approximately 70% of the total anthocyanin present in the extract.

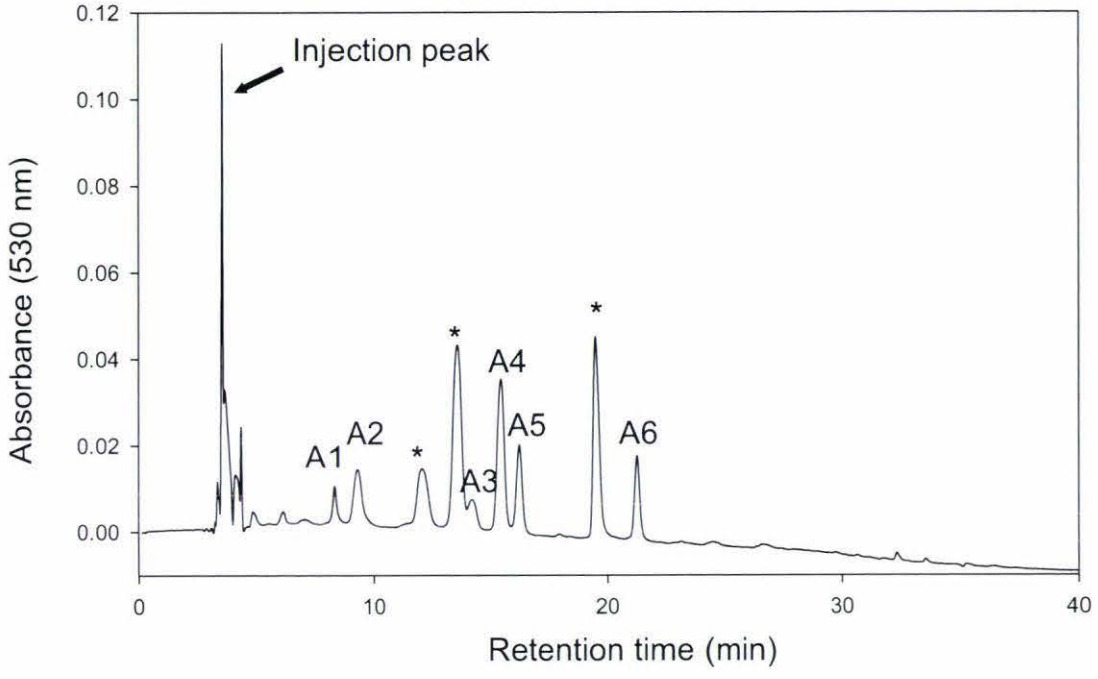


Figure 3.8: HPLC chromatagram of anthocyanins extracted from cymbidium petal/sepal tissues transformed by anthurium *DFR* (pAaDFR) and *Lc/C1* (pLc349 and pPN44). Three new anthocyanin peaks with spectral max at 501, 506 and 504 (from left to right) are marked (*).

3.3.3 Induction of anthocyanin pigment in cymbidium by pansy *F3'5'H*

Delphinidin-based anthocyanins are generally the basis for blue colour in flowers. Cymbidium lacks the ability to produce dephinidin-based anthocyanins by their endogenous anthocyanin pathway. A pansy *F3'5'H* driven by the *CaMV35S* promoter was introduced into petals/sepals of the JFDP cultivar by biolistic transformation to test the possibility of producing delphinidin-based pigments. Transient expression of pansy *F3'5'H* alone was only able to up-regulate pigment synthesis in a small numbers of cells (Figure 3.6 E). The locations of coloured foci was similar to that observed in transient expression of the anthurium *DFR* alone, and the foci were around petal/sepal veins of the shot zone and an extended exposure time was required to see pigmentation using the Leica camera. Upon closer inspection, cells transformed with pansy *F3'5'H* had a slight purple colour but the number of coloured cells was very low, with only about eight foci observed in each petal/sepal.

Lc/*C1* were co-introduced with pansy *F3'5'H* to see maximal pigment accumulation. Overexpression of a pansy *F3'5'H* together with *Lc*/*C1* resulted in increased coloured foci and a change from a pink (Figure 3.6E) to a dark purple colour (Figure 3.6F). The number of coloured foci was greatly increased with the inclusion of *Lc*/*C1*. There were hundreds of coloured foci in each petal/sepal, and formation of the shot zone can be seen clearly.

At higher magnification, the coloured cells of the petals/sepals transformed with *Lc*/*C1*+pansy*F3'5'H* produced a wider range of colour foci, exhibiting red, pink, dark purple and light purple (Figure 3.7E). The colour of all transformed cells intensified over two weeks, similar to the pattern seen in tissue treated with *Lc*/*C1* alone (Figure 3.7 A and B). A greater change in colour intensity was observed in cells transformed with pansy *F3'5'H* compared with colour changes in cells transformed with *Lc*/*C1*+anthurium *DFR*. The cell colour turned dark purple by the seventh day and no red coloured foci could be seen (Figure 3.7F).

The presence of delphinidin-based anthocyanins in the purple cells was analysed by TLC. An anthocyanidin extract from the petals/sepals transformed with pansy *F3'5'H* was run on the same TLC plate. Three bands were resolved (Figure 3.4, sixth lane). One band ran at the same level as the delphinidin standard (Figure 3.4, third and sixth lanes). The other two bands displayed had the same RF values as those observed for the anthocyanin extract from cells transformed with *Lc/C1*, indicating that they were cyanidin and peonidin. The amounts of cyanidin and peonidin present appeared similar to those from the tissues transformed with *Lc/C1* alone (Figure 3.4, sixth lane), and a relatively small amount of delphinidin was observed.

HPLC analysis was carried out to confirm the TLC result and identify the anthocyanidins produced. The analysis of cells transformed with *Lc/C1*+pansy*F3'5'H* showed there were one major and three minor new peaks (Figure 3.9). These new peaks have spectral maxima of 526 and 531 nm, indicating tri-hydroxylated delphinidin-based anthocyanins. These new peaks represent about 10% of the total anthocyanidin extracted.

A comparison of the shooting patterns from all constructs: *GFP*, *Lc/C1* and *Lc/C1*+anthurium *DFR* and *Lc/C1*+pansy *F3'5'H* is shown in Figure 3.10. The colour differences between each combination of constructs can be picked up using a standard digital camera. No colour induction is seen in petal/sepal tissue transformed with *GFP* alone.

3.3.4 Silencing of pansy *F3'5'H* activity

Engineering the anthocyanin biosynthesis pathway to produce different anthocyanins may require blocking endogenous anthocyanin biosynthetic enzyme activities. RNAi technology has shown that it can be an effective way to silencing gene expression in an organ-specific manner (Davuluri et al., 2005). The effects of down-regulating enzyme activities by using RNAi technology in a transient expression system has been demonstrated using excised *Antirrhinum* petals (Shang et al, 2007). Such a system was tested in cymbidium petals/sepals using a pansy *F3'5'H* RNAi construct to assess colour modification strategies in cymbidium. This system was assessed by using an expression

construct of pansy *F3′5′H* as a functional gene source, and a pansy *F3′5′H* RNAi construct, which would trigger RNAi silencing.

The 290 bp pansy *F3′5′H* fragments were successfully amplified by PCR as described in Section 3.2.5.1 for cloning an RNAi construct (Figure 3.11A). The pansy *F3′5′H* RNAi fragment was cloned into pDAH2 in sense and antisense orientations, to form a hairpin structure (Figure 3.11B). This RNAi construct was tested in both the JFDP and Virgin cultivars. In the control experiment, petals/sepals of JFDP and Virgin were transformed with *Lc*/*C1*+ pansy *F3′5′H* and formed purple coloured foci by the fourth day post-transformation, as expected (Figure 3.12A and C). When the pansy *F3′5′H* RNAi was co-induced with pPansy*F3′5′H*#40 in the transformation, the coloured foci were red rather than purple (Figure 3.12B and D). This indicates that pansy F3′5′H activity introduced to petals/sepals was successfully down-regulated by pansy *F3′5′H* RNAi, which prevented production of delphinidin-based anthocyanin.

3.4 Discussion

A transient gene expression system was successfully used to examine anthocyanin production in cymbidium floral tissues. Pigment synthesis and possible redirection of the anthocyanin pathway could be observed in mature flower tissue within days of the introduction of the gene constructs by biolistic transformation. This expression system accommodated multiple gene constructs for transient expression, therefore enabling the study on the effect of multiple gene manipulation in cymbidium floral tissues. Down regulation of genes of interest can also be studied using this system. An RNAi system for gene silencing was shown to work successfully in cymbidium. Wounding can be a factor for triggering pigmentation. In this study, however, transformation of *CaMV*::*GFP* (pPEP-GFP) in JFDP petals/sepals did not produce any pigment spots. This control demonstrated that, under the conditions of this study, wounding alone does not cause cymbidium petals/sepals to synthesise pigment. The transient gene expression system is timesaving and a useful way to study the genetic manipulation of the anthocyanin pathway in cymbidium flowers, and predict likely outcomes when stably transformed plants are developed.

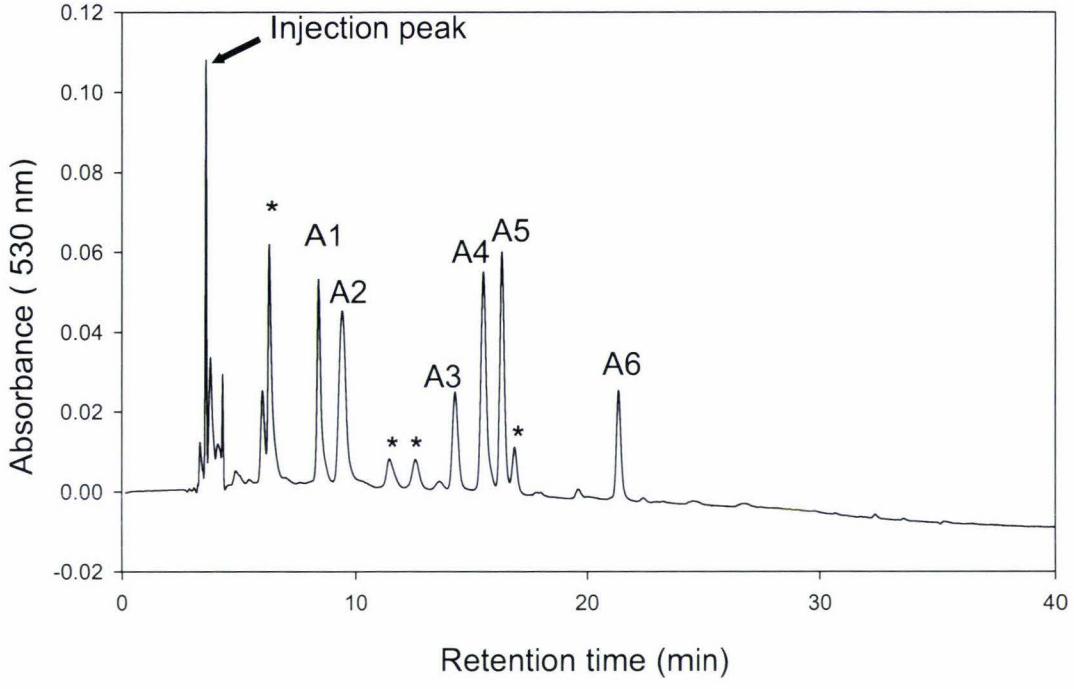


Figure 3.9: HPLC chromatagram of the anthocyanin extracted from cymbidium petal/sepal tissues transformed by pansy *F3'5'H* (pPansyF3'5'H#40) and *Lc/C1* (pLc349 and pPN44). The new anthocyanin peaks with spectral maxima at 526 (first peak) and 531 nm (three smaller peaks) are marked (*).

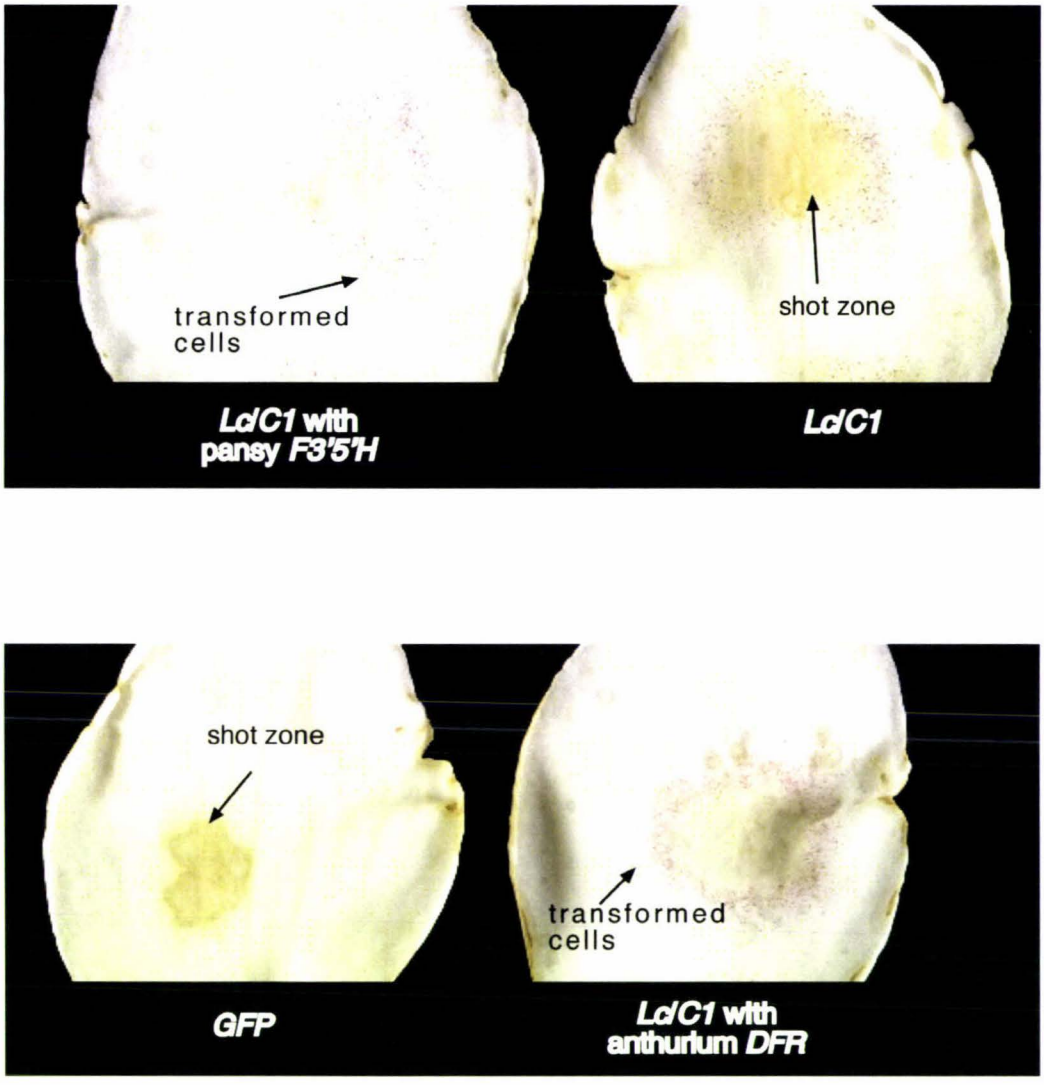


Figure 3.10: Whole petal view of JFDP petals, biolistically transformed with anthocyanin and reporter genes, each controlled by the *CaMV35S* promoter. A comparison in induced colour is made between *Lc/C1* (pLc349 and pPN44), *Lc/C1* with anthurium *DFR* (pAaDFR), *Lc/C1* with pansy *F3′5′H* (pPansyF3′5′H) and GFP (pPEP-GFP) alone.

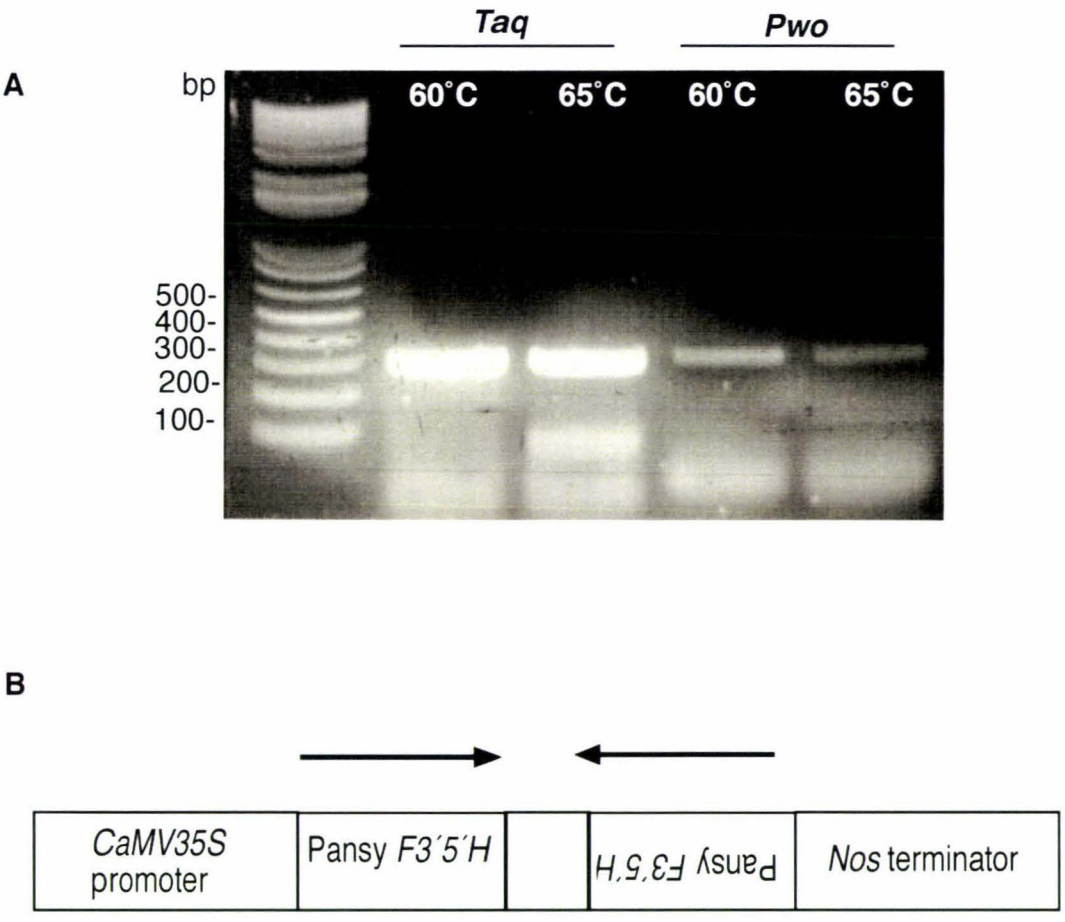


Figure 3.11: Cloning of pansy *F3′5′H* RNAi construct. **A**, PCR amplified 290 bp *F3′5′H* fragments for cloning into the RNAi construct; **B**, the RNAi construct consists of a *CaMV35S* promoter, an inverted repeat of the pansy *F3′5′H* fragment separated by a spacer, and a *Nos* terminator.

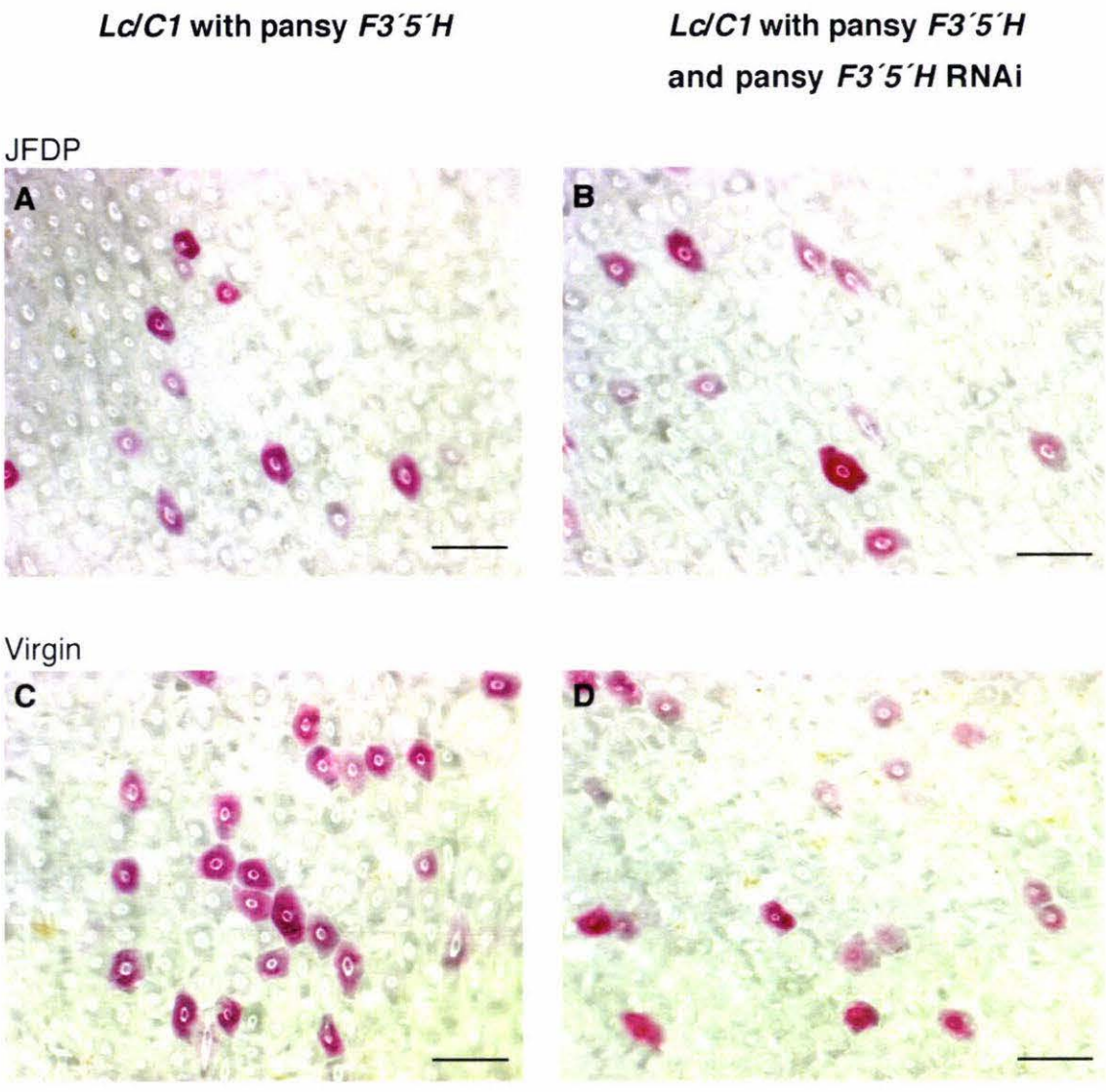


Figure 3.12: Inhibition of pansy *F3′5′H* activity in cymbidium petal/sepal tissues using transient RNAi. Petals cultured *in vitro* are shown three to five days after the biolistic introduction of the positive control *Lc/C1* (pLc349 and pPN44) and pansy *F3′5′H* (pPansyF3′5′H#40), in JFDP, **A** and Virgin, **C**. Silencing of pansy *F3′5′H* activity using pPansyF3′5′HRNAi in JFDP, **B** and Virgin, **D**. Scale bar = 100 μm.

The results showed that the best cultivar for transient expression assays was JFDP, as it has a white background that enables the easy observation of novel colour production and has the strongest pigmentation upon induction by *Lc/C1*. Although pink cultivars also produced strong pigmentation upon induction by *Lc/C1*, these cultivars are not ideal for transient studies on pigment manipulations. This is because petals/sepal of pink cultivars are made up of a mixture of pink and white cells, which makes it difficult to identify transformed cells and assess the colour of the foci. The fact that pink cultivars contain cells of mixed colours also indicated that the regulation of anthocyanin production is regulated differently in different cells. Transient gene expression results may therefore differ between individual cells. Pink cultivars, however, are likely to be the better candidate for development of stable transgenic cymbidium because genes involved in pigment biosynthesis and regulation are generally more active. Thus, *Lc/C1* may not be required in stable transformation. Green and yellow cultivars have not been tested in this experiment, but the lack of anthocyanin content in petals and sepals of these coloured cultivars means the anthocyanin biosynthetic pathway is not being turned on. The existing carotenoid and chlorophyll pigments would also mask or modify the colour of any anthocyanins produced.

The induction of anthocyanin formation by *Lc/C1* indicates that the white cymbidium cultivars used in this study are probably anthocyanin regulatory mutants, rather than there being a block in one of the biosynthetic genes. The results are consistent with studies in two other white flower orchids, *Doritis pulcherrima* and phalaenopsis (Griesbach and Klein, 1993; Ma et al., 2008). The level of pigmentation induced by *Lc/C1* did vary between the two white cultivars examined. Although both JFDP and Virgin are white cultivars with pink-coloured lips, petals/sepals of JFDP cultivar have some dark pink patches at the bases and a hint of pink colour on the abaxial side of the sepal, whereas petals and sepals of Virgin are pure white. Foci colour induced by *Lc/C1* in JFDP is generally darker than foci appearing in Virgin. This difference in pigmentation between the two white cultivars correlated with the physical colour appearance of the petals and sepals. It is possible that JFDP and Virgin are different kinds of regulatory mutant and that transcription factors are present in JFDP, but are only expressed in the coloured parts of the petals/sepals and are silenced in the white parts. Virgin, on the other hand, might be a mutant that lacks these transcription factors completely in petals/sepals.

HPLC analysis of pigments from petal/sepal tissues of the JFDP cultivar, induced by activation with *Lc/C1*, have shown the same pattern of six anthocyanin peaks as that reported in other studies (Sugiyama et al., 1977; Woltering and Somhorst, 1990; Tatsuzawa et al., 1996; D. Lewis, unpublished data). It appears that cymbidium only produces cyanidin- or peonidin- based anthocyanins. This relatively narrow range of anthocyanin production is the reason for missing colour groups in cymbidium flowers and has prompted this study to investigate if different anthocyanins can be induced.

Expression of anthurium *DFR* and pansy *F3'5'H* with *Lc/C1* transcription factors has shown that re-direction of pigment synthesis in cymbidium petals/sepals is possible. This indicates that new colours can be formed in cymbidium flowers if suitable enzymes are introduced into the pathway. Overexpression of these genes without *Lc/C1* did not allow any firm conclusions to be drawn. This result could mean that when the pathway was not up-regulated by *Lc/C1*, only limited amounts of substrate in a small numbers of epidermal cells were available for the high levels of transgene-derived recombinant enzymes introduced to the cell via biolistic bombardment, and therefore only a small amount of anthocyanin was produced due to deficiency of substrate. An alternative possibility is that since most genes in the pathway are not up regulated, the large amount of unstable leucoanthocyanins generated by highly active anthurium DFR and pansy F3′5′H are not able to be efficiently converted to more stable anthocyanidin by the low activities of late pathway enzymes such as ANS and UFGT. White cultivars are therefore not the best to use to achieve a novel colour by modifying anthocyanin production. Coloured cultivars with high levels of anthocyanins appear more suitable as the anthocyanin pathway is already active.

Anthocyanin content and potentially flower colour were successfully modified in cymbidium petal/sepal tissue by the introduction of heterologous anthoycanin biosynthetic gene constructs. Introduction of the anthurium *DFR* with *Lc/C1* into cymbidium petals/sepals shifted the anthocyanin profile from 100% cyanidin/peonidin to 70% pelargonidin and cell colour appeared redder. It would appear that the introduction of this DFR has overcome the inability of the cymbidium DFR to use DHK as a substrate (Johnson et al., 1999) and allowed the accumulation of pelargonidin-based anthocyanins. To the author's knowledges, this is the first time that anthurium *DFR* has been used to test for pelargonidin formation in flowers. Anthurium DFR was

chosen for re-direction of anthocyanin biosynthesis in cymbidium because anthurium spathe tissue accumulates both pelargonidin and cyanidin-derived anthocyanins and appears to have similar substrate specificity to maize and gerbera DFR, which can convert either DHK or DHQ (Iwata et al., 1979, 1985; Meyer et al., 1987; Collette, 2002). The fact that the anthurium DFR has been able to promote pelargonidin accumulation indicates that DHK substrate is available in cymbidium, despite no record of pelargonidin based anthocyanin being detected. The accumulation of predominantly pelargonidin anthocyanins may be because the anthurium DFR is present in many copies because of the biolistic bombardment, and the activity of endogenous F3′H and FLS may not be as strong as that of the recombinant anthurium DFR. Although transient gene expression results may not provide as accurate picture when compared with stable transgenic plants, this preliminary study shows that anthurium *DFR* appears to be a good heterologous DFR source to promote pelargonidin accumulation in cymbidium.

Some pelargonidin-accumulating plants have been generated by overexpressing a heterologous *DFR* in host plants deficient in F3′H, F3′5′H or FLS activity. For example, The transformation of maize *DFR* in the *Arabidopsis* TT7 mutant (a *F3′H* mutant), caused the accumulation of pelargonidin (Dong et al., 2001). Strategies successful in promoting pelargonidin accumulation generally require that the F3′H activity is low or absent. This ensures the DHK substrate is available. Petunia does not accumulate pelargonidin-type anthocyanins, because petunia DFR is unable to reduce DHK. Transgenic petunia accumulating pelargonidin-based anthocyanins have been developed by overexpressing rose *DFR* genes and down regulating endogenous *F3′H* genes (Tsuda et al., 2004). The introduction of a DFR that has a preference for DHK as substrate does not however, always effectively cause accumulation of pelargonidin in the transgenic plants. The transformation of *Gerbera hybrida* DFR and *Fragaria x ananassa* DFR, for example, into *Osterospermum hybrida* did not lead to an increase in pelargonidin, probably due to competition from the endogenous F3′5′H (Seitz et al., 2007). If a *F3′H* mutant line is not available, an alternative strategy of silencing endogenous activity is necessary to promote pelargonidin production and RNAi is one possibility. Therefore, suppression of endogenous F3′H activity by RNAi technology in combination with

overexpression of the anthurium *DFR* may be required for the accumulation of pelargonidin in cymbidium flower tissue.

Introduction of the pansy *F3'5'H* with *Lc/C1* resulted in the formation of some purple coloured foci in excised cymbidium petal/sepal tissue, and pigment analysis of the transformed cells showed formation of delphinidin-based anthocyanins. These results are consistent with the formation of delphinidin in substrate feeding experiments and confirm that the cymbidium endogenous DFR is capable of using DHM to produce delphinidin, and the main reason for the lack of delphinidin is likely to be the absence of a functional F3′5′H in cymbidium. These experiments have clearly shown that it is possible to manipulate the anthocyanin biosynthetic pathway in cymbidium to accumulate delphinidin and to obtain a purple flower colour. However, the trihydroxylated anthocyanin formed by overexpressing pansy *F3'5'H* with *Lc/C1* made up only ~10% of the total anthocyanin. The remaining 90% of total anthocyanins were still the cyanidin/peonidin-based anthocyanins. The relatively small amount of delphinidin-based anthocyanins formed indicates that the cymbidium DFR may not process DHM as efficiently as DHQ or that pansy F3′5′H might not be able to work efficiently in cymbidium. Further experiments will need to focus on how delphinidin anthocyanin accumulation can be maximized.

A suitable *F3'5'H* source is important for effective delphinidin-based anthocyanin accumulation. Although genes from different plants may encode enzymes with the same function, they do not always work with the same efficiency as in the original plant. Expression of petunia and gentian *F3'5'H* in tobacco resulted in only 20-35% of the total anthocyanin content being in the tri-hydroxylated anthocyanin form (Shimada et al., 1999). In contrast, the accumulation of delphinidin derivatives increased up to 99% when the *F3'5'H* gene from Canterbury bells (*Campanula medium*) was transformed into tobacco (Okinaka et al., 2003). Thus, the use of *F3'5'H* cDNAs from appropriate genetic resources for cymbidium will help with creating blue flowers. Carnation and rose are two transgenic ornamental crops accumulating delphinidin exclusively, and in both cases, viola *F3'5'H* (the same as pansy *F3'5'H*) has been the best source for re-directing delphinidin formation in the pathway. We have not tested the effectiveness of

other *F3′5′H* sources in this study. Determining the best *F3′5′H* source for "blue cymbidium" generation is important for future studies.

The only genetically modified floriculture crops available commercially in the world currently are the blue carnation and rose developed by Florigene and Suntory. Both used a combination of introduced F3′5′H and DFR activity to promote delphinidin accumulation. They have shown that overexpression of viola *F3′5′H* alone was insufficient to convert the metabolic flux towards delphinidin in rose and carnation, due to the relatively high level of cyanidin synthesized by endogenous DFR (Tanaka, 2006). A high level of accumulation of delphinidin in carnation was achieved by a combination of overexpression of viola *F3′5′H* and iris *DFR* in a white *dfr* mutant background (Fukui et al., 2003; Tanaka et al., 2005). Suntory have also reported that the "Blue rose", which accumulates delphinidin exclusively, was achieved by a combination of down-regulation of endogenous *DFR* using RNAi technology with overexpression of viola *F3′5′H* and iris *DFR* (Katsumoto et al., 2007). Endogenous DFR and F3′H activities might be the limiting factors for high-level delphinidin accumulation in cymbidium. While it is hard to obtain *dfr* and *f3′h* cymbidium mutants, a similar strategy to that used for generating the blue rose could be applied in cymbidium to achieve exclusive delphinidin-based anthocyanin production. This study has shown that RNAi technology can work effectively in cymbidium. Biolistic bombardment is able to accommodate multiple gene expression vectors for transient gene expressions. Transient co-expression of viola *F3′5′H* and iris *DFR* along with down-regulation of endogenous *DFR* and *F3′H* via RNAi can therefore be tested.

A pansy *F3′5′H* RNAi construct was used to verify the activity of our pansy *F3′5′H* construct, and the RNAi construct was shown to prevent purple foci formation. The pansy F3′5′H activity of the original transgene had been reduced, leaving the endogenous F3′H enzyme to catalyse DHQ formation, ultimately leading to pink coloured foci due to cyanidin accumulation. This evidence showed that the formation of delphinidin was indeed the result of overexpression of the pansy *F3′5′H* construct. It also demonstrated that RNAi technology could be ultilised in cymbidium orchids.

The possibilities for redirecting anthocyanin formation in cymbidium flower tissue have been demonstrated using a transient gene expression system. Results indicate that generating cymbidium flowers that accumulate delphinidin or pelargonidin exclusively may require a combination of overexpression of heterologous genes (such as pansy *F3'5'H* and anthurium *DFR*) and down-regulation of endogenous genes (such as *F3'H*), as was found in other flower crops. The results presented in this chapter indicate that the strategies to modify anthocyanin production and flower colour in *Cymbidium* orchids are feasible.

Chapter 4

Isolation of a flavonoid 3′- hydroxylase

4.1 Introduction

The later steps of the flavonoid biosynthetic pathway (as illustrated in Figure 1.4 and 3.1), can produce three types of anthocyanins: pelargonidin, cyanidin and delphinidin-based anthocyanins, providing red/orange, pink/mauve and purple/blue colours, respectively. Derivatives of pelargonidin have only one hydroxyl group at the 4′ position of the B-ring. Cyanidin-based anthocyanins have two hydroxyl groups, at the 3′ and 4′-position of the B-ring. The addition of a hydroxyl group at the 3′ position of the B-ring is catalyzed by flavonoid 3′-hydroxylase (F3′H). Delphinidin-based anthocyanins have three hydroxyl groups, at the 3′, 4′, 5′ positions of the B-ring, and the hydroxylation reaction is catalyzed by flavonoid 3′ 5′-hydroxylase (F3′5′H). An increased number of hydroxyl groups on the B-ring of the anthocyanin molecule is associated with a colour shift from red to blue. Thus the activities of F3′H and F3′5′H are crucial in determining the anthocyanin form in plant tissues and are therefore primary determinants of tissue colour (Forkmann and Martens, 2001; Tanaka, 2006).

Cyanidin/peonidin-based anthocyanins are the only anthocyanins found in pink/red cymbidium floral tissues (Sugiyama et al., 1977; Woltering and Somhorst, 1990; Tatsuzawa et al., 1996; Lewis et al., 2001). The formation of 3′ 4′-hydroxylated flavonoid intermediates is one of the key steps leading to production of cyanidin, and thus the activity of the F3′H enzyme is necessary and important for cyanidin formation in cymbidium. The possibilities for synthesis of other anthocyanins by expressing heterologous flavonoid biosynthetic genes in cymbidium sepals/petals were demonstrated in Chapter Three. Despite the fact that other anthocyanins can form when

appropriate biosynthetic genes are induced, a large proportion of substrate was still channeled to the formation of cyanidin-based anthocyanins. The presence of cyanidin suggests that the endogenous F3′H is competing with the introduced transgenes for the same substrate. Therefore, silencing of the endogenous *F3′H* may also be necessary in order to increase the content of other anthocyanins.

A *F3′H* gene was first isolated in petunia (Brugliera et al., 1999), but now has been cloned from a number of species including carnation (Holton et al., 2003), torenia (Ueyama et al., 2002) and grape (Jeong et al., 2006). Earlier enzyme studies have shown that the activity of the F3′H is controlled by the *Ht1* and *Ht2* loci in petunia floral tissue (Wiering, 1974; Stotz et al., 1985). A petunia *F3′H* cDNA clone corresponding to the *Ht1* locus has been shown to be expressed at high levels in petals of early floral developmental stages, but the *Ht2* locus has not been cloned (Brugliera et al., 1999). Studies with several other plant species have revealed the presence of multiple *F3′H* genes in the plant genome, with three *F3′H* genes cloned in sorghum and two in *Brassica napus* (Shih et al., 2006; Xu et al., 2007).

F3′H genes have been used to manipulate the flavonoid pathway and generate novel colours in other commercial flowers. A high level of accumulation of cyanidin was achieved in torenia by overexpressing torenia *F3′H* and suppressing endogenous *F3′5′H* genes (Ueyma et al., 2002). On the other hand, red-flowered tobacco plants containing high amounts of pelargonidin were generated by co-suppression of tobacco endogenous *FLS* and *F3′H* and expression of the gerbera *DFR* gene (Nakatsuka et al., 2007).

F3′H genes have been cloned from a number of dicotyledonous plants, but only a few from monocotyledons, and no *F3′H* genes have been reported from orchids. This chapter reports the isolation of a partial cymbidium *F3′H* sequence using by PCR-based techniques. This information will contribute to our understanding of anthocyanin production in cymbidium, and potentially in other orchid species too. The sequence obtained can also be used for designing constructs for silencing endogenous *F3′H* genes.

4.2 Materials and methods

4.2.1 Plant materials

Fresh flower stems of a maroon cymbidium cultivar, Clarissie Austin South Pacific (CASP) and a white cultivar, Jung Frau dos Pueblos (JFDP) were obtained from a commercial grower. Individual flower stems were held in a vase life room as described in Section 2.3. Flowers were graded into six developmental stages for harvest as shown in Figure 2.1 and the sampling of floral tissues was carried out as described in Section 2.3. Total RNA was extracted from stage four sepals, petals and lips of CASP cultivar. The RNA was used for subsequent cDNA synthesis. Combined stages of flower tissues from JFDP and CASP cultivars were collected for gDNA extraction.

4.2.2 Cloning a partial cymbidium *F3'H* cDNA

4.2.2.1 Design of degenerate primers for RT-PCR amplification

Proteins belonging to the P450 family have highly divergent primary sequences, but secondary and tertiary structures are conserved (Nelson et al., 1996). Degenerate primers were therefore designed based on the AGTDTS and DMEEAY conserved amino acid domains of CYP75 proteins. Protein sequence alignments consisting of all monocotyledon F3′H protein sequences as well as a phalaenopsis F3′5′H sequence, was carried out to choose regions for designing degenerate primers. Dicotyledon F3′H sequences were excluded because, based on phylogenetic analysis, sequences from monocotyledons are a more closely related group than are sequences from dicotyledons (data not shown). Phalaenopsis F3′5′H protein (Wang et al., 2006) was included in the sequence alignment because F3′5′H is another CYP75 protein (F3′H and F3′5′H are closely related), and *Phalaenopsis* is another orchid species. All sequences were obtained from NCBI GenBank (http://www.ncbi.nlm.nih.gov/), and sequences used in alignments are listed in Table 4.1. The sequence alignment was carried out using the CLUSTAL W program (Thompson et al., 1994) via the EMBL-EBI web site (http://www.ebi.ac.uk/Tools/clustalw2/index.html). Alignments of the DNA sequences that encode the AGTDTS and DMEEAY amino acid domains were also carried out.

The variations in the nucleotide sequences were taken into consideration for reducing levels of the degeneracy in primer sequences.

The forward primer was designed based on an alignment of all monocotyledon protein sequences (all monocot *F3'H* degenerate-F). The reverse primer was designed based on an alignment of *Allium cepa* F3'H and phalaenopsis F3'5'H (OP *F3'H* degenerate-R primer) as shown in Table 4.2.

4.2.2.2 First-strand cDNA synthesis

Total RNA extracted from flower tissues of stage four of CASP cultivar was used for the reverse transcription (RT) reaction. An AnXpT long adaptor primer consisting of a short nucleotide region upstream of a poly(T) region (10 μM, Table 4.2)was used for priming the RT reaction. This region provides an anchor region for the subsequent PCR amplification. The procedure for first strand cDNA synthesis was as described in Section 2.4.1 and incubations were performed using a Bio-Rad PCR thermocycler. First-strand cDNA was used as template for PCR amplification of a partial *F3'H* fragment using the degenerate primers and a subsequent 3'RACE experiment.

4.2.2.3 RT-PCR amplification of *F3'H* sequence

The initial *F3'H* fragment was amplified by PCR using the degenerate primers 'all monocot *F3'H* degenerate-F' and 'OP *F3'H* degenerate-R'. Three negative PCR controls were performed: the first reaction had no template control, which checked for contamination in PCR reactions; the second and third reactions contained a single primer only (either forward or reverse primer) which was checked for non-specific binding of the primers. Three different reactions were carried out, all amplified by *Taq* DNA polymerase (Roche), using the following thermocycling conditions: pre-denaturation at 94°C for 2 min, 35 cycles of amplification at 94°C for 30 s, annealing at 50°C, 55°C or 60°C for 30 s and extension at 72°C for 1 min, followed by a final extension at 72°C for 5 min. All PCR products were analyzed on agarose gels to detect positive bands of the predicted size.

Table 4.1: List of F3′H protein sequences used for alignment to design degenerate primers.

Plant species	Common Name	Number of amino acids	GenBank accession number	Anthocyanin biosynthetic gene
Phalaenopsis	Lady's slipper	506	AAZ79451	F3′5′H
Oryza sativa	rice	526	AAM00948	F3′H
Allium cepa	onion	510	AAS48419	F3′H
Allium ascalonicum	onion	510	AAZ86072	F3′H
Sorghum bicolor	Sorghum	517	AAV74194	F3′H
Sorghum bicolor	Sorghum	517	AAV74195	F3′H
Sorghum bicolor	Sorghum	517	ABG54321	SbF3′H3
Sorghum bicolor	Sorghum	517	ABG54320	SbF3′H2
Sorghum bicolor	sorghum	517	ABG54319	SbF3′H1

Table 4.2: List of primers used to amplify cymbidium *F3′H*.

Primer	**Sequence (5′-3′)**	**Tm**	**GC (%)**
All monocot *F3′H* degenerate-F	TCDYKGCKGGSACDGACAC	56.1	47.3
OP *F3′H* degenerate-R	CCAYAWGCCTCTYCCATRTC	60.8	45
Cym*F3′H* 3′RACE-1	AATCTAGATCGAGTGGCTATTATC TGAGCTCCTTC	69	48
Cym*F3′H* 3′RACE-2	CGCTTGATCTCACAGTCAGATCTA CC	67.9	50
AnXpT	GAAATTCATCGATGGATCC(T_{19})	ND	ND
AnX	GAAATTCATCGATGGATCC	63.0	42

ND, not determined

4.2.2.4 Rapid amplification of 3′ cDNA Ends (3′-RACE) of *F3′H*

The down-stream *F3′H* sequence was obtained by 3′-RACE. Two gene-specific RACE primers were designed based on the partial sequence obtained from RT-PCR. The 3′-RACE product was amplified in two rounds of reactions. First round PCR was amplified with a sense gene-specific primer, Cym*F3′H* 3′RACE-1, and the AnX short adaptor primer, which binds to the anchor region of the cDNA. The diluted CASP cDNA with the tagged anchor sequence was used as template. The PCR product from round one was used as template in the second round of PCR. A nested primer 'Cym*F3′H* 3′RACE-2' and the AnX short adaptor primer were used in this reaction. All primer sequences are listed in Table 4.2. Three negative controls were also performed for this experiment, as described in the RT-PCR experiment (Section 4.2.2.3). The 3′-RACE reactions were carried out in a total volume of 50 µL containing 1.5 units of *Taq* DNA polymerase (Roche). Both PCR reactions were carried out in the following thermo-cycling conditions: 94°C for 2 min, 35 cycles of amplification of 94°C for 30 s, annealing at 60°C for 30 s and extension at 72°C for 1 min and 30 s, followed by a final extension at 72°C for 7 min. All PCR products were analyzed on agarose gels as described in Section 2.5.6, checking for bands of the predicted size in experimental but not control amplificaitons.

4.2.2.5 PCR products detection, subcloning and sequence analysis

PCR products were recovered and quantified before being cloned into pGEM-T Easy (Promega) for sequencing as described in Section 2.5. The partial cymbidium *F3′H* nucleotide sequence and deduced protein sequence was aligned against *F3′H* sequences from other species using BLAST analyses on the NCBI website (http://www.ncbi.nlm.nih.gov/). The LaserGene program was used for all DNA analysis. The alignment of protein sequences and phylogenetic trees were carried out using the CLUSTAL W (Thompson et al., 1994) program via the EMBL-EBI web site (http://www.ebi.ac.uk/Tools/clustalw2/index.html).

4.2.3 Southern analysis of partial cymbidium *F3'H*

Genomic DNA was extracted from flower tissue of JFDP and CASP cultivars using the urea method as described in Section 2.6.2. Sixty μg aliquots of gDNA from the JFDP and CASP cultivars was digested with *Eco*RI, *Eco*RV, *Hin*dIII, and *Xba*I (Roche). Fully digested gDNA was separated on a 1% agarose gel, and transferred onto a positively charged Hybond N+ membrane (Amersham, Section 2.7.2). The membrane blot was detected with a ^{32}P-labelled probe at 60°C. The ^{32}P DNA probe was made by labelling the 566-bp DNA fragment, derived from the 3′-RACE, through the use of ^{32}P-dATP and random prime labelling kit (Roche). DNA gel blot hybridisation was performed as described Section 2.7.3.

4.3 Results

4.3.1 Partial cymbidium *F3'H* fragment amplification

A partial *F3'H* cDNA sequence has been isolated using a combination of RT-PCR and 3′-RACE techniques. The cymbidium CASP cultivar was chosen for *F3'H* amplification due to high levels of cyanidin-based pigments in the petals and sepals, which suggests that it should also therefore contain high levels of *F3'H* transcripts.

Two sets of degenerate primers were designed based on conserved regions of all plant F3′H sequences, one set is based on the both dicotyledon and monocotyledon sequences, and a second set based on monocotyledon F3′H sequences only. These were used in attempts to amplify a partial length *F3'H* from cymbidium. However, sequences amplified using these primer sets did not show similarity with the available *F3'H* sequences in GenBank. Sequence analysis of all plant CYB75 proteins showed that *Allium cepa* F3′H and phalaenopsis F3′5′H are the most closely related among monocotyledons (data not shown). Hence, degenerate primers (OP *F3'H* forward and reverse primers) were designed based on these two sequences. However, no PCR product was amplified using these primers. PCR reactions using combinations of all

available degenerate primers were then tried to amplify cymbidium *F3'H*, but only the combination of 'All monocot *F3'H* degenerate-F' primer with 'OP *F3'H* degenerate-R' primer amplified a PCR fragment of the predicted size that showed similarity with *F3'Hs* in the GenBank database.

The results of gradient PCR amplification of cymbidium *F3'H* using a range of annealing temperatures are shown in Figure 4.1. PCR fragments were amplified at three different annealing temperatures. A strong product with the expected size of 550 bp was obtained when the PCR reaction was carried out at an annealing temperature of 50°C. As the annealing temperature increased to 55°C, a second band around of 450 bp was also amplified, but the intensity of both bands was reduced. The larger band was not amplified when the same PCR was carried out at a higher annealing temperature of 60°C, and only the smaller PCR product was seen. This smaller band was also seen in the no template control (-ve) and forward primer only control (F). There was no PCR product obtained in the reverse primer only control (R).

The sequencing results of both bands showed that the larger band was 566 bp in length and the smaller band was 420 bp in length. Both sequences were checked for homology using BLAST search at the nucleotide level in the NCBI database, and the larger sequence showed ~ 66% identity with *F3'H* of *Pelargonidium×hortorum* (AF315465), *Verbena hybrida* (AB234901) and *Sorghum bicolor* (DQ787855, DQ787856, AY675076). The smaller band did not match with any functional sequence, showing that the larger PCR product is the putative cymbidium *F3'H*.

The downstream region of the *F3'H* sequence beyond the known 566 bp fragment was obtained by 3'-RACE, using CASP cDNA and two gene specific primers. '*F3'H* RNAi-F' and '*F3'H* 3'RACE-2' that were designed to anneal 30 bp and 120 bp from the beginning of the 5' end of the known sequence, respectively. The results of the 3'-RACE amplification is shown in Figure 4.2. At the end of the first round of PCR, smears of non-specific PCR products were amplified in both the experimental sample (labelled as CASP) and in the forward primer only control (F). However, a weak band of between 650 bp and 850 bp can be seen in the experimental lane (Figure 4.2A).

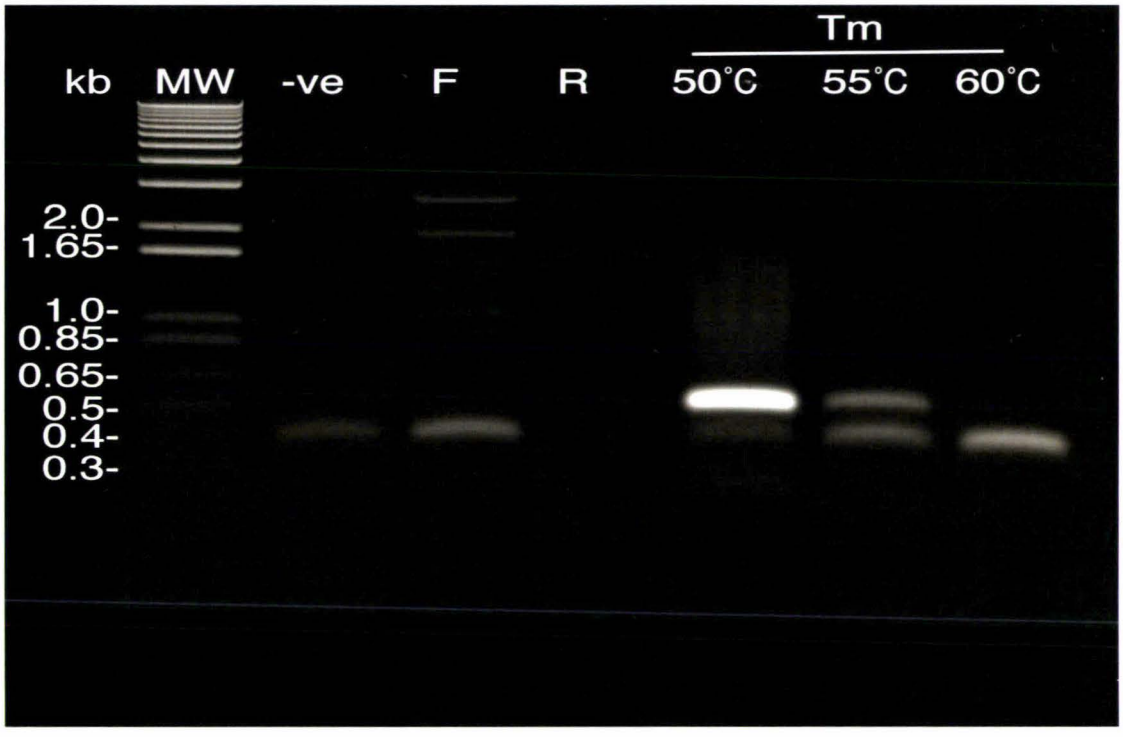
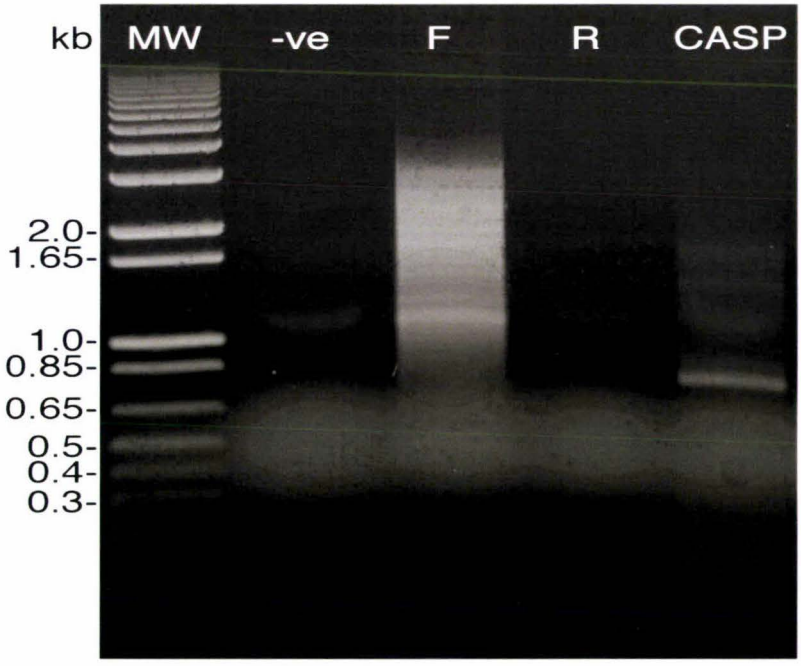


Figure 4.1: PCR amplification of a partial *F3'H* fragment. The fragment was amplified from cDNA of CASP cultivar, using degenerate primers designed to the conserved AGTDTS and DMEEAY regions. From the left: 1 Kb plus ladder; -ve, no template control; F, forward primer only control; R, reverse primer only control; 50°C, 55°C and 60°C indicating the annealing temperature at which sample PCR fragment was amplified.

A Cymbidium *F3′H* 3′RACE- first round PCR



B Cymbidium *F3′H* 3′RACE- second round PCR

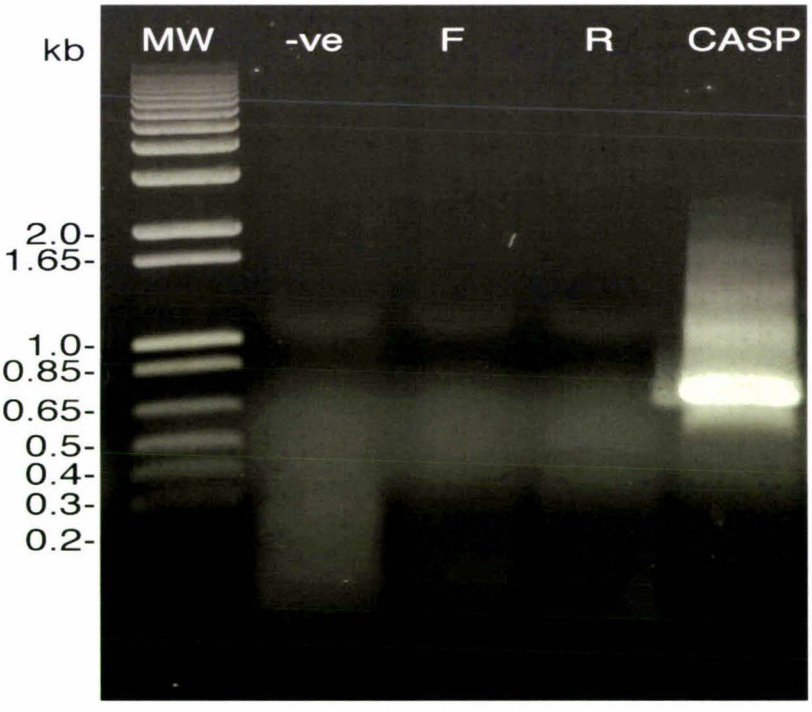


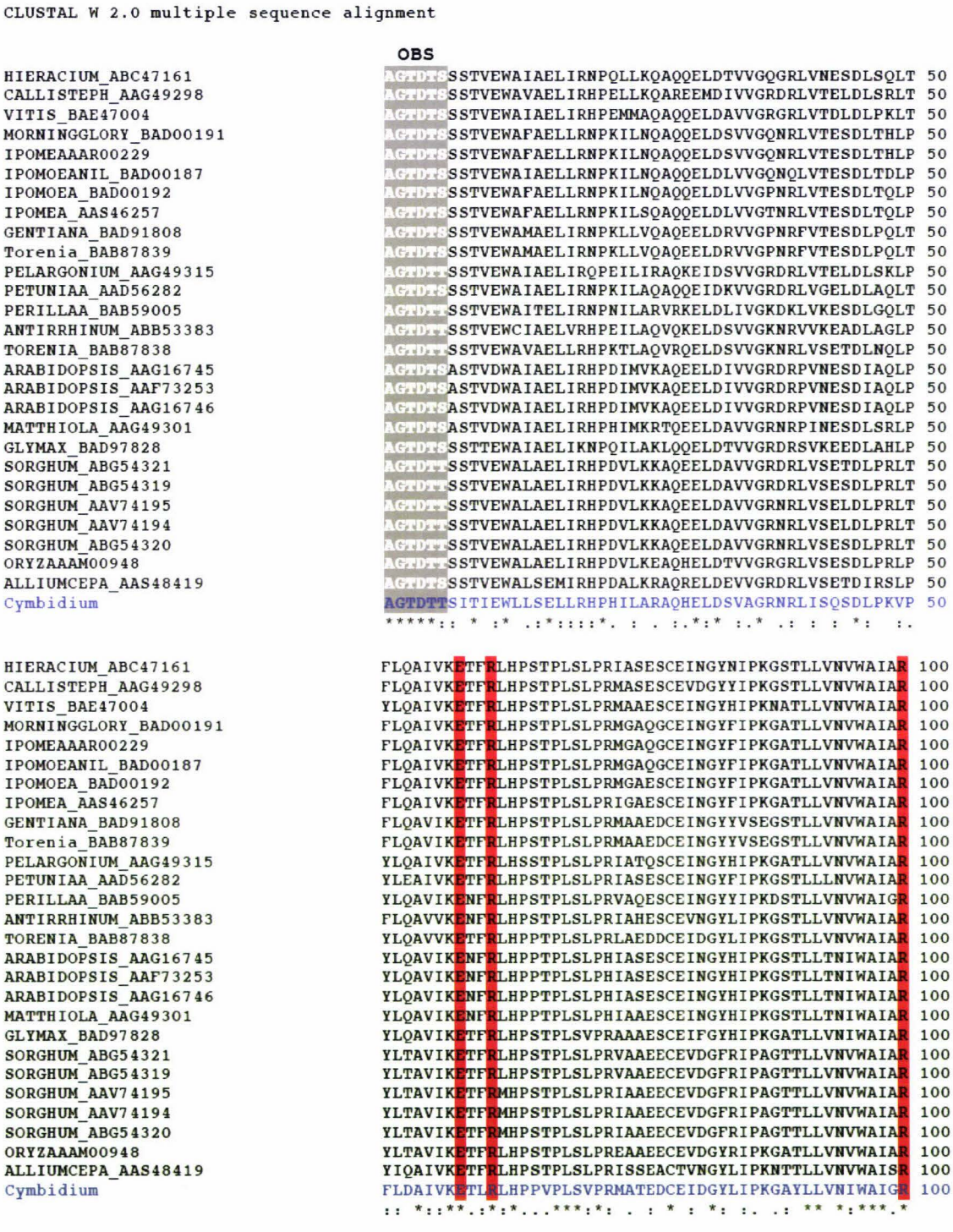
Figure 4.2: Amplification of the 3′ end of cymbidium *F3′H* cDNA using 3′-RACE. Fifty µL of each PCR was run on 1% (w/v) TBE gel. **A**, 3′RACE, round 1. **B**, 3′RACE round 2. The molecular weight ladder is labelled. -ve, no template control; F, forward primer only control; R, reverse primer only control; CASP, cDNA from CASP cultivar as template.

A second round of PCR showed a strong PCR product of ~ 700 bp, which is in agreement with the predicted length based on *F3'H* genes from other plants (Figure 4.2B). There was no PCR product amplified in the no template (-ve) control or in the forward- and reverse- primer only controls (labelled as F and R, respectively). Sequencing of the 3'-RACE product revealed a 673 bp PCR product, which was shown to be the same gene that had been amplified previously as a 566 bp PCR product.

In summary, the two PCR results showed a partial cymbidium *F3'H* cDNA that is 792 bp long, and contains a putative open reading frame of 736 bp encoding 210 amino acid residues. The *F3'H* cDNA sequence is shown in Appendix 3. Overall, the cymbidium *F3'H* cDNA sequence has 65-70% similarity with *F3'H* of *Allium cepa*, *Arabidopsis TT7s*, *Sorghum bicolor*, *Brassica napus*, *Ipomoea* and *Pelargonium x hortorum*. It also has 68% similarity with *F3'5'H* of *Dendrobium* orchid and *Phalaenopsis* orchid.

The deduced protein sequence was aligned with selected published F3'H enzymes (Figure 4.3). Domains conserved among many cytochrome P450s are found in the cymbidium sequence. These regions include a heme-binding site (HBS), FXXGXRXCXG, the oxygen-binding pocket site (OBS), AGTDTS (both are labelled in grey boxes) and the pocket locking motif E-R-R triad (labelled in a red box; Hasemann et al., 1995; Chapple, 1998; Werck-Reichhart et al., 2002). Identifying sequences that have previously been reported to be present in other dicotyledon F3'H proteins such as VDVKG and GGEK (labelled in yellow and green boxes, respectively) are also found in the partial cymbidium F3'H protein sequence (Brugliera et al., 1999; Toda et al., 2002; Boddu et al., 2004).

The phylogenetic tree based on the deduced amino acid of partial cymF3'H and other plant F3'H showed cymbidium F3'H is in a group with other monocotyledon F3'H, but is quite distinct from the other monocot sequences, including onion (*Allium cepa*) and rice (*Oryza sativa*) (Figure 4.4).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GENTIANA_BAD91808 AGTDTSSTVEWAMAELIRNPKLLVQAQEELDRVVGPNRFVTESDLPQLT 50
Torenia_BAB87839 AGTDTSSTVEWAMAELIRNPKLLVQAQEELDRVVGPNRFVTESDLPQLT 50
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Cymbidium AGTDTTSITIEWLLSELLRHPHILARAQHELDSVAGRNRLISQSDLPKVP 50
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CALLISTEPH_AAG49298 FLQAIVKETFRLHPSTPLSLPRMASESCEVDGYYIPKGSTLLVNVWAIAR 100
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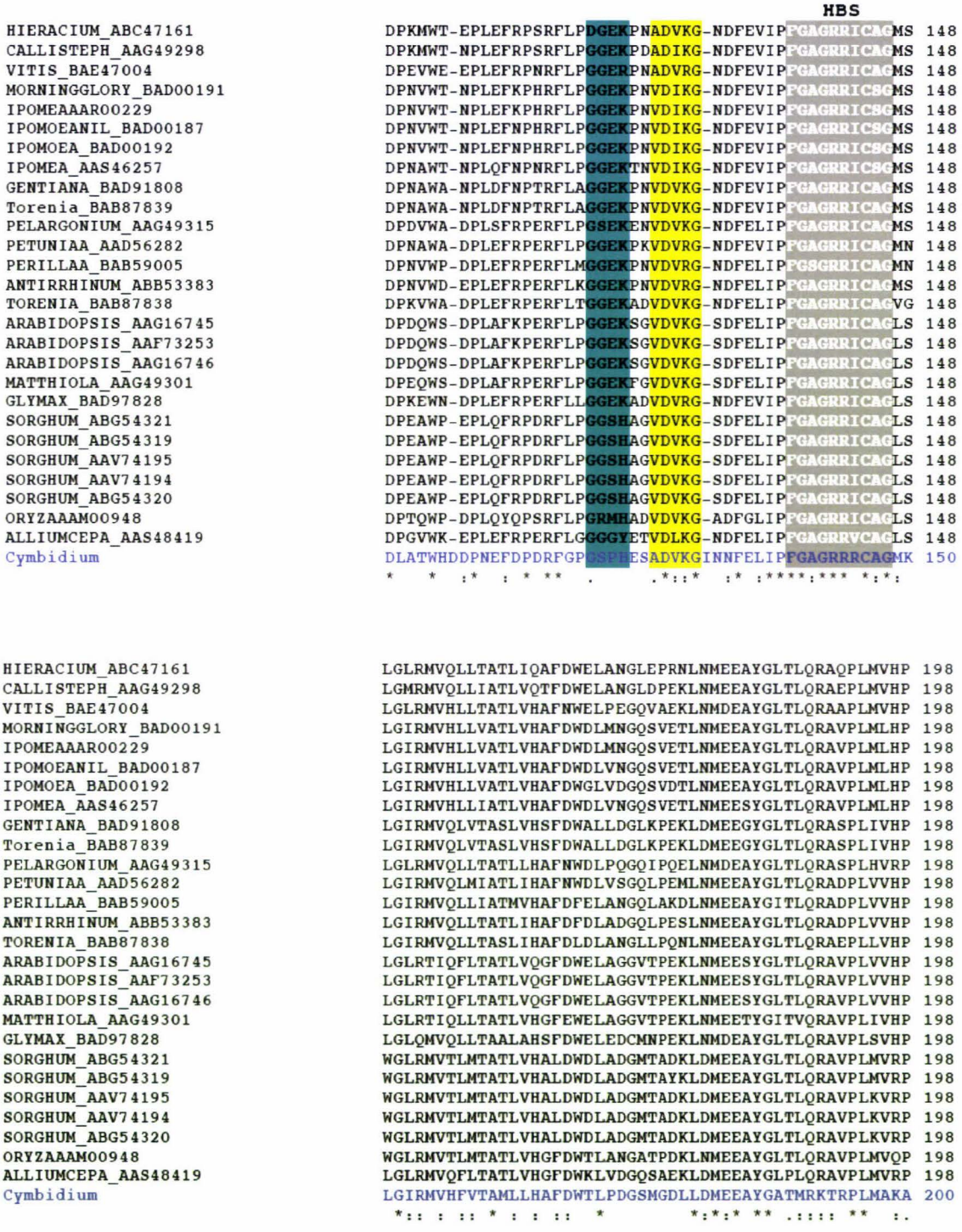
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                                    **     :
```

Figure 4.3: Sequence characterization of partial-length cymbidium F3′H deduced protein sequence. Multiple sequence alignment of deduced amino acid sequences of F3′H from cymbidium and other plant species was carried out using the Clustal W program. The plant species name is followed by their GenBank accession number. The cymbidium sequence is listed in blue colour font. The F3′H specific sequence, GGEK, is highlighted in green boxes; the VDVKG is highlighted in yellow boxes; the pocket locking motif E-R-R triad is labelled in a red box. The conserved domains found in the CYP450 family proteins are highlighted in grey boxes with white font. The conserved amino acids are labelled with asterisks, the highly conservative changes are labelled with double dots, and the conservative changes are labelled with dots.

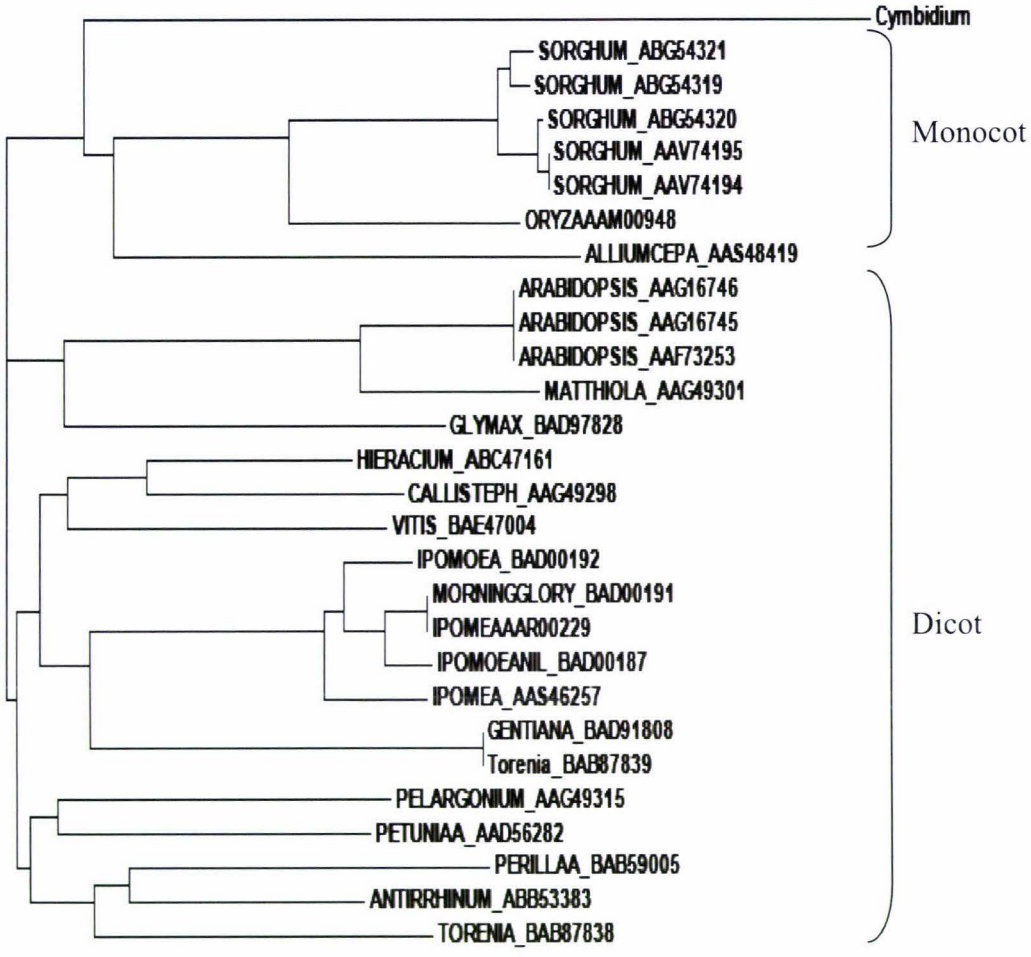


Figure 4.4: Phylogenetic tree of cymbidium F3′H and plant F3′Hs. The proteins from various plants are listed along with their GenBank accession numbers. The grouping of monocotyledon and dicotyledon plants is indicated.

4.3.2 Southern analysis

The complexity of the *F3'H* gene family in the genome of cymbidium CASP and JFDP cultivars was determined (Figure 4.5). Southern hybridization was carried out using a 566-bp fragment obtained using 3'-RACE as the probe. There was no *Bam*HI, *Eco*RI and *Eco*RV sites within the probe sequence, but the probe contains two *Hin*dIII sites. The result showed signals in the *Hin*dIII library of both cultivars was too weak to determine the numbers of bands. The *Eco*RI and *Eco*RV libraries of JFDP. The *Eco*RI and *Xba*I libraries of CASP, and *Xba*I of JFDP have three bands. This result indicates there are possibly up to three *F3'H* genes in the cymbidium genome.

4.4 Discussion

F3'H activity is important in determing if cyanidin-based anthocyanins can be produced. F3'H enzymatic activity has been studied intensively in petunia, *Arabidopsis* and perilla, but little is known about F3'H in monocotyledon ornamentals (Brugliera et al., 1999; Schoenbohm et al., 2000; Kitada et al., 2001). The aim of this work was to isolate a *F3'H* gene from cymbidium, and to determine its role in anthocyanin accumulation in cymbidium flowers.

A partial-length cymbidium *F3'H* cDNA sequence was isolated using a PCR-based strategy. The putative F3'H was related to other F3'H and F3'5'H proteins, since its deduced amino acid sequence showed 65% -70% identity with both F3'H and F3'5'H protein from other species. Phylogenetic analysis revealed that cymbidium F3'H is homologous to other monocot F3'Hs, but is more divergent than expected. Further work is needed to obtain a full-length clone and to confirm its functionality as a F3'H enzyme.

The amino acid sequence deduced from the putative *F3'H* DNA shows clear homologies to plant P450 proteins, indicating this sequence does belong to a P450 protein. The amino acid sequences of P450s are highly divergent, but the protein

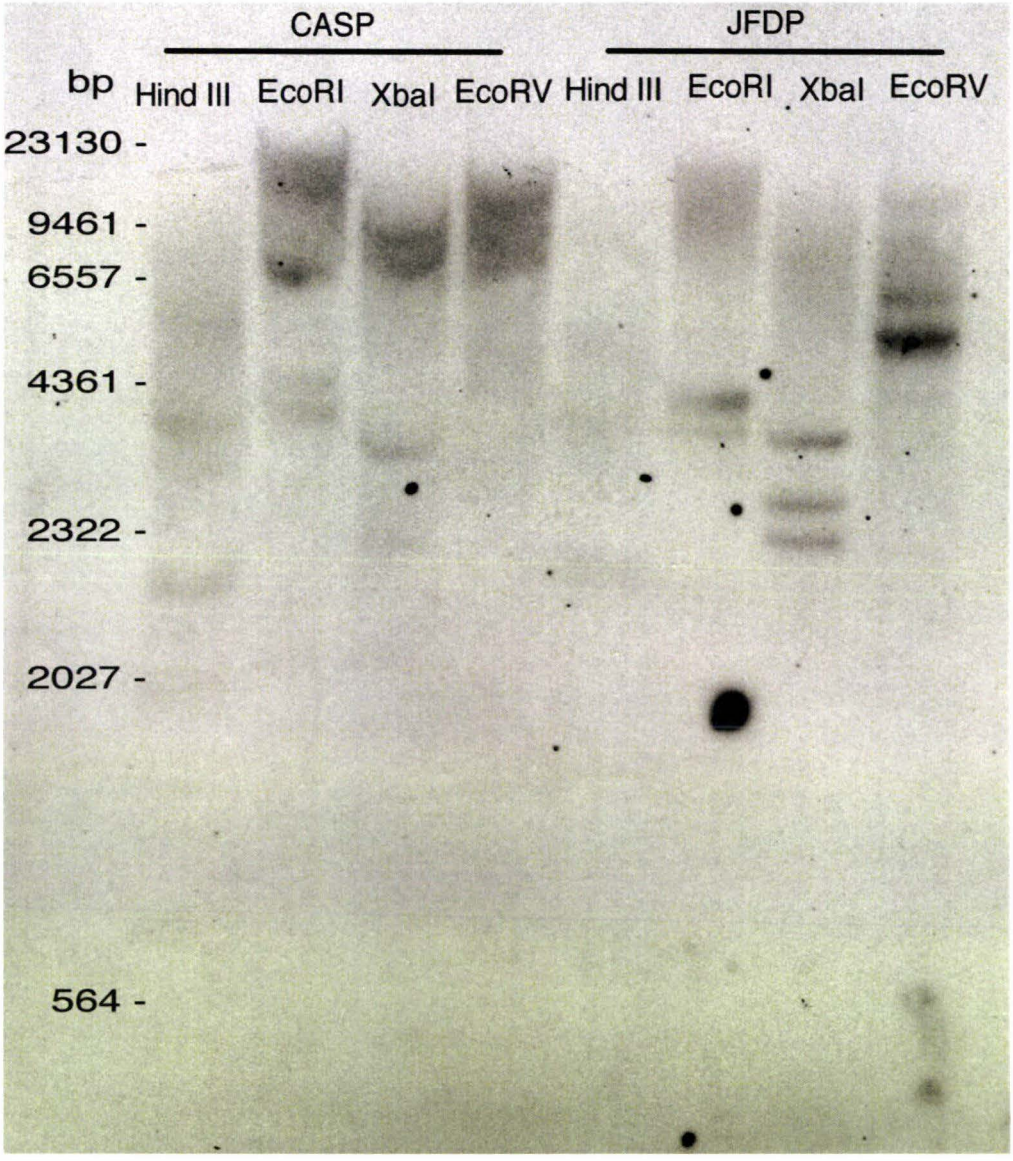


Figure 4.5: Southern analysis of cymbidium *F3′H*. Gel blot of genomic DNA extracted from CASP and JFDP cultivars and digested with various enzymes, hybridized with a labelled 3′ end of *F3′H* fragment as probe. High molecular weight markers are indicated for sizing the hybridized fragments.

folding and architecture of P450s remain conserved throughout evolution (Hasemann et al., 1995; Nelson et al., 1996; Rupasinghe et al., 2003). Members belonging to the P450 family all share some common motifs, and the functions of these conserved motifs have been identified. These include the AGXDTS motif that forms a threonine-containing oxygen-binding pocket (Hasemann et al., 1995), the heme binding domain, where the conserved cysteine act as a ligand to the heme iron (Chapple, 1998), and the pocket locking motif E-R-R triad (Werck-Reichhart et al., 2002). All of those motifs were found in the partial cymbidium F3′H protein sequence (Figure 4.3).

F3′H and F3′5′H have similar hydroxylation reactions, and they are closely related among the P450 anthocyanin biosynthetic pathway genes. F3′H belongs to the CYP75B and F3′5′H belongs to the CYP75A subfamilies (Ayabe and Akashi, 2006). Some short sequence motifs such as VDVKG and GGEK, and VVVAA motifs are unique to F3′H and have been reported to distinguish F3′H enzyme from F3′5′H (Brugliera et al., 1999; Boddu et al., 2004; Xu et al., 2007). Although the functional significances of these motifs are not known, both VDVKG and GGEK motifs are found in the cymbidium putative F3′H protein. The GGEK motif, which has shown to be conserved only in dicotyledons, has been modified to GGSH in several monocot F3′H enzymes (Boddu et al., 2004). Our alignment with other F3′H indicated that this motif is more diverse within monocotyledon sequences. In cymbidium the motif comprised GSPH, in onion it was GGGY and one gene of rice contained a GRMH motif.

P450s are thought to be associated with the endoplasmic reticulum (ER). The hydrophobic helix near the N-terminus is the major mechanism that anchors the protein to the ER membrane (Chapple 1998). Immediately after the N-terminal hydrophobic helix, it contains a proline-rich PPGPXPXP motif, which is considered to be able to destabilize the helices and act as a "hinge" for orientation of the enzyme (Yamazaki et al., 1993; Murakami et al., 1994). A PPGPXPXP motif could not identified without the N-terminal region of the cymbidium F3′H sequence. However, this motif is expected to be found in the protein sequence when the cymbidium F3′H full-length sequence is obtained. The VVVAA motif that is unique to F3′H protein was also unable to be identified in cymbidium sequence due to the same reason.

The initial Southern analysis showed that potentially multiple *F3'H* homologues are present in the cymbidium genome. This result raises the possibility that F3'H activity in cymbidium is controlled by a small gene family. Previous studies showed that multiple *F3'Hs* are present in other plants, such as soybean, gentian, grapes, sorghum and *Brassica* (Toda et al., 2002; Nakatsuka et al., 2005; Castellarin et al., 2006; Shih et al., 2006; Xu et al., 2007). The members of a multigene family may not all be functional. In sorghum, for example, only *SbF3'H1* and *SbF3'H2* are functional. Overexpression of both genes are able to restore the function of F3'H in *Arabidopsis tt7* mutants, but *SbF3'H3* had no detectable expression in the tissues examined (Shih et al., 2006).

Northern analysis was carried out to study the gene expression pattern for *F3'H* in developing cymbidium flowers, but no expression was detected (data not shown). It is possible that cymbidium *F3'H* is expressed at a very low level or is not expressed in the developmental stages of petal, sepal and leaf examined in this study. *F3'H* expression is relatively weak in some plants such as torenia (Nakatsuka et al., 2005). Studies in ornamental plants have shown that *F3'H* expression is most active in early developmental stages of flowers from petunia, torenia, and gentian (Brugliera et al., 1999; Ueyama et al., 2002; Nakatsuka et al., 2005). Since anthocyanin formation starts from very early stages of flower development in *Cymbidium* hybrids, *F3'H* expression would be expected from the early stages of flower development, and the expression should be in line with other anthocyanin biosynthetic genes such as *DFR*. The same probe used to hybridize to the Southern blot was also used for the RNA blot, and future work should use more sensitive techniques, such as real time-PCR, to detect *F3'H* mRNA. Isolation of the 5′ end of the cymbidium *F3'H* gene may require 5′-RACE (rapid amplification of 5′ cDNA end). RACE techniques have an advantage over genome walking in that they avoid long introns, which means it is easier to amplify the upstream sequence. A cymbidium cDNA library may be needed to isolate and characterize other copies of *F3'H* in the genome.

This is the first time a partial *F3'H* gene has been reported for the orchid family. The results presented in this chapter extend our knowledge of the flavonoid biosynthetic pathway in a monocot species and allow a *F3'H* RNAi construct to be made to test silencing of the endogenous F3'H activity in petal and sepal tissues of cymbidium.

Chapter 5

Dihydroflavonol 4-reductase expression and promoter isolation

5.1 Introduction

Dihydroflavonol 4-reductase (DFR) is a key enzyme during the later steps of the anthocyanin biosynthetic pathway. It catalyzes the NADPH-mediated reduction of dihydroflavonol to leucocyanidin, leading to anthocyanin or condensed tannin formation (Figure 3.1). DFRs from some plants exhibit substrate specificity and restrict accumulation to a particular type of anthocyanindin. Thus DFR in these plant species is important in determining which anthocyanins accumulate in the flower.

Cymbidium DFR preferentially uses DHQ as its substrate, which causes cymbidium flowers to accumulate cyanidin-based anthocyanins. Cymbidium DFR can also utilize DHM (D. Lewis, pers. comm.), although the lack of a functional F3′5′H limits the synthesis of delphinidin-based anthocyanin derivatives. It has been reported that the scarcity of orange-pelargonidin accumulation in cymbidium flowers is because cymbidium DFR cannot reduce DHK efficiently (Johnson et al., 1999). Petunia DFR can also not reduce DHK, due to its substrate specificity being is altered in a single amino acid change from an asparatic acid residue to an asparagine residue at position 134 (Forkmann and Ruhnau, 1987; Johnson et al., 2001). *Cymbidium* and *Dendrobium* orchids also have asparagines residues in the same position, in common with DHK-accepting DFR in other plants, and yet they cannot reduce DHK. Therefore, the substrate specificity may not be caused by the change in amino acid sequence, but rather could be due to competition between DFR and F3′H enzymes (Johnson et al., 2001; Mudalige-Jayawickrama et al., 2005; Hieber et al., 2006).

The substrate specificity of cymbidium DFR is well understood, although the activity of DFR throughout flower development has not been characterized. Therefore, identifying the temporal and spatial expression pattern of the cymbidium *DFR* gene in different coloured cymbidium cultivars is the first objective of this chapter. Understanding the *DFR* expression pattern in cymbidium will help to gain knowledge of anthocyanin biosynthesis in cymbidium, as well as the regulation of anthocyanin biosynthesis in monocotyledonous plants in general.

Novel flower colour can be achieved by altering enzymes involved in anthocyanin biosynthesis; however, this can lead to alterations in other plant functions too. Expressing genes in a tissue-specific manner can limit these potentially negative effects. Thus a tissue specific promoter is useful in terms of engineering flower colour. DFR activity appears to be confined to floral tissues, and activities in vegetative tissue have not been observed. Thus the *DFR* promoter may be a suitable candidate for a flower-specific promoter. Therefore, the second objective of this chapter was to isolate the cymbidium *DFR* promoter, and test promoter activity in various cymbidium tissues.

5.2 Methods

5.2.1 Plant materials and extraction of RNA and gDNA

Fresh floral stem and leaf tissues of the following cymbidium cultivars were obtained from commercial growers:

1) White cultivars: Jung Frau dos Pueblos (JFDP) and Winter Bride Peter's Choice (PC).

2) Pink cultivars: Narella Jennifer Gail (NJG) and Lisa Rose Flamingo (LRF)

3) Maroon cultivar: Clarissie Austin South Pacific (CASP)

4) Green cultivar: Vangard Mas Beauty (VMB)

5) Leaf tissues of green cultivars: Forty Niner Alice Anderson (AA), VMB, Big Chief Kirawee (BCK), Piedmont (Pd) and Lymonese (Ly).

6) Leaf tissues of the pink cultivar: NJG

All cut stems and leaves had water tubes attached and were held in a controlled temperature vase life room prior to experiments as described in Section 2.3. Floral tissue was collected at the six defined developmental stages as shown in Figure 2.1. Samples were collected as described in Section 2.3 and stored at -80C.

Total RNA was extracted from stage four sepals of the CASP cultivar, petals and lips, using the hot borate method (Section 2.6.1). RNA was used for subsequent cDNA synthesis by reverse transcription.

Genomic DNA was extracted for the downstream genome walking experiment from the flowers of CASP. Floral tissues from the JFDP and LRF cultivar were collected for genomic DNA extraction and Southern analysis.

Floral and leaf tissues were collected from a range of developmental stages and cymbidium cultivars for RNA extraction and subsequent northern analysis specifically:
1) Sepal, petal and lip tissues of each floral developmental stage from JFDP.
2) Sepal, petal and lip tissues at stage one, four, five and six from VMB.
3) Sepal and petal tissues combined at developmental stages one to three, and combined at stages four to six from NJG and PC. Lip tissues at combined developmental stages one to three, and combined at stages four to six from NJG and PC.
4) Leaf tissues from AA, NJG, VMB, BC, Pd, and Ly. Leaf tissues were taken as young leaves from this season's growth, approximately two to three months old.

Petals and sepal tissues at stage five and six from JFDP and LRF, and leaf tissue of JFDP were used for the transient expression studies of the *DFR* promoter activity. All tissues were surface sterilized before used as described in Section 2.8.

5.2.2 Isolation of cymbidium *DFR*

The ORF sequence of cymbidium *DFR* was amplified by reverse transcription followed by polymerase chain reaction (RT-PCR) as described in Section 2.4 prior to the *DFR* promoter isolation experiment.

The ORF sequence of cymbidium *DFR* was isolated from cDNA synthesized from RNA extracted from floral tissues of stage four CASP cultivar, using primers designed based on published sequences (listed in Table 5.1). A negative control lacking template was also carried out for checking contamination in the PCR reactions. The following thermo-cycling conditions were used for *DFR* fragment synthesis: denaturation at 94°C for 2 min, 35 cycles at 94°C for 30 s, 60°C for 30 s and 72°C for 2 min, followed by extension of 72°C for 5 min. PCR products were analyzed on agarose gels, recovered and then cloned into the pGEM T-Easy vector (Promega) before being sequenced by the Allan Wilson Centre sequencing service (Section 2.5.8). The *DFR* sequence was aligned with the published cymbidium *DFR* using the CLUSTAL W (Thompson et al., 1994) program via the EMBL-EBI web site (http://www.ebi.ac.uk/Tools/clustalw2/index.html).

5.2.3 Northern and Southern analysis of cymbidium *DFR*

The urea method was used to extract gDNA from petal tissue of LRF and JFDP cultivars as described in Section 2.6.2. Sixty micrograms of gDNA from LRF and JFDP cultivars was digested with *Bam*HI, *Eco*RI, and *Hind*III enzymes. The DNA fragments were separated on a 1% (w/v) agarose gel and blotted onto N+ membrane as described in Section 2.7.2.

The hot borate method was used for RNA extraction from various cymbidium floral and leaf tissues (see Section 2.6.1). A total of 20 µg of each RNA sample was loaded for northern analysis. The RNA samples were separated on 1.2% (w/v) agarose denaturing gels and blotted onto N+ membrane as described in Section 2.7.1.

The full-length cymbidium *DFR* cDNA fragment was used as a probe to hybridize to the Southern and northern blot. Probe labeling, hybridization and washing conditions were as described in Section 2.7.3. The images of the Southern and northern analysis were obtained using both the FLA phosphorimager and X-ray film (Kodak) (Section 2.7.3.4).

5.2.4 Isolation of the cymbidium *DFR* promoter

The promoter region of cymbidium *DFR* was isolated by genome walking. All the procedures for this experiment followed the GenomeWalker™ Universal Kit user manual, but the adaptor primers used were designed in-house. The enzyme used for the PCR reaction was Triple Master PCR *Taq* polymerase (Roche) rather than the recommended Advantage 2 Polymerase Mix.

Table 5.1: List of primers for cymbidium *DFR* ORF, genome walking and *DFR* promoter amplification (Tm = annealing temperature).

Primer Name	Sequence (5′-3′)	Tm (°C)
DFR start codon	ARGGAGACTGAGAGGAAGGGTC	64.8
DFR stop codon	TCACTTAACAGCAATTTGTTCTTTTAC	62.2
DFR 5′GW-2	TGACCTCATAACCCTTTTGAAGAAGC	67.8
DFR 5′GW-1	GTTTCGTCAATGTCATTGAGATCTGC	68.5
DFR promoter-F	AAGAGCTCGGCTGGTACTATTTGTAAGCTAT TTTGC (*Sac*I)	73.3
DFR promoter-R	AGAATTCTTCTTCTCTCTCCCCCTAGCTTC (*Eco*RI)	69.8
DFR promoter sequencing-F	AAGGAATAACAAATTTGAACACTC	59.0
DFR promoter sequencing-R	AACGGTAGAATGCAATTTAGG	59.5
Genome walker adaptor-1	GTAATTCGCATCACTATAGCTCACCGCTGGT CGACGGCCCGGGCTGGT	-
Genome walker adaptor-2	PO_4-ACCAGCCC-H_2N	-
Adaptor primer 1	GTAATTCGCATCACTATAGCTC	-
Adaptor primer 2	ACTATAGCTCACCGCTGGT	-

5.2.4.1 Genomic DNA enzymatic digest

The gDNA extracted from cultivar CASP was digested with blunt cutter enzymes: *Sca*I, *Eco*RV, *Stu*I, *Dra*I and *Hpa*I (Roche). A total of 2.5 μg of CASP gDNA was digested to completion with 130 units of enzymes in 150 μL of the recommended 1× buffer (Roche). The digestion was carried out at 37°C, overnight. Five microlitre aliquots of each digestion reaction were separated on a 1% (w/v) agarose gel to check for completion of digestion (Section 2.5.6). The fully digested gDNA was purified with phenol and re-precipitated by adding 0.1 volume of 3M NaOAc, 20 μg of glycogen and two volumes of 100% ethanol. The pellet was dissolved in 20 μL of TE.

5.2.4.2 Adaptor ligation

A Genome Walker Adaptor was made by annealing the longer 'Genome Walker adaptor-1' sequences to the shorter 'Genome Walker adaptor-2' sequences: heating primers at 70°C for 5 min and then cooling to room temperature for 30 min. The reaction for annealing contained 20 μL of 'Genome Walker adaptor-1' (10 μM), 20 μL of 'Genome Walker adaptor-2' (10 μM), 9.5 μL of H_2O and 0.5 μL of 5 M NaCl (50 mM). The adaptor was ligated to the purified DNA fragment followed the GenomeWalker™ Universal Kit user manual. Both adaptor sequences are listed in Table 5.1.

5.2.4.3 Gene walking to amplify *DFR* up-stream coding region and promoter

Two reverse gene-specific primers were designed based on the *DFR* ORF sequence following the guidelines given in the GenomeWalker™ Universal Kit user manual. The '*DFR* GW 5′-1' and '*DFR* GW5′-2' primers were designed at 167 bp and 52 bp from the start codon. Primer sequences are listed in Table 5.1.

The genome walking was performed through two rounds of PCR reactions. The first round was amplified using '*DFR* GW 5′-1' and 'Adaptor primer-1' primers. The second round PCR was amplified using '*DFR* GW 5′-2' and 'Adaptor primer-2' primers. Sequences of 'Adaptor primer-1 and -2' are listed in Table 5.1. The PCR product from

the *Sca*I library was excised from the gel, extracted and cloned into pGEMT-Easy for sequencing. The full-length promoter sequence was obtained by sequencing with T7 and SP6 primers, as well as two gene-specific primers, *DFR* promoter sequencing-F and R. The two DFR promoter-sequencing primers are listed in Table 5.1.

The promoter sequence was analysed using the PLACE database (www.dna.affrc.go.jp/PLACElsignalscan.html) and TFsearch ver 1.3 programme (Yutaka, 1995, Bioinformatics Center, Institute for Chemical Research, Kyoto University, www.cbrc.jp/research/ab/TFSEACH.html).

5.2.5 Transient expression test of cymbidium *DFR* promoter

5.2.5.1 *DFR* promoter amplification

The *DFR* promoter without the ORF was amplified by PCR for construction of the pDFRpromoter-GFP construct. Two gene-specific promoters (*DFR* promoter-F and *DFR* promoter-R) were designed using the sequence of the *DFR* promoter obtained from genome walking (Table 5.1). The *DFR* promoter forward primer contains a *Sac*I restriction site and the reverse primer contains an *Eco*RI site for cloning. Both restriction endonuclease digestion sites are underlined in Table 5.1. The promoter was amplified from the gDNA *Sca*I library using Triple Master *Taq* polymerase. The thermo cycling reaction conditions were: 94°C for 2 min, 35 cycle of 94 °C for 30 s, 62°C for 30 s, 72°C for 2 min, and final extension at 72°C for 5 min. The PCR fragment was analysed on a 1% (w/v) agarose gel, then extracted for cloning (Section 2.5).

5.2.5.2 Assembly of *DFR* promoter::*GFP* construct

A *DFR* promoter::*GFP* reporter construct was made to enable the *DFR* promoter to be tested using transient expressions assays. The *DFR* promoter was cloned into a pART7-based vector, which contains a *GFP* gene (pPEP-GFP). The cloning strategy involved removing the *CaMV35S* promoter from pPEP-GFP by *Sac*I and *Eco*RI digestion, and replacing it with *DFR* promoter fragment using the same cloning sites. The ligation reaction was carried out using the rapid ligation kit instruction (Roche), and putatively

ligated vector were transformed into XL-1Blue competent cells (Section 2.5.3). Colonies were picked randomly, grown up and the plasmid DNA extracted by miniprep extraction (Section 2.5.4). The positive clones were confirmed using an *Eco*RI and *Sac*I digest (Section 2.5.5).

5.2.5.3 Transient expression of the cymbidium *DFR* promoter

A total of 4 μg of pDFRpromoter-GFP plasmid was biolistically introduced to the petals of the JFDP and LRF cultivars, and the leaves of the JFDP cultivar. The experimental details for biolistic bombardment and GFP and colour foci assessment are described in Section 2.8.

A control experiment was carried out using 2 μg of pPEP-GFP bombarded into all tested plant tissues to ensure the bombardment conditions were right for obtaining most effective transient gene expression.

A study to determine if the *DFR* promoter was regulated by transcription factors was carried out using the maize *Lc* and *C1* (both genes are driven by the *CaMV35S* promoter). A plasmid DNA mixture containing 2 μg of pLN44 (*C1*) and 2 μg of pLc349 (*Lc*) and 4 μg of pDFRpromoter-GFP was mixed with gold particles, and biolistically introduced to the petals of the JFDP and LRF cultivars, and leaf tissues of the JFDP cultivar.

A second control experiment was set up to test if the *CaMV35S* promoter was also able to influence the activity of the *DFR* promoter. A mixture of pART7 plasmid DNA (2 μg) was mixed with 4 μg of pDFRpromoter-GFP and transformed into the same materials listed previously.

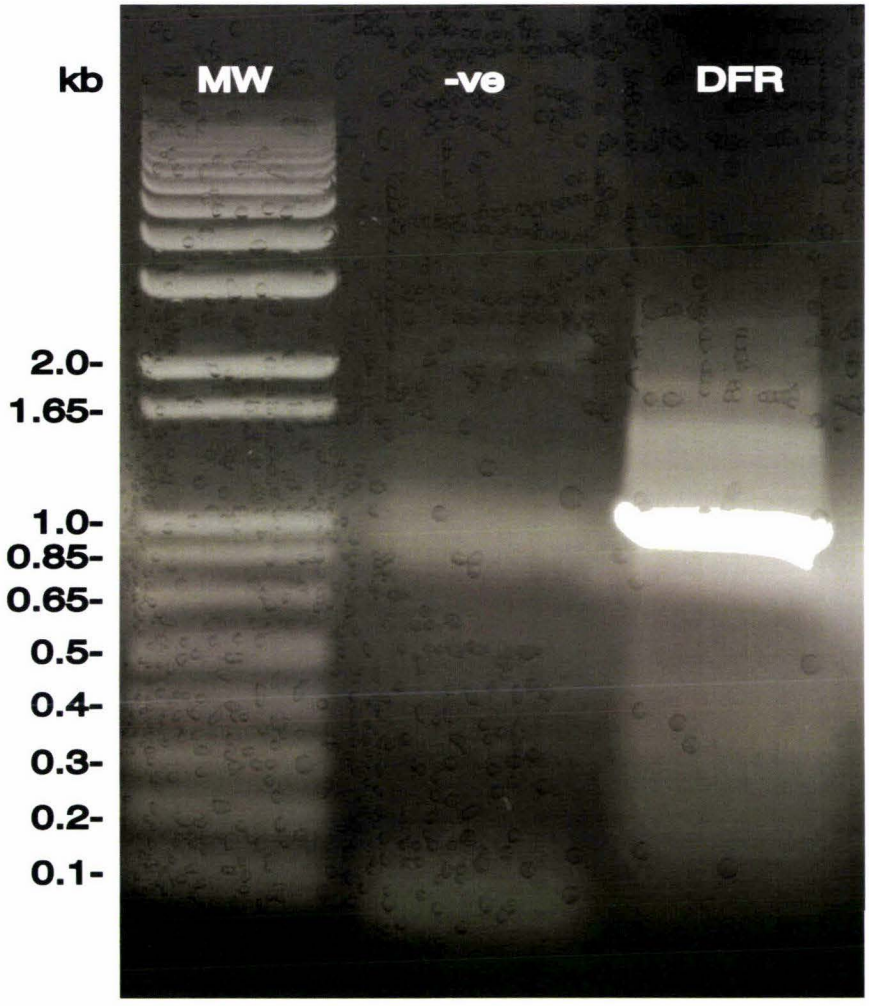


Figure 5.1: PCR amplification of the coding region of cymbidium *DFR*. cDNA from the maroon cultivar CASP was used as the template in the PCR reaction. –ve, no template control. The 1kb+ molecular weight markers are indicated.

5.3 Results

5.3.1 Cloning and molecular characterization of cymbidium *DFR*

5.3.1.1 Comparison of cymbidium *DFR* sequence with published data

A cymbidium *DFR* cDNA was amplified by RT-PCR. The PCR product was approximately 1 kb in length (Figure 5.1), which is consistent with the predicted size of the coding region based on published data. There were no PCR products present in the template-free control.

The sequence of the PCR-amplified cymbidium *DFR* (Figure 5.1) was then compared against the NCBI database, using BLAST searching and the sequence matched with the published cymbidium *DFR*. This sequence was then aligned with the published data (Figure 5.2). The alignment showed there are a few nucleotides different from the published *DFR* sequence (labeled in red). However, the majority of the sequence is identical to the published data.

5.3.1.2 Temporal and spatial expression of cymbidium *DFR* mRNA

Cymbidium *DFR* gene expression was examined at various floral developmental stages (stage one to six), in different cultivars (white- JFDP and PC, pink- NJG and green-VMB) and in leaves (Figure 5.3). *DFR* mRNA was most strongly expressed in the NJG compared to the JFDP and PC and VMB cultivars. Strong *DFR* expression occurred during early floral development (stage one to three) of NJG. *DFR* continued to be expressed strongly in lip tissues in later stages (stage four to six), while no expression was detected in sepals and petals at these later stages. Expression in the sepal and petal tissues of the VMB or JFDP and PC cultivars was undetectable at all stages tested. *DFR* expression, however, was detected in the lip tissues of all three of these cultivars, although the expression levels differed among the cultivars in relation to their developmental stages. This is consistent with the presence of anthocyanin in lip tissues, but not in petals or sepals of these cultivars.

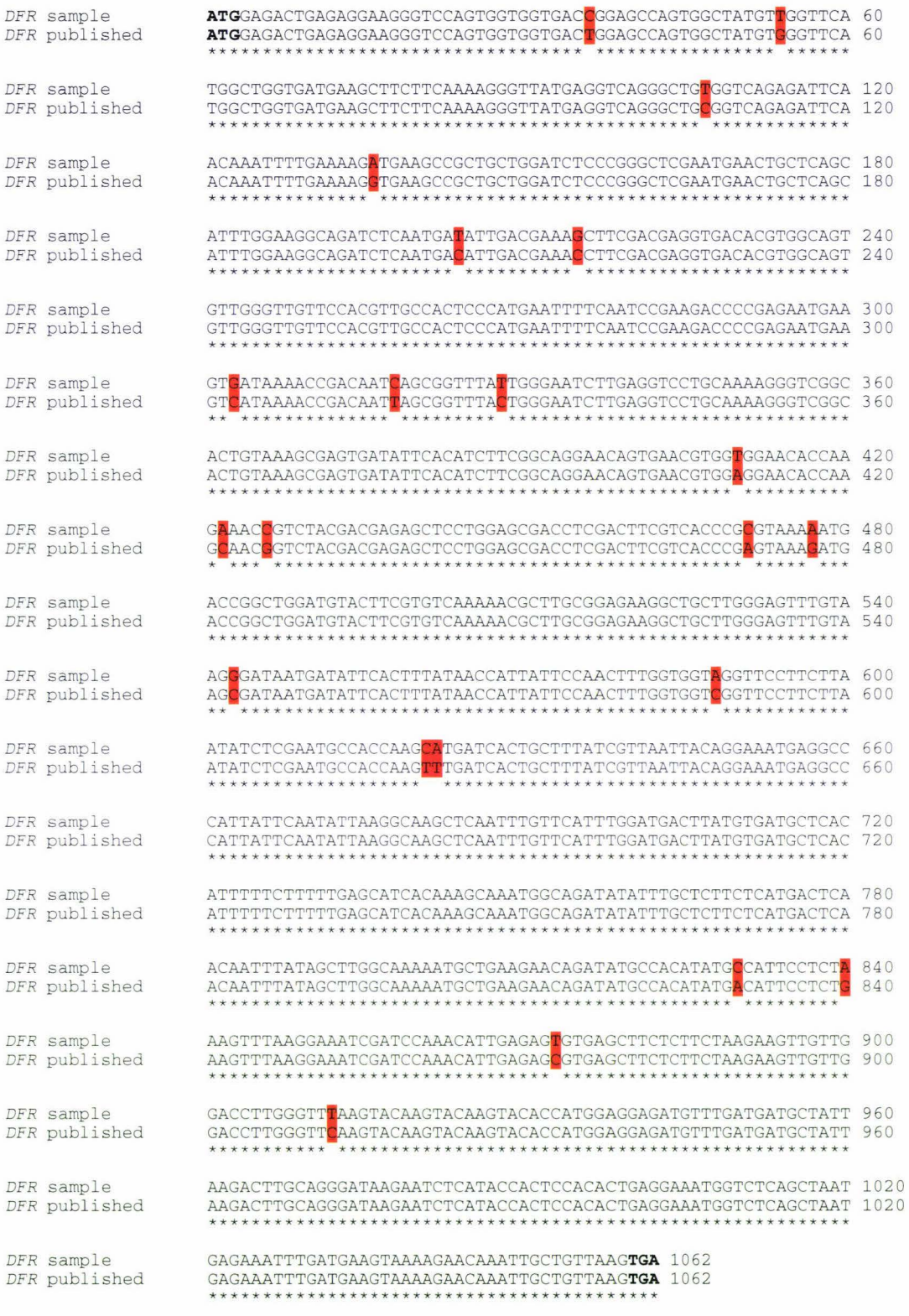


Figure 5.2: Alignment of the *DFR* published sequence (AF017451) with the amplified *DFR* sequence. Asterisks indicate conserved nucleotides. Different nucleotides between the amplified *DFR* sequence (Figure 5.1) and published *DFR* sequence are highlighted in red. The start codon and stop codon are in bold.

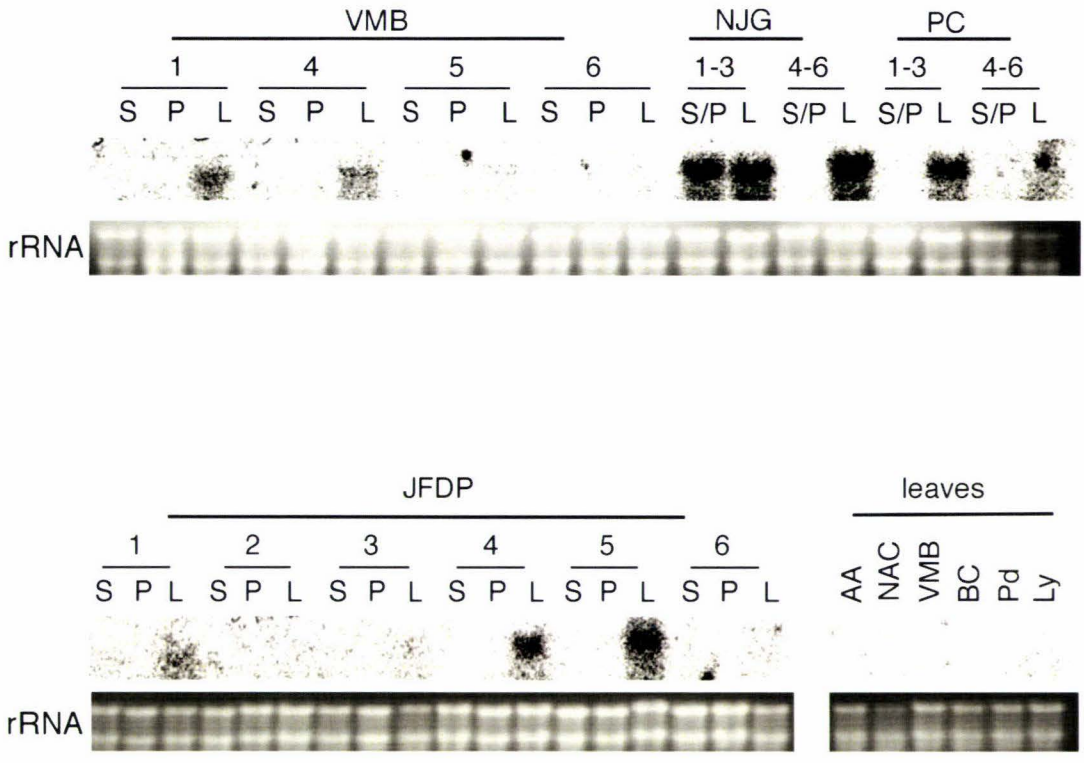


Figure 5.3: Northern blots showing temporal expression of cymbidium *DFR* in flower and leaf tissues of various cymbidium cultivars. Flower development stages are indicated by numbers. S, sepal; P, petal; L, lip; S/P, combined sepal and petal. VMB, Vanguard Mas Beauty; NJG, Narella Jennifer Gail; PC, Winter Bride Peter's Choice; JFDP, Jung Frau dos Pueblos; NAC, Narella Adam's Choice; AA, Alice Anderson; BC, Big Chief Kirawaee; Pd, Piedmont; Ly, Lymonese. The membrane was hybridized at 60°C and washed at 60°C in 0.5×SSC+1%SDS.The bottom panels indicate the loading of RNA using ethidium bromide staining of rRNA bands.

The VMB and JFDP and PC cultivars all have anthocyanin pigmentation on the lip although their petals and sepals do not contain anthocyanins. Lip tissues from the green VMB cultivar showed moderate *DFR* expression was shown in the stages one and four (Figure 5.3). The expression declined at stage five, with only very weak signal detected, and no expression was detected in stage six lip tissues. The expression level of *DFR* in lip tissue of white PC cultivar was stronger compared to VMB lip tissues at the early developmental stages (combined stage one to three), but no expression was detected in the later developmental stages (combined stage four to six). The *DFR* expression pattern in JFDP lip tissues was different from the expression pattern in VMB and PC. *DFR* was active in lip tissue of developmental stage one, but not at stage two. At stage three, *DFR* expression increased again in lip tissues, and the expression level increased progressively from stage three. The highest expression level was detected at stage five. There was no expression detected in stage six lip tissues.

No expression was detected in leaf tissues of any of the cultivar tested (Figure 5.3).

5.3.1.3 Southern blot analysis

Southern blot analysis was performed to investigate the copy number of *DFR* genes in the genomes of the cymbidium cultivars LRF and JFDP. The full coding region of the cymbidium *DFR* was used as a probe for hybridization of the Southern blot. A similar pattern of *DFR*-hybridized bands was obtained from both the pink (NJG) and white (JFDP and PC) cultivars (Figure 5.4). There were no *Bam*HI or *Eco*RI restriction sites within the *DFR* sequence, which gives one hybridizing fragment per *DFR* gene. There is one *Hind*III site within the *DFR* DNA at 82 bp from start codon, which means two hybridizing fragments are expected per *DFR* gene. Two bands were observed in both the *Eco*RI and *Bam*HI digests. Three to four bands were observed in the gDNA digested with *Hin*dIII lane of JFDP and LRF cultivars.

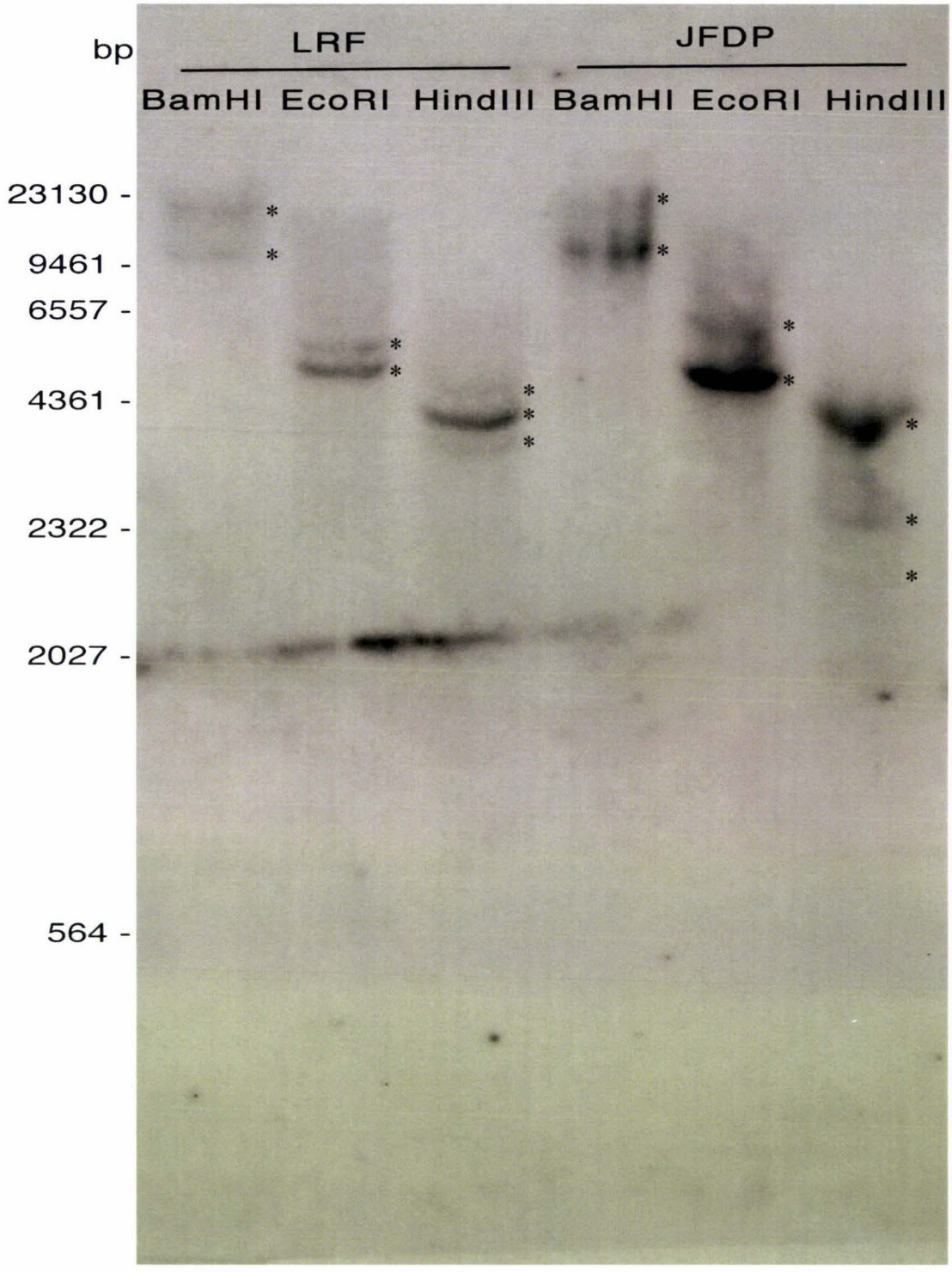


Figure 5.4: Southern blot analysis of *DFR* in two cymbidium cultivars.
Genomic DNA of two cymbidium varieties was digested with the enzymes as indicated and the blot hybridized with a radioactively labeled probe consisting of the cDNA of the ORF of *DFR*. LRF, Lisa Rose Flamingo; JFDP, Jung Frau dos Pueblos. Asterisks indicate the major bands observed.

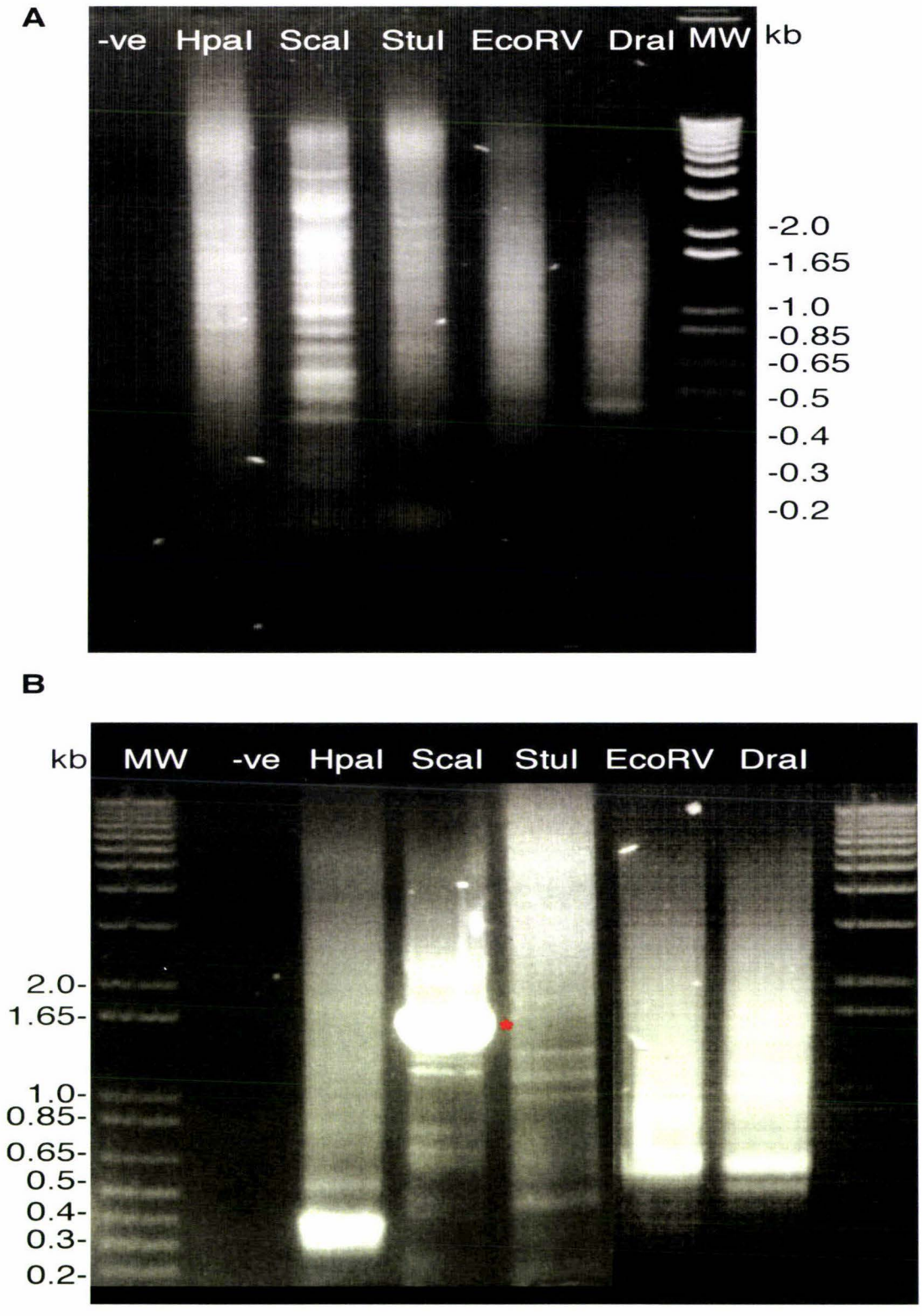


Figure 5.5: *DFR* promoter isolation by genome walking. The separation of the PCR products is shown **A**, result of first round PCR; **B**, result of second round PCR. The gDNA libraries are labelled. -ve is the no template control. The band isolated for sequencing is marked with an asterisk. The molecular weight of 1Kb ladder is indicated.

5.3.2 Cymbidium *DFR* promoter characterisation

5.3.2.1 Promoter isolation

Attempts to clone the up-stream region of the cymbidium *DFR* were carried out using *Hpa*I, *Sca*I, *Stu*I, *Eco*RV and *Dra*I 'gDNA libraries' via genome walking (Figure 5.5). The first round PCR reactions showed a smear of non-specific bands in all libraries (Figure 5.5A). A strong band of around 1.5 kb was synthesized using the *Sca*I library after the second round of PCR (Figure 5.5B). Each of *Hpa*I, *Eco*RV and *Dra*I libraries showed smaller PCR products with sizes of ~300 bp and ~550 bp (for both *Eco*RV and *Dra*I libraries), respectively. No clear product was amplified from the *Stu*I library. The negative controls (no template control) from both PCR reactions showed no contamination in these PCR reactions. The PCR product from the *Sca*I library was chosen for cloning and sequencing because it contains the longest putative promoter region. A full-length promoter region could not be fully sequenced using the T7 and SP6 primers, due to the large size of the fragment. Thus, two *DFR* promoter-sequencing primers (forward and reverse) were designed and used to complete the sequencing of the promoter (primer sequences shown in Table 5.1). Sequencing results showed a 1544 bp promoter fragment (Figure 5.6). This fragment was identified as the *DFR* promoter, since the primer sequence used for gene walking overlaps with the coding region. The positions of primers used in genome walking PCR are indicated in Figure 5.6.

5.3.2.2 *DFR* promoter sequence

The 5′ upstream region of *DFR* was analyzed using the online programs, PLACE and TFSEARCH, and several characteristic motifs identified in the sequence are listed in Table 5.2 and are labeled in Figure 5.6.

5.3.2.3 Promoter activation experiments

The *DFR* promoter was obtained using two *DFR* promoter-specific primers (highlighted in pink in Figure 5.6). An expected a ~1.5 kb band was amplified (Figure 5.7). This *DFR* promoter fragment was then cloned into *Sac*I and *Eco*RI sites of the pPEP-GFP

vector backbone. The clone containing the *DFR* promoter is named 'pDFRpromoter-GFP'.

A pPEP-GFP construct, which contains the constitutive *CaMV35S* promoter driving *GFP*, was introduced into petals of JFDP and LRF, and JFDP leaves as a positive control. Many strongly-expressed GFP foci were observed in the JFDP and LRF petal tissues (Figure 5.8 A and B), and *GFP* expression was observed in the shot zone of leaf

Table 5.2: List of possible transcription factor recognition sites of the *DFR* promoter.

Putative DNA binding-motifs	**Putative function**	**References**
TACpyAT repeats	organ-specific expression	van der Meer et al., 1992
Petunia Myb transcription factor, Ph3, binding site	petal epidermis-specific transcription factor	Solano et al., 1995
Maize MYB-homologus P gene-binding site	3-deoxyflavonoid and phlobaphene biosynthesis	Grotewold et al., 1994
ACGT-containing element (ACE)	transcription activation of *DFR* genes	Grotewold et al. 1994
MYB core binding sites	promoter activation	Blackwell and Weintraub, 1990
bHLH protein binding site	represented by the CANNG motif	Heim et al., 2003
Dof3 zinc finger transcription factors	tissue specificity and light regulation	Yanagisawa and Schmidt, 1999
GATA motifs	light dependent and nitrate dependent control of transcription	Reyes et al., 2004

tissue (Figure 5.8C). Localization of GFP protein throughout the cell and in the nucleus can be seen clearly. The black spot in the centre of the shot zone is where cells were damaged by bombardment under high-pressure conditions.

The activity of the *DFR* promoter, as the pDFRpromoter-GFP construct, was tested in white JFDP petals, pink LRF petals and JFDP leaves in the same way as the positive control. Northern analysis of cymbidium *DFR* showed that *DFR* was expressed at the highest levels in pink coloured petal tissues, but not in white flowers or leaves (Figure 5.3). Therefore it was expected that *DFR* promoter activity should be stronger in pink petals as compared with white petals, and therefore stronger reporter gene (*GFP*) expression is expected in pink petals. Transient expression results showed that no GFP foci appeared in petals from the pink LRF cultivar (Figure 5.9C). However, the *DFR* promoter was able to activate *GFP* in the white petals, displaying a few GFP foci (Figure 5.9A). A few green dots were visible in Figure 5.9E, although these are unlikely to be GFP expression, because they were not as bright as the GFP control (Figure 5.8C). These green spots are likely to be unknown particles on the surface of leaf tissues that reflect green light. The number of foci and extent of *GFP* expression driven by the *DFR* promoter was much weaker in the JFDP cultivar compared to *GFP* driven by the *CaMV35S* promoter, and this shows the *DFR* promoter is not as strong as the *CaMV35S* promoter.

DFR promoter activity was further investigated by co-expressing two transcription factors, *Lc* and *C1*, with the pDFRpromoter-GFP construct in cymbidium petals and leaves (Figure 5.9 B, D, F). *GFP* expression and the number of foci were increased in all tissues tested when *Lc/C1* were co-introduced with the *DFR* promoter::GFP construct (Figure 5.9B). The number of foci in the JFDP cultivar was increased compared to Figure 5.9A. There were no GFP foci observed in the LRF petals and JFDP leaf tissue when shooting the *DFR* promoter construct alone (Figure 5.9 C and E), but many GFP foci were observed when the *DFR* promoter was co-expressed with *Lc/C1* (Figure 5.9 D and F). The results showed that the *DFR* promoter appears to be activated by *Lc* and *C1* transcription factors.

A control experiment was carried out to ensure that the activation of the *DFR* promoter

DFR PROMOTER-F

-1544 GACGGCCCGGGCTGGTACTATTTGTAAGCTATTTTGCATTTAGTAGATTCCTAGCCAGGGG

ACE

-1483 CGGCTAGGCGTGAAGATAGGCAGGTTTTGAGATTTGGCCAAACCACGTAAAAATTGTATGC

MYB

-1422 CTATTGTCTCTCTTTACTTTACTGTACTTTTATTTTTACTGCGTTAAAAATTGTATCTTCT

-1361 CATTATGAAATCAAAAATTAATTTTCTACTAAAACTTAAAATTTAAGATTTAACTTTTAAA

-1300 ATTTTTAATATCACTCAATTCATCACCCCCCACCCCCTTAGGTGCTATAGTGACTCACACT

-1239 TTGCACTGTATTTTTTCCATCTTTCAAAAGCTTTCTATTTCTTTGGGTGGCTACTCTTTTT

ORGAN SPECIFIC EXPRESSION

-1178 GCCTTCACTAATGAAGACAACACAATACCATTATTTAACATTTTAAAACCCCTTATATATT

-1117 TTTTATTTTTATAGAAAAATATCTTTAGATTATAAATTTAACTATATTTCTTGAGTATAAT

BHLH

-1056 ATGTATTAGCTTATTCCTTGAGCATGTGTCACAGCCCCATTATATCAAAACATTTATATGT

BHLH

-995 ATATATATTTGATACTTGCCAAACTGTGGAGTTGAAGAGTTTGACCTCACACATCCAAATG

BHLH

-934 TTTTTTAAGATGAGAAGGAATAACAAATTTGAACACTCATGTGTGCGTGCGTGTGTGTGTG

-873 TGTGTGAGAGAGAGAGAGAGAGAGAGAGAGTATTTTTAATTCTAAGCAAAGGTTTAAACTA

-812 GTGGTTCATGTATATTTTGGAATATCTTTAGGAGCATTAATTTTATGGCCATATTATGAAA

Myb, PH3 BHLH

-751 GATGAATGCTATGATAACCTTTTTATATAACGGGATTGAATCACATATGCAAAAAATAATG

DOF

-690 AAAAATATGTTACTCTTTACTGAGCTGTGATTTAAGATCATAAGTGTTTAAAAGGTGTGCT

DOF BHLH

-629 TATGTGGAAGAATCTTAAAGTCTCTTACCACCCAACTGAAGATTTAAAATATCCGAGCATC

DOF

-568 GAGATCGAAAGTGCTCCTCTTTTTTATTTCATATACGTTTTTTTGCATCAGATTTTATTTT

DOF

-507 CCAAAGTGGTGGATGAAGATCTACGGAGAAGTTTTGGCACAGTAGGGGTGACCTTATTTTG

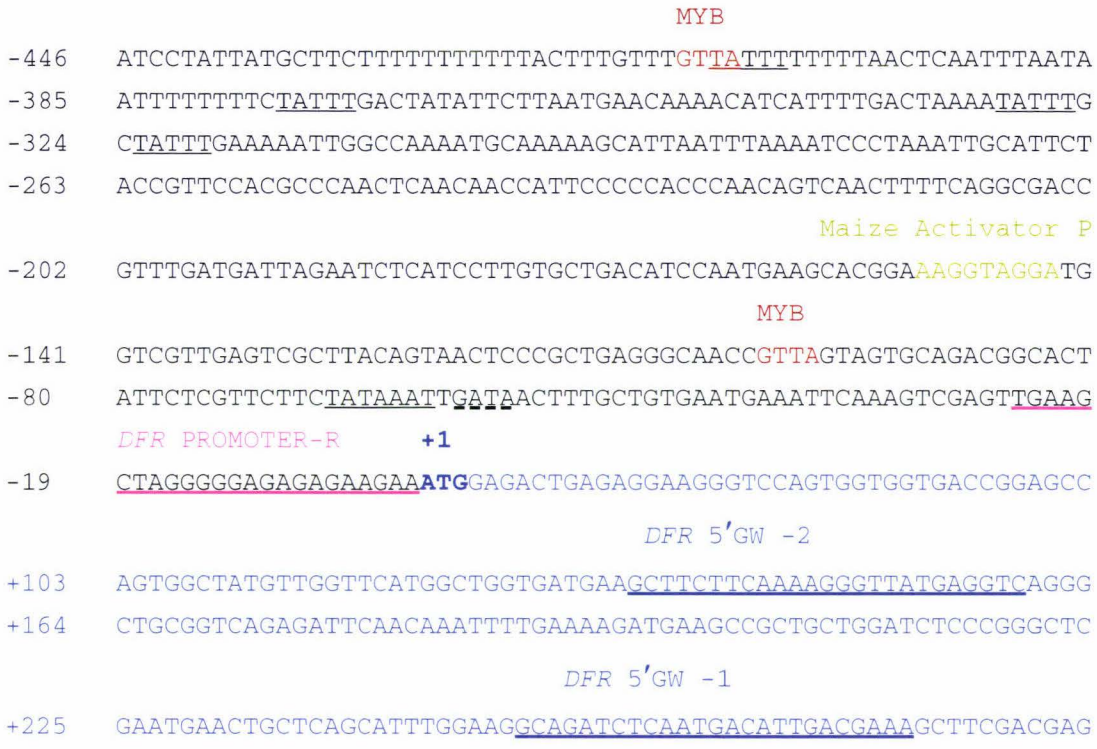


Figure 5.6: Sequence of the *DFR* promoter fragment isolated from floral tissues of the cymbidium cultivar. The *DFR* upstream region (1544 bp) and 225 bp of the partial coding region (in blue) are indicated. The TATA-box (underlined), the CATA-box (dash underlined) and the ATG codon (+1 in bold) are indicated. A TACpyAT box (organ-specific expression site) overlapping with the TATA-box is double underlined. The core MYB regulatory motifs are indicated and labelled in brown. The ACE element is labeled in orange. Dof3 elements for zinc finger transcription factor are labelled in green. The bHLH binding site or the R-motif is labelled in pink. The maize activator P of flavonoid biosynthetic genes is labelled in yellow/green; Myb transcription factors for petal epidermis-specific from petunia (PH3) are labelled in purple. Primers used to isolate the promoter region are underlined in pink, and primers used for gene walking to obtain the promoter region are underlined in blue.

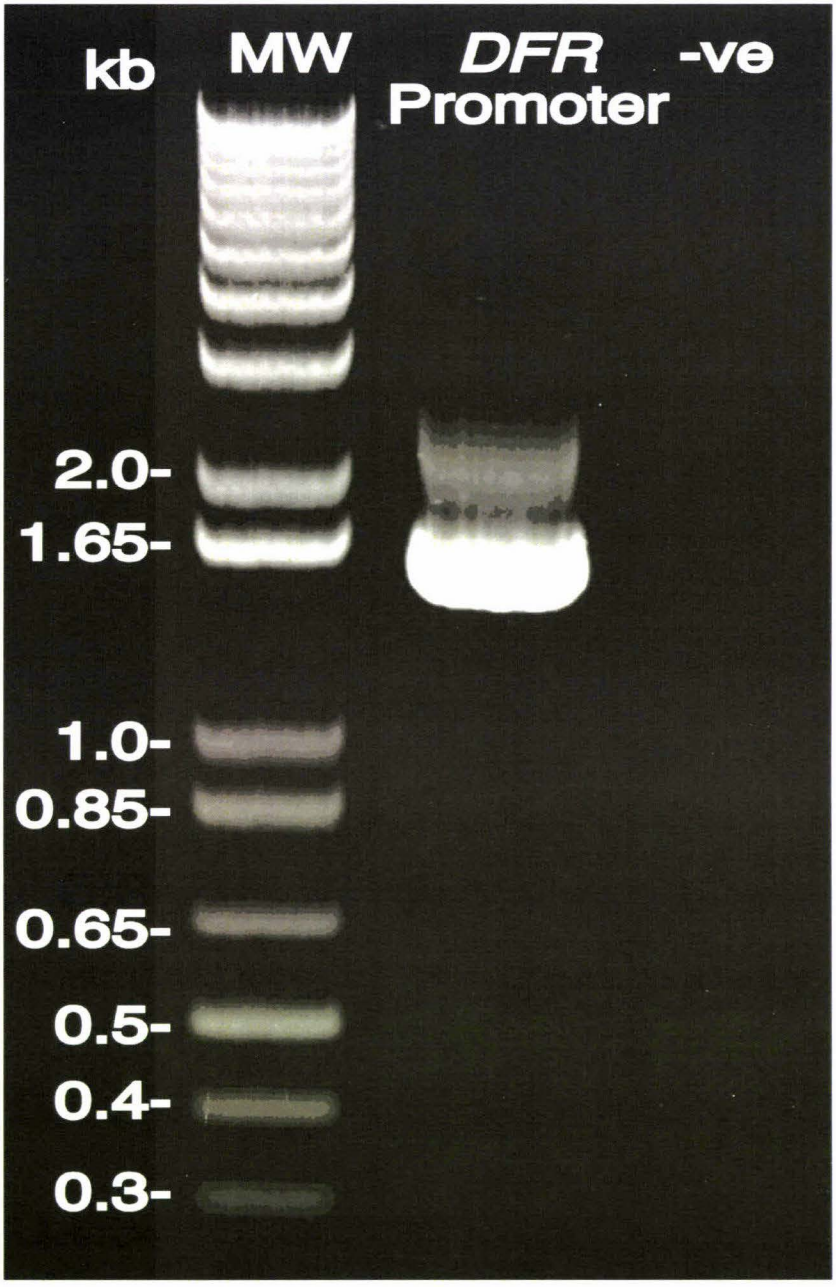


Figure 5.7: PCR amplification of the cymbidium *DFR* promoter region. -ve, no template control. Molecular weights of 1 Kb+ DNA ladder are marked.

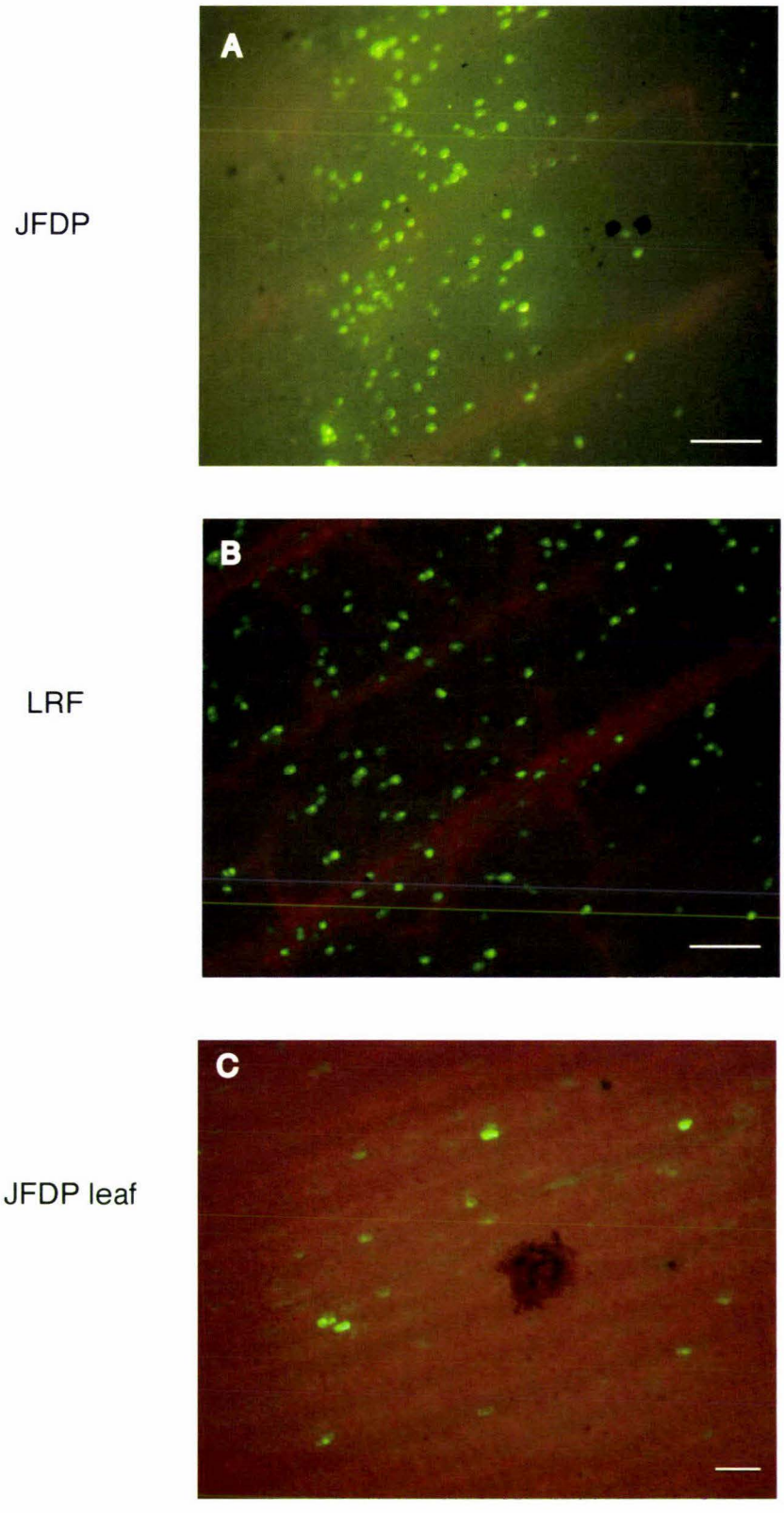


Figure 5.8: Transient expression of *GFP* driven by *CaMV35S* promoter (pPEP-GFP) in petal and leaf tissues of cymbidium orchid. **A**, petal tissue of JFDP, scale bar = 1 mm; **B**, petal tissue of LRF, scale bar = 1 mm; **C**, JFDP leaf tissue, Scale bar = 250 μm. All images were taken under the blue light at 48 h post transformation.

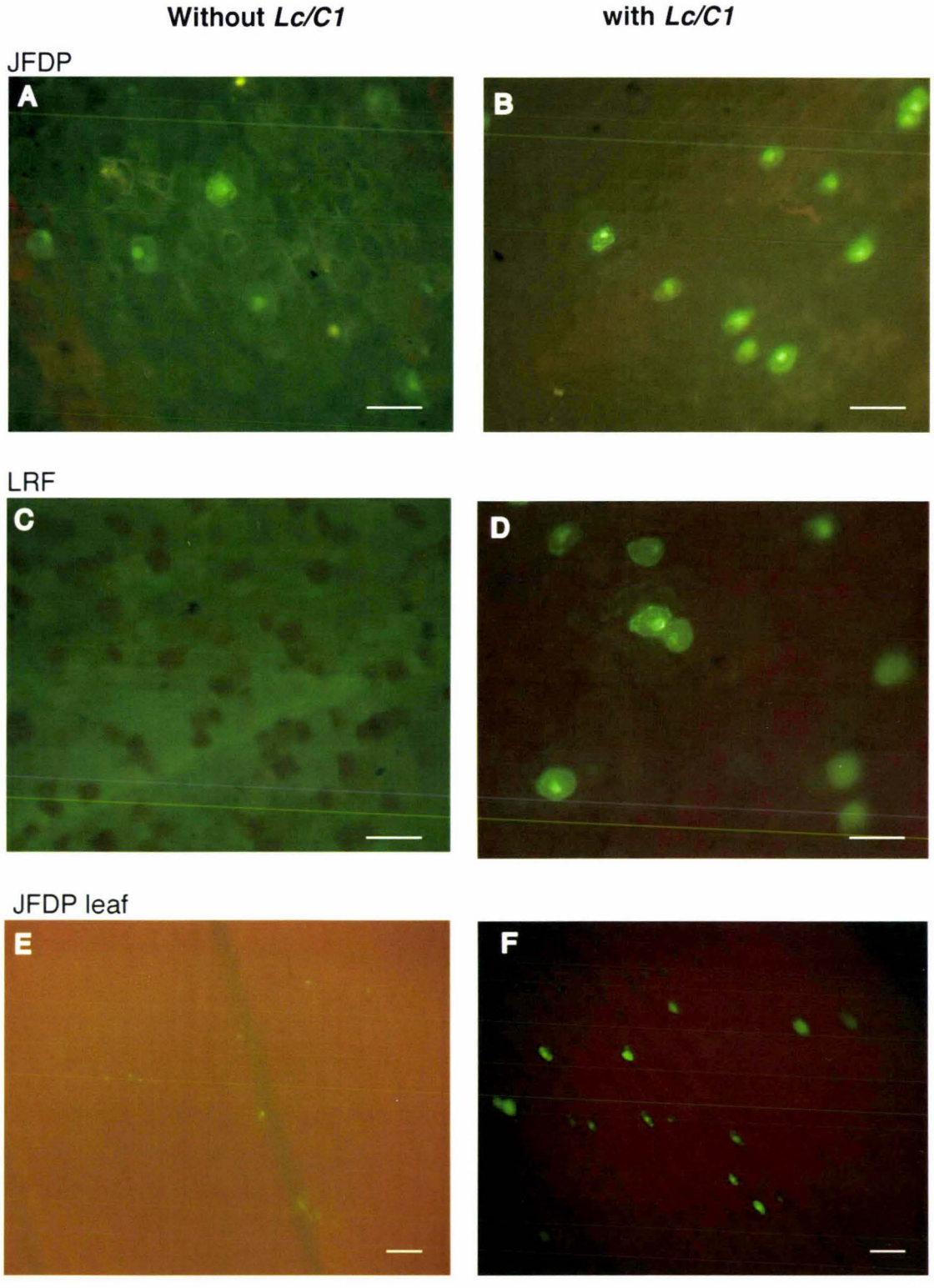


Figure 5.9: Transient expression of *DFR* promoter::*GFP* with and without co-expression of *Lc* and *C1* in petal and leaf tissues of cymbidium orchid. **A**,**B**, petal of JFDP; **C**,**D**, petal of LRF; **E**,**F**, JFDP leaf tissue. **A**, **C**, **E** expression of pDFRpromoter-GFP; **B**, **D**, **F** are co-expression of *Lc*/*C1* with pDFRpromoter-GFP. All images were taken under the blue light at 48 h post transformation. Scale bar = 250 μm. The red background in **E** is fluorescence from chlorophyll under long exposure to blue light.

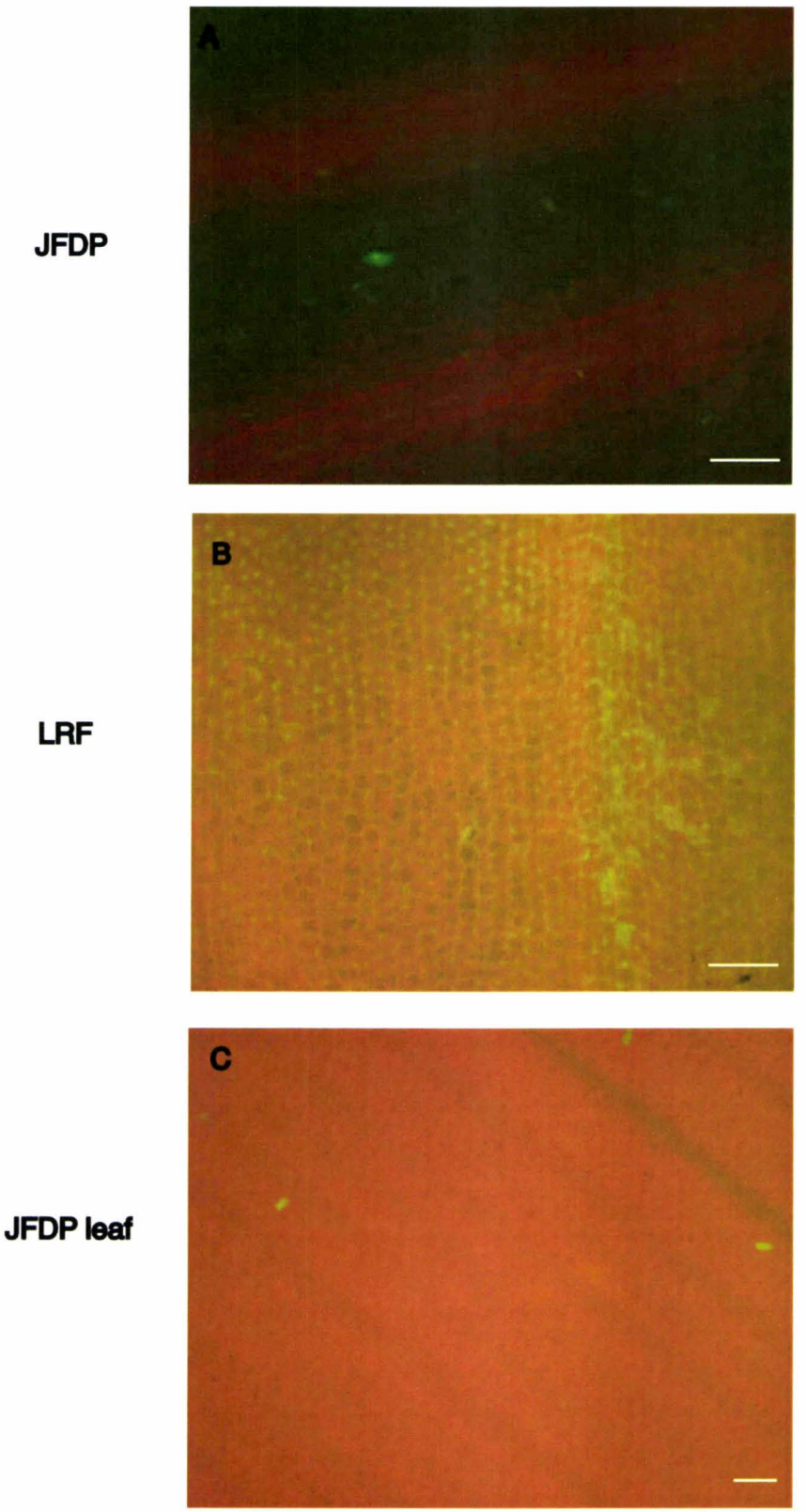


Figure 5.10: Transient expression of pART7+pDFRpromoter-GFP in floral and leaf tissues of cymbidium orchid. The red and green or yellow background shown in the images is fluorescence from chlorophyll and lignin under long exposure to blue light. **A**, JFDP petal tissue, scale bar = 1 mm; **B**, LRF petal tissue, scale bar = 1 mm; **C**, JFDP leaf tissue, scale bar = 250 μm.

was due to *Lc* and *C1* transcription factors, and was not affected by the presence of a *CaMV35S* promoter. The pART7 vector, which has a *CaMV35S* promoter but no *GFP* reporter gene, was co-introduced with the *DFR* promoter::GFP construct. In these assays, no GFP foci were observed in petals from LRF cultivar and leaf from JFDP cultivar, but the *DFR* promoter was able to activate *GFP* in the white petals from JFDP cultivar (Figure 5.10).

5.4 Discussion

A cDNA was isolated successfully from floral tissue of the cymbidium orchid cultivar, CASP, showing that *DFR* is present and expressed in the CASP flower. The sequence isolated from the CASP cultivar is consistent with a previously published *DFR* sequence (AF017451), with some minor differences between the two sequences probably due to the different cultivars studied.

Southern analysis of *DFR* in cymbidium indicated two *DFR* homologues might be present in the genome of the JFDP and LRF cultivar, based on DNA digested with *Eco*RI and *Bam*HI. The *DFR* probe has one internal *Hin*dIII recognition site, and so in the *Eco*RI and *Bam*HI digests, four hybridization fragments in the *Hin*dIII digest were expected. Only three bands, however, were apparent in the blot. This result suggests there could be up to two *DFR* genes in the JFDP and LRF genome, although since commercial cymbidium cultivars are triploid or tetraploid, this may indicate a single gene per haploid genome. This is consistent with findings in the cymbidium cultivar 'Rosannagirl Mild', where a single *DFR* gene was found (Johnson et al., 1999). Similarly, a single *DFR* gene is present in the genome of *Bromheadia finaysoniana*, barley (*Hordeum vulgare*) and rice (*Oryza era*) (Kristiansen and Rohde; 1991; Chen et al., 1998; Liew et al., 1998b). The possibility, however, that the cymbidium genome contains multiple *DFR* genes cannot be ruled out. As *DFR* was isolated by RT-PCR from the floral tissues alone, there might be another *DFR* gene that is expressed in other parts of plant. In the orchid *Oncidium* Gower Ramsey, petunia, Japanese morning glory (*Ipomoea nil*) and *Gerbera hybrida*, *DFR* is present as three genes, but only one gene is active in flower and its expression pattern correlates with anthocyanin accumulation

(Beld et a., 1989; Helariutta et al., 1993; Inagaki et al., 1999; Hieber et al., 2006). The genus *Cymbidium* contains a range of chromosome numbers (Tomlinson, 1985), and hybridization during breeding may have caused further variation. The number of *DFR* genes in each cymbidium cultivar may therefore depend on the ploidy of the cultivar.

DFR expression correlated with the amounts of cyanidin accumulated in the plant tissue. Cymbidium *DFR* was most highly expressed in the floral tissues of the pink NJG cymbidium cultivar, which has the highest amounts of cyanidin/peonidin anthocyanins among the cultivars examined. There was moderate to low expression in the pink lip tissues of the VMB, JFDP and PC cultivars, while the white and green petal tissues of the PC, JFDP and VMB cultivars and leaf tissue did not exhibit *DFR* expression (Figure 5.3). This shows that anthocyanin accumulation is correlated with *DFR* expression. Temporal expression analysis during flower development also showed that the *DFR* expression pattern generally coincides with colour change. *DFR* had highest expression in the bud stages of the NJG cultivar, and expression was not detectable in sepals and petals of late developmental stages where anthocyanin was already present. This expression pattern is similar to that reported in flowers of the *Dendrobium* orchid (Mudalige-Jayawickrama et al., 2005). The *DFR* expression pattern in *Oncidium* Gower Ramsey, however, is differ as the *DFR* gene expression is also up-regulated in sepal, petals and the lip crest of fully open flowers (Hieber et al., 2006).

DFR expression in the lip is regulated differently compared to the petal and sepal tissues. *DFR* is continually expressed in late stages in lip tissues of the NJG and JFDP cultivar, whereas expression declined in sepal and petal at the later developmental stages. The lip turns more pink as senescence takes place, while the colour in the sepals and petals show no further development when those tissues have matured. This may be the reason that *DFR* was expressed in lip tissues throughout the later stages of flower development. The expression pattern in the lip of the VMB and PC cultivar is, however, different from the expression pattern observed in the lip of the NJG and JFDP cultivars, where no expression was detected in late stages. The reasons for differential *DFR* regulation in lip tissue of different cultivars are not known.

Results from this study showed that while *DFR* is expressed in floral tissue, it is not expressed in leaves. This is consistent with studies with other orchid genera. *DFR*

expression is not found in the root, pesudobulb or leaves of dendrobium (Mudalige-Jayawickrama et al., 2005), or leaves in oncidium (Hieber et al., 2006). Thus the expression profile of cymbidium *DFR* makes its promoter a possible candidate for tissue-specific expression of genes for flower colour manipulation.

The activity of the cymbidium *DFR* promoter was examined using a transient expression system to determine if it was suitable as a floral tissue specific promoter. The results obtained suggested that firstly the promoter was able to drive GFP expression but only relatively weakly. Expression was enhanced with the addition of the maize transcription factors, *Lc*/*C1*. Secondly, the *DFR* promoter activity is not constitutive. *DFR* gene expression is developmentally regulated and therefore will be stronger at certain stages of flower development. The stage five and six petals used for transient expression experiments were probably not ideal. *DFR* expression would possibly have been higher at earlier stages of development, when the *DFR* promoter would benefit from the promoter ofhigher endogenous transcription factor activities. Finally, transient expression is a crude method to assess promoter activity as the plant tissue is damaged in the transformation zone and many copies of the construct are introduced into each cell. Shooting conditions may mean that interactions between recombinant transcription factors and promoters are not likely to occur in the same way as normally.

It is possible that the *DFR* promoter may be useful for tissue occur *in vivo* specific modification of anthocyanin biosynthesis but only at certain stages of flower development. Further research is required. Currently, anthocyanin biosynthesis is believed to be regulated by transcription factor complexes that includes both a MYB and bHLH transcription factors (Griesbach and Klein, 1993; Ma et al., 2008). The results reported here, suggest such a model may apply in cymbidium too.

Analysis of the promoter sequence upstream of the coding region predicted several core sequences for bHLH and MYB protein binding sites, suggesting *DFR* may be regulated by MYB and bHLH type transcription factors. This included a maize MYB-homologous P gene binding site, which has been reported to recognize and bind to the sequence CCT/AACC, and activates transcription of the *A1* gene required for 3-deoxyflavonoid and phlobaphene biosynthesis in maize floral organs (Grotewold et al., 1994). The P

transcription factor site is found in rice, barley and wheat *DFR* promoters, and appears to be the key transcription factor necessary for activation of *DFR* in these plants (Nakai et al., 1998; Kristiansen and Rohde, 1991; Himi and Noda, 2004). The Dof3 zinc finger transcription factor binding sites found in the cymbidium *DFR* promoter sequence are also found in the *DFR* promoter of wheat and orange (Himi and Noda, 2004; Lo Piero et al., 2006), and have been reported to be involved in tissue specificity and light regulation (Yanagisawa and Schmidt, 1999). The presence of Dof3 domains indicated that *DFR* promoter activity might respond to light. The TACPyAT repeat in the promoter was found to be involved in the regulation of floral-specificity of the *chsA* gene in *Petunia hybrida* (van der Meer et al., 1992), and was also identified in orange and wheat *DFR* promoters (Lo Piero et al., 2006; Himi and Noda, 2004). It must be emphasized that these potential transcription factor binding sites identified using bioinformatics are only suggestions of what regulatory element might be involved in promoter activation. More detailed promoter analysis, such as promoter deletions, is needed to identify the function of elements involved with tissue-specificity and promoter activity.

In summary, this study showed that *DFR* gene expression is correlated with cyanidin-based anthocyanin accumulation in floral tissues of cymbidium orchid, and the expression of *DFR* is developmentally regulated. *DFR* is active in early flower development in both petal and sepal tissues but may remain active in lip tissue at later stages of development. The cymbidium flower has a complex developmental pattern in the flower which lip morphology and pigmentation pattern regulated separately from sepals and petals. Thus it is likely that *DFR* activity is regulated separate in the lip. Analysis of the *DFR* promoter sequence and the ability of the *Lc* and *C1* transcription factor to up-regulate *DFR* expression implicates these factors in the regulation of anthocyanin biosynthesis, but this would need to be examined in future studies.

Chapter 6

Isolation of chalcone synthase (*CHS*) cDNA and promoter region

6.1 Introduction

Chalcone synthase (CHS) is an important enzyme in the flavonoid biosynthetic pathway, providing the entry point to the pathway by catalysing the reaction for chalcone formation from which production of various flavonoids subsequently occurs (see Figure 1.4). The importance of CHS means it is the most studied enzyme in the phenylpropanoid pathway and is particularly well characterized in petunia (Koes et al., 1987), parsley (Kreuzaler et al., 1983) and pea (Ito et al., 1997). The crystal structure of CHS has been resolved indicating it is a thiolase-fold enzyme (Ferrer et al., 1999). The CHS protein structure is highly conserved, with conserved cysteine and histidine residues important for catalytic function (Tropf et al., 1995). Genetic modification of *CHS* expression have mostly focused on down-regulation of CHS activity, using either sense or anti-sense silencing constructs to create lighter coloured or white flowers in several plant species (Que et al., 1997; Aida et al., 2000b). Changes to CHS activity may lead to changes in anthocyanin accumulation in flowers, but other deleterious side effects have also been reported. Mutant maize and petunia that lack CHS activity, for example, often lack pigmentation in the flowers, pollen and seed coat, but also show reduced fertility (Franken et al., 1991; Mo et al., 1992; Napoli et al., 1999).

Flavonoids have multiple roles in plants including UV photoprotection, antibacterial defence and pollen fertility and all rely on CHS activity (Harborne and Turner, 1984; Dixon, 1986; Schmlezer et al., 1988; Taylor and Jorgensen, 1992). *CHS* expression can be induced by abiotic stress, UV light, wounding and pathogen infection, leading to the accumulation of anthocyanin or other flavonoids in the responsive tissues. Plants

contain multiple copies of *CHS* (Holton and Cornish, 1995), possibly to allow its diverse biochemical functions. Many different *CHS* genes may be expressed in different tissues of the plant, with each CHS protein potentially associated with different functions and exhibiting specific responses to developmental and environmental signals.

Orchid *CHS* genes have been identified in *Phalaenopsis*, *Dendrobium* and *Bromheadia finlaysoniana* (Han et al., 2005; Mudalige-Jayawickrama et al., 2005; Leiw et al., 1998a), but so far there is no information available for cymbidium. Therefore, the main objectives of this chapter are:

1) Isolate the coding region of the *CHS* gene and to characterize *CHS* expression and genomic organization in different cymbidium cultivars.
2) Isolate the cymbidium *CHS* promoter. *CHS* is usually expressed very strongly and therefore the promoter is potentially useful as a tissue-specific promoter.

6.2 Materials and methods

6.2.1 Plant materials and extraction of RNA and DNA

Fresh flower stems and leaf tissues of selected cymbidium cultivars were obtained from commercial growers, then kept in a controlled vase-life room and samples were collected as described in Section 2.3.

Floral tissues of the pink cultivar, Narella Jennifer Gail (NJG), were collected as combined stages one to three and combined stages four to six, while sepals and petals tissues were pooled during the harvest, and lip tissues were collected separately. Total RNA was extracted from all collected samples and subsequently used for cDNA synthesis (Section 2.6.1). For northern analysis experiments, floral and leaf tissues were collected from a range of developmental stages of different cymbidium cultivars (Section 5.2.1). Genomic DNA for genome walking experiments was extracted from floral tissue at all stages from the maroon cultivar, Clarisse Austin South Pacific (CASP). Genomic DNA for Southern analysis was extracted from floral tissues of white cultivar, Jung Frau dos Pueblos (JFDP), and pink cultivar, Lisa Rose Flamingo (LRF).

6.2.2 Isolation of cymbidium *CHS* and promoter

6.2.2.1 Reverse transcription PCR (RT-PCR)

RT-PCR was used to isolate a partial *CHS* fragment. Total RNA was isolated using the hot borate method as described in Section 2.6.1. cDNA from the petal and sepal tissues of the NJG cultivar was synthesized with the KSDT primer (listed in Table 6.1) as described in Section 2.4.1. A pair of degenerate primers and a nested degenerate primer were designed from consensus amino acids domains, YQQGCFAG and DWNSIFW, based on an alignment of CHS protein sequences from the following monocotyledonous plants: dendrobium (CAL15003, AAU93767, ABE77392), bromheadia (AAB62874, AAB62875, AAB62876), phalaenopsis (AAY83389, AAV70116, AAP34702, AAX54693), *Lilium* (AAD49355, BAB40787, AAD49353, BAB40786) and *Z. mays* (CAA42764, AAW56964, CAA42763). An alignment at the nucleotide level corresponding to the chosen conserved amino acid regions was taken into consideration for designing degenerate primers to decrease degeneracy. The sequences of degenerate primers are listed in Table 6.1.

The partial *CHS* fragment was isolated in two rounds of PCR reactions. The first round of PCR amplification used '*CHS* degenerate-F' and '*CHS* degenerate-R' primers, and 1 µL of cDNA from the NJG cultivar (5 × dilution) as template. One µL of PCR product from the first round was used as template for the second round PCR. Primers '*CHS* degenerate nested' and '*CHS* degenerate-R' were used for amplification. An *Antirrhinum* aurone mutant cDNA was used as template for a positive control of *CHS* amplification. Negative controls included no template and single primer controls. *Taq* polymerase was used in PCR experiments, with the following conditions: 94°C for 2 min, 35 cycles of 30 s at 94°C, 30 s at 50°C, 30 s at 72°C, followed by a 5 min extension at 72°C. PCR products were cloned into pGEM-T Easy and sequenced as described in section 2.5.

6.2.2.2 Rapid amplification of cDNA ends (3′-RACE)

The downstream region of the cymbidium *CHS* sequence was obtained using 3′-RACE. The '*CHS* 3′ RACE-1' and '*CHS* 3′ RACE-2' primers were designed at 823 and 859 bp of the partial coding sequence obtained from RT-PCR. One µL of 'stage one to three cDNA from the NJG cultivar' (5 × dilution) was used as template for the first round of 3′ RACE, and '*CHS* 3′ RACE-1' and 'KS adaptor' primers were used for the amplification. The second round PCR was carried out using 1 µL of undiluted PCR product from the first round, and '*CHS* 3′RACE-2' and 'KS adaptor' primers for the amplification. Both RACE reactions were carried out under the following conditions: 94°C for 2 min, 35 cycles of 30 s at 94°C, 30 s at 55°C, 1 min at 72°C, followed by 5 min at 72°C. All primer sequences are listed in Table 6.1.

6.2.2.3 Genome walking to amplify the 5′ end of the gene and the promoter

Genomic DNA was digested with *Hpa*I, *Sca*I, *Stu*I, *Eco*RV and *Dra*I blunt end cutter enzymes, and the resulting DNA fragments were ligated to the gene walker adaptor as described in Section 5.2.4.2. Two antisense gene-specific primers were designed based on the *CHS* sequence obtained from the RT-PCR. The '*CHS* 5′ GW -1' and '*CHS* 5′ GW-2' primers (Table 6.1) were used in the first round and second round of genome walking PCR, respectively. All the PCR amplifications were carried out following the GenomeWalker™ Universal Kit user manual.

6.2.2.4 Cloning the *CHS* ORF

The ORF of the *CHS*stuI and *CHS*scaI genes were amplified from cDNA from the NJG cultivar using PCR. Two separate gene specific forward primers '*CHS*stuI -F' and '*CHS*scaI -F' were designed based on sequence identified by genome walking, and were used to amplify *CHS*stuI and *CHS*scaI, respectively. The primer '*CHS*-R' was used as the reverse primer for both amplifications. Both *CHS*stuI and *CHS*scaI primers started from the start codon of *CHS*, and '*CHS*-R' reverse primer started from the stop codon of the coding region (Table 6.1). The PCR reaction using *Pwo* polymerase was as follows:

94°C for 2 min; 35 cycles of 30 s at 94°C, 30 s at 65°C, 2 min at 72°C; and 5 min at 72°C.

Table 6.1: Primers used for the amplification of the *CHS* coding region and the *CHS* promoter using PCR.

Primer name	Sequence (5′-3′)	Tm (°C)
KSDT	CGGTACCGATAAGCTTGAT$_{19}$	58.5
KS	CGGTACCGATAAGCTTGA	
CHS degenerate nested	ARCARGGNTGUTTUGCNG	56.8
CHS degenerate-F	TAYCARCARGGNTGYTTYG	54.0
CHS degenerate-R	CCARAADATNSWRTTCCARTC	54.4
CHS 3′RACE-1	TTCTACATCTCAAGTCTTACTGC	56.9
CHS 3′RACE-2	TATTGGAGGCCATGTAAGTGAG	62.7
CHS 5′GW-1	ACCCACTATAAGTGCGGAAGC	64.0
CHS 5′ GW-2	ATAAAGCTTGGGTAACAAGGTC	59.9
*CHS*scaI-F	ATGCCGAGCCTTGAATCCAC	68.4
*CHS*stuI-F	ATGCCGAGCCTTGAATCCGTT	70.7
CHS-R	TTAAATAGGGACACTATGAAGAACGACAG	65.7
*CHS*scaI promoter-F	TGAGCTCACTTTCTCGTGCTTAAAAAGG (*Sac*I)	70.4
*CHS*scaI promoterR	TCTCGACTGTGGGAAGTTTTGTGATT (*Xho*I)	69.4
*CHS*stuI promoter-F	TGAGCTCGCGGCCGCCCTAGCCCAGTAAGAATGT TCCAC (*Sac*I *Not*I)	87.8
*CHS*stuI promoter-R	TGAATTCGGTGGGAGGTTTTGATAATTTTTG (*Eco*RI)	73.2

6.2.2.5 Cloning the *CHS* promoter sequence

Two promoter-specific primers (*CHS*stuI promoter-F and R primers) were designed according to the sequence of information from the genome walking to amplify the *CHS*

promoter from the *Stu*I generated CASP gDNA library and uncut gDNA. The forward primer has *Sac*I and *Not*I sites, and the reverse primer has an *Eco*RI site at the 5′-end of the primer for the purpose of subsequent cloning (Table 6.1). *CHS*scaI promoter-F and R primers were designed to amplify the *CHS*scaI promoter from the *Sca*I generated CASP gDNA library and uncut gDNA. The forward primer has a *Sac*I site, and the reverse primer has an *Xho*I site at the 5′-end of the primer for cloning (Table 6.1). The negative controls set for PCR comprises amplification without template. All PCR reactions were performed using *Pwo* polymerase using the following conditions: 94°C for 2 min; 35 cycles of 30 s at 94°C, 30 s at 60°C, 3 min at 72°C; and 5 min at 72°C.

6.2.3 Molecular analysis of *CHS* gene

The RNA and genomic DNA blots used for northern and Southern analysis, respectively, were the same membranes probed as described in Chapter Five. The blots were stripped and hybridised with a labelled probe derived from the PCR fragment of *CHS* generated uisng 3′-RACE. The probe labelling, hybridization and washing are described in Section 2.7.3. The image of the blot was developed as described in Section 2.7.3.4.

6.2.4 Assembly of *CHS* promoter::*GFP* construct

The activation of the *CHS*stuI and *CHS*scaI promoters were tested transiently in petals of the pink NJG cymbidium cultivar and the white JFDP cultivar and leaf tissues of the JFDP cultivar. The *CHS* promoters were cloned into a pART7-based construct, pPEP-GFP, which contains a *GFP* reporter gene. The *CHS*stuI promoter replaced the *CaMV35S* promoter in the pPEP-GFP construct using *Sac*I and *Eco*RI restriction sites, and the *CHS*scaI promoter replaced the *CaMV35S* promoter using *Sac*I and *Xho*I sites. The resulting constructs were termed pCHSstuIpromoter-GFP and pCHSscaIpromoter-GFP. The cloning procedures were described in Section 5.2.5.2. The transient gene expression of the pCHSstuIpromoter-GFP was carried out as described in Section 5.2.5.3, but 4 μg of plasmid DNA was used, in the presence and absence of maize *Lc* and *C1* transcription factors. GFP foci were assessed as described in section Section 2.8.6.

6.3.1 Cloning of partial *CHS* cDNA from cymbidium

A partial *CHS* cDNA was isolated by RT-PCR using degenerate primers (Figure 6.1). There was no visible PCR product after initial PCR amplification (data not shown). A PCR product (~ 400 bp) was amplified in the second round of PCR from the cDNAs transcribed from the RNA of the floral tissues collected as the early developmental stages (stage one to three), but no product was amplified from cDNA amplified from the later stages of floral development. The positive control, which used an *Antirrhinum* aurone mutant cDNA as template, displayed a strong band, indicating that degenerate primers and the PCR conditions were both suitable for *CHS* amplification. No PCR product was amplified from the 'no template' and 'single primer' controls, which indicated that the PCR product obtained using cDNA from developmental stages 1-3 of the NJG cultivar was not due to contamination or to non-specific primer binding. DNA sequencing showed the partial sequence was 422 bp in length, and NCBI BLAST searching showed that this partial sequence shares ~90% identity with *Oncidium* Gower Ramsey *CHS1* and *CHS2* genes, between 80%-89% identity with phalaenopsis *CHS*, phalaenopsis *bibenzyl synthase*, *Oncidium* Gower Ramsey *CHS3*, and *Bromheadia finlaysoniana bibenzyl synthase* genes, and ~70% identity to dicotyledon *CHS* such as *Gerbera hybrida*. This result indicates that the partial fragment isolated is likely to be *CHS*. The same set of degenerate primers was used to isolate partial *CHS* cDNA fragments from the CASP and LRF cultivars as well, and DNA sequences from both varieties were identical to those amplified from cDNA from the NJG cultivar (data not shown).

The downstream sequence of the known *CHS* fragment (see above) was amplified by 3′-RACE using the same cDNA as template. A band of approximately 650 bp was amplified in the second round of PCR (Figure 6.2). There was no specific PCR product amplified from round one (data not shown). The DNA sequence showed this fragment was 619 bp in length, and the overlapping region of the two amplified sequences was found, confirming this 3′-RACE fragment was from the same cDNA of the known fragment. The 3′-RACE sequence was used as the query sequence is BLAST searching

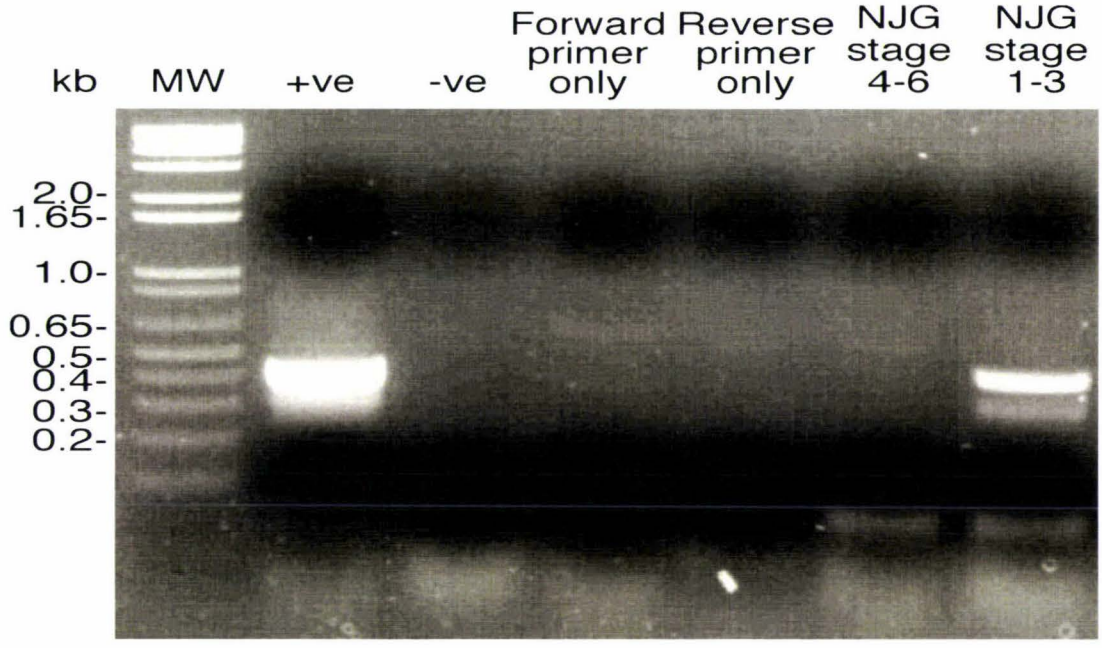


Figure 6.1: PCR amplification of partial *CHS* cDNA using *CHS* degenerate primers. Lanes from left to right: 1kb+ molecular weight marker; +ve, *Antirrhinum* aurone mutant cDNA as template positive control; -ve, no template control, Forward primer only control; Reverse primer only control; PCR using stage 4-6 cDNA from the NJG cultivar as template; PCR using stage 1-3 cDNA from the NJG cultivar as template.

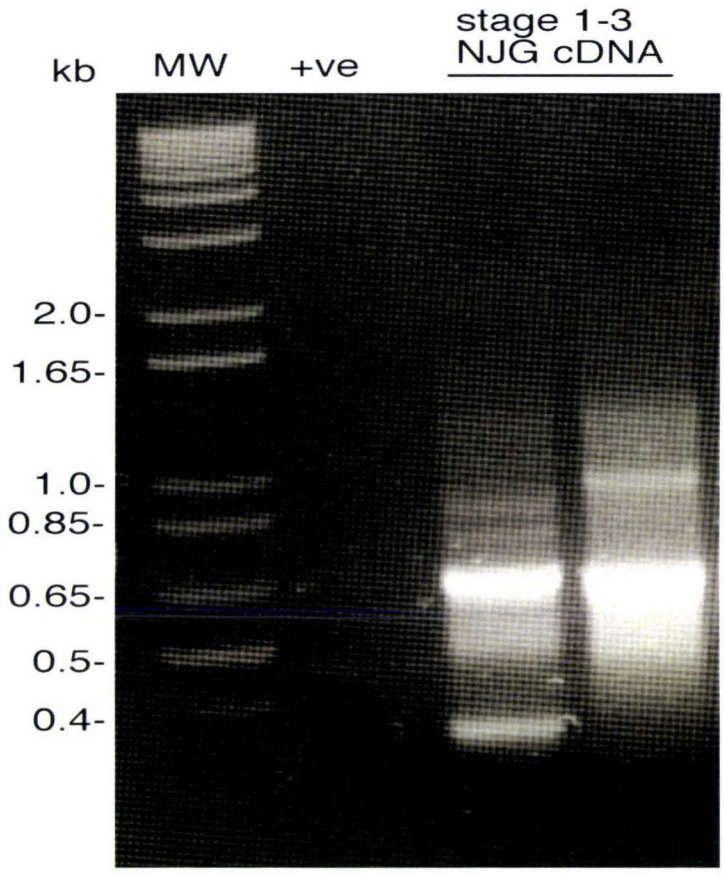


Figure 6.2: PCR amplification of downstream sequence of *CHS* cDNA by 3′RACE. cDNA of the NJG cultivar synthesised from combined RNA extracts from stage one to three flower tissues was used as template. A 1kb+ molecular weight marker is labelled; -ve, no template control.

in NBCI databases, and the result showed a high identity (~90%) to oncidium *CHS* with a percentage identity similar to the BLAST result obtained from the RT-PCR fragment.

6.3.2 Upstream coding region and the promoter of *CHS* from gDNA

The upstream coding sequence of the *CHS* as well as the promoter region was amplified by genome walking. Many non-specific PCR products were present in all libraries at the end of the first round of PCR (Figure 6.3A), while the second round of amplification amplified more specific PCR products. Two major bands with sizes of ~ 2.2 kb (band i) and 1.7 kb (band ii) were amplified from the *Sca*I library, two bands of ~1.8 kb (band iii) and ~700 bp were also amplified from the *Stu*I library, a band ~ 400 bp was amplified from the *Hpa*I library, a ~1 kb band was amplified from the *Eco*RV library and a PCR product of ~850 bp was amplified from the *Dra*I library (Figure 6.3B). The larger PCR products (bands i, ii and iii) were selected and cloned into pGEM-T Easy. The screening of positive clones was carried out by digest the pGEM-T Easy clones with *Eco*RI, which allows the drop out of the PCR fragment. Screening for positive clones by *Eco*RI digestion did not identify any positive clones from band i of the *Sca*I library. The positive clones identified from band ii of the *Sca*I library showed there are two internal *Eco*RI sites, while the positive clone from band iii of the *Stu*I library did not have an internal *Eco*RI site. The DNA sequences of band ii and band iii clones were slightly different but both were predicted to be part of a *CHS* gene. The primer and the known sequence obtained from RT-PCR were found in both sequences.

The DNA sequence of *CHS*stuI and *CHS*scaI obtained from genome walking showed both sequences shared a high degree of similarity in the upstream coding region, but were not identical. The two clones differed from each other in particular in the first 282 bp of coding sequence. The intron of *CHS*scaI is 105 bp, which is slightly longer than the intron of *CHS*stuI (99 bp). The *CHS*stuI and *CHS*scaI protein sequences differ from each other at nine amino acid residues. Cymbidium *CHS*stuI and *CHS*scaI promoter fragments (without coding region) were also amplified using two different sets of promoter-specific primers. *CHS*stuI had a larger PCR product ~ 1.5 kb, whereas *CHS*scaI was ~1.2 kb (Figure 6.4). The promoter sequences are very different over the two sequences (see Appendix 4).

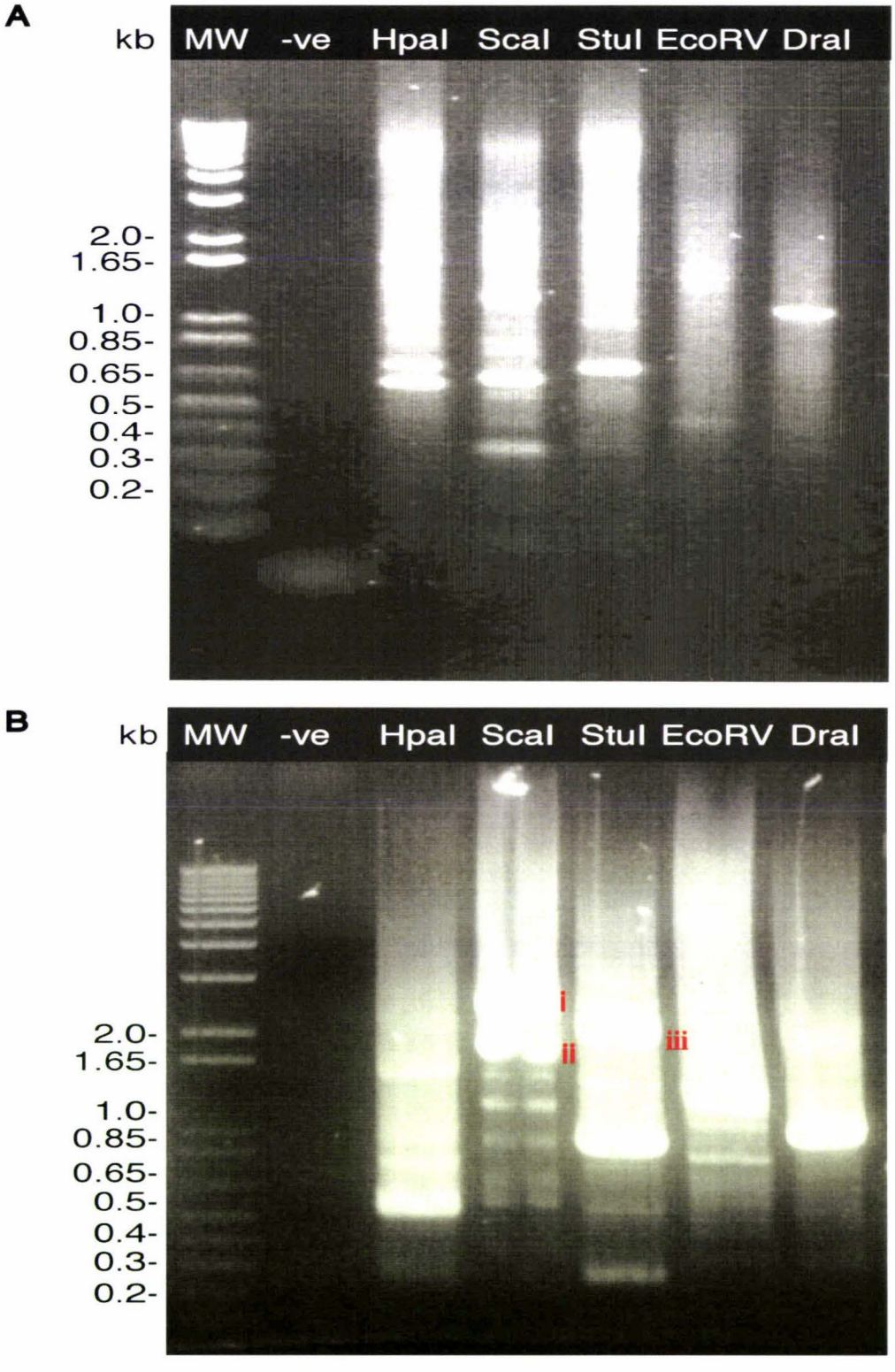


Figure 6.3: Genome walking amplified upstream regions of cymbidium *CHS* coding sequence and promoter. **A**, result of the first round PCR reaction; **B**, result of the second round PCR reaction. -ve, no template control. The genomic DNA libraries used for walking are labelled. Size of the molecular ladder is indicated. Bands labelled with numbers are the ones that were cloned.

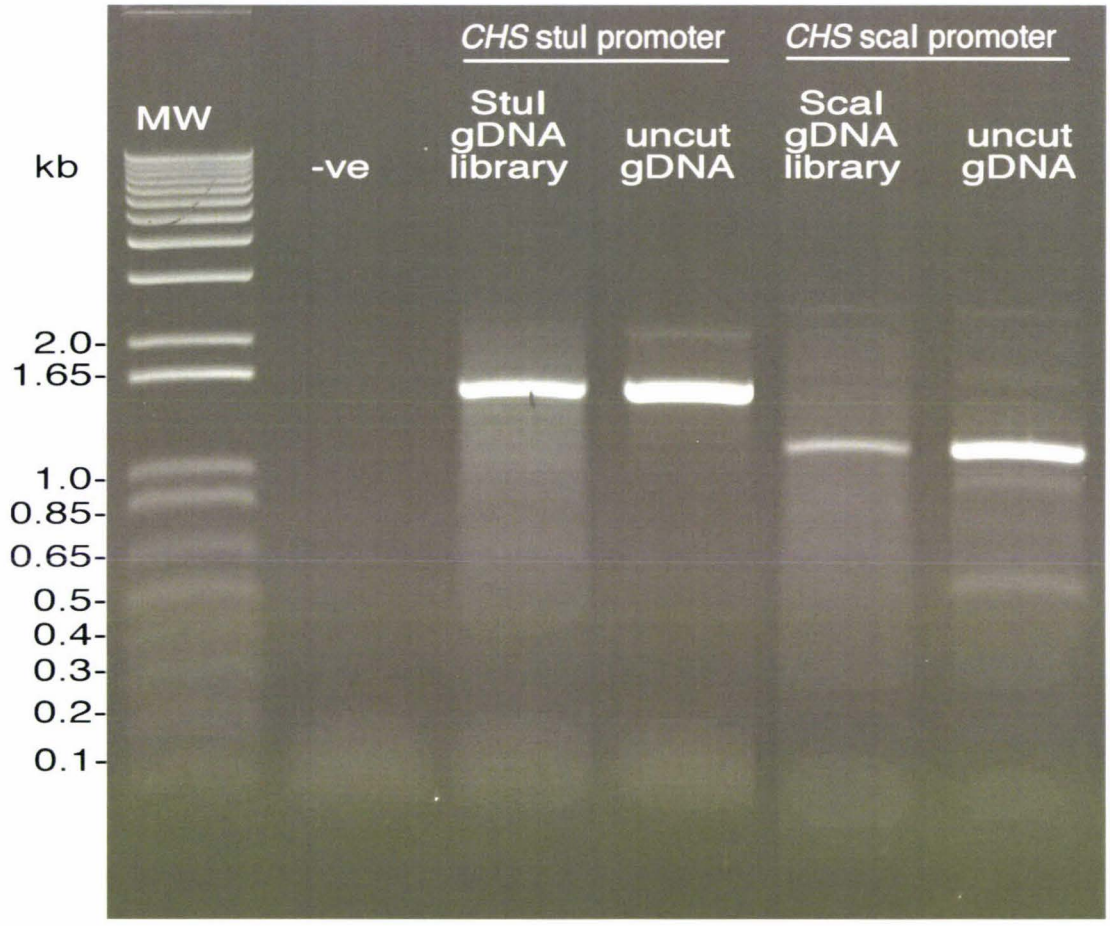


Figure 6.4: PCR amplified promoter region of *CHS*stuI and *CHS*sca*I*. -ve is no template control. The position of DNA markers and their size in kb are indicated.

Analysis of the promoter fragment revealed that the putative initiation site for transcription (CCAGGCC) was located at position -144 (Figure 6.5). A TATA-box was localised at position –168, 24 bp upstream from the putative transcription initiation site. A plant MYB transcription factor recognition site was present at -545 bp. Other putative *cis* elements were also found in the *CHS* promoter region, including one G-box located at position -266, one E-box located at -90, two ASF-1 binding sites and several W-box, Dof and MYC core recognition sites.

6.3.3 Cloning ORF from the cDNA derived from cymbidium flowers

The complete ORF of *CHS* was amplified by PCR from cDNA synthesised from total RNA isolated from stage one to three floral tissues from the NJG cultivar. The PCR primers were designed to include the ATG start codon and the translation stop codon. Amplification of the coding region of *CHS*scaI and *CHS*stuI both showed a ~1.2 kb band (Figure 6.6). The DNA sequence of *CHS*scaI clone, however, was identical to *CHS*stuI, meaning that only *CHS*stuI amplification was successful.

The full length of the *CHS* coding sequence is 1173 bp in length and the 3′-UTR region (245 bp) contains one polyadenylation site (Figure 6.7). The cymbidium *CHS* ORF encodes 390 deduced amino acids. Most conserved amino acid residues preventing identified in other CHS proteins, such as Cys164, Phe215, Ser133 and His304 (Ferrer et al., 1999; Han et al., 2005), were found in cymbidium *CHS*. A BLAST search of the DNA sequence of cymbidium *CHS*stuI showed identities of ~85% with *Oncidium* Gower Ramsey CHS2 (DQ118024), *Phalaenopsis* × *Doritaenopsis* hybrid cultivar CHS (AY28575), *Phalaenopsis* hybrid cultivar *chalcone synthase* pesudogene (AY825503), *Phalaenopsis* hybrid cultivar CHS (AY954515), *Oncidium* Gower Ramsey *CHS3* (DQ118022), *Oncidium* Gower Ramsey CHS1 (DQ118023) and *Phalaenopsis* sp,'pSPORT1' bibenzyl synthase (X79903 and X79904).

6.3.4 *CHS* expression pattern

The *CHS* fragment (partial coding region with 3′-UTR) obtained from 3′-RACE was used as a probe to study *CHS* expression patterns in floral tissues at different


SacI and NotI
CHSstuI promoter-F
-1561 TGAGCTCGCGGCCGCCCTAGCCCAGTAAGAATGTTCCACTATGTATTTCTTTTAGAA
ASF-1 binding site
-1504 TACAGGATCTTCCCGATGACGGGATTACATAATAATATGTGAGAAGGGAACAACAAT
MYC
MYC
-1447 TATGTAGTCACGTTCATGTTGTAACAGTTGCTAGTTATAGCAGATGATGGTGAGCGG
MYC ASF-1 binding site
W-box
-1390 CGGGGGACATCAACTGACGATGAAGGTCGTCGGGATGATGTTAACGACGATGTTGAC
W-box
W-box
W-box
-1333 GGTGATGTTGACGACGATGTTGACGGCGATGTCGACGACGATGTTGACGACGATGTC
W-box
W-box
W-box
-1276 GACGAGGATGTTGACGGCGATGTTGACGACGATGTCGAAGATGATGTGCATGACGAT
W-box
W-box
-1219 GTCGACGACGATGTTGACGACGATGTCGACGATGATGTTGACGATGATGTCGACAAC
W-box
-1162 GATGTCGACCATGATGTCCACAATGATGTGGACGATGATGTCCATGACGACGAGGAT
W-box
-1105 CAGCGGCGGCCGACGGTGGTGGTTGACGACCGTCCAGTGGTGGTGCGATTGTGCTCG
-1048 GAGGATCTTCAGGCTTTACAACTCTCTCCTCCCTCTTGCTTAGGCTCTCATTCTTGC
MYC
MYC
-991 GTCGCTTGGCCTCCCCTCATGTGAAGAATGAGGGCTTATTTATACATTTGAGGAGTG
-934 GTGTCCTTGTAAATACATGCGATCCCCGTACACTCCACTTTATCGGATGGCTCAGA
MYC
MYC
-877 TTAATTCCTTTGATCATGTGATGTATTTCGAGTATTCGAATGCTCAAATGAAGTCAG
-820 AATTATGAGATCTTGGGCTTGTTAGATCCGGCTCGTCGAGATGCTCGATTTGACTAT
-763 AAACACTTTATATTTAAGAGTCATTTAGGGATTTAATTGATATCTCAAAATTTCTAA
W-box
-706 TGATATTTTAAGTGCTTGATCTCGACTAGTATTGACTCAGGATCAAATTGCTTTCGA
-649 CATCAAGATTCGTGCTTCGACTAGTAGTATTTTGAGTGTCATATCATAAATAATTCT
Plant Myb binding site
-592 TAAAATAAATAAATTTATTTTAAAATAAGTGTAATAAGTTAATTACGCACCTAACGC
-535 TTCTTAGCCCTAAATTTTCGAAGTAACCAAAATTTATTCCACATTTAATCATAAAAA
-478 TATAAAAATATATTGAGAATTTATTTTTTAGTAGGAGCGATTAAAACGCTATAGATG
W-box
Dof
-421 ATATTTGTTGTATTTGAGAAATGTTTTTTTATTGACCGACTTAATTAAAAGTTGGAT
Dof
-364 TTAATTGAGTTTAAATAATTTTTTAAAGATTCTTCCATCAATCGTAGTCAGTCAACA
Dof
-307 ATGCACACCAACTTAATTCGCGGAAAGAGGGGAGGATCAAATTACAGAACTGCGATA
G-box
-250 ACCGAAGTAGAAGACCACGTGGAATATCTATGGTAGATGTGGATTAATTCCATCTAC

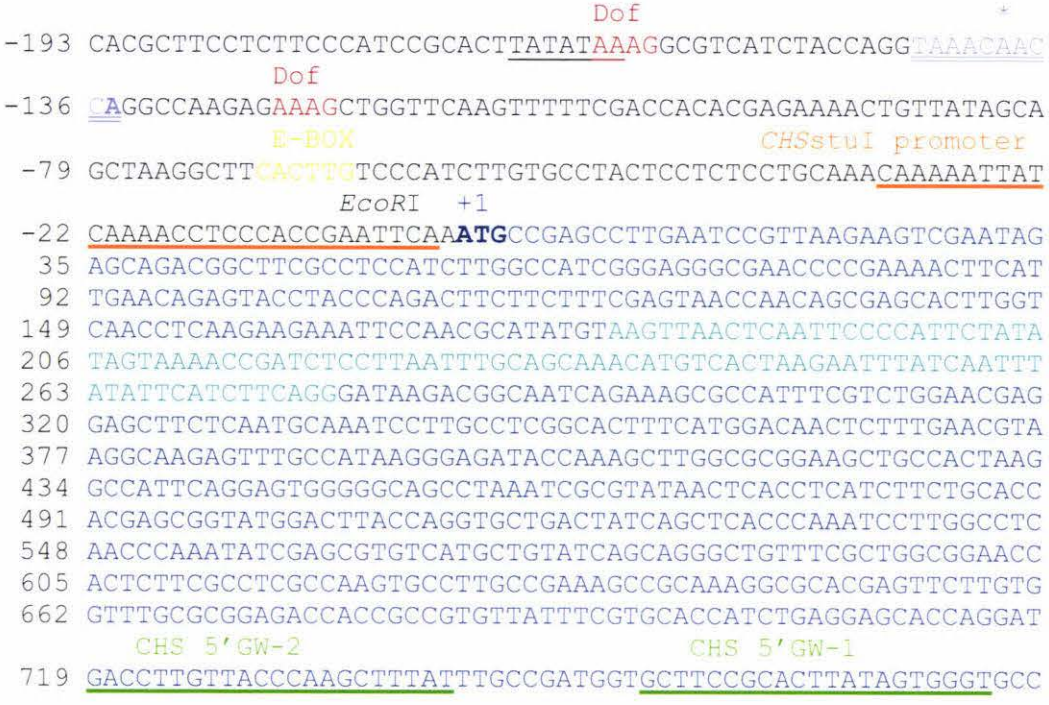


Figure 6.5: A detailed description of the cymbidium *CHS* promoter and partial coding sequence. The nucleotide sequence positions are marked at the left margin. The promoter region is in black. The identified TATA box and CAAT box are underlined, the transcription initiation site is marked with an asterisk in blue grey and double underlined, the G-box is labelled in blue, an E-box is labelled in yellow and a W-box is labelled in green. A MYC recognition site is labelled in red, ASF-1 binding sites are labelled in purple and Dof core recognition sites are labelled in brown. A plant MYB binding site is highlighted in a pink box. The coding region is in dark blue. Translation start 'ATG' is in bold and the intron region is labelled in blue/green. Primers used for *CHS* genome walking and promoter amplification are labelled.

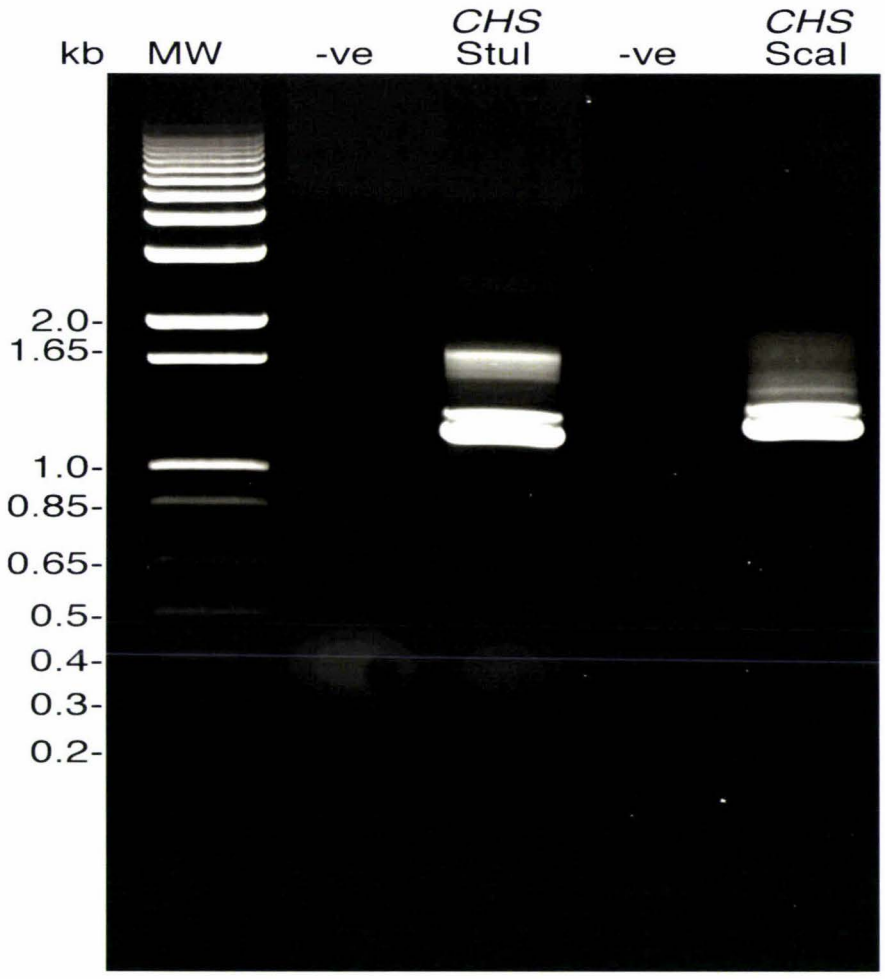


Figure 6.6: Amplification of full length coding region of *CHS*stuI and *CHS*scaI, using gene specific primers. *CHS* fragments were amplified from genomic DNA from the CASP cultivar digested with *Stu*I (labeled as *CHS*stuI) and *Sca*I (labelled as *CHS*scaI), respectively. Sizes of the 1 kb+ molecular marker are marked; -ve, is no template control.

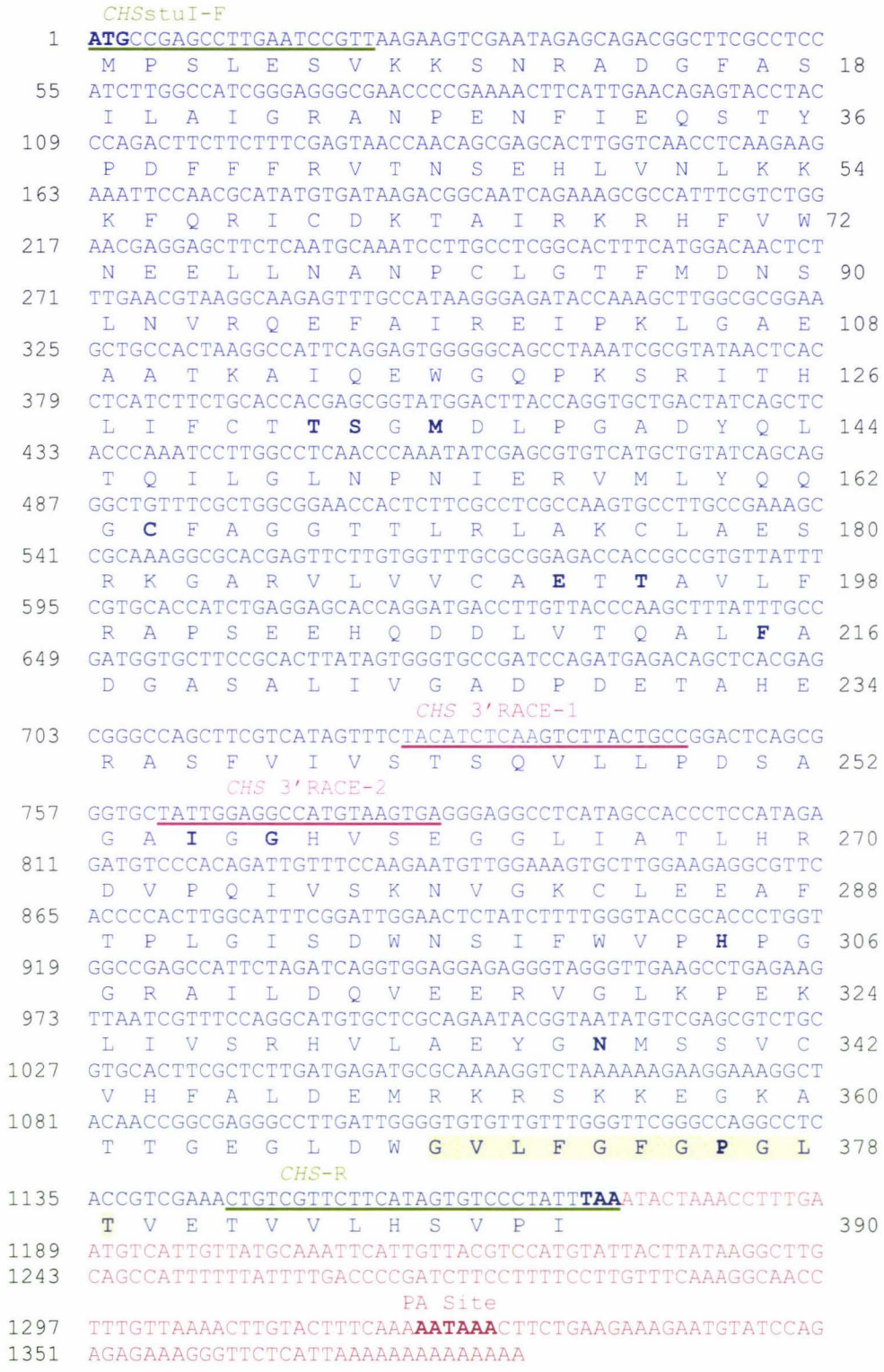


Figure 6.7: Full length coding sequence of cymbidium *CHS* and its deduced polypeptide sequence. The nucleotide sequence positions are marked at the left margin and the amino acid residue positions are marked at the right margin. The coding region (1173 bp) is in dark blue and the translation start 'ATG' and stop codon 'TAA' are marked in bold. The 3′-UTR, which consist of 245 bp, is marked in purple. A polyadenylation (PA) site is identified (marked in bold). The CHS active sites are in bold. The family signature (GVLFGFGPGLT) is indicated with a grey box. Primers used for *CHS* 3′RACE and full length coding sequence amplification are labelled.

developmental stages of selected cymbidium cultivars as well as in leaf tissues of various cultivars. The expression pattern of *CHS* is shown as Figure 6.8. *CHS* transcripts are most abundant in sepal and petal tissues from the middle to late stages of flower development for VMB (stage four to six) and in leaves of all cultivars, except the Alice Anderson (AA) cultivar. The *CHS* expression level in leaves varies between cultivars, with highest expression in leaf tissues of the VMB cultivars. *CHS* transcripts were detected at very weak levels in floral tissues of the white JFDP cultivar at stages two to four, then the expression declined at stage five and was below a detectable level by stage six. There was no *CHS* expression detected in the white PC or pink NJG cultivars.

6.3.5 *CHS* genes in the cymbidium genome

The number of genes encoding *CHS* in the JFDP and LRF cultivars were investigated by Southern analysis. There was no *Hin*dIII, *Bam*HI and *Eco*RI digestion site within the amplified *CHS* 3′-RACE partial sequence, meaning that each *CHS* gene or its allele should be represented by one band on the blot. The results showed that digested gDNA from both the LRF and JFDP cultivars displayed similar patterning, with multiple bands hybridised to the *Bam*HI- and *Hin*dIII-digested gDNA (Figure 6.9). There were at least five bands hybridised to the *Bam*HI digested gDNA, and at least eight bands present in *Eco*RI digested gDNA. Only a single band was present in *Hin*dIII digested gDNA from both LRF and JFDP cultivars, probably because the gDNA was not fully digested. This result indicates that *CHS* is encoded by a small multigene family of more than three members in cymbidium orchid.

6.3.6 Cymbidium *CHS* promoter activity

The cymbidium *CHS*stuI promoter fragment was cloned into the pPEP-GFP vector backbone to produce a p*CHS*stuIpromoter-GFP construct for testing the promoter activity in petals of the JFDP and LRF cultivars and leaf tissue of the JFDP cultivar (Figure 6.10A, C, E, respectively). The *CHS*stuI promoter was able to induce GFP expression in petals of the JFDP cultivar, as a few GFP foci were observed.

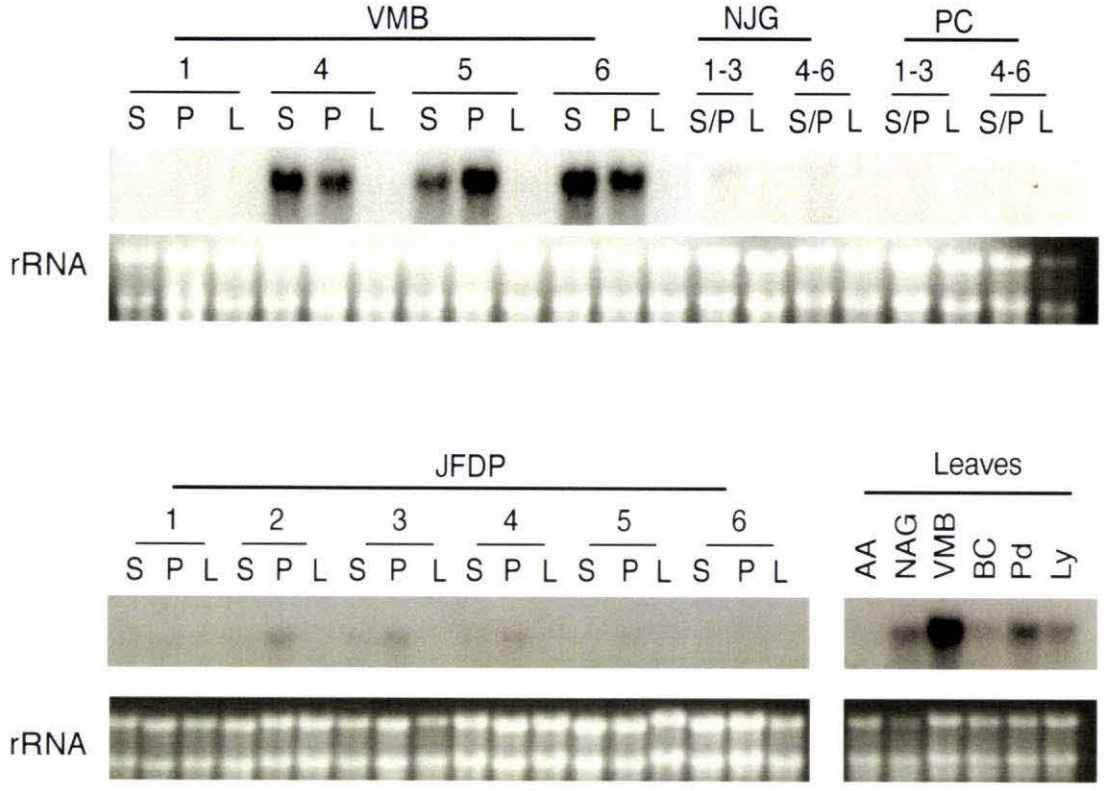


Figure 6.8: Spatial and temporal expression of cymbidium *CHS* expression in cymbidium floral and leaf tissues. VMB, Vanguard Mas Beauty; NJG, Narella Jennifer Gail; PC, Winter Bride Peter's Choice; JFDP, Jung Frau dos Pueblos; AA, Alice Anderson; Pd, Piedmont; Ly, Lymonese; BC, Big Chief Kirawee; S, sepal; P, petal; L, Lip. Developmental stages are labelled 1 to 6. Cymbidium *CHS* 3′UTR was used as probe. The hybridisation was carried out at 60°C overnight and washed in 0.5×SSC+1%SDS. The bottom panels indicate the loading of RNA stained using ethidium bromide staining of rRNA bands.

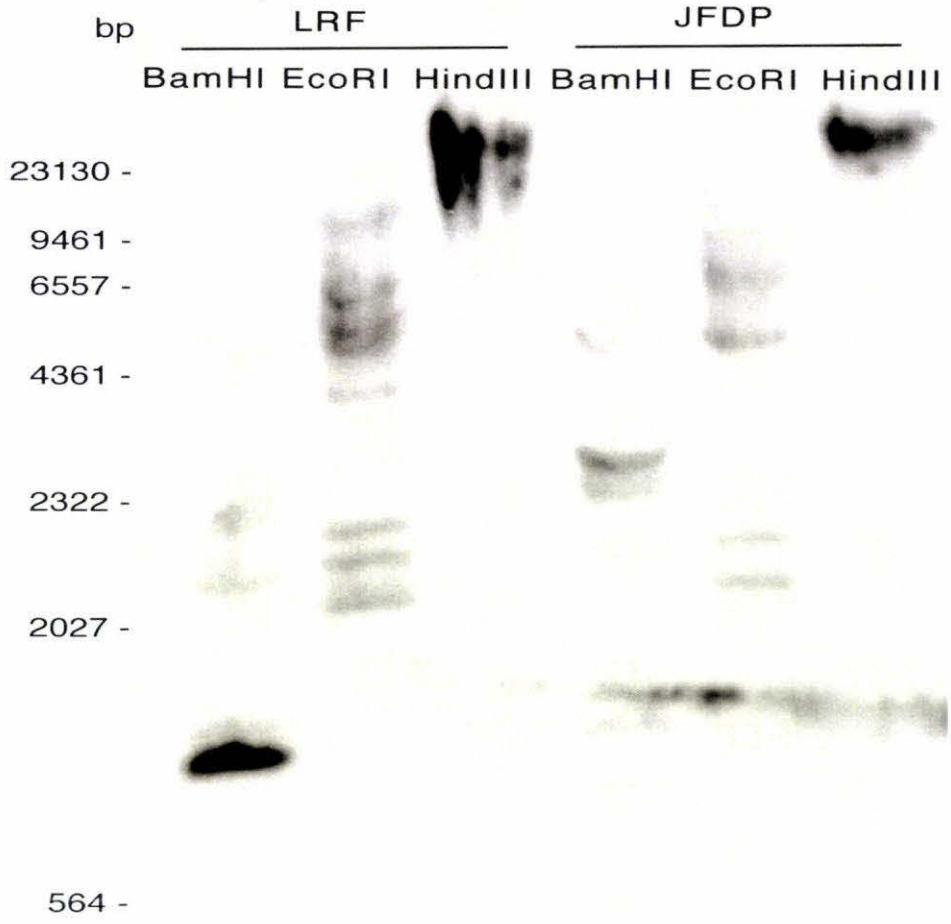


Figure 6.9: Southern blot analysis of *CHS* in two cymbidium cultivars. LRF, Lisa Rose Flamingo and JFDP, Jung Frau dos Pueblos. gDNA was digested with *Bam*HI, *Eco*RI and *Hin*dIII as described. Hybridization was carried out with a labelled *CHS* 3′RACE fragment as probe. The position of the DNA markers are indicated.

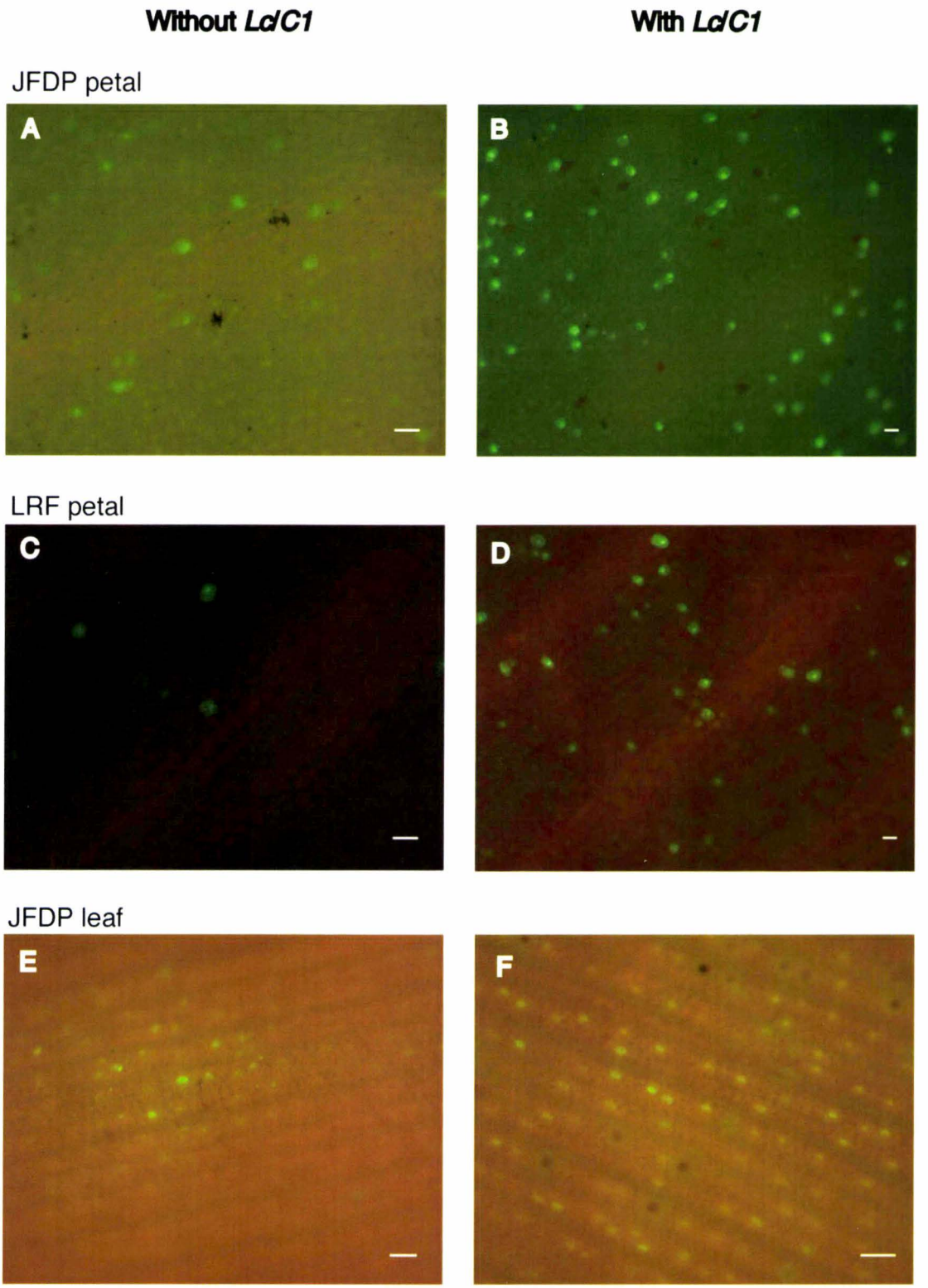


Figure 6.10: Transient expression of p*CHS*stuIpromoter-GFP with and without *Lc* and *C1* in petal and leaf tissues of the cymbidium cultivar as indicated. **A, B**, JFDP petal; **C, D**, RFL petal; **E, F**, JFDP leaf. **A**, **C** and **E** expression of p*CHS*stuIpromoter-GFP; **B**, **D**, **F** are co-expressed of *Lc/C*1. All images were taken under blue light, 48 h post transformation. **A-D**, scale bar = 100 μm; **E**,**F**, scale bar = 250 μm.

The *CHS*stuI promoter was also able to activate GFP expression in petals of the LRF cultivar and leaf tissues of the JFDP cultivar, but the number of foci (expression level) was not as strong as in petals from the JFDP cultivar. When maize transcription factors *Lc* and *C1* were co-introduced with p*CHS*stuIpromoter-GFP into the same cymbidium tissues, an increased number of GFP foci were observed in both petals of JFDP and LRF cultivar (Figure 6.10B, D, F). In contrast, in leaf tissues of the JFDP cultivar the strength of *GFP* expression and the number of foci observed did not change when the promoter was co-expressed with *Lc/C1*. A control experiment was carried out to ensure that the GFP expression observed was not due to the *CaMV35S* promoter in the *Lc* and *C1* constructs. A pART7 construct was co-introduced with the p*CHS*stuIpromoter-GFP construct into petals and leaves, and the result was similar to that has been observed in the p*CHS*stuIpromoter-GFP only experiment. That is no increase in the number of GFP foci in both JFDP petals and leaves (data not shown). The *CHS*scaI promoter activity was also tested with and without *Lc/C1* in the same cymbidium tissues. However, no *GFP* expression was observed in any tissues in both experiments (data not shown). It appears that the *CHS*scaI promoter is unable to activate *GFP*.

6.4 Discussion

A *CHS* cDNA sequence was isolated from cymbidium orchid flower tissues. Analysis of the *CHS* cDNA sequence and deduced amino acid sequence has shown that it has some of the features that are conserved in all *CHS* genes. *CHS* has a 1173 bp coding region, with one intron. The length of the coding region and intron number is in agreement with most *CHS* sequences reported to date, with only the *Antirrhinum majus CHS* having two introns (Sommer and Saedler, 1986). The *CHS* sequence showed a high sequence homology with other published orchid *CHS* genes, with the active sites identical to those active sites found in phalaenopsis PCHS1 (Han et al., 2006a,b). The CHS protein contained the CHS family signature (GVLFGFGPGLT) as well as the active site residues that are identified in CHS proteins. This result indicated that it is likely to be a true *CHS* gene. The *CHS* sequence, however, also showed high homology with bibenzyl synthase (Preisig-Müller et al., 1995). Bibenzyl synthase is also a member of the PKS III superfamily, which is believed to be evolved from CHS (Tropf

et al., 1994). The cDNA sequence of bibenzyl synthase shares high identity to *CHS* and catalyses similar chemical reactions to CHS (Justin and Noel, 2003). Thus without functional confirmation, it is difficult to confirm that the *CHS* gene is definitely encoding a CHS enzyme simply based on the sequence data obtained in this chapter. Further analysis to study the function of *CHS* function is required.

CHS has undergone gene duplication, with the duplicated genes diverging and acquiring biochemical and developmental differences. *CHS* is present as multiple copies in most plants, except for parsley, snapdragon and *Arabidopsis*, which have only one *CHS* in their haploid genome (Holton and Cornish, 1995). Studies on the other orchid species *Bromheadia finlaysoniana* and *Phalaenopsis hybrid* have shown three copies of *CHS* present in the genome (Liew et al., 1998a; Han et al., 2006a). The observations that different *CHS* clones were isolated from floral genomic DNA, and multiple hybridizing bands detected by Southern analysis suggests that *CHS* in cymbidium is likely to be represented by a gene family consisting of at least three members (Figure 6.9). However, due to the sequence similarity between *CHS* and *CHS*-related genes, the possibility of cross-hybridisation with *CHS* related genes in the Southern blot analysis cannot be ruled out, despite the relatively stringent washing conditions used. The increased ploidy of cymbidium cultivars may also explain the number of *CHS* genes detected by Southern analysis.

Expression of the isolated cymbidium *CHS* clone does not correspond with anthocyanin biosynthesis in cymbidium flowers. Pigmentation is initiated in the early developmental stages in cymbidium flower, and cymbidium *DFR* expression was highest in early flower development (Chapter Five). CHS is the first enzyme of the anthocyanin production pathway, so expression at the early floral developmental stages is expected. Although the CHS gene family comprises many members in most plant species, usually only a few members are responsible for anthocyanin biosynthesis. Previous studies showed that an increase in *CHS* expression corresponded to anthocyanin production in the flower of gentian, petunia and snapdragon (Koes et al., 1989; Martin et al., 1991; Nakatsuka et al., 2005). In monocotyledon plants, such as *Asiatic* hybrid lily, *LhCHS A* and *B* were expressed from the early stages of flower development, right though development. The pattern of *CHS* expression was correlated with that of *LhDFR* (Nakatsuka et al., 2003). Dendrobium *CHS* had strongest expression in the early floral

developmental stages, but no expression was observed in the later stages. The expression pattern of dendrobium *CHS* also correlates with *DFR* expression (Mudalige-Jayawickrama et al., 2005). The *CHS* gene identify in this study is highly active in green floral tissues and leaves, especially in the VMB cultivar, and is weakly expressed in the white JFDP floral tissues, but it is not highly expressed in the NJG cultivar where it was isolated from. It is also expressed late in flower development, as expression pattern that is not similar to *DFR*. The primary role of this isolated *CHS* gene appears unlikely to be in anthocyanin synthesis in cymbidium flowers. It may be involved in other secondary metabolite biosynthesis. Further investigation of different *CHS* genes in cymbidium is required to identify those involved in anthocyanin biosynthesis and to definitively exclude the gene isolated in this study.

The expression pattern of cymbidium *CHS* indicated that its promoter could be useful for colour manipulation in leaf and green floral tissues, which prompted the isolation of the promoter region. The activity of the *CHS*stuI promoter was transiently tested in cymbidium white and pink petal and leaf tissues. The promoter of cymbidium *CHS*stuI is able to drive *GFP* expression. The activity of the *CHS*stuI promoter seems to be stronger than that of cymbidium *DFR* (Chapter 5), as the cymbidium *CHS*stuI promoter is able to activate *GFP* expression not only in JFDP petal but also in petals of the LRF cultivar and leaves of the JFDP cultivar, whereas the *DFR* promoter could only activate *GFP* expression in JFDP petal. When the *CHS*stuI promoter was co-expressed with maize *Lc* and *C1*, enhanced *GFP* expression was also shown in petals of the white JFDP and pink LRF cultivars. This result is similar to observations with the *DFR* promoter, and shows that *CHS* promoter activity is also able to be up-regulated by the MYB and bHLH type transcription factors. Surprisingly, no enhanced *GFP* expression was observed in leaves when *Lc* and *C1* were co-introduced with the *CHS*stuI promoter. The strength of promoter activation may be different in different tissues, but the binding of transcription factors should be the same across different tissues. Thus it is harder to explain the result observed. However, the nature of biolistic bombardment experiments means inconsistent result can sometimes be obtained. The biolistic experiments in leaves need to be repeat further than the duplications to confirm the observations.

CHS expression is induced by different environmental and developmental signals. Analysis of the *CHS*stuI promoter has identified several *cis* element and transcription-

binding sites that could potentially be sites that are responsive to different signals. The W-boxes distributed in the *CHS*stuI promoter can be induced by elicitors, suggesting *CHS* might be induced by pathogens (Rushton and Somssich, 1998; Eulgem et al., 1999). The ASF-1 binding site may be involved in transcriptional activation of genes by auxin or salicylic acid. The G-box is a light- and elicitor-inducible element and is normally located near the TATA box. It has been shown to be an essential tissue-specific regulatory element in a French bean *CHS* (Faktor et al., 1997 a &b). A G-box is found in the *CHS*stuI promoter, but is not located near the TATA box, so may not be a real regulatory element. A plant MYB binding site was also found in the *CHS*stuI promoter. This site is related to the P-box in phenylpropanoid biosynthetic genes and it has been shown that a floral-specific MYB protein can activate transcription of phenylpropanoid biosynthetic genes *via* interacting with this site (Sablowski et al., 1994; Tamagnone et al., 1998). The E-box and MYC recognition sites identified in the promoter are known as R-response elements, which are recognized by the *C1* and *Sn* transcription factors to activate *CHS* (Hartmann et al., 2005). It has been suggested that enzymes in the earlier part of the phenylpropanoid and flavonoid pathway are encoded by multiple gene family members, because flavonoids serve multiple metabolic functions in plants (Sparvoli et al., 1994). Different *CHS* genes may respond to different environmental stimuli, such as UV light, pathogen infection and wounding, thus allowing more diverse control of gene expression to serve multiple functions (Arioli et al., 1994). The promoter analysis suggests *CHS*stuI may be induced by different environmental stimuli and regulatory genes.

The study of *CHS* has contributed to an understanding of the anthocyanin biosynthetic pathway in cymbidium. The molecular analysis showed *CHS* is encoded by a multigene family. It appears that the isolated *CHS* clone is not responsible for anthocyanin biosynthesis in cymbidium flower. However, it is the first full-length cymbidium *CHS* cDNA clone reported. The *CHS* in cymbidium is likely to be involved in reactions for synthesis of other phenylpropanoids. A potential green-tissue specific promoter has been isolated, since *CHS* is highly expressed in green tissues. Future work requires identification of the *CHS* that is involved in flower anthocyanin biosynthesis. Since the same *CHS* fragment was amplified from cDNA made from different cultivars, using existing degenerate primers, a new set of degenerate primers with higher degeneracy is needed to allow amplification of the other *CHS* genes in cymbidium.

Chapter 7

General discussion and conclusions

The anthocyanin biosynthetic pathway has been extensively studied in many plant species (Koes et al., 1994). However, anthocyanin research in orchid is scanty (Hieber et al., 2006). Development of novel colours in cymbidium is commercially valuable and requires knowledge on anthocyanins and their biosynthetic pathway in this species. The overall aim of this thesis was to understand more about anthocyanin production in cymbidium and examine whether the anthocyanin pathway in this species could be redirected with the introduction of selected heterologous flavonoid biosynthetic genes.

Three key outcomes were achieved:

1) Modification of anthocyanin biosynthesis in cymbidium flower can be achieved by introducing suitable heterologous genes: pelargonidin and delphinidin production was induced in floral tissues that normally only form cyanidin or peonidin.

2) A full-length chalcone synthase (*CHS*) cDNA and a partial sequence of flavonoid 3′ hydroxylase (*F3′H*) cDNA were isolated from cymbidium, and analysis of their expression pattern has been carried out. However, *CHS* expression pattern does not correlate with anthocyanin accumulation in cymbidium floral tissues, while *F3′H* expression was not detected. Cloning and characterization of important flavonoid biosynthetic genes such as *CHS*, *DFR* and *F3′H* from cymbidium is necessary for the generation of novel flower colour, and the information obtained from this study contributes to the understanding of gene resource in cymbidium and orchids in general. Further, two promoters were also isolated, one from *CHS* and one from *DFR*. Both promoters have shown able to activate *GFP* and both are potentially useful for tissue specific expression.

3) The maize transcription factors, *Lc* and *C1*, were able to up-regulate anthocyanin production in cymbidium petal tissues. This provided a useful model system to study anthocyanin production. This result also indicates that white flower cultivars of

cymbidium are likely to be anthocyanin regulatory gene mutants, and that MYB/bHLH transcription factors are important factors in anthocyanin production in cymbidium orchids.

Transient gene expression studies have demonstrated that cymbidium has the potential to produce delphinidin- and pelargonidin- type anthocyanins, which are naturally absent in cymbidium flowers. The first genetically modified flower crop, purple carnations, were successfully developed by Florigene Ltd. and Suntory Ltd. by introducing viola *F3'5'H* (also pansy *F3'5'H*) and petunia *DFR* into a carnation mutant that lacks DFR activity (Fukui et al., 2003). The same companies have developed the blue-hued rose, by over-expressing viola *F3'5'H* and Iris *DFR* and down regulating rose endogenous *DFR* (Katsumoto et al., 2007). The transgenic rose accumulated up to 95% delphinidin, and exhibited violet flower colour. In this study on cymbidium, the same gene responsible for blue colour, pansy *F3'5'H*, was tested for delphinidin production in cymbidium and did support the possibility of generating purple colour through delphinidin accumulation. A reduction in endogenous F3'H activity appears necessary to allow sufficient delphinidin derivatives to accumulate to give a purple flower colour. Past research in rose and *Dianthus* showed that generating a true blue flower colour involves other factors such as co-pigmentation and appropriate vacuolar pH, and choosing the best recipient cultivars is also essential for blue flower generation (Katsumoto et al., 2007). This would appear to be the same in the case of developing novel colour in cymbidium.

The effective accumulation of pelargonidin anthocyanin in the floral tissues during transient gene expression has strongly suggested that *Anthurium DFR* is an excellent candidate in molecular breeding for red colour in cymbidium. This is the first time the *Anthurium DFR* (Collette et al., 2004) has been investigated as a heterologous *DFR* source for promoting pelargonidin formation in cymbidium. The fact that pelargonidin made up 70% of accumulated anthocyanin in the petals/sepals indicates that *Anthurium* DFR is more efficient in converting its substrate than the cymbidium endogenous DFR. Although these results cannot represent the final outcome of stable transformants, the knowledge gained from these experiments should be valuable in assessing the strategies for generating novel flower colour in cymbidium.

F3′H activity is required for cyanidin synthesis and so silencing F3′H activity should block cyanidin production in cymbidium. This hypothesis was unable to be tested as no *F3′H* expression was detected on the northern blot, and also because of the difficulties in obtaining a *F3′H* clone. The results of re-directing anthocyanin biosynthesis by expressing heterologous genes indicate that strong endogenous F3′H activity might be a barrier, as cyanidin production in the petals/sepals was still evident. Cymbidium flowers that exclusively accumulate pelargonidin or delphinidin, will probably require the silencing of the endogenous *F3′H*. The partial *F3′H* fragment obtained from this study will allow for construction of a *F3′H* RNAi vector, and a block of cyanidin production can be tested using the transient gene expression system (Shang et al., 2007).

Heterologous expression of biosynthesis pathway genes to help redirection of anthocyanin production is a common strategy for generating flowers with novel colour. The gene source, however, is important because genes from different plant sources, despite encoding the same enzyme, may have different efficiencies in the host plant (Tanaka et al., 1998). The gerbera *DFR* gene for example, showed a stronger and more stable expression than a maize *DFR* gene in transgenic petunia (Elomaa et al., 1995). Introduction of *F3′5′H* from Canterbury bells in tobacco also gave a greater amount of delphinidin as compared to the petunia or lisianthus *F3′5′H* (Okinaka et al., 2003). The common plant sources for a gene encoding a heterologous DFR, that can catalyze the reduction of DHK to give pelargonidin, are maize (Schwarz-Sommer et al., 1987), gerbera (Helariutta et al., 1993), and rose (Tanaka et al., 1995). The transient gene expression system offers a useful way to test the suitability of flavonoid biosynthetic genes from different plant sources.

However, it is important to understand how anthocyanins are naturally synthesized in cymbidium before re-direction of the flavonoid pathway is considered. This study reports on the isolation of *CHS* and *F3′H* from cymbidium for the first time. The sequences analysis for both *CHS* and *F3′H* has shown high homology with published genes from other species, and the characteristic-conserved regions were found in both protein sequences. Although the *DFR* isolated in this study was from a different cymbidium cultivar (CASP) from the one used in a previously published study (Johnson et al., 1999), the sequence showed that both *DFRs* are the same.

The expression pattern of *DFR* showed the highest mRNA accumulation in early stages of cymbidium flower development, which correlates with colour development in cymbidium flowers. This result is also consistent with a study of *DFR* in *Dendrobium* orchid (Mudalige-Jayawickrama et al., 2005). *DFR* is regulated differently in the flower lip, compared to the petals and sepals. *DFR* in the lips was expressed later in development, just before the flowers opened. There are no other studies documenting the *DFR* expression pattern at different developmental stages for lip tissues. The findings suggest a sophisticated anthocyanin regulatory mechanism for the different parts of the cymbidium flower. The expression of *CHS* did not correlate with anthocyanin production in cymbidium flowers. The expression pattern of *CHS* reported in this study is not consistent with studies on flowers of other orchid species or other plants (Martin et al., 1991; Koes et al., 1989; Nakatsuka et al., 2005; Mudalige-Jayawickrama et al., 2005; Han et al., 2006a,b), indicating that the *CHS* gene obtained was not responsible for anthocyanin production in flowers, and further investigations are needed. *F3'H* expression was not detected. Interestingly, both *CHS* and *F3'H* appear to comprise small gene families in cymbidium. Studies on other flavonoid gene families suggest that the different members of the gene family allow the plant to respond to different developmental and environmental signals and/or may be expressed differentially in different tissues (Arioli et al., 1994; Sparvoli et al., 1994). Clarifying the expression pattern of the different members of the gene families and obtaining an anthocyanin-related clone is an important next step for studies on cymbidium.

Two promoters have been isolated, from the cymbidium *CHS* and *DFR* clones. The *DFR* promoter is potentially active only in the flowers, whereas *CHS*stuI promoter is potentially active in both leaves and flowers. Transient expression studies showed that both promoters could activate GFP expression, but they are weaker than the *CaMV35S* promoter. Northern analysis indicates that the *DFR* promoter could be useful in the flower, and the *CHS* promoter could be a good promoter for green tissues.

It has been suggested previously that the white orchid flower phenotypes are regulatory gene mutants, since expressing maize transcription factor *C1* and *B* or *R* genes induced pigmentation in the white petals of two other orchid genera *Doritis* and *Phalaenopsis* (Griesbach and Kilen, 1993; Ma et al., 2008). Transient expression of maize *Lc* and *C1* transcription factors also increased anthocyanin production in cymbidium petals as

shown in this study. This is consistent with white flowered cymbidium cultivars being flavonoid regulatory gene mutants. A single biosynthetic gene will not induce anthocyanin accumulation, and therefore white flower cultivars are probably not suitable as targets for modified flower colour. The fewer the transgenes required to induce a colour change, the less likely it is that other problems, such as gene silencing, will occur.

The regulation of anthocyanin biosynthesis in cymbidium flower tissues was not a major area of work for this thesis, but the results from this study and from work on other orchids have demonstrated that MYB and bHLH transcription factors may also control anthocyanin production in orchids, similar to the proposed model in petunia (Mol et al., 1998; Chiou and Yeh, 2008; Ma et al., 2008). Pink pigmented spots or patches in petals of phalaenopsis and oncidium orchids are the result of up-regulation of flavonoid biosynthetic genes by MYB-type transcription factors (Ma et al., 2008; Chiou and Yeh, 2008). A MYB-type regulatory gene, *MYB1*, has been isolated from oncidium, and activation of the oncidium *MYB1* was shown to induce pigmentation by up-regulating expression of two biosynthetic genes *CHI* and *DFR* (Chiou and Yeh, 2008). The maize transcription factors, *Lc* and *C1*, enhanced *CHS* and *DFR* promoter activity in cymbidium. A similar response is seen in oncidium (Chiou and Yeh, 2008). This is consistent with the idea that anthocyanin biosynthetic genes are controlled by MYB- and bHLH-type transcription factors. An understanding of the regulation of pathway genes at the transcription level is another component of understanding anthocyanin production in cymbidium. The isolation of MYB and bHLH genes from cymbidium is clearly needed in future studies.

Future work for this project includes isolating the full-length cymbidium *F3'H* and isolating the *CHS* gene that is responsible for anthocyanin biosynthesis. A cymbidium cDNA library may be required for isolation of the different members of the *F3'H* and *CHS* gene families. The cymbidium *F3'H* sequence is very divergent from dicotyldedon sequences, which mean it is difficult to be amplified using a PCR approach. Given the lack of knowledge about cymbidium flavonoid genes, isolating the members of the *CHS* gene family and characterizing their expression patterns will be helpful in understanding overall flavonoid biosynthesis in cymbidium orchid. Isolating other

anthocyanin biosynthetic genes in the pathway would also be useful. The control of anthocyanin biosynthetic genes at a transcription level can be investigated. All lines of information are important in understanding anthocyanin biosynthesis in cymbidium, an attractive but complex commercial flower crop. This study to date provides important background information for generating novel cymbidium flower colours, and has also broadened the current understanding on anthocyanin biosynthesis in cymbidium orchid.

Appendix 1

Buffers, media and solutions

1.1 Media, antibiotics and reagents for bacterial culturing

Luria-Bertani (LB) broth and media

The LB media comprised 10 g L^{-1} of bactotryptone, 5 g L^{-1} yeast extract, 10 g L^{-1} of sodium chloride.

Ampicillin plates

An agar solution were prepared including all ingredients for making LB broth as well as 1.5% (w/v) agar (Difco) for the media to set. The agar solution was melted in the microwave and then left to cool until the container was only warm to touch. A 1/1000 volume of Ampicillin (100 mg/mL) was added to the agar solution to give a final concentration of 100 mg/L, mixed well and poured into plates. The agar plates were left in the hood to set and then stored at 4°C.

Ampicillin solution (100 mg mL^{-1})

Ampicillin solution at 100 mg mL^{-1} was made by dissolving 2 g of the sodium salt of ampicillin into 20 mL of milliQ water. The ampicillin solution was filter sterilized (0.2 μm) and then stored at -20°C.

IPTG (200 mg/ml)

IPTG was prepared by dissolving 2 g IPTG in a final volume of 10 ml of sterile water. One μL into aliquots were dispensed into eppendorf tubes wrapped in foil, and then stored at -20°C.

X-Gal (20 mg/ml)

X-Gal was prepared by dissolving 200 mg of X-Gal into a final volume of 10 ml of N, N'-dimethyl formamide. One mL into aliquots was dispensed into eppendorf tubes wrapped in foil, and then stored at -20°C.

1.2 Polymerase Chain Reaction mix

PCR reaction mixture was made up as follows:

1 µL of template

3 µL of forward primer (10 µM)

3 µL of reverse primer (10 µM)

0.5 µL of polymerase (either *Pwo* [Roche] or *Taq* [invitrogen])

5 µL of 10 × buffer (with $MgCl_2$)

1 µL of 10 mM dNTPs

36.5 µL of H_2O

50 µL

1.3 Alkaline lysis miniprep solutions

Solution I- Re-suspension solution

Solution I comprised 50 mM of glucose, 25 mM of Tris HCl (pH 8) and 10 mM of EDTA (pH 8). This solution was autoclaved before use, and stored at 4°C.

Solution II- Lysis solution

Solution II comprised 1% (w/v) SDS and 0.2 N NaOH dissolved in sterile water. Since the SDS degrades with time, solution II was made up fresh each time before use.

Solution III- Neutralisation solution

Solution III comprised 60 ml of 5 M potassium acetate and 11.5 ml of glacial acetic acid, make up to 100 mL with sterile water, autoclaved and stored at 4°C.

1.4 Electrophoresis reagents

10 × TBE buffer

10 × TBE comprised 0.89 M Tris-base, 0.89 M boric acid and 20 mM EDTA.

10 × MOPS buffer

10 × MOPS buffer comprised 0.2 M MOPS, 50 mM sodium acetate and 10 mM EDTA with the pH adjusted to 7.0 with NaOH. The solution is autoclaved and stored at room temperature.

10 × loading dye

10 × loading dye comprised 30% (w/v) glycerol, 0.1 M EDTA (pH 8.0), 1% (w/v) SDS, 0.25% (w/v) bromophenol blue, and 0.25% (w/v) xylene cyanol made up with sterile water.

1.5 RNA and genomic DNA extraction, blotting and hybridization reagents

Extraction (XT) Buffer for the Hot Borate Method

XT buffer comprised 0.2 M di-Na tetraborate, 30 mM EGTA, 1% (w/v) SDS and 1% (w/v) deoxycholate. The buffer was adjusted to pH 9.0 with 4 M NaOH, and then kept in room temperature.

Urea Buffer for genomic DNA extraction

Urea buffer comprised 7 M urea, 0.3 M NaCl, 50 mM Tris (pH 8), 20 mM EDTA and 1% (w/v) N-lauroyl sarcosine.

Phenol/chloroform/IAA

Phenol/chloroform/IAA comprised 600 ml of phenol, 576 ml of chloroform and 24 ml of isoamyalcohol (25:24:1 by vol.).

10 × SSC

10 × SSC comprised 1.5 M NaCl and 0.15 M tri-sodium citrate made up with water. The pH is adjusted to pH 7 prior to autoclaving.

Church and Gilbert buffer (Church and Gilbert, 1984)

Church and Gilbert buffer comprised 7% (w/v) SDS, 0.5 M phosphate buffer pH 7.2 and 10 mM EDTA. The solution was sterilized by filter through a 0.45 μM filter.

TE buffer

TE buffer comprised 10 mM Tris (pH 8.0) and 1 mM EDTA.

10 × RP buffer

10 × RP buffer contains 1 M MOPS-NaOH (pH 6.6), 50 mM $MgCl_2$, 10 mM DTT, 0.5 mg ml^{-1} BSA and 0.2 μM dCTP, dGTP and dTTP.

1.6 Tissue culture/ transformation media

Media 2

Media 2 contains 1/2 × MS macrosalts (Murashige and Skoog, 1962), 1 × MS micro salts, 1 × MS iron, 1 × LS vitamins (Linsmaier and Skoog, 1965), 3% (w/v) sucrose and 7.5% (w/v) agar.

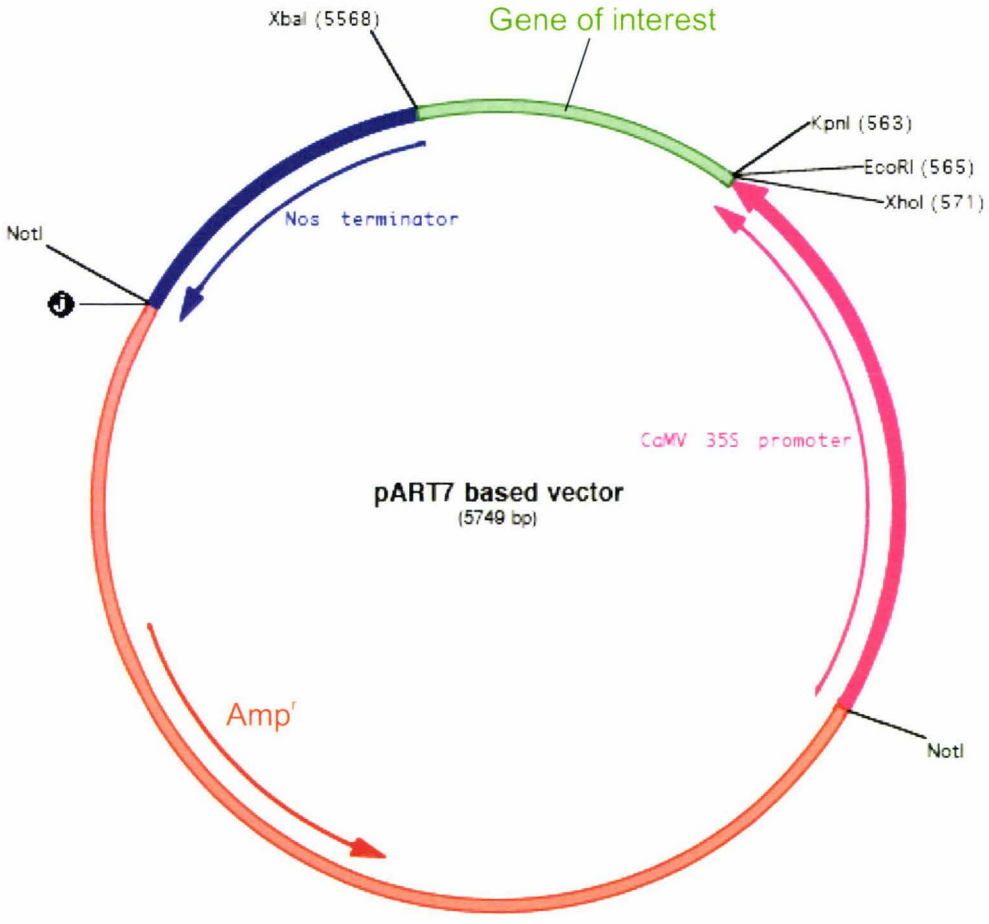
1.7 Flavonoid extraction solutions

90% (v/v), 80% (v/v) and 70% (v/v) acidified methanol was prepared by diluting methanol, as appropriate, with 10% (v/v) acetic acid in water.

Appendix 2

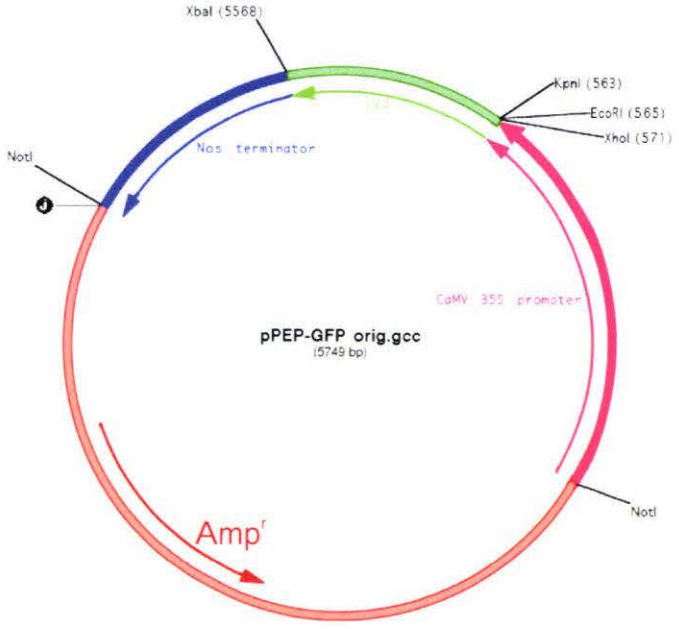
Plasmid Maps

2.1 Plasmid maps for pLN44, pLc349, pAaDFR and pPansyF3′5′H#40



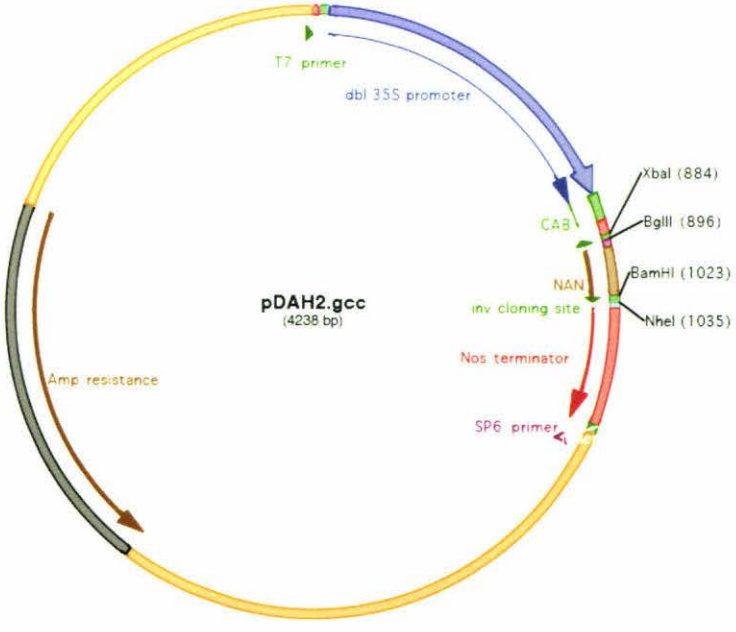
pART7 vector with *Lc* (pLc349), *C1* (pPN44), anthurium *DFR* and pansy *F3′5′H* ORFs. *E. coli* selection: Amp 100 mg L^{-1}

2.2 Plasmid map for pPEP-GFP



GFP in a pART7 based vector. *E. coli* selection: Amp 100 mg L^{-1}

2.3 Plasmid map for pDAH2



E. coli selection: Amp 100 mg L^{-1}

Appendix 3

Cymbidium *F3'H* partial sequence

```
GCGGGGACGGACACTACTTCCATCACCATCGAGTGGCTATTATCTGAGCT 50
CCTTCGTCATCCTCATATCCTCGCTCGAGCACAGCATGAGCTCGACTCCG 100
TCGCCGGCCGCAACCGCTTGATCTCACAGTCAGATCTACCAAAAGTTCCC 150
TTCCTCGATGCCATTGTCAAGGAGACCTTACGCCTCCATCCCCCAGTCCC 200
TCTATCCGTCCCCCGCATGGCAACCGAAGATTGTGAGATCGACGGCTACC 250
TCATTCCCAAAGGTGCTTACCTCCTCGTCAACATATGGGCCATCGGTCGG 300
GACCTCGCTACTTGGCACGACGACCCCAATGAATTTGATCCCGATCGATT 350
CGGCCCGGGGTCGCCGCATGAGAGTGCCGACGTTAAGGGGATCAATAATT 400
TCGAGCTAATACCGTTCGGCGCAGGGCGAAGAAGATGTGCGGGGATGAAG 450
CTTGGCATTCGTATGGTTCACTTCGTCACGGCTATGCTGCTGCATGCCTT 500
TGATTGGACATTACCGGATGGTTCTATGGGCGATCTACTGGACATGGAAG 550
AGGGTTATGGGGSGACGATGCGCAAGACAAGGCCGCTGATGGCTAAGGCG 600
ACGCCCCGATTGGCACCTCAAGCTTATCTTTAGTCCTTTTTTTTTTCTTT 650
CATGTGATACTGATTTTGCATGAATTTAGTTGGCCAAAAGGATGATGTTA 700
TCGAACAAATATTTGTGTAATGTGGAAACTAGTTGGGGGTAATGTTATAT 750
GGTGATTTTAGTTAAAAAAAAAAAAAAAAAAAAAAAAAAA          792
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CAAATCCTTGGCCTCAACCCAAATATCGAGCGTGTCATGCTGTATCAGCAGGGCTGT
 Q  I  L  G  L  N  P  N  I  E  R  V  M  L  Y  Q  Q  G  C
TTCGCTGGCGGAACCACTCTTCGCCTTGCCAAGTGCCTTGCCGAAAGCCGCAAAGGY
 F  A  G  G  T  T  L  R  L  A  K  C  L  E  S  R  K  G  A
GCACGAGTTCTTGTGGTTTGCGCGGAGACCACCGCCGTGTTATTTCGTGCACCATCT
 R  V  L  V  V  C  A  E  T  T  A  V  L  F  R  A  P  S  E
GAGGAGCACCAGGATGACMTGGTTACCCAAGCTTTATA
 E  H  Q  D  D  X  V  T  Q  A  L
```

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Erratum

p. 2, L. 13: Provide *should read* provided.

p. 13, L. 9: "all class of" *should read* "all classes of".

p. 18, L. 8: "developmental signal" *should read* "developmental signals".

p. 21, L. 3: "genes resource" *should read* "gene resource".

p. 27, L. 4: "using the general procedures are stated below" *should read* "using the general procedures stated below".

p. 44, L. 16: "(Katsumoto et al., 2007)" *should read* "(Katsumoto et al., 2007).".

p. 91, L. 14: "functional significances" *should read* "functional significance".

p.118, L. 18: "is differ" *should read* "is different".

p.119, L. 17: *omit* "preventing".