Identification and expression analysis of flavonoid biosynthetic genes in the genus *Clivia*

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Declaration

I, the undersigned, hereby declare that the work contained in this dissertation is my original work and that I have not previously in its entirety or in part submitted it at any university for a degree. Furthermore, I waive my rights as author in favour of the University of the Free State.

______________________ Date: ______________________

Marius C. Snyman

Table of Contents

Table of Contents iii

List of Abbreviations vi

Acknowledgements x

1. General Introduction 1

2. Literature Review 3

A. Biochemistry and Genetics of Anthocyanins 3

2.1 Introduction 3

2.2 Anthocyanin biosynthesis 4

2.2.1 Chalcone synthase (CHS) 7

2.2.2 Chalcone isomerase (CHI) 8

2.2.3 Flavanone 3-hydroxylase (F3H) 8

2.2.4 Flavonoid 3'-hydroxylase (F3'H) and Flavonoid 3',5'-hydroxylase (F3'5'H) 9

2.2.5 Dihydroflavonol 4-reductase (DFR) 10

2.2.6 Anthocyanidin synthase (ANS) and UDP-glucose:flavonoid 3-O-glucosyltransferase (3GT) 12

2.3 Anthocyanin structure and modification 12

2.4 Regulation of anthocyanin biosynthesis 14

2.5 Cellular localization, transport and accumulation of anthocyanins 17

2.6 Factors influencing anthocyanin stability and colour in flowers 19

2.6.1 pH 19

2.6.2 Co-pigmentation 21

2.6.3 Metal complexes 22

2.7 Important biological functions of anthocyanins in plants 22

2.7.1 Pigmentation 22

2.7.2 Stress protection 23

2.8 Genetic engineering in floriculture 23

B. Notes on the genus *Clivia* Lindl. 25

2.9 Introduction 25

2.10 Morphological characterization and distribution 26
2.10.1 *Clivia miniata* (Lindley) Regel (1854) 27
2.10.2 *Clivia nobilis* Lindley (1828) 29
2.10.3 *Clivia caulescens* Dyer (1943) 30
2.10.4 *Clivia gardenii* Hooker (1856) 32
2.10.5 *Cilvia robusta* Murray *et al*. (2004) 33
2.10.6 *Clivia mirabilis* Rourke (2002) 34
2.11 *Clivia* floral pigmentation 36
2.12 Aims of this study 40

3. Materials and Methods **41**
3.1 Identification of *Clivia* flavonoid biosynthetic gene sequences 41
3.1.1 Plant material 41
3.1.2 Total RNA isolation 41
3.1.3 Degenerate primer design 42
3.1.4 First-strand cDNA synthesis 43
3.1.5 PCR reaction setup 44
3.1.6 Agarose gel electrophoresis 44
3.1.7 Sequencing of PCR products 44
3.1.8 Sequence assembly and analysis 45
3.1.9 Phylogenetic analysis 46
3.2 Expression analysis of *CHS* and *DFR* in *Clivia miniata* flowers 47
3.2.1 Plant material 47
3.2.2 Total RNA isolation 48
3.2.3 Amplification of *Clivia miniata 18S* ribosomal RNA (rRNA) 49
3.2.4 Primer design 49
3.2.5 First-strand cDNA synthesis 50
3.2.6 Real-time quantitative PCR (qPCR) 51
3.2.7 Data analysis 51
3.3 Total anthocyanin determination 53
3.3.1 Plant material 53
3.3.2 Anthocyanin extraction 53
3.3.3 Spectrophotometry 53
3.3.4 Statistical analysis 54

4. Results and Discussion **55**

4.1 Identification of *Clivia* flavonoid biosynthetic genes 55

4.1.1 Degenerate primer design and PCR amplification 55

4.1.2 Identification of *Clivia CHS*, *CHI*, *F3H* and *DFR* genes 59

4.1.3 Sequence analysis 61

4.1.4 Phylogenetic analysis 63

4.1.5 Towards gene characterization: Isolation of the full-length gene sequences 64

4.2 Expression analysis of *CHS* and *DFR* in *Clivia miniata* flowers 70

4.2.1 Introduction 70

4.2.2 Efficiency of the qPCR assay 72

4.2.3 Analysis of relative gene expression with real-time qPCR involving SYBR Green chemistry 74

4.2.4 Expression of flavonoid biosynthetic genes in *Clivia miniata* var. *miniata* ‘Plantation’ 77

4.2.5 Expression of flavonoid biosynthetic genes in *Clivia miniata* var. *citrina* ‘Giddy’ 80

4.2.6 Future prospects concerning regulation of anthocyanin pigmentation in *Clivia* 82

4.3 Total Anthocyanin determination 85

4.3.1 Total anthocyanin determination 85

4.3.2 Gene expression vs. Anthocyanin production 87

4.3.3 Future considerations for total anthocyanin determination 90

5. Concluding remarks **91**

6. Summary **94**

7. Opsomming **96**

8. Literature Cited **98**

Appendices **127**

List of Abbreviations

°C	Degree Celsius
µg	Microgram
µl	Microlitre
µM	Micromolar
2-ODD	2-oxogluturate dependent dioxygenase
4CL	4-coumaroyl:CoA-ligase
A	Adenine
AAT	Anthocyanin acetyl transferase
AFLP	Amplified fragment length polymorphism
A_{max}	Maximum absorbance
ANOVA	Analysis of variance
ANR	Anthocyanidin reductase
ANS	Anthocyanidin synthase
ATP	Adenosine triphosphate
AVI	Anthocyanic vacuolar inclusion
bHLH	Basic helix-loop-helix
BLAST	Basic local alignment search tool
bp	Base pairs
C	Cytosine
C_{15}	15 Carbons
C4H	Cinnamate 4-hydroxylase
cDNA	Complementary DNA
CHI	Chalcone isomerase
CHR	Chalcone reductase
CHS	Chalcone synthase
cm	Centimeter
Cm18S rRNA	*Clivia miniata* 18S ribosomal ribonucleic acid gene
CmCHS	*Clivia miniata* chalcone synthase gene
CmDFR	*Clivia miniata* dihydroflavonol 4-reductase gene
CODEHOP	Consensus degenerate hybrid oligonucleotide primer
C_t	Cycle threshold

Cy	Cyanidin
DEPC	Diethylpyrocarbonate
DFR	Dihydroflavonol 4-reductase
dH_2O	Distilled water
DHK	Dihydrokaempherol
DHM	Dihydromyricetin
DHQ	Dihydroquercitin
Dicot	Dicotyledon
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
dNTP	Deoxynucleotide triphosphate
DTT	Dithiothreitol
Dp	Delphinidin
EBG	Early biosynthetic gene
EDTA	Ethylene diamine tetra-acetic acid
ER	Endoplasmic reticulum
***et al*.**	'And others'
EtOH	Ethanol
F3'5'H	Flavonoid 3',5'-hydroxylase
F3'H	Flavonoid 3'-hydroxylase
F3H	Flavanone 3-hydroxylase
FLS	Flavonol synthase
FNS	Flavone synthase
FW	Fresh weight
g	Gram
G	Guanine
g.	Gravitational force
GMO	Genetically modified organism
GSP	Gene-specific primer
GT	Glucosyl transferase
HCl	Hydrochloric acid
HPLC	High-performance liquid chromatography
IFS	Isoflavone synthase
IPCR	Inverse polymerase chain reaction

ISSR	Inter-simple sequence repeat
IUPAC	International Union of Pure and Applied Chemistry
KCl	Potassium chloride
LAR	Leucoanthocyanidin reductase
LBG	Late biosynthetic gene
log	Logarithm
MAS	Marker-assisted selection
MatGAT	Matrix global alignment tool
$MgCl_2$	Magnesium chloride
min	Minute
ml	Millilitre
mm	Millimetre
mM	Millimolar
M-MuLV	Moloney Murine Leukemia virus
Monocot	Monocotyledon
mRNA	Messenger ribonucleic acid
MRP	Multidrug resistance-associated protein
Mv	Malvidin
NCBI	National Center for Biotechnology Information
NJ	Neighbor-joining
nm	Nanometre
NMR	Nuclear magnetic resonance
OMT	O-methyltransferase
p	Statistical significance / “probability”
PAL	Phenylalanine ammonia-lyase
PAP1	Production of anthocyanin pigment 1
PCR	Polymerase chain reaction
Pg	Pelargonidin
pH	“Power (or potential) of hydrogen”
pmol	Pico-mole
Pn	Peonidin
Pt	Petunidin
qPCR	Quantitative polymerase chain reaction
qRT-PCR	Quantitative reverse transcriptase polymerase chain reaction

QTL	Quantitative trait loci
R	Pearson correlation coefficient
R^2	Coefficient of determination
RACE	Rapid amplification of cDNA ends
RAPD	Randomly amplified polymorphic DNA
RFLP	Restriction fragment length polymorphism
RNA	Ribonucleic acid
rRNA	Ribosomal ribonucleic acid
RT	Rhamnosyl transferase
RT-PCR	Reverse transcriptase polymerase chain reaction
SDS	Sequence detection system
sec	Second(s)
STS	Stilbene synthase
T	Thymine
Ta	Annealing temperature
TAE	Tris, acetic acid, EDTA
Taq	*Thermus aquaticus*
Tm	Melting temperature
U	Units
UV	Ultraviolet
V	Voltage
v	Version
v/v	Volume per volume
w/v	Weight per volume

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Chapter 1

GENERAL INTRODUCTION

Plant life not only provides us with important nutritional resources, but also nourishes our souls with its beauty and endless array of colours. For more than a century, extensive work on the topic of plant colouration added new solutions and techniques which enabled some advances in molecular biology. Flower pigmentation has grasped the attention of hundreds of researchers, unravelling the mysteries and establishing models for plant colouration. At present much is known about the chemical compounds that provide colour and how they infer certain health-promoting qualities. Due to knowledge of the genetics and biochemistry of these compounds the execution of biotechnological projects were possible, thereby changing the production of these compounds towards the increased phytochemical value of a plant, or changing a plant's colour according to aesthetic demand.

The next chapter contains reviews of the biosynthetic steps, genetics, regulation and cellular localisation of the flavonoid biosynthetic pathway, with reference to the well-established anthocyanin biosynthetic branch. The important end-products, particularly the anthocyanin pigments, with their chemical structure and properties as colouring agents are also discussed. For the purpose of this study, the main emphasis was on how sequential gene expression finally produces anthocyanin pigmentation in flowers and how the cellular environment of anthocyanins influences their photochemical properties. To prevent confusion all gene and cDNA names are shown in italics throughout the text.

The literature review is accompanied by a section where the genus *Clivia* and its species are briefly described in terms of morphology and distribution. Clivias are currently the subject of considerable floricultural attention among conventional breeders who are trying to introduce new and exciting flower colours into the market, thus broadening the colour range. Despite all this attention, very little is known regarding the biochemistry and genetics of anthocyanin biosynthesis in *Clivia* flowers. Therefore the principle objective of this study was initiating molecular research to understand and elucidate *Clivia* anthocyanin biosynthesis with its ultimate long-term goal the acquisition of the necessary information for biotechnological applications. According to the outcomes of studies conducted during the past 20 years, genetic engineering can be considered a more attractive and efficient approach towards obtaining new *Clivia* flower colours.

Following the literature review, the third chapter describes the general materials and methods used to address the predetermined objectives of this study. Selected molecular techniques, reagents, composition of solutions, and computer software used are mentioned and/or described. Some methods are briefly referred to in the fourth chapter, which comprises the findings of each investigation in combination with relevant discussions. Finally, concluding remarks are made in an attempt to answer some of the questions that were initially asked regarding the aims of this study.

Chapter 2

LITERATURE REVIEW

Section A: BIOCHEMISTRY AND GENETICS OF ANTHOCYANINS

2.1 Introduction

Anthocyanins (Greek: *anthos* meaning flower, and *kyanos* meaning blue) are probably the most important group of plant pigments visible to the human eye (Kong *et al.*, 2003). They are naturally occurring, water-soluble compounds that have gained a great deal of attention for nearly five centuries because they fulfil a wide range of biological functions including their contribution to the beautiful and diverse pigmentation throughout the plant kingdom (Harborne and Williams, 2000). Anthocyanins are members of a widespread class of phenolic compounds collectively known as the flavonoids. They are the most conspicuous and provide most of the orange, red, blue and purple cyanic pigmentation in flowers, fruits, vegetables and leaves (Mol *et al.*, 1998; Tulio *et al.*, 2008).

Although anthocyanins are the major flower pigments, other phytochemical compounds known as the carotenoids and the betalains also contribute to the colouration in flowers, fruits and vegetables (Mol *et al.*, 1998). Carotenoids are generally responsible for flower colours in the yellow to orange range. Some species that belong to the Asteraceae are examples of plants that exhibit a wide range of petal colours due to a combination of both anthocyanins and carotenoids (Kishimoto *et al.*, 2007). Betalains, which are usually associated with red leaf colour, are restricted to the suborder Chenopodineae within the Caryophyllales and have not been found together with anthocyanins in the same plant (Manetas, 2006).

There are three types of flavonoids synthesised by virtually all higher plants that contribute to pigmentation: anthocyanins, mentioned above; flavonols, which provide yellow colour in some plants; and proanthocyanins (or condensed tannins) that provide brown pigmentation for a variety of plant seeds. Other major subgroups include chalcones, flavones, flavandiols (Winkel-Shirley, 2002). Finally, specialised forms of flavonoids also exist, such as aurones, which also provide bright yellow colouration in the flowers of snapdragon (*Antirrhinum* *majus*) and dahlia (*Dahlia variabilis*) (Ono *et al.*, 2006), and the 3-deoxyanthocyanins that provide red pigmentation in the kernels of plants such as maize and sorghum. Two other important classes, the flavanones and isoflavonoids, do not contribute to plant pigmentation, but play other essential roles (Winkel-Shirley, 2002).

Since Gregor Mendel's experiments on flower and seed coat colour, among others, in peas during the early 19^{th} century the striking pigmentation provided by flavonoids has resulted in extensive research that unravelled some of the basic principles of genetics and biochemistry and therefore contributed enormously to the advances in modern biology. "*The remarkable diversity of form and function of flavonoids in present-day plants has provided a rich foundation for research in areas ranging from genetics and biochemistry to chemical ecology and evolution to human health and nutrition*" (Winkel, 2006). The focus of the following sections will mainly be on genetics and biochemistry that affect each step of central flavonoid biosynthesis, especially the well-established anthocyanin biosynthetic pathway.

2.2 Anthocyanin Biosynthesis

The flavonoids are located within the cellular cytosol and vacuole or on the surfaces of different plant organs (Beld *et al.*, 1989; Stobiecki and Kachlicki, 2006). Their classical chemical structures are based on a C_{15} (C_6-C_3-C_6) skeleton, commonly consisting of an aromatic A -and B-ring as well as one heterocyclic C-ring containing one oxygen atom (Figure 2.1). Flavonoids may be modified by hydroxylation, methoxylation or *O*-glycosylation of the hydroxyl groups as well as by C-glycosylation directly to a carbon atom of the flavonoid skeleton (Stobiecki and Kachlicki, 2006).

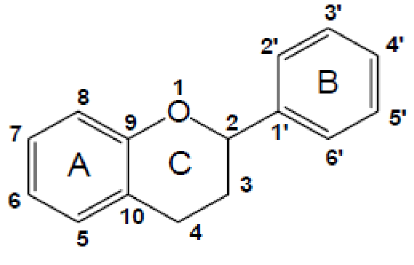


Figure 2.1: The basic flavonoid structure including the numbering system.

Flavonoids are only one class of the thousands of phenolic compounds that are produced through the phenylpropanoid pathway and its specific branching reactions. The phenylpropanoid pathway is exclusively located in the cytoplasm and catalyses the conversion of the amino acid phenylalanine (Phe), which is derived from the shikimate pathway in the plastids and serves as the base for the flavonoid B-ring (Winkel-Shirley, 1999). Phenylalanine ammonia-lyase (PAL) catalyses the conversion of Phe to the precursor for chalcone synthesis, coumaroyl-CoA (Weisshaar and Jenkins, 1998). The central flavonoid pathway (Figure 2.2) that ultimately leads to anthocyanin biosynthesis was extensively studied with the use of maize (*Zea mays*), snapdragon (*Antirrhinum majus*), petunia (*Petunia x hybrida*) and *Arabidopsis* (Holton and Cornish, 1995; Winkel-Shirley, 2001b). In the following subsections the enzymes that catalyse the reactions in anthocyanin biosynthesis, as well as the corresponding structural genes (Table 2.1) will be discussed briefly.

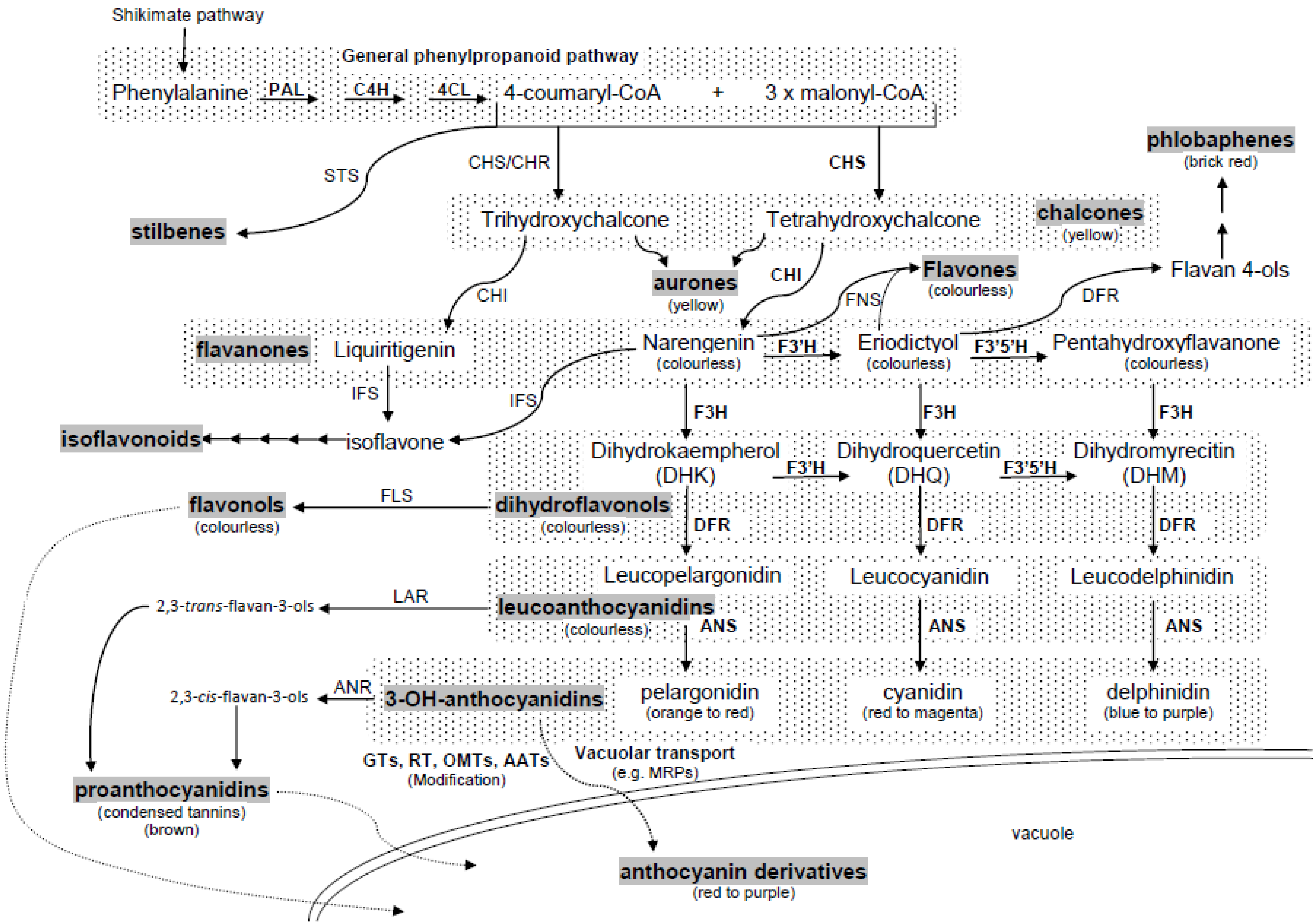


Figure 2.2: Schematic of the flavonoid biosynthetic pathway showing the enzymatic steps leading to the major classes of end products (highlighted in grey). Names of the major classes of intermediates are given. Enzymes are indicated with standard abbreviations: AATs, anthocyanin acetyl transferases; ANR, anthocyanidin reductase; ANS, anthocyanidin synthase (also known as leucoanthocyanidin dioxygenase); C4H, cinnamate-4-hydroxylase; CHI, chalcone isomerase; CHR, chalcone reductase; CHS, chalcone synthase; 4CL, 4-coumaroyl:CoA-ligase; DFR, dihydroflavonol 4-reductase; F3H, flavanone 3-hydroxylase; FLS, flavonol synthase; FNS, flavone synthase; F3'H and F3'5'H, flavonoid 3' and 3'5' hydroxylase; IFS, isoflavone synthase; LAR, leucoanthocyanidin reductase; MRPs, multidrug resistance-associated proteins; OMTs, O-methyltransferases; PAL, phenylalanine ammonia-lyase: GTs, glucosyl transferases; RT, rhamnosyl transferase; STS, stilbene synthase.

Table 2.1: The genetic loci of model plant species encoding the enzymes of the central flavonoid biosynthetic pathway leading to coloured anthocyanidin 3-glucosides (Obtained from: Holton & Cornish, 1995; Winkel-Shirley, 2002; Chopra *et al.*, 2006).

		Structural loci				
Gene product		**Maize**	**Petunia**	**Snapdragon**	***Arabidopsis***	**Morning Glory**
Chalcone synthase	(CHS)	*c2, whp*	*chsA, chsJ*	*niv*	*tt4*	*R1, A*
Chalcone isomerase	(CHI)	*chi1*	*po*		*tt5*	*Sp, Cr*
Flavanone 3-hydroxylase	(F3H)		*an3*	*inc*	*tt6*	
Flavonoid 3'-hydroxylase	(F3'H)	*pr1*	*ht1, 2*		*tt7*	*Mg, P, Fuchnia*
Flavonoid 3',5'-hydroxylase	(F3'5'H)		*hf1, 2*			
Dihydroflavonol 4-reductase	(DFR)	*a1*	*an6*	*pal*	*tt3*	*A3, Pearly*
Anthocyanin Synthase	(ANS)	*a2*		*candi*	*tt18*	*R3*
UDP-Glc:anthocyanidin 3-O-glucosyltransferase	(3GT)	*bz1*			*fgt-1*	*Dk*

2.2.1 Chalcone synthase (CHS)

A chalcone synthase (*CHS*) cDNA clone from parsley was the first flavonoid biosynthetic gene to be isolated (Kreuzaler *et al.*, 1983). CHS provides the entry point and catalyses the stepwise condensation of one *p*-coumaryl-CoA and three malonyl-CoA molecules, which is formed via acetyl-CoA metabolism, to yield narengenin chalcone, the precursor for a large number of flavonoids (Weisshaar and Jenkins, 1998; Claudot *et al.*, 1999; Lunkenbein *et al.*, 2006). Chalcones and dihydrochalcones are considered to be the primary precursors and constitute the main intermediates for flavonoid synthesis (Marais *et al.*, 2006).

Analyses of *CHS* genes has shown that the enzyme is encoded by a multigene family in which the copy number varies among plant species and functional divergence and gene duplication appear to have occurred repeatedly. For example, the *CHS* genomic copy number in grapevine (*Vitis vinifera*) was estimated at three to four (Goto-Yamamoto *et al.*, 2002), eight members have been identified in both *Petunia* strain V30 (*ChsA*, *B*, *D*, *F*, *G*, *H*, *J*, *L*) (Koes *et al.*, 1989) and Soybean (*Glycine max*) (Tuteja *et al.*, 2004). In *Petunia*, *ChsA* and *ChsJ* are the only genes transcribed to a significant extent in flower tissue (Holton and Cornish, 1995; O'Dell *et al.*, 1999). Southern hybridization results indicated about seven copies in barley (Christensen *et al.*, 1998), and six genes are present in Morning Glory (*Ipomoea purpurea*) (Durbin *et al.*, 2000). *Antirrhinum* and *Arabidopsis* are known to carry single copies of the gene (Fukada-Tanaka *et al.*, 1997).

2.2.2 Chalcone Isomerase (CHI)

Chalcone isomerase (or chalcone flavanone isomerase) (CHI) converts the yellow chalcones into the corresponding flavanones, in this case the colourless narengenin, by an intramolecular reaction during which the C-ring is closed (Grotewold and Peterson, 1994), thus accelerating a stereo-chemically-defined intramolecular cyclisation reaction yielding a biologically active (S)-isomer (Jez and Noel, 2002). The first *CHI* cDNA clone was isolated from French bean (*Phaseolus vulgaris*) by antibody screening of mRNA extracted from elicitor-treated bean cells (Mehdy and Lamb, 1987). Two CHI isozymes have been identified: (1) the more common CHI1-type that can utilise 6'-hydroxychalcone substrates, and (2) the CHI2-type that can catalyse the isomerisation of both 6'-hydroxy- and 6'-deoxychalcones. Tandem gene clusters of both types are found in *Lotus japonicus* and it was suggested that type 2 *CHIs* evolved from an ancestral type 1 *CHI* by gene duplication (Shimada *et al*., 2003; Ralston *et al*., 2005). The growing interest for developing food products with increased health benefits has been illustrated by a transgenic approach where a *Petunia hybrida CHI* gene was transformed into, and over-expressed in tomato fruit, producing elevated levels of peel flavonols (Muir *et al*., 2001).

2.2.3 Flavanone 3-hydroxylase (F3H)

Flavanone 3-hydroxylase (F3H) hydroxylates narengenin at carbon 3 of the flavonoid structure to provide dihydrokaempherol (DHK), which is one of the dihydroflavonols. Dihydroflavonols are the precursors for many classes of flavonoid compounds (Pelletier and Shirley, 1996; Holton and Cornish, 1995). F3H is a soluble nonheme 2-oxogluturate dependent dioxygenase (2-ODD) that has 14 conserved amino acids, including those that play a role in Fe^{2+} and 2-oxogluturate binding (Britsch *et al*., 1993). Martin *et al*. (1991) isolated the first *F3H* cDNA clone, corresponding to the *incolorata* locus in *Antirrhinum*, by means of differential screening.

2.2.4 Flavonoid 3'-hydroxylase (F3'H) and Flavonoid 3',5'-hydroxylase (F3'5'H)

The hydroxylation pattern of the B-ring at the C (carbon)-3' and C-5' positions of flavonoids is determined by the presence and activity of flavonoid 3'-hydroxylase (F3'H) and flavonoid 3',5'-hydroxylase (F3'5'H) (Figure 2.3). Both these enzymes belong to the cytochrome P450 proteins and have shown to hydroxylate a wide range of flavonoid substrates. Anthocyanin colour shifts towards blue due to this increased hydroxylation. Most violet/blue flowers contain delphinidin-based anthocyanins (3',4',5'-hydroxy anthocyanins). In the central flavonoid biosynthetic pathway, F3'H catalyses the 3'-hydroxylation of DHK to form dihydroquercitin (DHQ), and F3'5'H catalyses the 3',5'-hydroxylation of DHK to form dihydromyricetin (DHM). F3'5'H can also convert DHQ to DHM (Seitz *et al.*, 2006; Togami *et al.*, 2006).

The genes and cDNAs for both these enzymes, sometimes referred to as the red (*F3'H*) and blue genes (*F3'5'H*), have been cloned and characterised from *Petunia*. *F3'5'H,* for example, was isolated via PCR with degenerate oligonucleotides that were designed based on the conserved P450 heme-binding domain. Restriction fragment length polymorphism (RFLP) mapping and complementation of mutant petunia lines showed that the *F3'5'H* genes correspond to the genetic loci *Hf1* and *Hf2* (Holton *et al.*, 1993; Toguri *et al*., 1993; Brugliera *et al*., 1999). De Vetten *et al.* (1999) showed that the activity of F3'5'H, but not F3'H, is reduced in *difF* ($cytb_5$ gene) mutant *Petunia* lines, resulting in altered flower colour and therefore indicating the required activation role of cytochrome b_5. *F3'H* and *F3'5'H* are both very important genes used in engineering flower colour.

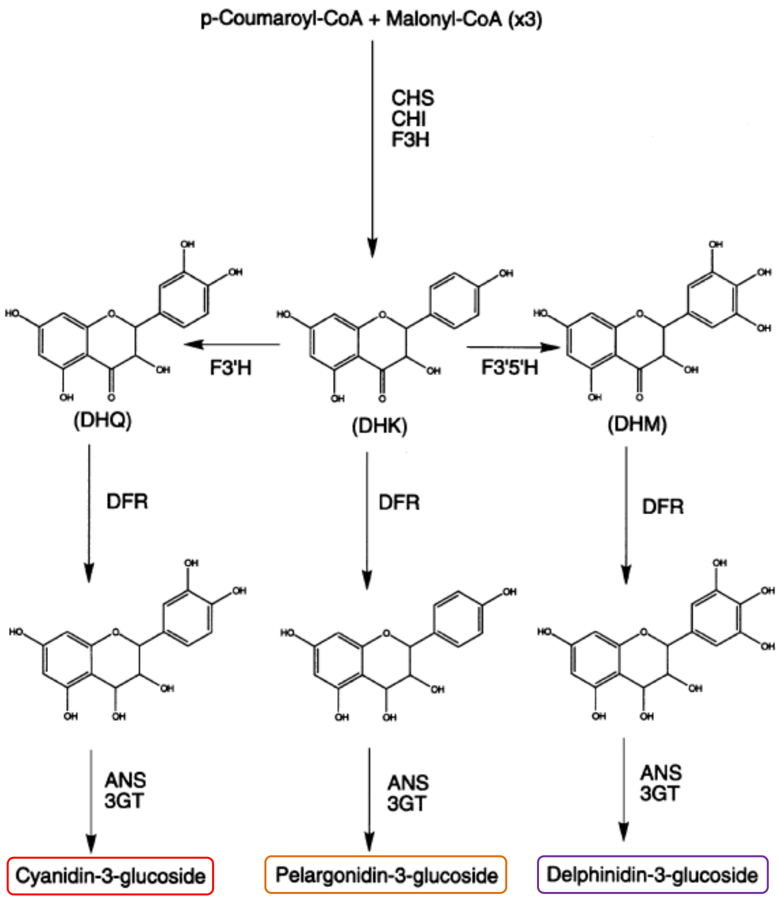


Figure 2.3: A schematic diagram showing the topology of the branching point in the anthocyanin biosynthetic pathway where Flavonoid 3'-hydroxylase (F3'H), Flavonoid 3',5'-hydroxylase (F3'5'H), and dihydroflavonol 4-reductase (DFR) play important roles in anthocyanin determination (modified from Johnson *et al*., 2001).

2.2.5 Dihydroflavonol 4-reductase (DFR)

The next entry step, ultimately leading to anthocyanin biosynthesis, is catalysed by dihydroflavonol 4-reductase (DFR). DFR is located in an important regulatory branching point in the pathway and also catalyses the reactions upstream of proanthocyanidin and phlobabene production (Himi and Noda, 2004). It is a key enzyme responsible for the NADPH-dependent reduction of the dihydroflavonols (DHK, DHQ and DHM) to colourless leucoanthocyanidins (flavan-3,4-cis-diols). These substrates are very similar in structure and DFRs from different species can utilise all three substrates (Liu *et al.*, 2005), whereas the preference in other species varies markedly.

In particular, pelargonidin-based pigments rarely accumulate in *Arabidopsis thaliana*, *Vaccinium macrocarpon*, *Cymbidium hybrida*, *Gentiana triflora* and *Petunia hybrida* because none of these species can efficiently reduce DHK (Meyer *et al.*, 1987; Johnson *et al.*, 1999; Polashock *et al*., 2002; Zufall and Rausher, 2003). DFRs from *Callistephus chinensis*, *Dianthus caryophyllus* and *Dahlia variabilis*, in contrast, can accept all three dihydroflavonols as substrates (Martens *et al*., 2002; Yu *et al*., 2006). These are only a few examples where DFR enzymes are either substrate generalists or substrate specialists, partly determining the nature of anthocyanins being produced. Substrate specificity appears to be based on a 26 amino acid region of the DFR polypeptide where any variability or even a single amino acid change can alter enzyme specificity (Johnson *et al*., 2001). Alteration of *DFR* expression levels has been used to modify flower colour in ornamental plants (Aida *et al*., 2000a, 2000b).

The first DNA sequences for *DFR* were identified in *Zea mays* and *Antirrhinum majus* by transposon tagging (O'Reilly *et al.*, 1985; Holton and Cornish, 1995). Since then many other full-length *DFR* sequences, single or multiple gene(s), from a number of plant genomes have been cloned and characterised (listed in Shimada *et al*., 2005). The number of *DFR* genes, as with *CHS*, is variable in the genomes of different plants, some having replicated versions of the gene and others having only single copies. The use of southern analyses and molecular cloning has proven that small *DFR* gene families occur in some plants. For example, two different sequences for *DFR* are presented at two loci in *Vaccinium macrocarpon* and *Zea mays* (Bernhardt *et al.*, 1998; Polashock *et al*., 2002). Three *DFR* genes are present in hexaploid *Triticum aestivum* and *Petunia* (Beld *et al.*, 1989; Himi and Noda, 2004). After structural and functional characterisation, five *DFR* genes, the largest number so far, were found to be located in tandem at a single locus in the genome of *Lotus japonicus* (Shimada *et al.*, 2005). Two orchid species, *Cymbidium hybrida* and *Bromheadia finlaysoniana* are known to carry a single copy of *DFR* in their genomes (Liew *et al*., 1998; Johnson *et al*., 1999).

2.2.6 Anthocyanidin synthase (ANS) and UDP-glucose:flavonoid 3-O-glucosyltransferase (3GT)

Leucoanthocyanidins, formed previously through dihydroflavonol reduction by DFR, are the direct precursors of the coloured anthocyanidins. Anthocyanidin is hardly detected in plant tissues because of its instability at physiological pH. The 2-oxoglutarate-dependent oxidation of leucoanthocyanidin to 2-flavan-3,4-diol, which can then be readily converted to anthocyanidin 3-*O*-glycoside (or coloured “anthocyanin 3-glucoside”) is catalysed by the action of anthocyanidin synthase (ANS) and UDP-glucose:flavonoid 3-*O*-glucosyltransferase (3GT) (Saito *et al.*, 1999; Nakajima *et al*, 2001). 3GT catalyses the transfer of glucose from UDP-glucose to C-3 of anthocyanidins and flavonols, increasing water solubility and improving stability by external hydrogen bonding of sugar residues with the surrounding water molecules in the vacuole (Yu *et al.*, 2006). According to Kong *et al*. (2003), cyanidin 3-glucoside is the most widespread anthocyanin in nature.

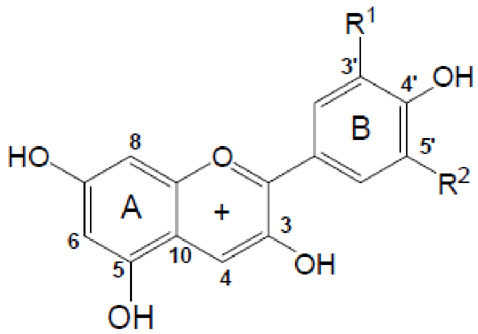
DNA sequences for *ANS* were first identified and cloned from mutant maize line generated through transposon tagging of the *A2* mutant (Menssen *et al.*, 1990). The *A2* mutation blocked the enzymatic conversion of leucoanthocyanidins to anthocyanidins. Based on homology, the *A2* sequence enabled successful identification of the *candi* locus in snapdragon and the petunia *ant17* locus (Holton and Cornish, 1995).

2.3 Anthocyanin structure and modification

Anthocyanins consist of an aglycone (anthocyanidin, also known as an anthocyanin chromophore), with a sugar moiety (mainly attached at position 3 on the C-ring or at position 5 or position 7 on the A-ring (Prior and Wu, 2006). The nature of the sugar (e.g. glucose - glc, arabinose - ara, rutinose - rut, sambubiose - samb), acylated or not, and its position in the aglycone skeleton are important structural factors that affect the hue of these pigments (de Freitas and Mateus, 2006).

In solution at a very acidic pH ($pH < 2$), anthocyanins exist primarily as positively charged equilibrium forms known as the stable flavylium cation. Approximately 90% of all anthocyanins in higher plants are based on the six most common anthocyanidins acting as central chromophores of anthocyanins: cyanidin (Cy), pelargonidin (Pg), delphinidin (Dp), petunidin (Pt), peonidin (Pn), and malvidin (Mv) (Kong *et al.*, 2003; Prior and Wu, 2006). They only differ depending on the hydroxylation and methoxylation pattern on their B-rings (Figure 2.4).

Currently there are 25 naturally occurring anthocyanidins, including pyranoanthocyanidins. According to Kong *et al*. (2003), the three non-methylated anthocyanidins (Cy, Dp and Pg) are the most widespread in nature, being present in 80% of pigmented leaves, 69% of fruits and 50% of flowers. In general, cyanidin-based pigments impart a pink to red colour, pelargonidon-based pigments a brick-red to orange colour, and delphinidin-based pigments are required for a blue to purple colour (Winkel-shirley, 2001a).



	Substitution pattern	
	R^1	R^2
Cyanidin (Cy)	OH	H
Pelargonidin (Pg)	H	H
Delphinidin (Dp)	OH	OH
Petunidin (Pt)	OCH_3	OH
Peonidin (Pn)	OCH_3	H
Malvidin (Mv)	OCH_3	OCH_3

Figure 2.4: The structures of the most common anthocyanidins occurring in nature, depicted by the flavylium cation on the left and the possible R^1 and R^2 substitutions shown in the table.

Diversity within anthocyanins is achieved by certain enzymes responsible for glycosylation and methylation of the hydroxyl groups, and aromatic and/or aliphatic acylation of the core anthocyanin structure. These enzymes are discussed in great detail in review articles by Holton and Cornish (1995), Yu *et al*. (2006) and Winkel (2006). The functioning of these enzymes are responsible for the establishment of four common classes of anthocyanidin glycosides: 3-monosides, 3-biosides, 3,5-diglycosides and 3,7-diglycosides (Kong *et al.*, 2003). Therefore the variety of colours in plants is derived through the action of these modification enzymes.

In addition to 3GT, UDP-glucose:anthocyanin-glucosyltransferases also catalyse glycosylation at C5, C7 and C3' of anthocyanidins and are therefore known as flavonoid 5-*O*-glycosyltransferase (5GT), flavonoid 7-*O*-glycosyltransferase (7GT) and flavonoid 3'-*O*-glycosyltransferase (3'GT), respectively. In *Petunia*, snapdragon and many other species the anthocyanins 3-glucosides, formed through the action of 3GT, serve as the substrate for the *Rt* encoded enzyme UDP-rhamnose:anthocyanin-rhamnosyltransferase (3RT), which adds a rhamnose to the glucose at C-3 to create rutinoside (Brugliera *et al*., 1994; Kroon *et al*., 1994).

Anthocyanins can also be acylated by a group of enzymes known as anthocyanin acyltransferases (AATs). AATs catalyse the transfer of aliphatic or aromatic acyl groups from a CoA-donor molecule to the hydroxyl residues of anthocyanin sugar moieties. This increases anthocyanin stabilisation and water solubility through intermolecular stacking, also making them bluer. The third type of modification can occur through the action of *O*-methyltransferases (OMTs). The OMTs in flowers usually catalyse specific late steps in anthocyanin biosynthesis. In *Petunia hybrida*, *Fuchsia*, *Plumbago*, and *Torenia*, for example, anthocyanin OMTs acted on the 3', or the 3' and 5' hydroxyls of delphinidin 3-*O*-glucoside and delphinidin 3-*O*-rutinoside to produce 3'- or 3',5'-*O*-methylated derivatives (Brugliera *et al*., 2003).

The last known modifying enzymes are flavonoid-specific peroxidases, which occur at the final destination in the cell wall or vacuole. They are involved in the oxidation of anthocyanins to become brown or colourless (Winkel, 2006).

2.4 Regulation of anthocyanin biosynthesis

Flavonoid biosynthesis involves many structural genes and several alternative branches from common precursors and intermediates leading to the great variety of flavonoid types and other compounds. The type of species, the developmental stage of a tissue, as well as the enormous diversity of intrinsic and environmental signals such as hormones, sugar and stress factors (high-intensity light, UV light, temperature, pathogen infection, wounding, drought, and nutrient deficiency), are all factors that add to the complexity of the anthocyanin biosynthetic pathway (Chalker-Scott, 1999; Shan *et al*., 2009). Therefore fine-tuned regulation is required to allow alteration of flux as conditions vary. The activity of the anthocyanin structural genes is mainly regulated at the transcriptional level. Their intensity and expression patterns are therefore generally controlled by the expression patterns of regulatory genes (Table 2.2) (Mol *et al.*, 1998; Koes *et al.*, 2005).

These regulatory loci were identified in numerous plant species through analysing mutants in which anthocyanin biosynthesis was blocked or completely reduced. These loci encoded transcriptional activators that include members of the R2R3-MYB and the bHLH (basic helx-loop-helix) type proteins (Mol *et al*., 1998; Grotewold, 2006).

Table 2.2: Regulatory loci of the anthocyanin biosynthetic pathway characterised in different plant species (Obtained from: Springob *et al*., 2003; Morita *et al*., 2006; Gonzalez *et al*., 2008).

Type	**Maize**	**Petunia**	**Snapdragon**	***Arabidopsis***	**Morning Glory**	***Perilla***
MYB	*C1*, *Pl*	*AN2*	*MYB305*	*TT2*	*C1*	*MYB-P1*
	P	*AN4*	*MYB340*	*PAP1,PAP2*		
bHLH	*R*, *B*	*AN1*	*Delila*	*TT8*	*IVS*	*MYC-F3G1*
	IN1	*JAF13*		*GL3, EGL3*		*MYC-GP/RP*
WD40	*PAC1*	*AN11*		*TTG1*	*Ca*	*PFWD*

This was first revealed in *Zea mays* where anthocyanin accumulation was regulated by pairs of duplicated transcription factors, i.e. C1 (COLOURED ALEURONE1) and PL1 (PURPLE PLANT1), which are closely related MYB DNA-binding domain proteins, and R1 (RED1) and B1 (BOOSTER 1) (R/B family), which are bHLH proteins. The C1 or PL1 transcription factors require the presence of a member of the R/B family to be fully functional. A physical interaction occurs within the transcriptional activation complex between the R3 repeat of the MYB domain and the N-terminal region of the bHLH protein (Goff *et al.*, 1992; Cone *et al.*, 1993; Winkel-Shirley, 2002). This interaction facilitates a stabilised complex to permit transcriptional activation of anthocyanin biosynthetic genes. In addition, P, another MYB maize paralog, and C1 can also activate the expression of common flavonoid genes such as *A1* (*DFR*) by binding DNA through discrete *cis*-regulatory elements in the target gene promoters (Hernandez *et al*., 2004). P can also induce the expression of the structural anthocyanin genes independently without binding to a bHLH partner (Bruce *et al.*, 2000).

The anthocyanin biosynthetic genes in maize appear to be co-ordinately controlled as a single module (Irani *et al*., 2003).

Other species in which the regulatory mechanisms controlling anthocyanin pigmentation are still in the process of being fully elucidated are the dicot species *Antirrhinum majus*, *Petunia hybrid*a (primarily for floral pigmentation). The transcription factors of these species independently control the late biosynthetic genes (LBG), starting at *F3H*, from the early biosynthetic genes (EBG) (Mol *et al*., 1998; Nesi *et al*., 2000). Several MYB and bHLH proteins have been identified in dicot plants that regulate anthocyanin biosynthesis and exhibit high sequence similarity to C1 and R of maize: petunia AN2 and JAF13; *Arabidopsis* PAP1/PAP2 and TTG8; *Perilla frutescens* MYP-P1 and MYC-RP/GP (Springob *et al*., 2003).

In petunia there are two bHLH proteins, e.g. AN1 and JAF13 that can interact with AN2. A physical interaction between AN1 and AN2 or AN4 is necessary to activate a structural gene such as *DFR*. Transient expression assays (TEAs) have shown that both the petunia bHLH proteins, in combination with AN2, are sufficient to form an active transcription complex at the *DFR* promoter (Spelt *et al*., 2000). The MYB-type proteins of snapdragon, MYB305 and MYB340, are similar to the maize P protein in that they can activate the early biosynthetic steps without interacting with a bHLH partner (Springob *et al*., 2003). In *Ipomoea tricolor* ‘Blue Star’ the mutable *IVS* allele confers modified flower and seed pigmentation caused by an intragenetic tandem duplication of a bHLH-encoding gene (Park *et al*., 2004).

Borevitz *et al*. (2000) used activation tagging by *Agrobacterium*-mediated transformation to acquire the *PAP1* (production of anthocyanin pigment1) transcription factor gene, which encode a MYB-type protein. Over-expression of *PAP1* resulted in a PAP1-D mutant that exhibited purple pigmentation throughout the whole plant due to the widespread activation of the phenylpropanoid pathway. Many flavonoid-related mutants have been isolated from *Arabidopsis thaliana*, all on the basis of changes in seed coat colour, therefore referred to as transparent testa (*tt*) mutants (Springob *et al*., 2003). Cloning and sequencing of the *TT8* gene, which is a bHLH-type maize *R1* ortholog, and the *TT2* gene, which is an MYB-type gene, were permitted through isolation of T-DNA-tagged *Arabidopsis* mutants. In the presence of TT8, TT2 was able to induce temporal and spatial expression of *DFR* and the

BANYULS gene (putative leucoanthocyanidin reductase) in immature seed (Nesi *et al.*, 2000; Nesi *et al.*, 2001).

Although R2R3-MYB and bHLH transcription factors have been extensively identified and characterised in dicots and Poaceae species, only a few studies have dealt with these regulators in the flowers of monocot species. Recently the *OgMYB1* gene was identified in *Oncidium* Gower Ramsey, and during the study it was demonstrated that differential expression of the gene was critical for the unique floral colouration pattern (Chiou *et al.*, 2008). Nakatsuka *et al*. (2009) cloned and characterised the first monocot bHLH genes, *LhbHLH1* and *LhbHLH2*. The latter's expression paralleled anthocyanin accumulation in leaves during different light exposures.

Another subgroup of regulators known as the WD40 repeat proteins also participates in the regulation of anthocyanin genes. The first known WD40 gene, *AN11*, was discovered in petunia and encodes a small protein with five to six conserved WD repeats. It was shown that *AN11* mutants that lacked flower pigmentation were partially rescued by *AN2*/*AN1* over-expression in the petals (de Vetten *et al.*, 1997). *AN11* orthologs, i.e. *TTG1*, *PFWD*, *Ca* and *PAC1* (pale aleurone color1), have been isolated from *Arabidopsis, Perilla frutescens*, *Ipomoea nil* (Japanese morning glory), and maize, respectively (Springob *et al.*, 2003; Morita *et al.*, 2006; Selinger and Chandler, 1999).

2.5 Cellular localisation, transport and accumulation of anthocyanins

Immuno-localisation experiments indicate that flavonoid biosynthetic enzymes are loosely bound to the endoplasmic reticulum (ER) within a multi-enzyme complex, whereas the pigments themselves (anthocyanins and proanthocyanins) occur within the vacuole (Koes *et al.*, 2005). Anthocyanins are able to confer their colouration as soon as they reach the acidic environment within the vacuolar lumen (discussed in section 2.6). After biosynthesis in the cytosol they need to be effectively transported into the cellular vacuoles where they must be stabilised for prolonged accumulation. Certain mechanisms, although not as well-understood as anthocyanin biosynthesis, do exist and portray vacuolar deposition and sequestration.

The acidic environment in the vacuolar lumen and the more neutral pH of the cytosol create a pH gradient across the vacuolar membrane. This gradient fuels the movement of compounds across the membrane via tonoplast-localized ATP- or pyrophosphate (PPi)-powered proton pumps (Maeshima, 2001). A transporter that is dependent on this pH gradient has been suggested by Hopp and Seitz (1987) after investigating anthocyanin uptake into carrot vacuoles. Klein *et al*. (1996) proposed the existence of an isovitexin/H^+-antipoter in barley after investigating the vacuolar uptake of a radiolabeled flavone glucoside, isovitexin.

The presence of glutathione *S*-transferase (GST) proteins, known to be involved in xenobiotic detoxification systems, were first suggested by Marrs *et al*. (1995) to participate in the last genetically-defined step in anthocyanin biosynthesis in maize. They cloned the maize *Bz2* gene after identifying a *bz2* (bronze-2) mutant that is deficient in anthocyanins, leading to bronze kernel pigmentation. The *Bz2* gene encodes a type III GST protein required for the vacuolar uptake of anthocyanin-glutathione conjugates. A comparable step in the petunia anthocyanin biosynthetic pathway is controlled by the *An9* (Anthocyanin9) gene which encodes a type I GST protein. A petunia *an9* mutant has acyanic petals (Alfenito *et al.*, 1998). Similarly, a mutated GST encoding gene in *Arabidopsis* leads to a *tt19* mutant, which has reduced anthocyanin and proanthocyanin accumulation in seedlings and seed, respectively (Kitamura *et al*., 2004).

Another class of proteins, the multidrug resistance-associated proteins (MRPs), is known to facilitate vacuolar transport and sequestration of anthocyanin-glutathione conjugates in plants. The *Arabisopsis* MRP transporters, AtMRP1 and AtMRP2, mediate the *in vitro* vacuolar uptake of anthocyanin-glutathione conjugates in heterologous yeast (Lu *et al*., 1997; Lu *et al*., 1998). Another MRP protein found on the tonoplast membrane in maize is encoded by *ZmMRP3* and appears to also play a role in the vacuolar accumulation of anthocyanins. The expression of *ZmMRP3* correlated with the anthocyanin accumulation and was also co-regulated with the anthocyanin structural genes (Goodman *et al*., 2004).

In addition to anthocyanins existing in solution within the vacuole, they have been observed in the anthocyanin containing epidermal cells of red-cabbage plants and many angiosperm species in association with intensely pigmented structures called anthocyanoplasts. It was proposed that an anthocyanoplast is a membrane-bound intracellular compartment containing the late enzymes of anthocyanin biosynthesis (Small and Pecket, 1982). More recent reports, however, indicate that these structures may be protein matrices and that they do not possess a membrane or internal structure (Markham *et al.*, 2000). Similar structures known as "anthocyanic vacuolar inclusions" (AVIs) have been observed in the vacuoles of adaxial epidermal cells. These inclusions have a profound effect on the colour and intensity of carnation and lisianthus petals. AVIs contain proteinacious matrices with a high degree of specificity for anthocyanins (Markham *et al.*, 2000).

2.6 Factors influencing anthocyanin stability and colour in flowers

2.6.1 pH

The earliest discovered factor known to influence colour in flowers is vacuolar pH. The vacuolar pH varies greatly among different species and may also be different depending on the tissues or developmental stage, but is generally between 4 and 6 (Stintzing and Carle, 2004; Yu *et al*., 2006). Anthocyanins can undergo structural transformations depending on the pH of the surrounding aqueous solution, ensuring dynamic equilibrium (McGhie and Walton, 2007). It has been shown that four major anthocyanin forms exist at equilibrium: the abundant red flavylium cation, the blue quinoidal base, the colourless carbinol pseudobase (hemiketal form), and the colourless chalcone. These inter-conversions are summarised in Figure 2.5.

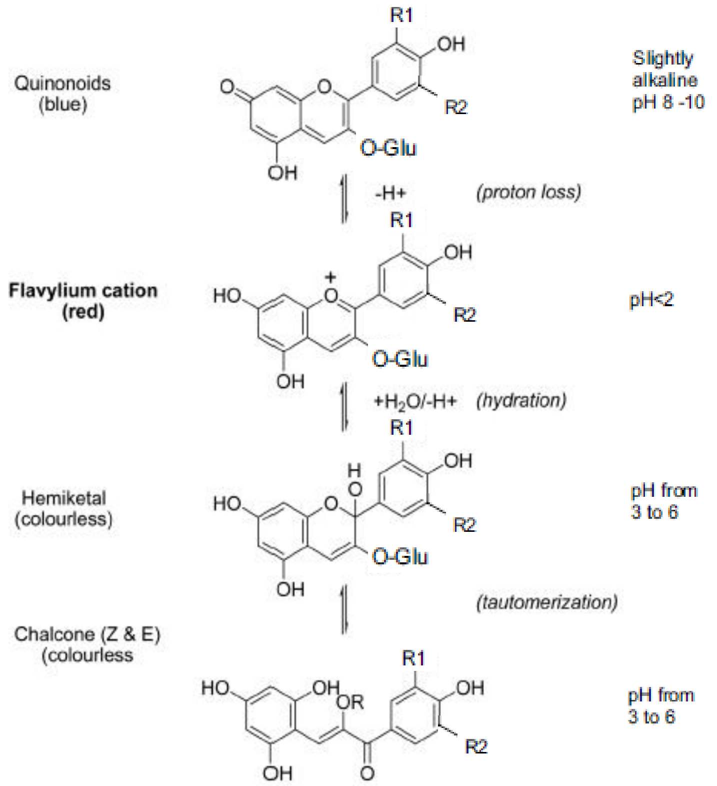


Figure 2.5: Different anthocyanin structural transformations known to take place upon pH changes (modified after: McGhie and Walton, 2007; Horbowicz *et al.*, 2008).

It is assumed that red flowers generally contain cyanidin derivatives and blue flowers mostly contain delphinidin derivatives. This is not always true and some exceptions do exist. For example, the red flower colour of *Petunia exerta* Stehman is the result of delphinidin, whereas the red flowered *Petunia* x *hybrid* Vilm. cultivars predominantly contain cyanidin (Ando *et al.*, 2000).

Differences in vacuolar pH can bring forth flowers that have the same anthocyanin but different colouration. Yamaguchi *et al.* (2001), for example, identified a recessive mutation in the purple (*PR*) gene of *Ipomoea* that leads to a slight decrease in vacuolar pH during flower development. This caused the red-purple buds of the *pr* mutant to change into purple open flowers instead of the blue wild-type flowers. The PR protein is a putative Na^+/H^+ pump believed to control the flux of sodium ions into and protons out of the vacuole resulting in a higher pH and blue colour (Fukada-Tanaka *et al*., 2000). In *Petunia hybrida*, seven loci (*PH1-PH6*) have been identified that, when in their recessive mutant forms, affect vacuolar pH and ultimately cause blueing of the flower (Mol *et al.*, 1998; van Houwelingen *et al.*, 1998).

Mutations in the petunia genes *AN1*, *AN2*, and *AN11* (mentioned in section 2.4) cause, besides a small decrease in anthocyanins, an increase in pH of petal extracts. This pH shift can be partly attributed to the increased vacuolar pH that was evident from the bluish flower colour due to the mutated *AN1* loci (formerly known as *PH6*) that lost the activity to activate vacuolar acidification, but could still stimulate transcriptional activation of anthocyanin biosynthesis (Spelt *et al*., 2002). The *PH4* gene of petunia was shown to be a R2R3-MYB domain protein expressed in the petal epidermis, and when mutated can still interact, like AN2, with AN1 and JAF13 to activate anthocyanin synthesis, but results in a bluer phenotype due to increased vacuolar pH (Quattrocchio *et al*., 2006).

2.6.2 Co-pigmentation

The stability of anthocyanins can be enhanced by a mechanism called co-pigmentation. When anthocyanins form chemical complexes with other flavonoids, either flavones or flavonols, the phenomenon is called 'intermolecular co-pigmentation'. This usually leads to a shift of the visible absorption maximum of the complex towards longer wavelengths to produce an increase in colour intensity (bathochromic shift) (Mol *et al.*, 1998; Horbowicz *et al.*, 2008). "*Assemblies of anthocyanins co-pigmented with flavone glycosides contribute to the colour of red, purple and blue flowers*" (Ellestad, 2006).

According to Harborne and Williams (2000), delphinidin is the most common anthocyanidin in blue flowers and co-pigmentation with a flavone co-pigment, and the occasional presence of one or more metal cations, shifts mauve coloured delphinidin glycosides toward blue. Wild-type carnations, on the other hand, cannot produce delphinidin in their flowers due to the lack of a functional *F3'5'H* gene. Fukui *et al*. (2003), however, concluded that the bluish hue in the marketed GMO violet carnation cv. Moonshadow was accounted for by: (1) heterologous *F3'5'H* gene expression that complemented the synthesis of analogous delphinidin-type anthocyanins, (2) the presence of a strong flavone co-pigment, and (3) a relatively high vacuolar pH of 5.5.

Another form of co-pigmentation known as 'intramolecular co-pigmentation' involves the intramolecular stacking between anthocyanin and aromatic acyl groups, thus stabilising the complex (Harborne and Williams, 2000). The intramolecular structure consists in sequences of glycosyl and aliphatic or aromatic acyl residues linked to a central flavylium chromophore. The remarkable capacity for folding between these planar molecules protects the central anthocyanin chromophore from hydrolyses and nucleophylic attack (Dangles *et* al., 1993; Figueiredo *et al*., 1999). This phenomenon not only induces distinct bathochromic and hyperchromic shifts, but also brings about stability at near neutral pH values (Stintzing and Carle, 2004).

2.6.3 Metal complexes

The term "metal complex", or better known as a metalloanthocyanin, refers to a super-molecular weight pigment composed of stoichiometric amounts of anthocyanins, flavones, and metal ions. The first structure of a metalloanthocyanin known as commelinin was elucidated in 1991 and was isolated from the deep blue flower *Commelina communis*. An x-ray crystallographic analyses confirmed of the structure to be a flattened spherical cluster consisting of six molecules of the anthocyanin malonyl-awobanin, six molecules of the flavones flavocommelin, and two central molecules of Mg^{2+} (Goto and Kondo, 1991, Mori *et al*., 2008). Commelinin and other metalloanthocyanins, including protodelphin, protocyanin, cyanosalvianin and nemophilin are discussed in detail by Ellestad (2006) and Yoshida *et al*. (2009), where the importance of these complexes to stabilise anthocyanins from hydration, especially in the case of blue colour development in flowers, is explained.

2.7 Important biological functions of anthocyanins in plants

2.7.1 Pigmentation

Flavonoids are able to absorb light over a wide range of the light spectrum. Their absorbance shifts towards longer wavelengths as the conjugation of the three planar ring structures increases and saturation decreases. The most highly modified forms are the anthocyanins, which have maximal absorbance across the visible spectrum (500 - 550 nm). Further maxima modification by the effect of pH and interactions with metal ions and co-pigments brings forth visual cues that undoubtedly promote the primary functions of flavonoids in flowers, seeds and fruits being the recruitment of pollinators and seed dispersers (Shirley, 1996). Plant colouration is also of great aesthetic value to humans and is therefore the encouragement for using conventional breeding, as well as biotechnology to create novel colours in flowers.

2.7.2 Stress protection

The ultra-violet (UV)-absorbing ability of flavonoids also points to the role of flavonoids in UV protection. The UV-absorbing characteristics in the epidermal layers of susceptible tissues have been proved to act as 'sunscreens' against harmful UV radiation (Steyn *et al.*, 2002; Manetas, 2006). Studies on petunia and *Arabidopsis* have shown that the synthesis of flavonols with higher hydroxylation levels is strongly induced by exposure to UV-B radiation, suggesting a UV stress response (Ryan *et al.*, 2001, 2002). Protection against UV-B radiation is also consistent with DNA shielding (Kootstra, 1994). Defective flavonoid biosynthesis in *Arabidopsis* mutants has shown to increase susceptibility to UV-induced damage of DNA (Li *et al.*, 1993; Lois and Buchanan, 1994).

Apart from the UV screening function, anthocyanins in leaves also function as indirect protection against excess light through their oxy-radical scavenging properties. After mechanical injury to the red and green portions of *Pseudowintera colorata* leaves, Gould *et al*. (2002) observed that a necrotic lesion and intense anthocyanic band had formed at these injured areas. Real-time imaging of the injured palisade mesophyll cells with fluorochromes showed that the red regions recovered rapidly due to enhanced rates of H_2O_2 scavenging attributed to the elevated anthocyanin levels.

2.8 Genetic engineering in floriculture

The economic importance of ornamental plants has increased globally and internationally the demand has expanded. This phenomenon contributes to the global competitiveness of the floriculture industry. Seven countries export 73% of the world-value of floricultural crops.

They are the Netherlands, Columbia, Italy, Belgium, Denmark, Ecuador and the United States. In 2002 the worldwide trade in floriculture products was estimated at a retail value of €27 billion in the USA, Japan and most of the populous European countries combined alone. Cut flowers made out a third of the ornamental horticulture market. The Netherlands are becoming the epicentre for world flower trading and, estimated in 2000, supplied almost 50% of floriculture products (Lawson, 1996; Chandler, 2003; BC Floriculture factsheet, 2003). The FloraHolland Auction alone had a turnover of €2,005 million in 2005 (www.floraholland.com).

Flower colour is one of the most important traits in the floriculture industry, adding to aesthetic value and therefore complying with the consumer's preference. Another trait is the increased emphasis on quality, related to post-harvest, which includes environmental influences on flower longevity as well as the influence of pathogenic micro-organisms (Lawson, 1996). With respect to the potential of genetic modification, there are important factors that contribute to breeding ornamental plants with novel cyanic colours:

i. The flavonoid pathway, which leads to anthocyanin biosynthesis, has been established.
ii. The genes encoding the pathway enzymes have been cloned from many plants and their sequences can be easily obtained from public DNA data bases.
iii. Transformation systems have been developed for economically important floricultural species (Tanaka *et al*., 2005).
iv. Great progress in the regulation of heterologous or endogenous genes in transgenic plants has been made, for example, there is an increasing *in silico* availability of candidates for tissue-specific promoters (Tanaka and Ohmiya, 2008).
v. Epigenetic mechanisms for silencing transgenes and endogenous genes via sense and antisense RNA inhibition are available for efficient down-regulation of target genes (Fagard and Vaucheret, 2000).

It should be kept in mind that the final visible colour of a flower is also dependent on other factors such as anthocyanin concentration, anthocyanin stacking and vacuolar pH (section 2.6). These factors are again regulated by a number of regulatory genes, many of which have been cloned and characterised. Petunia, tobacco and torenia have served as model species for flower colour modification studies, largely because they are easy to transform. The first genetic flower modification study involved the transformation of a mutant petunia line with a maize *A1* (*DFR*) gene construct, changing flower colour from pale pink to brick-red due to novel accumulation of pelargonidin derivatives (Meyer *et al*., 1987). A period of 22 years has passed since then and much pioneering work concerning transgenic plants with altered flower colours has been reported.

The much sought-after blue rose has more or less been accomplished. Although not quite blue, but violet, Florigene (Pty) Ltd and Suntory Ltd engineered a rose that exclusively accumulated delphinidin in its petals. This was achieved by selecting a host rose cultivar with the appropriate pH in its petal sap, down-regulating its endogenous *DFR* gene, and over-expressing the *Iris* x *hollandica DFR* in addition to the viola *F3'5'H* gene (Katsumoto *et al*., 2007). The most recent study involved the genetic engineering of ornamental gentian plants by changing their vivid-blue wild-type colour to lilac and pale-blue. Interestingly, the transgenic expression cassette that was used was expressed under the control of the *Agrobacterium rhizogenes rolC* promoter since the widely used cauliflower mosaic virus 35S (CaMV35S) promoter is silenced in gentian (Mishiba *et al.*, 2005; Nakatsuka *et al*., 2010).

There is a vast number of studies that encompasses flower colour manipulation. Although not discussed here, comprehensive reviews with examples of such cases involving the establishment of novel flower colours via genetic engineering are available (Tanaka *et al.*, 1998; Dixon and Steele, 1999; Mol *et al*., 1999; Forkmann and Martens, 2001; Winkel-Shirley, 2001a; Tanaka *et al*., 2005; Rosati and Simoneau, 2007; Tanaka and Ohmiya, 2008; Tanaka *et al*., 2009).

Section B: NOTES ON THE GENUS *CLIVIA* Lindl.

2.9 Introduction

Clivia Lindl. (1828) is a small, evergreen, rhizomatous genus endemic to southern Africa. It belongs to the sub-Saharan African tribe Heamantheae of the family Amaryllidaceae (Meerow *et al*., 1999). Currently the genus consists of six species, namely *Clivia nobilis* Lindley (1828), *Clivia miniata* Regel (1854), *Clivia gardenii* Hooker (1856), *Clivia caulescens* Dyer (1943), *Clivia mirabilis* Rourke (2002) and *Clivia robusta* (Murray *et al*., 2004). *Clivia miniata* is the most attractive and well-known and has gained the most attention among *Clivia* breeders in respect of producing vast varieties in different colours.

Numerous references mention that the English naturalist, William J. Burchell, first discovered a *Clivia* (*Clivia nobilis*) near the mouth of the Great Fish River in the Eastern Cape in 1815. Another intrepid pioneer, a botanical collector and Kew gardener, James Bowie, gathered plants of the same species during the early 1820s and sent them to the new director of the England Royal Botanical Gardens, William J. Hooker. In October 1828, another Kew botanist, John Lindley, described this plant flowering at Syon House, residence of the Duke of Northumberland, and named it *Clivia nobilis* in honour of the Duchess of Northumberland, Lady Charlotte Florentia Clive who first cultivated the type specimen in England (Lindley, 1828; Duncan, 1999; Koopowitz, 2002). Coincidently Hooker (1828) also named and described the same plant as *Imatophyllum aitoni* in an independent publication on the same day, a name that was later discarded (Duncan, 1992).

2.10 Morphological characterisation and distribution

Clivias belong to the Amaryllidaceae, a cosmopolitan family of petaloid monocotyledons, all originating from southern Africa (Meerow *et al*., 1999). The family includes approximately 59 genera, containing about 850 species (Meerow and Snijman, 1998). The genus *Clivia* is an evergreen, rhizomatous herb, characterised by distichous, firm, strap-shaped leaves, arranged in two ranks on a thick rhizome. Inflorescences are pseudo-umbels borne on umbellate solid scapes. Flowers are trumpet-shaped or pendulous and have short tubes extending to tepals. The stamens have long filiform filaments bearing versatile anthers and the style is terete and slender with a short, terminal, tricuspidating stigma. The ovary contains five to six ovules per locule. Usually the plant bears coloured, subglobose berries containing one to few turgid, ivory-coloured seeds embedded in soft yellow pulp (Meerow and Snijman, 1998; Koopowitz, 2002; Rourke 2002).

Although the genus *Clivia* comprises six species, *Clivia miniata* is readily distinguishable by its unmistakable large, trumpet-shaped flowers, arranged in an upright umbel. When observing the other five pendulous-flowered species they may look very similar at first glance although they can usually be distinguished when incorporating key features compiled and refined by Swanevelder (2003). The following subsections contain a brief description of each of the six *Clivia* species with the main emphasis on flower colour:

2.10.1 Clivia miniata (Lindley) Regel *(1854)*

The species *Clivia miniata* is also known by common names such as Bush lily, Boslelie (Afrikaans) and Umayime (Zulu). The Latin epithet *miniata* refers to the flowers supposedly having a red lead-like colour when it was first discovered in its natural habitat (Grove, 1992; Koopowitz, 2002; www.plantzafrica.com/plantcd/cliviaminiata.htm). Lindley originally described the plant as *Vallota*? *miniata* in 1854 (the question mark indicating his uncertainty), mislead by the erect funnel-shaped blooms not found in *Clivia nobilis*. In, 1864 Regel transferred the species to the correct genus known today as *Clivia miniata* (Koopowitz, 2002). Known populations of *Clivia miniata* occur within isolated areas within the Kei River and Transkei region in the south, through the Eastern Cape and KwaZulu-Natal Provinces, with the most northern localities into Swaziland and Mpumalanga on the Sondeza range mountains (Vorster, 1994; Duncan, 1999; Winter, 2000).

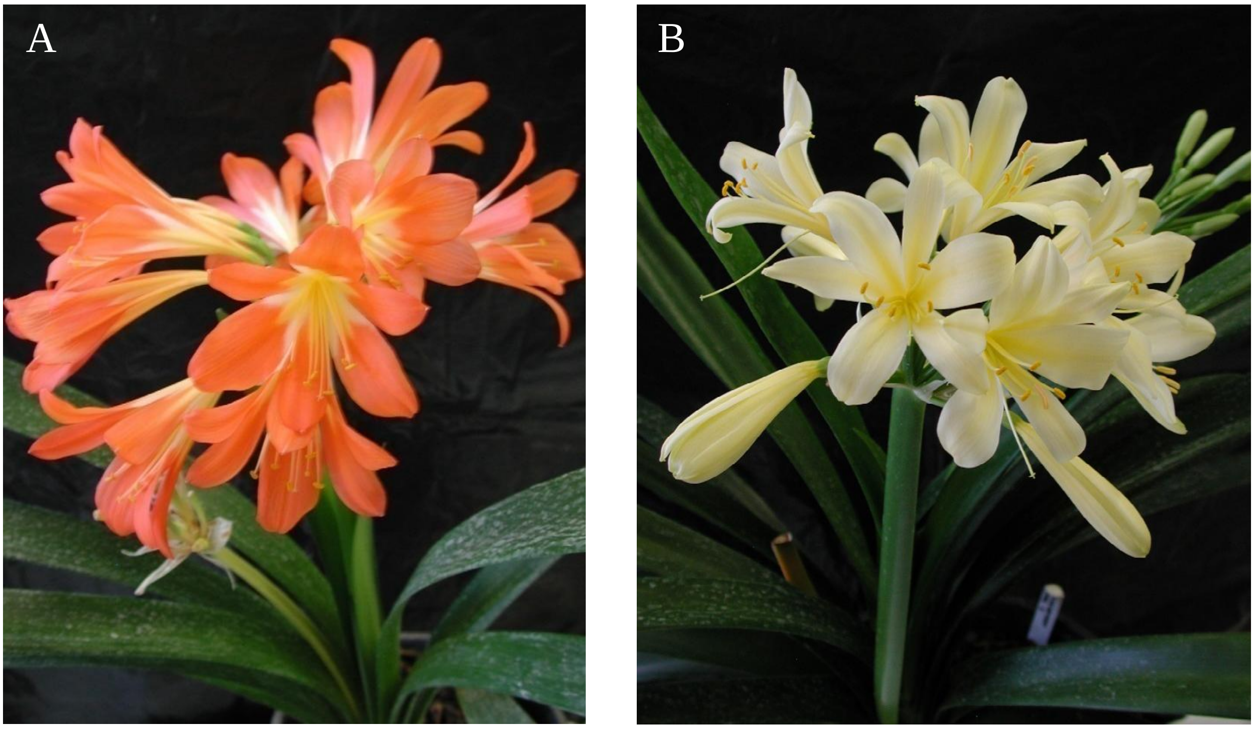


Figure 2.6: Wild-type *Clivia miniata* plants i.e. *Clivia miniata* var. *miniata* (A) and *Clivia miniata* var. *citrina* (B).

Clivia miniata are the most beautiful and easily identified species because of their 10 to 40 trumpet-shaped flowers borne on almost globose umbels. Their blooming season is from August to November (late winter until early summer) after a dormant period during the dry winter months. Flowers may appear sporadically throughout the year. They exhibit remarkable flower colours in the wild, ranging from cream to sporadic occurrences of pure yellow-flowered forms, (*Clivia miniata* var. *citrina*) described by Watson (1899), through to different pastel oranges, quite bright and dark oranges (Figure 2.6). Several forms of peach-coloured varieties also occur. Orange-coloured forms also exhibit a contrasting cream-yellow throat, with hints of green pigmentation that may vary in colour and extent. The berries that form at the tips of the pedicels after pollination may contain as much as 25 seeds with each seed being up to 15 mm in diameter (Grove, 1992; Duncan, 1999; Koopowitz, 2002; Swanevelder, 2003). "*All forms of Clivia miniata which have flowers in shades of orange or red will produce orange-red or red berries, while most forms of this species with cream or yellow flowers will produce yellow berries*" (Duncan, 1999) (Figure 2.7).

Figure 2.7: Fruits borne on umbels (left); seeds in soft pulp of a single fruit (middle); and seedlings (right) of *Clivia miniata* (www.hort.wisc.edu).

The leaves of wild *Clivia miniata* are long, narrow, smooth-edged, and strap-like with lengths between 600 and 1840 mm and widths rarely over 50 mm. A vast array of hybrid strains of cultivated *C. miniata* has arisen over the past century such as large, broad-leaved hybrids and the more recent dwarf, broad-leaved hybrids developed in Belgium, China and Japan during the late nineteenth and early twentieth century (Grove, 1992; Duncan, 1999; Koopowitz, 2002; Swanevelder, 2003).

2.10.2 Clivia nobilis Lindley *(1828)*

A short discussion considering the history behind *Clivia nobilis* was given earlier and mentions the first description of this species by Lindley in 1828. This plant has also been described in great detail by Vorster (1994). Together with *Clivia caulescens*, *Clivia gardenii*, *Clivia mirabilis*, *Clivia robusta* and *Clivia mirabilis* this species fall into the category of pendulous-flower-Clivias. *Clivia nobilis* occurs mainly in the Eastern Cape Province on the coast from the Alexandria forest northwards to the Nqabara River. It has been recorded that occasional populations occur inland as far as Grahamstown (Winter, 2000).

The inflorescences are large umbels of 20 to 50 narrow, pendulent, tubular flowers in shades of orange-yellow with contrasting green tepal tips (Figure 2.8). Blooming season usually occurs during August to January (late winter / spring to summer). Differences in flower colour intensity vary with prolonging exposure to sunlight or growth in the shade. For example, very pale, pastel apricot flowers have been witnessed to be growing in deeper shade, while the intensity of orange pigmentation was directly dependent on exposure to the sun. The age of the flowers also determines the extent of the green apices, which fade as the flowers become older. As said before, the flower of *Clivia nobilis* is shaped as a narrow tube with a long, straight, cylindrical appearance. The slight degree to which the style and anthers protrude beyond the perianth lobes may vary although this does not normally appear (Grove, 1992; Vorster, 1994; Duncan, 1999; Koopowitz, 2002). Fruits of *Clivia nobilis* are small, globular berries of 15 to 20 mm in diameter containing 1 to 6 seeds (per locule) of approximately 9 mm in diameter. The berries are dark red at maturity (Vorster, 1994; Swanevelder, 2003).

Figure 2.8: *Clivia nobilis* (Photo by courtesy of Dr. Loukie Viljoen).

The leaves of this species can be distinguished from others owing to the variation in leaf tip morphology. In most forms the leaf apex can either be bluntly rounded or notched to a greater or lesser extent, whereas other species have acute leaf apices. The strap-like leaves appear distichous and are sub-erect and leathery with leaf margin edges that are rough to the touch. Leaf sheaths usually have a grey-purplish colour. The leaves may reach lengths of between 300 and 1000 mm and widths of between 25 and 50 mm (Grove, 1992; Vorster, 1994; Duncan, 1999; Koopowitz, 2002; Swanevelder, 2003).

2.10.3 Clivia caulescens Dyer *(1943)*

Clivia caulescens, commonly known as a stem *Clivia*, was the fourth legitimate species that was named and described by R.A. Dyer (1943) and is primarily distributed within the eastern part of the Mpumalanga and the Northern Provinces (Vorster, 1994; Duncan, 1999; Winter, 2000; www.cliviasociety.org/clivia_caulescens.php). Winter (2000) recorded localities as far north as the Soutpansberg and the most southern localities in the Sodenza Range on the border of Swaziland and Mpumalanga.

The species also fall into the group with cylindrical, pendulous flowers. The most distinguishing feature of this species is its thickened aerial stem, present at a mature age, which may reach a length of 3000 mm. As the outer leaves fall off with age, transversely ringed leaf-scars appear at different intervals, depending on how fast the plants grow. Another characteristic of these plants is the presence of an occasional narrow, elongated rhizome that may reach considerable distances underground (Grove, 1992; Koopowitz, 2002; Swanevelder, 2003; www.cliviasociety.org/clivia_caulescens.php). These features are also apparent for some swamp forms of *Clivia robusta* (Murray *et al*., 2004).

Flowers mainly bloom during spring between September and November (southern hemisphere) although umbels in cultivation may be produced at any time of the year. Inflorescences are produced on umbels with 20 to 50 florets that are often carried at the same height as the leaves. The thin pedicels carrying the flowers are usually green and the flowers have an orange-red/red-salmon colour with shades of yellow on overlapping margins and contrasting green apices (Figure 2.9). The style and anthers are usually as long as the perianth lobes and may protrude slightly beyond them (Dyer, 1943; Duncan, 1999; Koopowitz, 2002; www.cliviasociety.org/clivia_caulescens.php).

Figure 2.9: *Clivia caulescens* (Photo by courtesy of Dr. Loukie Viljoen).

This species, as well as *Clivia gardenii* and *Clivia robusta* have green berries before ripening and may have berries of different colours at maturity, ranging from pale to dark red, orange-red, yellow, or a mixture of red, yellow and green (Duncan, 1999). A single berry may contain one to four seeds that are 12 mm in diameter (Swanevelder, 2003). The arching, smooth-margined leaves of *Clivia caulescens* have notched tips, a green/light red sheath colour and may reach a length of 1000 mm and are usually 50 mm wide (Koopowitz, 2002; Swanevelder, 2003)

2.10.4 Clivia gardenii Hooker *(1856)*

Clivia gardenii also falls into the drooping, pendulant flowering category of the genus, and 'gardenii' commemorates its collector and discoverer, Major Robert J. Garden, in 1855. After he sent the specimens to the Royal Botanical Gardens at Kew, Sir W. Hooker named the new species when it first flowered in 1856. The species is primarily distributed within the Maputuland-Pondoland Region of KwaZulu-Natal and its existence has also been recorded in the Eastern Cape and Swaziland (Grove, 1992; Duncan, 1999; Koopowitz, 2002; www. cliviasociety.org/clivia_gardenii.php).

Clivia gardenii is usually a large species of heights between 800 and 1300 mm with the presence of aerial stems and roots merely present due to a possible edaphic adaptation when forced to grow under marshy conditions. Their leaves also have sheaths of a green/light red colour and can often be differentiated from other pendulous flowering species because the leaves are softer than those of *Clivia nobilis* and have acute (pointed) apices. The leaves are also broader (35 - 60 mm) than those of *Clivia nobilis* but relatively have the same width as the leaves of *Clivia caulescens* (Duncan, 1999; Koopowitz, 2002; Swanevelder, 2003; www.cliviasociety.org/clivia_gardenii.php).

Figure 2.10: *Clivia gardenii* (Photo by courtesy of Dr. Loukie Viljoen).

According to Koopowitz (2002), the variability in plant size and flower colour has assisted greatly in making modern hybrids. Flower colour may range from dark orange-red forms to very pale dull orange forms, both exhibiting a merge into yellow and finally green apices which may turn yellow with age (Figure 2.10). Other unusual forms such as brick-red, pink and even yellow clones are also known. Inflorescences are produced on umbels with 10 to 20 florets. The perianth shape of the flowers is tubular and curved (thus being falcate) and not as pendulous as, and larger than *Clivia caulescens* or *Clivia nobilis*. Pedicels usually have a tinged red or orange colour and are stiff, erect or sub-erect and bend downwards as the fruits mature. Protrusion of the style and anthers from the flower tip is always prominent. Flowering time extends from late autumn to mid winter (May to July). A single berry may contain one or two large seeds of about 18 mm each in diameter (Duncan, 1999; Koopowitz, 2002; Swanevelder, 2003; www.cliviasociety.org/clivia_ gardenii.php).

2.10.5 Cilvia robusta Murray *et al*. (*2004)*

Clivia robusta is another pendulous-flowered species and is probably one of the tallest members of the genus (Swanevelder, 2006; www.plantzafrica.com/plantcd/cliviarobust.htm). In horticulture it is also known as the 'robust form' of *Clivia gardenii*, 'Swamp Forest *Clivia*' or 'Robust *gardenii*' (Hammett, 2002). Its unique morphology and distribution in tandem with a distinct karyotype and recently profiled DNA fingerprint distinguish these plants from *Clivia gardenii* and all other known *Clivia* species. Therefore these plants are recognised as a distinct taxon at species level (Ran *et al.*, 1999, 2001a, 2001b; Murray *et al*., 2004). These plants are confined to the Pondoland Centre of Endemism, South Africa, and occur in isolated populations distributed mainly from Port St John's, through to Port Edward and to a lesser extent as far north as the Oribi Gorge (Swanevelder, 2003, 2006; Murray *et al*, 2004).

Clivia robusta is a strong grower and thrives in swamp conditions and can grow to a height of 1600 mm in ideal conditions. Leaves of the plant are broad, strap-shaped with an obtuse-apiculate apex, and are planar in cross section. They are usually orientated in an arching to erect position. Leaf sheath colours may vary from green to light red (Swanevelder, 2003; Murray *et al*., 2004). Inflorescences are slightly globose umbels of variable forms with 15-40(-45) flowers. Pedicels are stiff, erect or sub-erect with variable colour but are usually green. The flowers have a drooping orientation and have a perianth shape that is tubular, somewhat falcate with an increasingly flaring apex (Swanevelder, 2003; Murray *et al*., 2004) (Figure 2.11). Flower colours may range from dark orange to red with red tips, through to pale orange to pink orange. Flowers in all shades of these colours belong to *Clivia robusta* var. *robusta*. In rare cases yellow-flowering forms with dark green apices are found comprising *Clivia robusta* var. *citrina* (Murray *et al*., 2004; Swanevelder, 2006) (Figure 2.11).

Figure 2.11: *Clivia robusta* var. *robusta* (A) and *Clivia robusta* var. *citrina* (B) (www.bulbsociety.org; www.cliviape.co.za).

Flowering time is extended over a five to six month period from late March to early August, i.e. early autumn to late winter (Southern hemisphere). The stigma barely protrudes and stamens occasionally extend to, and exceed the perianth mouth, and are therefore retained within the corolla tube. The berries of the plants are irregularly ovoid and globulose, containing one or two (- four) large seeds (largest in genus) and as said before the pericarp colour matures from a pale green through orange to bright red (Murray *et al*., 2004).

2.10.6 Clivia mirabilis Rourke *(2002)*

Clivia mirabilis is another member of the pendulous tubular-flowered *Clivia* species (Rourke 2002) and according to Dr John Rourke, retired head of Compton Herbarium at the

Kirstenbosch Research Centre, the epithet *mirabilis* reflects the astonishing or miraculous nature of its discovery (www.plantzafrica/plantcd/cliviamirabilis.htm#grow). Specimens were first collected in the Oorlogskloof Nature Reserve near Nieuwoudtville in the Northern Cape, which is an unusual distribution for *Clivia*, as Clivias are generally shade-loving and usually occur in summer rainfall areas. The Northern Cape, which is approximately 700 km north west away of the other five species in the Eastern Cape, have a semi-arid Mediterranean climate with a strictly winter rainfall. In contrast the rainfall in the Eastern Cape has a bimodal regime (spring to autumn) (Rourke 2002; Snijman, 2002-2003; www.plantzafrica/ plantcd/cliviamirabilis.htm#grow).

Clivia mirabilis corresponds morphologically most to *Clivia nobilis*. Its strap-like, distichous leaves are in a stiffly erect orientation and a distinct white-grey striation is visible in the midrib area on the upper surface. The leaf sheath colour is a deep dull green with a flushed carmine-maroon at the base. The leaf apex is obtuse-acute and lacks the distinctive apical notch and minute marginal serration found in *C. nobilis* (Rourke 2002; Snijman, 2002-2003; Swanevelder, 2003).

Inflorescences form tight umbels with 20 to 48 flowers each. Each flower has a drooping orientation with a tubular perianth shape that flares increasingly towards the apex (Rourke 2002; Snijman, 2002-2003; Swanevelder, 2003) (Figure 2.12). "*During the development of the flower, both perianth and ovary progress through a series of well-marked colour changes. The unopened bud is yellowish, but prominently green-tipped, and the ovary is also pale green*" (Rourke 2002) (Figure 2.12). When anthesis is reached, the tips of the tepals slowly changes from green to the same yellow shades of the basal half of the perianth. The drooping pedicels and the top half of the perianth are deep orange-red at this stage. After pollination occurred, both the perianth and ovary take on a uniform orange/red colour.

Flowering time may last up to six weeks between October and mid-November (late spring). Both the stigma and anthers are slightly exerted from the tip of the perianth mouth at anthesis. The berries of *Clivia mirabilis* mature more rapidly than in the other *Clivia* species. The epicarp changes from green through to yellow, orange to pink until it reaches a red colour prior to the onset of the winter rains when the berries are shed (Rourke 2002; Swanevelder, 2003).

Figure 2.12: *Clivia mirabilis* (www.bulbsociety.org).

2.11 *Clivia* floral pigmentation

A few investigations concerning pigment content in Clivias have been pursued and revealed the presence of two main pigment types i.e. anthocyanins and carotenoids. The green colour seen in the throats and tips of some *Clivia* flowers is caused by the presence of chlorophylls. Pigment concentration and the mixing and matching of colours between the anthocyanin-containing epidermal layer and the underlying carotenoid/chlorophyll-containing mesophyll layers greatly influence our observation of the flower colour of Clivias (Lötter, 1998; Lötter, 2006).

Most studies concerning in-depth anthocyanin analysis in the family Amaryllidaceae are limited to one genus i.e. *Hippeastrum.* Other members include: (1) *Lycoris* where the 3-glucoside and 3-xylosylglucoside of pelargonidin and cyanidin have been identified; and (2) *Nerine*, where the 3,5-diglucoside of cyanidin, peonidin and pelargonidin, the 3-glucoside of cyanidin and pelargonidin, cyanidin 3-sophoroside and two partly identified anthocyanins have been detected (Byamukama *et al*., 2006). Recently, a combination of chromatographic techniques has been used to isolate the anthocyanins, cyanidin 3-*O*-(6”-*O*-α-rhamnopyranosyl-β-glucopyranoside) and pelargonidin 3-*O*-(6”-*O*-α-rhamnopyranosyl-β-glucopyranoside) from the flowers of a Ugandan *Hippeastrum* cultivar (Byamukama *et al*., 2006).

A high-performance liquid chromatography (HPLC) study was done to analyse the anthocyanin content in the tepals, berries and leaves of *Clivia miniata*, *Clivia caulescens* and *Clivia nobilis*. Chromatographic profiles showed that two pelargonidin derivatives, i.e. pelargonidin-3-glucoside and pelargonidin-3-rutinoside, were present as the main pigments in three different colour varieties, ranging from light orange to red, of *Clivia miniata*. Cyanidin derivatives were also present in much lower quantities in the pendulent flowering species. The analysis of two typical orange flowered *Clivia miniata* cultivars also showed the presence of 14 different co-pigments (Koopowitz *et al*., 2003).

Many ornamentals such as marigold (*Tagetes*), daffodil (*Narcissus*), *Freesia*, *Gerbera*, *Rosa*, *Lilium*, *Adonis*, and *Calendula* are examples of plants where carotenoids are responsible for flower colour (Forkmann, 1991; Cunningham and Gantt, 2005). The carotenes (hydrocarbons) and their oxygenated derivatives, the xanthophylls, are most commonly associated with flower pigmentation (Cunningham and Gantt, 1998; Fraser and Bramley, 2004; Wurtzel, 2004; Grotewold, 2006). The use of spectrophotometry has shown that carotenoids do contribute to flower pigmentation in many species of the genus *Clivia* (Hammett, 2006). An early study indicated that a large number of carotenoid components exist in the flowers of both orange and yellow Clivias (Matsuno and Hirao, 1980; Koopowitz, 2002). They found that in all cases the flowers contained seven different carotenoids including taraxanthin, β-carotene and violaxanthin in the highest concentrations. Koopowitz (2002) suggested that a “block” in any of the early steps of the anthocyanin pathway (that may occur due to a mutation), can lead to the apparent yellow-coloured *Clivia*.

Since this study turns its attention to anthocyanin biosynthesis in Clivias, a putative biosynthetic pathway (Figure 2.13) that coincides with the end-products found during the above-mentioned pigment analysis is introduced. Molecular and genetic analyses of the pathway in the taxonomically disparate model organism’s maize, snapdragon, petunia, and *Arabidopsis* indicate that the pathway more or less consists of the same set enzymes in most, if not all, angiosperms (Dooner *et al*., 1991; Holton and Cornish, 1995; Mol *et al*., 1998). The pathway has been discussed (section 2.2).

As said before, the presence of F3’5’H catalyses the hydroxylation at C-3’ and C-5’ of the anthocyanidin B-ring which shifts colour towards blue and purple. Like roses (*Rosa* spp.), carnations (*Dianthus caryophyllus*), and *Lilium* spp., Clivias most probably lack F3’5’H activity in tepals and therefore do not present any natural blue-flowering varieties (Brugliera *et al*., 1999; Shimada *et al*., 2001). Especially genes encoding F3'5'H enzymes are desired for the genetic transformation of such species to establish lilac to blue colours based on delphinidin derivatives (Tanaka *et al*., 1998, 2005; Forkmann and Martens, 2001).

The lack of F3'5'H activity in *Clivia* will allow naringenin to serve as substrate for either F3H or F3'H, whereas DHK will serve as substrate for F3'H and DFR. When examining the topology of the pathway (Figure 2.13) two suggestions arise by which a mutation in the production of pelargonidin rather than cyanidin could result. Firstly, mutational or transcriptional inactivation of F3'H would prevent 3'-hydroxylation, leading to the production of pelargonidin 3-glucoside rather than cyanidin 3-glucoside. Alternatively, pelargonidin over cyanidin production can be achieved by a difference in substrate specificity of either one of the late biosynthetic enzymes, i.e. DFR or ANS.

As mentioned in section 2.2.5, DFR and ANS can be substrate generalists, thus utilising both hydroxylated and non-hydroxylated precursors to produce anthocyanidins (Holton and Cornish, 1995), while in other species these enzymes are substrate specialists. Moreover, in petunia a single amino acid change in DFR alters substrate specificity dramatically (Johnson *et al*., 2001). Therefore it is possible that F3'5'H activity may exist in *Clivia,* but the DFR enzyme has no substrate specificity for dihydromyricetin (DHM), which also blocks the synthesis of delphinidin derivatives, prohibiting any formation of blue colouration. Therefore, it would be advisable to have a closer look at this substrate-specificity region in the amino acid sequence of the *Clivia* DFR(s).

In conclusion, the fact that there are so many colour varieties of Clivias can either be the result of mutations in the structural or regulatory genes involved in the anthocyanin biosynthesis pathway or the existence of a spatial, organ-specific regulation of the late stages of the flavonoid pathway. For example, expression of *DFR* is under the control of developmental and environmental factors in a tissue-specific manner (Tanaka *et al*., 1995; Helariutta *et al*., 1993; Kubasek *et al*., 1998).

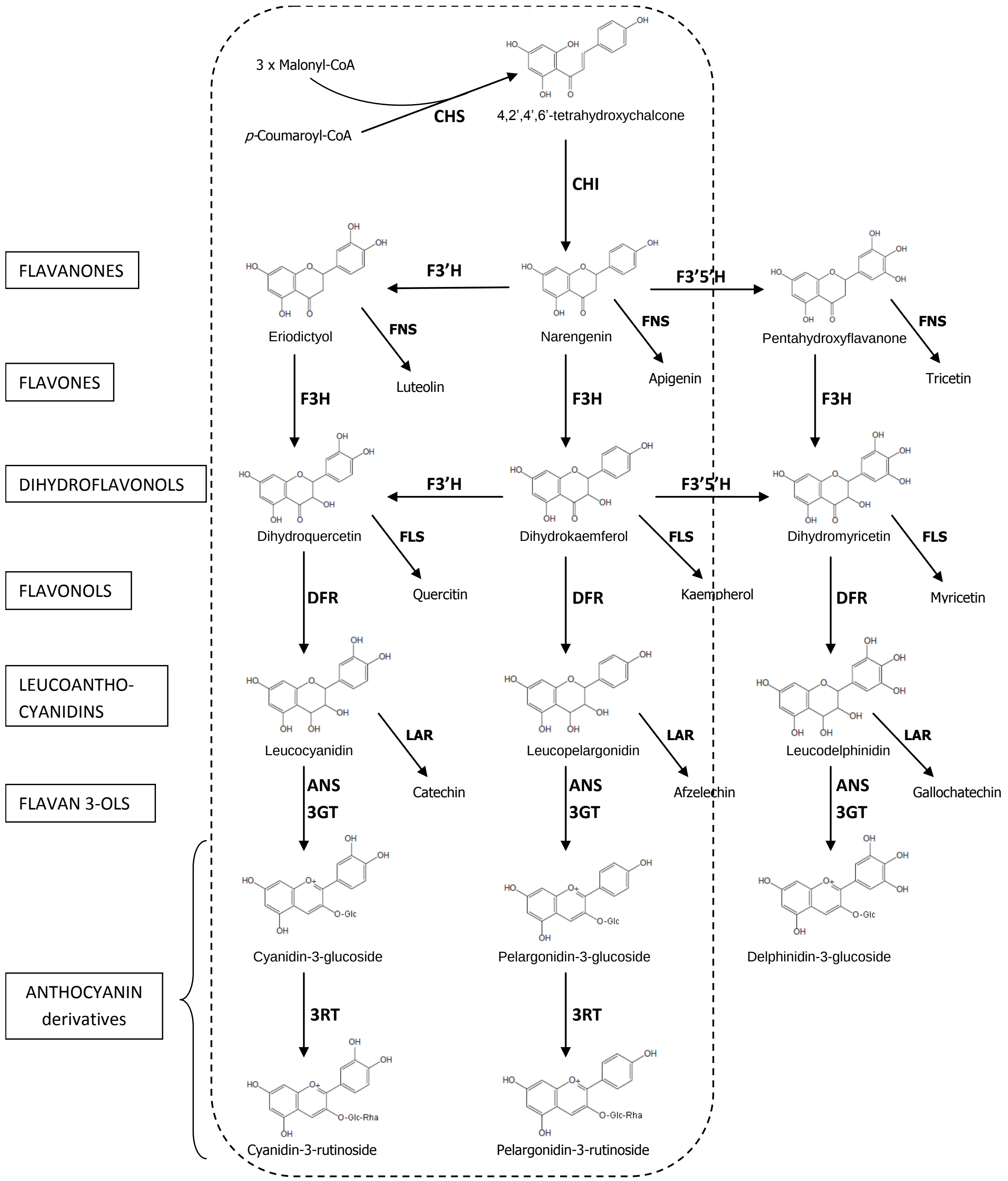


Figure 2.13: Diagram of flavonoid biosynthesis representing a putative anthocyanin biosynthetic pathway in *Clivia* (Area surrounded by dotted line). Enzyme abbreviations: ANS, anthocyanidin synthase CHI, chalcone isomerase; CHS, chalcone synthase; DFR, dihydroflavonol 4-reductase; F3H, flavanone 3-hydroxylase; FLS, flavonol synthase; FNS, flavone synthase; F3'H and F3'5'H, flavonoid 3' and 3'5' hydroxylase; GTs, glucosyl transferases; LAR, leucoanthocyanidin reductase; RT, rhamnosyl transferase.

2.12 AIMS OF THIS STUDY

The following topics are accompanied by questions, each referring to important methods used to investigate each objective of this study:

i. The identification of flavonoid biosynthetic genes in the genus *Clivia*. Can optimal primer pairs be designed to permit the isolation of partial cDNA fragments with conventional PCR? Are the acquired *Clivia* cDNA fragments related to the corresponding sequences of similar published cDNAs from other plant species?

ii. The investigation of temporal expression of flavonoid biosynthetic genes in different flower tissues of both orange and yellow varieties of *Clivia miniata*. Can the relative expression levels of the genes be analysed with real-time quantitative RT-PCR?

iii. The investigation of anthocyanin accumulation during tepal development of both orange and yellow varieties of *Clivia miniata*. Can the anthocyanin content at different tepal developmental stages be measured with UV-visible Spectrophotometry? Can statistical analyses be used to verify parallelism between anthocyanin accumulation and the expression of the flavonoid biosynthetic genes?

Chapter 3

MATERIALS AND METHODS

3.1 Identification of *Clivia* flavonoid biosynthetic gene sequences

3.1.1 Plant Material

Five *Clivia* varieties were obtained from the private collection of Prof J.J. Spies (Department of Genetics, University of the Free State) (Table 3.1) and a fully bloomed flower was harvested from each.

Table 3.1: The *Clivia* varieties from which tepal samples, necessary for total RNA isolation, were collected.

***Clivia* variety**	**Floral Colour**
Clivia miniata var. *citrina* 'Kirstenbosch Yellow'	Group 1 yellow
Clivia miniata var. *miniata* 'Teleurstelling'	Orange
Clivia miniata var. *citrina* 'Giddy'	Group 2 yellow
Clivia caulescens	Light orange
Clivia miniata var. *miniata* 'Plantation'	Dark orange

3.1.2 Total RNA isolation

The fresh tepals of each flower were removed and homogenised in liquid nitrogen to a fine powder with a sterile pre-cooled mortar and pestle. Approximately 0.1 g biomass of each sample was suspended in 800 µl TRizol® (Invitrogen). After addition of the TRizol® solution, the suspension was vortexed for 10 seconds every minute for 5 min at room temperature.

Thereafter, 150 µl of chloroform was added, the mixture vortexed briefly, and incubated for 3 min at room temperature. Samples were then centrifuged at 10 000 g. for 20 min at 4°C and the supernatant removed and precipitated by the addition of 400 µl isopropanol, followed by vortexing and incubation for 10 min at room temperature. The precipitate was centrifuged at 10 000 g. for 15 min at 4°C after which the supernatant was discarded and the precipitate washed with 1 ml 70% ethanol. The precipitate was dried in a SpeedVac condensator followed by resuspension in 50 µl DEPC-treated water. The RNA concentration of each sample was measured using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies). Only the RNA samples with a 260 / 280 nm absorbance ratio (an indication of protein contamination) between 1.9 and 2.1, and a 260 / 230 nm absorbance ratio (an indication of reagent contamination) greater than 2.0 were used for first strand cDNA synthesis. The integrity of the RNA samples and the absence of genomic DNA were also assessed by electrophoresis on a 1.5% agarose gel and ethidium bromide staining. The RNA was kept at -80°C until used.

3.1.3 Degenerate Primer design

The CODEHOP (Consensus-DEgenerate Hybrid Oligonucleotide Primer) (Rose *et al.*, 1998, 2003) approach was used to design primers for *CHS*. Published CHS amino acid and cDNA sequences of different monocot species were collected from GenBank (www.ncbi.nlm.nih.gov/sites/entrez). Multiple alignments of these sequences were performed in ClustalX v2.0 (Thompson *et al.*, 1997; Larkin *et al.*, 2007) under default alignment parameters. The amino acid alignment mentioned above was copied into the BLOCKMAKER interface (blocks.fhcrc.org/blockmkr/make_blocks.html). BLOCKMAKER identified potential conserved amino acid blocks in the form of two algorithmic motif-finding outputs i.e. GIBBS and MOTIF (Lawrence *et al.*, 1993; Steven *et al.*, 1995). The MOTIF output was chosen (Appendix A), from which degenerate primers ("CODEHOPs") were predicted under default settings, except for the degenerate core strictness that was set to 0.1 (blocks.fhcrc.org/blocks/codehop.html). A primer map was generated and the appropriate *CHS* primer set was selected (Appendix B). The cDNA alignment was used as a guide to optimize the 5' consensus clamp region (if necessary) by selecting the most appropriate nucleotides.

The *DFR* primer set was designed based on the highly conserved motifs, KDPENEVIKP and MTGWMYFVSK, within aligned *DFR* amino acid sequences from different monocot plants (Appendix C1). The corresponding areas in aligned cDNA sequences of these DFRs were used as reference from which DFR primers were finally selected (Appendix C2). The primer sets for *Clivia CHI* -and *F3H* were designed based on conserved areas within multiple alignments of monocot cDNA sequences (Appendices D and E). Both amino acid and cDNA sequences were obtained from GenBank and were all aligned in ClustalX v2.0.

The "Oligonucleotide Properties Calculator" (www.basic.northwestern.edu/biotools/oligocalc.html; Kibbe, 2007) was used to determine the melting temperature (Tm) and GC content for the *CHI*, *F3H* and *DFR* primers. Each primer was designed according to the following criteria: a Tm closest to 60°C, a GC content of at least 40%, low primer degeneracy, a primer length of not less than 20 nucleotides, and a minimum PCR product size of 200 bp. FastPCR Professional v5.0.73 (Kalendar, 2007) was used to determine the expected *in silico* PCR amplicon size for each primer set, and was also used to evaluate possible primer-dimer formation. Certain guidelines were also followed to simplify primer design (www1.qiagen.com/resources/info/guidelines_for_pcr.aspx). All primer sequences were submitted to Bioneer for commercial synthesis. After receiving the primers, concentrated stock solutions of 100 µM (100 pmol/µl) were prepared by dissolving each lyophilised pellet in TE buffer (10 mM Tris, pH 7.5; 1 mM EDTA, pH 8.0). 10 µM working solutions were prepared for use in PCR.

3.1.4 First-strand cDNA synthesis

RT-PCR (reverse transcriptase polymerase chain reaction) was used to synthesize first-strand cDNA. The reaction was prepared as follow: 1 µg RNA and 1 µl (100 µM) anchored oligo$(dT)_{23}$VN (custom-designed; Bioneer) was added, followed by addition of DEPC-treated water to a volume of 11 µl. The mixture was incubated at 70°C for 5 min and chilled on ice. Thereafter, the following reagents were sequentially added: 4 µl of 5x reaction buffer (250 mM Tris-HCl, pH 8.3; 250 mM KCl; 20mM $MgCl_2$; 50 mM DTT), 2 µl dNTP mix (1.0 mM each) (Applied Biosystems), 1 µl (20 U) GeneAMP® RNase inhibitor (Applied Biosystems) and DEPC-treated water to a volume of 19 µl. The reaction mixture was incubated at 37°C for 5 min after which 1 µl (200 U) RevertAid™ M-MuLV Reverse Transcriptase (Fermentas) was added. The mixture was then incubated at 42°C for 1 hour followed by heating at 70ºC for 10 min to inactivate the reaction after which the reaction was cooled on ice. All heating steps were performed in a Thermal Cycler 2720 (Applied Biosystems). Each cDNA sample was diluted by the addition of 5 µl dH_2O.

3.1.5 PCR reaction setup

Each PCR reaction mixture contained 5 µl 10x buffer (500 mM KCl; 100 mM Tris-HCl, pH 8.3), 8 µl $MgCl_2$ (4 mM), 8 µl dNTP mix (200 µM each) (Applied Biosystems), a forward and reverse primer (10 µM each), 5 µl first-strand cDNA, 0.5 µl AmpliTaq Gold Polymerase (2.5 U) (Applied Biosystems) and sterile 1x distilled water (dH_2O) to a final volume of 50 µl. Premixes were prepared beforehand and negative and positive controls were included. PCR was performed in a Thermal Cycler 2720 and amplification was carried out with an initial denaturation and 'hot start' at 95°C for 10 min, followed by 30 cycles of 95°C for 30 sec, 55°C for 30 sec, and 72°C for 30 sec. A final extension step at 72°C for 10 min followed, after which each PCR mixture was kept at 4°C.

3.1.6 Agarose Gel Electrophoresis

A 1.5% agarose gel was prepared by using electrophoresis grade agarose in 1x TAE buffer (Tris, acetic acid and EDTA, pH 8.0) and 2.5 µl of Ethidium Bromide (10 mg/ml) to a final concentration of 15 mg/ml. PCR product (5 µl) and 2 µl 6x loading dye solution (Fermentas) was mixed and loaded on the agarose gel. A GeneRuler 100 bp DNA Ladder (Fermentas) was loaded in the first lane as a molecular size standard. The PCR products were separated at 100 V for 45 min and visualised on a gel documentation system (Bio-Rad) under UV light.

3.1.7 Sequencing of PCR products

All PCR samples that had cDNA fragments of the correct size were purified with a purification kit (BioFlux gel extraction kit; Bioer). In some cases where non-specific secondary bands were present in a gel lane, the fragments of the correct size were excised and subsequently purified. A cycle sequencing reaction mixture of 10 µl was prepared for each template with a BigDye terminator v3.1 kit (Applied Biosystems), containing 0.5 µl Terminator mix, 1 µl sequencing primer (3.2 µM), 2 µl dilution buffer, and 6.5 µl template (which had a final concentration of ~10 ng/µl in the sequencing mixture). Reactions were performed in a Thermal Cycler 2720 under the following conditions: initial denaturation at

96°C for 1 min, followed by 25 cycles of 96°C for 10 s, 50°C for 5 s, 60°C for 4 min, and then storage at 4°C.

A post-reaction cleanup step with EDTA/Ethanol precipitation followed: Each sequencing reaction was adjusted to 20 µl with 1x dH_2O 5 µl 125 mM EDTA and 60 µl 95% EtOH added followed by vortexing for 5 sec and precipitation at room temperature for 15 min followed; The samples were centrifuged at 20 000 g. for 10 min at 4°C and the supernatant completely aspirated. The pellet was washed by the addition of 60 µl of 70% Ethanol by vortexing briefly and centrifugation for at 20 000 g. for 5 min at 4°C. The supernatant was completely aspirated and the pellet dried in a SpeedVac condensator for 5 min. The sample was then stored in the dark at 4°C until being analysed on the ABI Prism 310 Genetic Analyser (Applied biosystems) at the molecular biology division of the Department of Microbial, Biochemical and Food Biotechnology (University of the Free State).

3.1.8 Sequence assembly and analysis

Sequencing electropherograms were visualised with FinchTV v1.3.0 (Geospiza, Inc.) to ensure that the relative intensity of fluorescence was acceptable and that no ambiguous peaks were present. The forward sequences and the reverse compliment of each reverse sequence were aligned and analysed with ContigExpress (Vector NTI Advance v10.3.0, Invitrogen) to obtain a consensus sequence (or ‘contig’) for *CHS*, *CHI*, *DFR* and *F3H*, respectively. CAP3 (Huang and Madan, 1999) was used to report the obtained consensus sequences (Appendix F-I).

Each *Clivia* consensus sequence was used to perform a nucleotide BLAST (Basic local alignment search tool) comparison search within the National Center for Biotechnology Information (NCBI) database (Altschul *et al.*, 1990; blast.ncbi.nlm.nih.gov/Blast.cgi). The resulting homologous sequences from other plant species were aligned with the corresponding *Clivia* sequence in ClustalX v2.0. These alignments were exported to BioEdit v7.0 sequence alignment editor (Hall, 1999) to change all other sequences to the same length as the corresponding *Clivia* sequence. BioEdit was also used to translate nucleotide alignments to amino acid alignments. Similarity and identity to the abovementioned homologous sequences were assessed for each *Clivia* consensus sequence and were presented in the form of a percentage matrix with MatGAT v2.0 (Matrix Global Alignment Tool) (Campanella *et al.*, 2003). Similarity and identity each have a distinct meaning. Identity is defined by the extent to which two nucleotide sequences are invariant. Therefore, percent identity was given in terms of the fraction of nucleotides according to base-to-base level within an alignment between two sequences. Similarity is the extent to which the nucleotide sequences are related and were calculated by including sequence gaps and mismatches. A positive matrix score, using a more complex formula and a comparison look-up table, in terms of percentage was attributed to the similarity between two sequences (Campanella *et al.*, 2003; www.ncbi.nlm.nih.gov/education/BLASTinfo/glossary2.html).

3.1.9 Phylogenetic analysis

The nucleic acid alignments obtained during analysis were also subjected to phylogenetic analysis. Phylogenetic trees were constructed by the neighbor-joining (NJ) method with MEGA v3.1 software using default parameters (Saitou and Nei, 1987; Kumar *et al.*, 2004). The reliability of the trees was measured by bootstrap analysis with 1000 replicates (Felsenstein, 1985). Though the obtained overall topologies were comparable, phylogenetic analysis based on nucleic acid sequences led to more stable and reliable results than those based on amino acid sequences and were therefore preferred.

3.2 Expression analysis of *CHS* and *DFR* in *Clivia miniata* flowers

3.2.1 Plant material

Clivia miniata var. *miniata* ‘Plantation’ (dark orange) and *Clivia miniata* var. *citrina* ‘Giddy’ (group 2 yellow) plants were provided by Prof J.J. Spies (Department of Genetics, University of the Free State) from his private collection. Tepal, stamen (male reproductive organ) and carpel (female reproductive organ) samples (Figure 3.1) were collected at five different flower developmental stages defined as follows: stage 1 - unpigmented bud; stage 2 - slight pigmentation appears; stage 3 - pigmentation appears over about one third of surface; stage 4 - pigmentation covers almost two thirds of surface with buds just before anthesis (onset of opening to full bloom of flower); stage 5 - anthesis is active and darker pigmentation is visible; stage 6 - mature, fully pigmented flowers (Figure 4.7). All samples were frozen in liquid nitrogen and kept at -80°C until use.

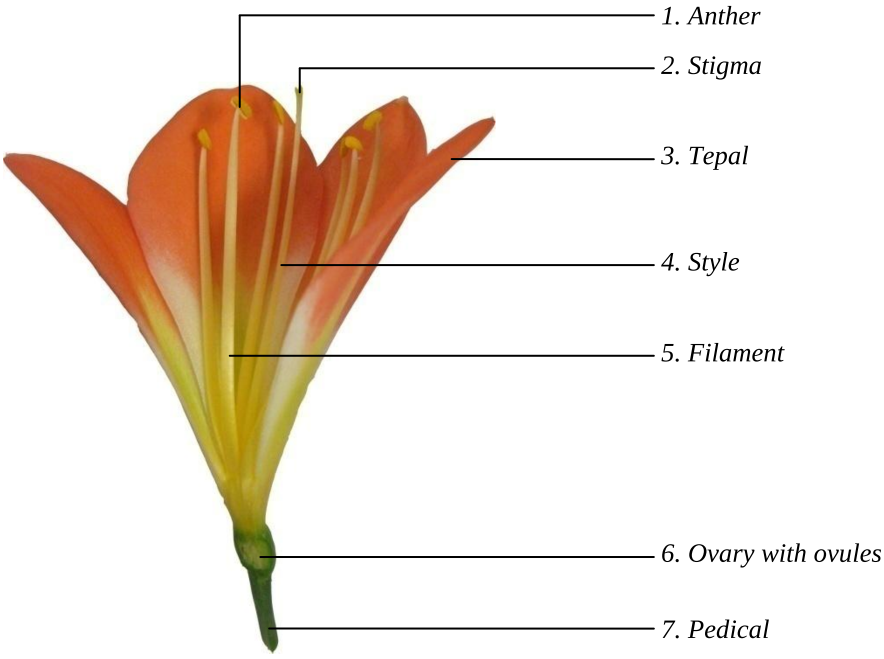


Figure 3.1: Anatomical diagram of a wild-type *Clivia miniata* flower. The parts that were used for organ-specific expression analysis include the tepals (3), stamen (1 and 5), and carpel (2, 4 and 6).

3.2.2 Total RNA Isolation

The collected tepal, stamen and carpel samples from flower developmental stages 2 to 6 were homogenised in liquid nitrogen to a fine powder with a sterile pre-cooled mortar and pestle. Approximately 0.1 g biomass of each sample was suspended in 800 µl TRizol® (Invitrogen). After addition of the TRizol® solution, the suspension was vortexed for 10 second every minute for 5 min at room temperature. Thereafter, 150 µl of chloroform was added, the mixture vortexed briefly, and incubated for 3 min at room temperature. All samples were then centrifuged at 10 000 g. for 20 min at 4°C and the supernatant removed and precipitated by the addition of 400 µl isopropanol, followed by vortexing and incubation for 10 min at room temperature (Figure 3.2). The precipitate was centrifuged at 10 000 g. for 15 min at 4°C after which the supernatant was discarded and the precipitate washed with 1 ml 70% ethanol. Drying in a SpeedVac condensator and resuspension in 50 µl DEPC-treated water followed.

The concentration of each RNA sample was measured using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies). RNA purity was assessed and samples with a 260 / 280 nm absorbance ratio (an indication of protein contamination) between 1.9 and 2.1 and a 260 / 230 nm absorbance ratio (an indication of reagent contamination) greater than 2.0 were used for first strand cDNA synthesis. The integrity of the RNA samples and the absence of genomic DNA were also confirmed by electrophoresis on a 1.5% agarose gel and ethidium bromide staining. All samples were kept at -80°C to preserve the RNA until used.

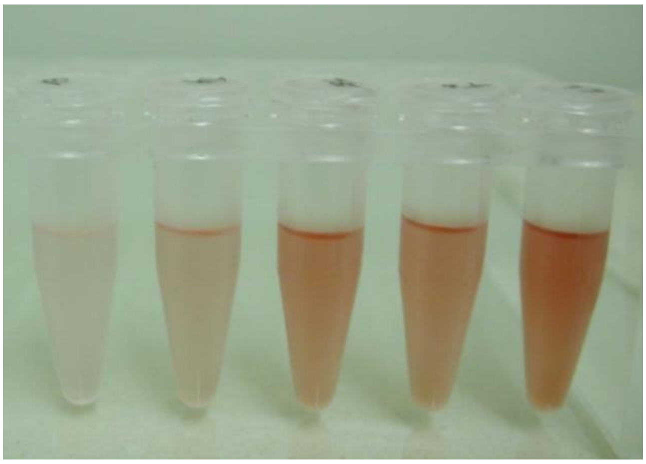


Figure 3.2: Eppendorf tubes containing the homogenization/precipitation mixture during total RNA extraction from orange tepals with the TRizol® method. Interestingly, an increase in the colour intensity can clearly be seen with each flower developmental stage.

3.2.3 Amplification of Clivia miniata 18S ribosomal RNA (rRNA)

Before attempting primer design for quantitative experimentation, an appropriate housekeeping gene, in this case the *Clivia miniata 18S* rRNA (*Cm18S* rRNA), was chosen as endogenous reference to normalize all expression data. 5 µl of the first-strand cDNA prepared from *Clivia miniata* var. *miniata* 'Plantation' in section 3.1.3 served as template in a 50 µl PCR reaction prepared the same as in section 3.1.5. A wheat-specific *18S* rRNA primer pair was used in the PCR reaction (Bovis249, 5'-TCAAGAACGAAAGTTGGGGG-3'; Bovis250, 5'CTCGTTGAATACATCAGTGTAGCG-3'; 10 µM each). The primer sequences were tested with FastPCR Professional v5.0 beforehand to obtain the *in silico* PCR fragment size while using the complete sequence of *Clivia nobilis 18S* rRNA (GenBank accession number: AF206889) as template. The *in vitro* PCR was performed in a Thermal Cycler 2720 under the following conditions: denaturation and 'hot start' at 95°C for 10 min, followed by 30 cycles of 95°C for 15 s, 53°C for 20 s, 72°C for 30 s, a final elongation step of 72°C for 5 min, after which the PCR mixture was conserved at 4°C. 5 µl of the sample was analysed by electrophoresis on a 1.5% agarose gel to confirm that a PCR product was obtained, followed by direct sequencing of the PCR fragment (Methods described in sections 3.1.6 and 3.1.7).

3.2.4 Primers design

Gene specific primers (GSPs) for real-time qPCR were designed with Primer Express v3.0 (Applied Biosystems). Primers were designed from the newly obtained *18S Clivia miniata* rRNA sequence, and the *CHS* and *DFR* consensus sequences obtained for *Clivia miniata* (now designated as *CmCHS* and *CmDFR* target genes, respectively) (See Appendices F and I). Primer parameters were set within specified criteria that were a Tm value of 58 to 60°C, a GC content of 40 to 60%, a primer length of 19 to 24 bp and a PCR amplicon length of 80 to 120 bp. The primers were chosen to exhibit the least possible formation of primer-dimers and other secondary structures, and were also designed according to specified properties outlined within the primer design algorithm compiled by Wang and Seed (2003). Selected primers were submitted to Bioneer for commercial synthesis (Table 3.2). After receiving the primers, concentrated stock solutions of 100 µM (100 pmol/µl) were prepared by dissolving each lyophilised pellet in TE buffer (10 mM Tris, pH 7.5; 1 mM EDTA, pH 8.0). 0.5 µM working solutions were prepared for use in real-time quantification.

Table 3.2: Gene specific primers used for detection of *CmCHS*, *CmDFR* and *Cm18S rRNA* with real-time quantitative PCR.

Expressed gene		Primer	Sequence (5' - 3')	Tm (°C)	Expected PCR amplicon length
Type	**Name**				
Target gene	*CmCHS*	QchsF	CAAGCGCCTCATGATGTATCA	58	114 bp
		QchsR	TCCGAGCAGACGACGAGAA	59	
Target gene	*CmDFR*	QdfrF	TGTAAGAAAGCAAGGTCAGTCCAA	59	80 bp
		QdfrR	GGCTTTTGACGTTCCTCCATAT	58	
Reference gene	*Cm18S rRNA*	18SF	ACTAGGGATCGGCGGATGTT	59	100 bp
		18SR	AGTTTCAGCCTTGCGACCAT	58	

3.2.5 First-strand cDNA synthesis

A reaction mixture was prepared for each RNA sample as follow: 2 µg Total RNA was added, followed by DEPC-treated water to a volume of 10 µl; The RNA solution was treated with a RNAase-free DNase I kit (Sigma-Aldrich); 1 µl (100 µM) anchored oligo(dT)$_{23}$VN (custom-designed; Bioneer) and 1 µl (50 µM) random hexamer (custom-designed; Bioneer) was added to anneal to the total RNA, and DEPC-treated water was added again to a volume of 17 µl; The mixture was incubated at 70°C for 5 min and cooled on ice.

A synthesis mix of 8 µl was finally added to each tube and contained 4 µl 5x reaction buffer (250 mM Tris-HCl, pH 8.3; 250 mM KCl; 20mM $MgCl_2$; 50 mM DTT), 2 µl dNTP mix (10 mM each) (Applied Biosystems), 1 µl (20 U) GeneAMP® RNase inhibitor (Applied Biosystems), and 1 µl (200 U) RevertAid™ M-MuLV Reverse Transcriptase (Fermentas). The final reaction mixture of 25 µl was incubated at 25°C for 10 min followed by incubation at 42°C for 60 min. Heating at 70ºC for 10 min inactivated the reaction and the tubes were subsequently cooled on ice. All heating steps were performed by using a Thermal Cycler 2720 (Applied Biosystems). Each sample of first-strand cDNA was diluted 1:10 before preparing the real-time PCR reactions.

3.2.6 Real-time quantitative PCR (qPCR)

Real-time amplification was performed using SYBR® Green I detection chemistry. The real-time qPCR assay was carried out according to the manufacturer's instructions in a total volume of 25 µl containing 12.5 µl of Maxima™ SYBR Green qPCR Master Mix (Fermentas), 0.5 µM of each specific primer, 2 µl template cDNA and nuclease-free water. Amplification was performed in a 96-well plate with an ABI 7500 Real-Time PCR SDS (Sequence Detection System) (Applied Biosystems).

Three reactions for each primer set without cDNA, known as 'no template controls' (NTC) were included on each reaction plate. To assess the efficiency of PCR amplification, reactions for a standard dilution series (32 ng, 3.2 ng, 0.32 ng, 0.032 ng) from the cDNA pool of tepal development stage 6 (T6) were prepared for each target gene (*CmCHS* and *CmDFR*) and the *Cm18S* rRNA reference gene. This was done for the orange and yellow flower variety. A 'PCR set-up sheet' was used to organize the pipetting of plates as well as to coordinate the data during the analysis.

The PCR reaction mixtures were subjected to the following thermal profile: pre-incubation at 95°C for 10 min, followed by 50 cycles of denaturation at 95°C for 15 sec, annealing at 55°C for 40 sec, and extension at 72°C for 30 sec. Baseline and threshold cycles (C_t) were automatically determined using the 7500 SDS Software (Applied Biosystems).

3.2.7 Data analysis

Standard curves for *CmCHS*, *CmDFR* and *Cm18S* rRNA were prepared in Microsoft Excel® 2007 by plotting the C_t read-outs of the abovementioned diluted standards, versus the logarithm of the samples' initial template concentration. In order to assess the data, a linear regression line with its corresponding equation and *R*-squared value (coefficient of determination) were selected to be displayed on each chart (ABI Prism 7700 SDS User Bulletin no.2, 2001). The relative quantitative gene expression of the *CmCHS* and *CmDFR* target genes at each flower developmental stage was calculated using the comparative C_t method (Wong and Medrano, 2005; Schmittgen, 2006). Raw C_t values from the real-time qPCR analysis were exported into Microsoft Excel® 2007 where further processing was done.

ΔC_t-values were calculated for each sample by subtracting the *Cm18S* rRNA C_t from the C_t of each target gene. Finally, the comparative expression levels of the target genes were determined by the formula $2^{-\Delta\Delta Ct}$, where $\Delta\Delta Ct$ was calculated by subtracting the calibrator ΔC_t value from the ΔC_t of the sample (Livak and Schmittgen, 2001; Schmittgen, 2006). Line graphs were created by plotting the values for $2^{-\Delta\Delta Ct}$ against the corresponding developmental stages of each flower organ to observe how changes in relative gene expression occurred from one stage to the next (therefore observing the temporal expression).

The correlation between the relative gene expression values of *CmCHS* and *CmDFR* in each tissue type for each *Clivia miniata* variety was calculated using BioStat 2009 Professional for Windows (AnalystSoft, Inc.). Each result was expressed as a Pearson correlation coefficient (R). Furthermore, one-way ANOVA (analysis of variance) was performed to test if there were significant differences between the three tissue types concerning temporal expression of each target gene. The null hypothesis, stating that there was a statistically significant difference between different groups of data, was tested with the p-level set at 0.05.

3.3 Total anthocyanin determination

3.3.1 Plant Material

Tepal samples previously collected from developmental stages 2 to 6 (see section 3.2.1) from *Clivia miniata* var. *miniata* 'Plantation' and *Clivia miniata* var. *citrina* 'Giddy' were used to determine anthocyanin content.

3.3.2 Anthocyanin extraction

Extraction procedures were carried out according to the combined methods of Sparvoli *et al.* (1994), Mato *et al.* (2000), Nunes *et al.* (2006), and Nakatsuka *et al.* (2008). Tepals were grinded to a fine powder in liquid nitrogen with a clean mortar and pestle. A 1:10 dilution was prepared in plastic test tubes by suspending approximately 200 to 400 mg of tissue from each developmental stage in methanol acidified with 1% HCl (v/v). Each extraction was prepared in triplicate. The test tubes were covered with alumina foil to minimize light exposure and shaken overnight at 4°C in the dark. After exactly 16 hours the samples were put on ice followed by centrifugation at 10 000 g. for 10 min. The supernatants were used for further analysis.

3.3.3 Spectrophotometry

The total anthocyanin content was measured with UV-visible spectrophotometry. 1 ml of each supernatant was pipetted into a plastic cuvette with a 1 cm pathlength which was placed into a Cary 100-Bio UV-visible spectrophotometer (Varian, Inc.). Maximum absorbance (A_{max}) was measured at 530 nm. All absorbance settings and readings were controlled with the use of Cary WinUV analysis software. The three absorbance values for each developmental stage were used to calculate the anthocyanin concentration expressed as the average absorbance at 530 nm ($\bar{A}_{530nm}$) / 100 mg fresh weight (FW) of tissue (Table 7.1). These averages were then used to report any changes in anthocyanin content during the five developmental stages.

3.3.4 Statistical analysis

Correlation was determined between the relative gene expression values obtained for the two flavonoid biosynthetic genes, *CmCHS* and *CmDFR*, and the absorbance values obtained during anthocyanin quantification. This was done by assessing the linear correlation between two data sets, i.e. *CmDFR* or *CmCHS* expression versus anthocyanin concentration at each developmental stage, and expressing the result as a Pearson correlation coefficient (*R*). A statistical analysis program called BioStat 2009 Professional for Windows was used (AnalystSoft, Inc.). In order to report any correlation, graphs were generated in SigmaPlot v11 for Windows (Systat software, Inc.). One-way ANOVA was also performed with BioStat 2009 to support any possible correlation, using a *p*-level of 0.05.

Chapter 4

RESULTS AND DISCUSSION

4.1 IDENTIFICATION OF *CLIVIA* FLAVONOID BIOSYNTHETIC GENES

4.1.1 Degenerate primer design and PCR amplification

Degenerate primers were designed to PCR amplify cDNA segments of the *CHS*, *CHI*, *F3H* and *DFR* genes in four varieties of *Clivia miniata* and *Clivia caulescens* (Table 4.1). Amplicon fragment sizes for the different genes were similar to what had been determined *in silico* (Figure 4.1).

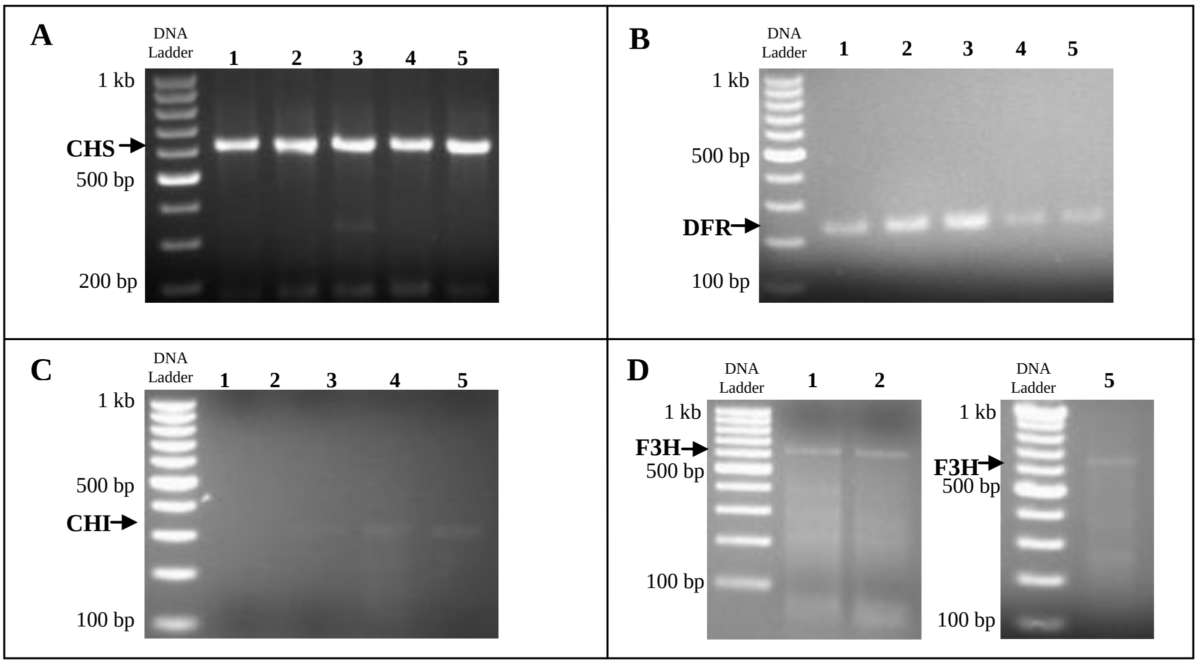


Figure 4.1: Photographs of agarose gels stained with ethidium bromide and visualized under UV light to resolve the PCR products of *Clivia* for *CHS* (A), *DFR* (B), *CHI* (C), and *F3H* (D). Lanes 1 to 5 represents different C*livia* cultivars in the following order: lane 1, *Clivia miniata* var. *citrina* 'Kirstenbosch Yellow'; lane 2, *Clivia miniata* var. *miniata* 'Teleurstelling'; lane 3, *Clivia miniata* var. *citrina* 'Giddy'; lane 4, *Clivia caulescens*; lane 5, *Clivia miniata* var. *miniata* 'Plantation'. A 100 bp DNA ladder was used to indicate the sizes of amplified fragments.

Table 4.1: Degenerate primers used to amplify flavonoid gene fragments of *Clivia*. IUPAC symbols for degeneracy: Y=C/T; R=A/G; M=A/C; S=C/G; N=A/T/C/G.

Primer	**Sequence (5'-3')**	**Length (nt's)**	**%GC**	**Tm (°C)**	**Degeneracy**	**Expected PCR fragment size (*in silico* PCR)**
CHS-f	GACATGCCGGGCGCNGAYTAYCA	23	57-70	61	16	615 bp
CHS-r	CGCAGGCGCTCGACATRTTNCCRTA	25	52-64	63	16	
CHI-e2f	GTTCACGGCCATCGGMGTSTACYTGGA	27	56-63	62	8	327 bp
CHI-e3r	TGAGTGAAGAGAATGGAGGMRCCMGG	26	50-62	61	8	
F3H-f	CAGGTGGTGGACCAYGGMGTSGA	23	61-70	61	8	625 bp
F3H-r	CCACCGCCTGGTGRTCYGCRTT	22	59-73	62	8	
DFR-f	CAAAGATCCGGAGAACGAAGTGATMAARCC	30	43-50	61	4	227 bp
DFR-r	TCTTGGATACAAAGTACATCCATCCNGTCAT	31	39-42	60	4	

The alignments of published amino acid and cDNA sequences of the *CHS*, *CHI*, *DFR*, and *F3H* genes of different monocot species including *Hordeum vulgare*, *Oryza sativa*, *Zea mays*, *Anthurium andreanum*, *Lilium speciosum*, *Allium cepa*, *Agapanthus praecox*, *Triticum aestivum*, *Lilium speciosum*, *Bromheadia finlaysoniana*, *Cymbidium hybrid*, *Secale cereal* and *Thinopyrum ponticum* revealed appropriate regions for designing degenerate primers (Appendices A, C, D and E). Monocot sequence data was used since Clivias are monocots and most studies have shown that flavonoid biosynthetic genes share high sequence similarity among different plant species in terms of phylogenetic grouping (Reddy *et al*., 1996; Nakatsuka *et al*., 2003).

The degenerate primers used to amplify the *CHS*, *CHI*, *F3H* and *DFR* gene fragments in *Clivia* were designed to have certain characteristics (see section 3.1.3) (Table 4.1). The "nearest neighbour method" acknowledged as the most accurate method to calculate oligo thermodynamic stability was used to determine primer Tm (Breslauer *et al*., 1986). The Tm of each primer in a primer pair was very similar, differing with 1 or 2°C. All primers had acceptable lengths of between 22 and 31 bases, respectively. The primers were designed with a 16-fold or less degeneracy (the number of nucleotides needed to cover all combinations of nucleotides) since the higher the degeneracy, the lower the primer concentration, which has an effect on lowering the primer Tm. Furthermore, all primers had a GC content of 40 to 70%, which is known to facilitate stronger primer-to-template annealing (McPherson and Møller, 2006).

The design of primers for the PCR amplification of the *Clivia* flavonoid genes was extremely difficult due to a lack of sequence data. In addition, no published *Clivia* genomic sequences were available to generate a codon usage table that may have assisted in predicting specific codon sequences for this organism. Thus, in order to design primers for these genes the following methods were used: 1) a web-based degenerate primer design software program known as CODEHOP; 2) visual identification of conserved areas within nucleotide alignments of published sequences and designing primers based on these regions; and 3) known primer sequence information from previous studies (Table 4.2). CODEHOP primers (Appendix B) were used to PCR amplify the *CHS* gene segment by using either genomic DNA or cDNA. Since *CHS* primers produced the expected amplification result using cDNA as template, it was assumed that the forward and reverse primers annealed within the same exon. The *DFR* primers were designed according to highly conserved protein motifs KDPENEVIKP and MTGWMYFVSK (Appendix C1), also used to design degenerate primers for the amplification of *DFR* fragments from other plant species, including *Gerbera hybrid*, *Forsythia x intermedia* and *Allium cepa* (Helariutta *et al.*, 1993; Rosati *et al.*, 1997; Kim *et al.*, 2004). However, the *DFR* primer set used in the current study was custom-designed, based on highly conserved areas in published monocot *DFR* cDNA sequences, in an effort to produce PCR primers that would amplify the *DFR* region in *Clivia* (Appendix C2). The *F3H* and *CHI* primer sequences were based on conserved areas visually identified in nucleotide alignments of published cDNA sequences (Appendices D and E), but did not result in amplification for some *Clivia* samples (Figure 4.1).

When comparing the amount of successful PCR amplifications for each of the target cDNA sequences, certain observations, when closely inspecting each primer sequence, could be made. From triplet codon assignments it is evident that the genetic code is degenerate since a set of codons may specify the same amino acid, as the early researchers predicted (Table 4.3). According to McPherson and Møller (2006), the complexity of a degenerate primer mixture can be reduced by "*identifying very pronounced codon bias and including such codons as unique rather than degenerate sequences*". This implied that primers can be designed by relying on conserved motifs within amino acid alignments of related published protein sequences. However, a conserved motif should harbour a stretch of eight to ten amino acids, referred to as a "corresponding peptide" (Table 4.2), where each amino acid should preferably exhibit restricted codon usage (McPherson and Møller, 2006). Such amino acids are listed in Table 4.3. Unfortunately, this method of design was not considered for this study. The corresponding peptides for *CHS* were automatically selected by the CODEHOP program (Appendix B), whereas the corresponding peptides for *DFR* were already known from previous studies mentioned earlier (Helariutta *et al.*, 1993; Rosati *et al.*, 1997; Kim *et al.*, 2004). In the case of *CHI* and *F3H*, nucleotide alignments between the *Clivia* consensus sequences and published homologous sequences from GenBank were translated to amino acid alignments with BioEdit to reveal the corresponding peptides given in Table 4.2.

Table 4.2: Summary of methods used to design the degenerate primers used to isolate *Clivia* gene fragments of *CHS*, *CHI*, *F3H* and *DFR*. Their corresponding peptides are also shown.

Primer	Web-based design or Visual identification	ClustalX alignment used as reference		Conserved areas in amino acid alignment		
				Corresponding peptide	Conserved amino acids *	
		Protein	Gene		%	Considered when assigning degeneracy
CHS-f	web-based (CODEHOP)	yes	yes	**D M** P G A **D Y Q**	62.5	yes, possibly by program
CHS-r				**Y** G **N M** S S A **C**	50.0	
CHI-e2f	visual identification	-	yes	**F** T A I G V **Y** L **E**	33.3	-
CHI-e3r				P G A S I L **F** T	12.5	
F3H-f	visual identification	-	yes	**Q** V I **D H** G V **D**	50.0	-
F3H-r				**N** A **D H Q** A V V	50.0	
DFR-f	visual identification	yes (used in other studies)	yes	**K D** P **E N E** V I **K** P	60.0	-
DFR-r				**M** T G **W M Y F** V S **K**	60.0	

* Conserved amino acids (shown in bold) refer here to amino acids with restricted codon usage; In this case an optimal amino acid is not coded by more than 2 codons. Single-letter database codes (SLC) for amino acids are shown Table 4.3.

The CHS-f primer designed by using the CODEHOP software would be considered an ideal primer for PCR for the following two reasons: 1) the primer contains more than 60% conserved amino acids in its corresponding peptide (Table 4.2). For example, Aspartic acid (D), Tyrosine (Y), and Glutamine (Q) are each encoded by two possible codons, while Methionine (M) is encoded by a single codon (Table 4.3); 2) amino acids such as Leucine (L), Serine (S), and Arginine (R) were avoided because each is encoded by six codons (Table 4.3). In comparison, primers CHI-e2f and CHI-e3r cannot be regarded as optimal primers due to the low presence of restricted codons and the occurrence of Leucine (L) and/or Serine (S) in their corresponding peptides (Table 4.2). According to these observations, the main reason for constantly obtaining either no amplification or very low yields for some of the samples, especially in the case of *CHI*, can be mostly ascribed to primer pairs not having optimal sequences.

Table 4.3: Amino acids, their single-letter database codes (SLC), and their corresponding DNA codons. IUPAC symbols for degeneracy: Y=C/T; R=A/G; M=A/C; W=A/T; H=A/C/T; S=C/G; N=A/T/C/G; D=A/G/T.

Amino Acid	**SLC**	**DNA codon(s)**	**Codon(s) -'degenerate position(s)' shown**	**Complement (for reverse primer)**
Methionine	M	ATG	ATG	TAC
Tryptophan	W	TGG	TGG	ACC
Phenylalanine	F	TTT, TTC	TTY	AAR
Tyrosine	Y	TAT, TAC	TAY	ATR
Cysteine	C	TGT, TGC	TGY	ACR
Glutamine	Q	CAA, CAG	CAR	GTY
Asparagine	N	AAT, AAC	AAY	TTR
Histidine	H	CAT, CAC	CAY	GTR
Glutamic acid	E	GAA, GAG	GAR	CTY
Aspartic acid	D	GAT, GAC	GAY	CTR
Lysine	K	AAA, AAG	AAR	TTY
Isoleucine	I	ATT, ATC, ATA	ATH	TAD
Valine	V	GTT, GTC, GTA, GTG	GTN	CAN
Alanine	A	GCT, GCC, GCA, GCG	GCN	CGN
Glycine	G	GGT, GGC, GGA, GGG	GGN	CCN
Proline	P	CCT, CCC, CCA, CCG	CCN	GGN
Threonine	T	ACT, ACC, ACA, ACG	ACN	TGN
Leucine	L	CTT, CTC, CTA, CTG, TTA, TTG	YTN	RAN
Serine	S	TCT, TCC, TCA, TCG, AGT, AGC	WSN	WSN
Arginine	R	CGT, CGC, CGA, CGG, AGA, AGG	MGN	KCN

Based on guidelines for optimal primer design, an optimal degenerate primer should be designed from a corresponding peptide harbouring amino acids of which at least 50% exhibit restricted codon usage. If a corresponding peptide for a primer includes Methionine and Tryptophan, and is devoid of Leucine, Serine, and Arginine, a more improved PCR product yield for subsequent amplifications can be expected.

4.1.2 Identification of Clivia CHS, CHI, F3H and DFR genes

The application of two-step PCR (Reverse Transcriptase Polymerase Chain Reaction) resulted in the amplification of a putative *Clivia* cDNA fragment for *CHS*, *CHI*, *DFR* and *F3H* when using degenerate primers and a tepal cDNA template (Figure 4.1). The size of the PCR amplified fragments for *CHS* and *DFR* in all five *Clivia* varieties was similar to the expected sizes determined *in silico*. The PCR fragments were purified, sequenced and analysed further. Each *CHS* electropherogram revealed that single nucleotide polymorphisms (SNPs) were present in equal quantity at certain positions suggesting the presence of heterozygous *CHS* alleles expressed in the flower tepals (Figure 4.2). Degenerate bases were assigned to these positions (Appendix F).

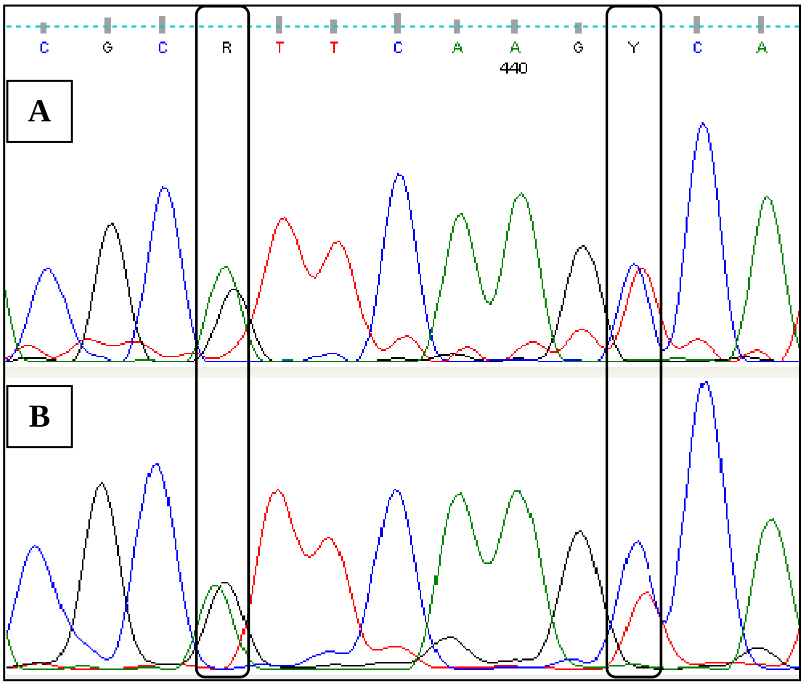


Figure 4.2: Sections of electropherograms obtained after sequencing the *CHS* cDNA fragment in *Clivia miniata* var. *miniata* 'Teleurstelling' (A) and *Clivia miniata* var. *citrina* 'Giddy' (B). Ambiguities are present at position 435, with a R (G or A), as well as position 442 with a Y (T or C).

In the case of *CHI*, PCR amplification for *Clivia caulescens* and *Clivia miniata* var. *miniata* 'Plantation' produced amplification fragments of the expected *in silico* length (Figure 4.1). Unfortunately, the PCR product yield for *Clivia caulescens* was too low for use in sequencing (Figure 4.1). In the agarose gel shown for *F3H*, PCR with samples from *Clivia miniata* var. *citrina* 'Kirstenbosch Yellow', *Clivia miniata* var. *miniata* 'Teleurstelling' and *Clivia miniata* var. *miniata* 'Plantation', successfully amplified cDNA fragments similar to the expected *in silico* length after PCR optimisation (Table 4.1; Figure 4.1). PCR reactions were optimized to reduce the number of background bands through the use of a modified primer annealing temperature (Ta) of 58°C.

PCR with genomic DNA produced either non-specific PCR fragments, no amplification or very low yields of the expected fragment, except in the case of *CHS* where successful amplification was obtained from genomic DNA, but was not used for further study. A number of factors may have contributed to the lack of PCR amplification using genomic DNA as template, including miss-priming due to the use of degenerate primers, the presence of multiple primer binding sites, primer binding at intron/exon junctions or the presence of an intron within the target region, as well as unidentified inhibitory substances that might have inhibited the *Taq* DNA polymerase. Whenever multiple PCR fragments were visualised on a gel, the expected size fragment was excised, gel-purified and re-amplified with the same primer set. Unfortunately, re-amplification of the *CHI*, *F3H* and *DFR* gene fragments repeatedly failed to produce expected results. It was for this reason that the use of a cDNA template was preferred throughout this study.

4.1.3 Sequence analysis

The PCR products of the different genes, *CHS*, *CHI*, *F3H* and *DFR* respectively, were purified and sequenced, followed by a data analysis using the software program, ContigExpress. A consensus cDNA sequence of 586 bp, 326 bp, 510 bp and 225 bp was obtained for *CHS*, *CHI*, *F3H*, and *DFR*, respectively (Appendices F-I). Global alignments in MatGAT were used to assess both the similarity and identity to the corresponding cDNA fragments of other higher plants and revealed high percentages ($\geq$ 60%) on the nucleotide level. No differences were found between different *Clivia* varieties for each of the respective genes analysed. According to the predicted similarity and identity values, the consensus cDNA sequence for *Clivia CHS* displayed a closer relationship to other monocot *CHS* sequences. These values were the highest for the *CHS* sequence of *Lilium speciosum* (~77% and ~76%, respectively), *Allium cepa* (~74% and ~73%, respectively) and *Oryza sativa* (~75% and ~74%, respectively) (Appendix J), suggesting the expression of a *CHS* orthologue in *Clivia* tepals. In an amino acid alignment with the deduced *Clivia* CHS fragment, the four conserved residues (Cys164, Phe215, His303, and Asn336) that define the catalytic mechanism of known CHS-related enzymes were identified (Ferrer *et al*., 1999; Jez *et al*., 2001) (Appendix N). The presence of these active site residues supports the existence of a functional CHS enzyme in *Clivia*. With regard to the cDNA sequence of *CHI* in *Clivia miniata* var. *miniata* ‘Plantation’, identity and similarity comparisons revealed values that were similar on average (≥ 59%), and therefore both dicot and monocot sequences were evolutionarily related (Appendix K). The *Clivia* sequence was the most homologous to the sequence of the monocot *Allium cepa* (similarity, ~74%; identity, ~75%). Additionally, when comparing the translated *Clivia* sequence with the corresponding region of CHIs from other plants, the amino acid identity values were relatively low for most dicots compared to monocots (data not shown). On amino acid sequence level *Clivia miniata* also showed the closest relationship to *Allium cepa* (similarity, ~89%; identity, ~75%). A similarity and identity analysis of the *Clivia F3H* consensus sequence with the corresponding region in other plants also proved to be more homologous to the monocot *F3H* sequences of *Allium cepa* (~81%) and *Lilium speciosum* (~78%) (Appendix L). Unexpectedly, the *Clivia* sequence was also very similar to dicot sequences, which might have not been the case if complete cDNA sequences were compared. An alignment of the deduced *Clivia* F3H amino acid sequence with other F3H amino acid sequences of the corresponding size displayed three of five strictly conserved motifs, i.e. motifs 2, 3 and 4 (Appendix O). F3H is a member of the class of 2-oxoglutarate-dependent dioxygenases (2-ODDs) meaning it is a non-heme iron enzyme, dependent on binding typical cofactors such as Fe^{2+}, molecular oxygen, 2-oxoglutarate, and ascorbate (Lukacin and Britsch, 1997) (see section 2.2.3). Based on the amino acid sequence deduced from the cDNA sequence, motif 2 and motif 3 contained three prolines, which were strictly conserved and were predicted to have important roles in the folding process of the polypeptide. Conserved histidine and aspartate residues in motif 4 necessary for ligating ferrous iron at the active site are also present (Britsch *et al*., 1993; Lukacin and Britsch, 1997). These observations suggest the existence of a functional *Clivia* F3H enzyme. A nucleotide sequence analysis between the cDNA consensus sequence of the *Clivia DFR* gene and the corresponding cDNA region of other published monocot *DFR* sequences, confirmed higher similarity and identity values for *Allium cepa* (~80% and ~81%, respectively), *Agapanthus praecox* (~78% and ~79%, respectively), and *Lilium speciosum* (~77% and ~78%, respectively) (Appendix M). These observations suggested that a *DFR* orthologue was present in all *Clivia* tissues. From an alignment of the deduced DFR amino acid sequence from *Clivia* and the corresponding amino acid sequences from other *DFR*-encoded sequences it was possible to identify a previously proposed amino acid region that determines substrate preference of DFR (Appendix P) (Beld *et al*., 1989). A mutation analysis confirmed that a single amino acid (134^{th} residue) within this region of the *Gerbera* DFR affected substrate preference (Johnson *et al*., 2001). DFR in certain plants lacks the ability to efficiently reduce DHK (dihydrokaempherol) to form orange-coloured pelargonidin derivatives (See Figure 2.2). The DFR of petunia and DFR5 of *Lotus japonicus*, for example, do not efficiently catalyse the reduction of DHK to leucopelargonidin (Johnson *et al*., 1999; Shimada *et al*., 2005). DFRs of both these plants have an Aspartic acid (Asp) at the position corresponding to the 134th residue of *Gerbera* DFR and are referred to as "Asp-type DFRs" (Shimada *et al*., 2005). Some plants such as *Gerbera hybrida*, *Zea mays* and *Rosa hybrida* contain DFRs that have an Asparagine (Asn) at this position (Meyer *et al*., 1987; Helariutta *et al*., 1993; Tanaka *et al*., 1995). These DFRs can utilise all three dihydroflavonols (DHK, DHQ and DHM) and are referred to as "Asn-type DFRs". However, an exception to this is the *Cymbidium* orchid DFR, which has an Asn at the previously mentioned position but was reported to lack the ability to reduce DHK (See Figure 2.2). Heterologous expression of the *Cymbidium* DFR in *Petunia* demonstrated that it prefers DHQ over DHK, suggesting that orchid DFR substrate specificity is not determined by this proposed amino acid position (Johnson *et al*., 1999). The deduced amino acid sequence of the *Clivia* DFR-like region also contains an Asn at the specified position, suggesting the presence of an Asn-type DFR in *Clivia* (Appendix P). An HPLC analysis has shown that orange-coloured pelargonidin derivatives were the major pigments in *Clivia* tepals, although red-coloured cyanidin derivatives were also found, mostly in small quantities (Koopowitz *et al*., 2003). This is an important discovery since it may imply that *Clivia* DFR activity favours the reduction of DHK to form leucopelargonidin (see Figure 2.13). Biochemical characterisation would therefore be of vital importance to gain future insight into the substrate preference of *Clivia* DFR(s).

4.1.4 Phylogenetic analysis

To investigate the evolutionary relationships among the isolated *Clivia* flavonoid gene sequences (*CHS*, *DFR*, *CHI* and *F3H*, respectively) and other genes involved in the biosynthesis of flavonoids in plants, phylogenetic trees were constructed with MEGA v3.1 applying the neighbour-joining method. The results are shown in Figures 4.3 to 4.6 and include bootstrap values of 50% and higher. Monocots and dicots were grouped into different clusters in each analysis. The putative *Clivia* gene fragments were clustered together with sequences from different lily cultivars, some orchid species, onion and cereal crops such as rice, barley, wheat and maize. Clustering was expected since these are all monocot plants. Furthermore, amino acid alignments with each translated *Clivia* cDNA consensus sequence also suggested that each gene shares a common evolutionary ancestor with other homologues based on conserved structure and sequence characteristics such as amino acid identities and conserved motifs.

4.1.5 Towards gene characterisation: Isolation of the full-length gene sequences

The isolated cDNA fragments for genes involved in the *Clivia* central flavonoid biosynthetic pathway were sequenced directly. Sequences ranged in length from 227 and 586 bp. Since these fragments showed such a high degree of similarity with genes from other species, they can be used for the design of homologous (or heterologous) probes and gene-specific primers (GSPs). A homologous probe would be helpful during screening of a newly constructed *Clivia* cDNA library and would also assist in hybridisation to genomic DNA fragments of interest when performing Southern blotting.

Subjecting full-length cDNA or amino acid sequence data to a sequence -and/or phylogenetic analysis has the potential to add increased resolution to the final results. When attempting to isolate full-length *Clivia* cDNA sequences, screening of a cDNA library or subjecting isolated mRNA to 3'- and 5'-rapid amplification of cDNA ends (3'- and 5'-RACE) can prove useful. Screening cDNA libraries with homologous probes was done to detect members of the *CHS* multi-gene family in the flowers of *Bromheadia finlaysoniana* (Liew *et al.* 1998), for the isolation of three *CHSs* and one *DFR* from the tepals of two Asiatic hybrid lily cultivars (Nakatsuka *et al*., 2003), and for the isolation of clones harbouring *F3H*, *ANS* and *F3'H* from the petals of *Gentiana triflora* (Nakatsuka *et al.*, 2005). RACE with degenerate primers for nested PCR have been used to isolate full-length *DFR* cDNAs from members of the *Caryophyllales* (Shimada *et al*., 2004), and to isolate F3'H -and F3'5'H cDNA from *Vitis vinifera* (Bogs *et al*., 2005). This implies that the *CHS* and *DFR* primers designed for the present study can be used in RACE-PCR to isolate the full-length *Clivia* cDNA sequences. RACE-PCR have also been performed with nested GSPs to isolate *DFR* cDNAs from *Lotus Japonicus* (Shimada *et al*. 2005), to generate a full-length cDNA of *Ginko biloba F3H* (Shen *et al*., 2006), and in obtaining the full-length coding sequences of *ANS* and *F3H* from *Fragaria x ananassa* (Almeida *et al*., 2007).

The characterisation of the genes involved in the *Clivia* flavonoid biosynthetic pathway with regard to the identification of introns and regulatory regions will require the isolation of full length genomic DNA sequences. Studies on flavonoid biosynthesis have confirmed two general methods used in this regard. The first method concerns the construction of a genomic library by cloning genomic DNA fragments into an appropriate vector such as a Lambda phage vector and screening the colonies with a cDNA probe. Helariutta *et al*. (1996), for example, isolated two novel *CHS* genes from *Gerbera* by screening the genomic library with a homologous cDNA probe. In another study three genomic clones of the *CHS* gene were obtained from *Vitis vinifera* through plague hybridisation with a labelled carrot cDNA clone of *CHS* (Goto-Yamamoto *et al*., 2002). An additional method that could be used to isolate a genomic DNA sequence of a gene involves conventional PCR with gene-specific primers. This was achieved by De Schepper *et al*. (2001), who were able to obtain a 4 kb amplification product followed by direct sequencing of the genomic fragment of *DFR*. Kim *et al*. (2004) used a similar approach except that two separate PCR reactions were carried out by first predicting the possible splicing sites (intron-exon boundaries) in the cDNA sequence by performing a BLAST search, followed by designing two sets of primers that were used to amplify two overlapping *DFR* sequences respectively. The fragments, however, were sub-cloned for the convenience of sequencing.

There are methods that also allow the isolation of unknown DNA fragments. One important example is the isolation of upstream or downstream regulatory regions, including promoters. Such methods include inverse PCR (IPCR) and the genome walking strategy, of which the latter is much more novel than the other methods (Ochman *et al*., 1988; Ashoub and Abdalla, 2006). Other advantages include the reliability of each procedure without the need to spend time on constructing DNA libraries, therefore also avoiding the use of radioactive probes. IPCR were used to isolate full-length genomic clones of the *Phalaenopsis CHS* and the *Vinca major F3'5'H*. The 3' and 5' flanking regions of these genes were also characterised during a sequence analysis (Mori *et al*., 2004; Han *et al*., 2005). Genome walking was used to obtain the upstream and downstream genomic sequences of five *Vitis vinifera FLS* fragments (Fujita *et al*., 2006). The genomic sequences of *ANR*, *FLS*, *DFR*, *3GT*, *F3H* and *ANS* of *Fragaria* x *ananassa* were also obtained by using genome walking (Almeida *et al*., 2007).

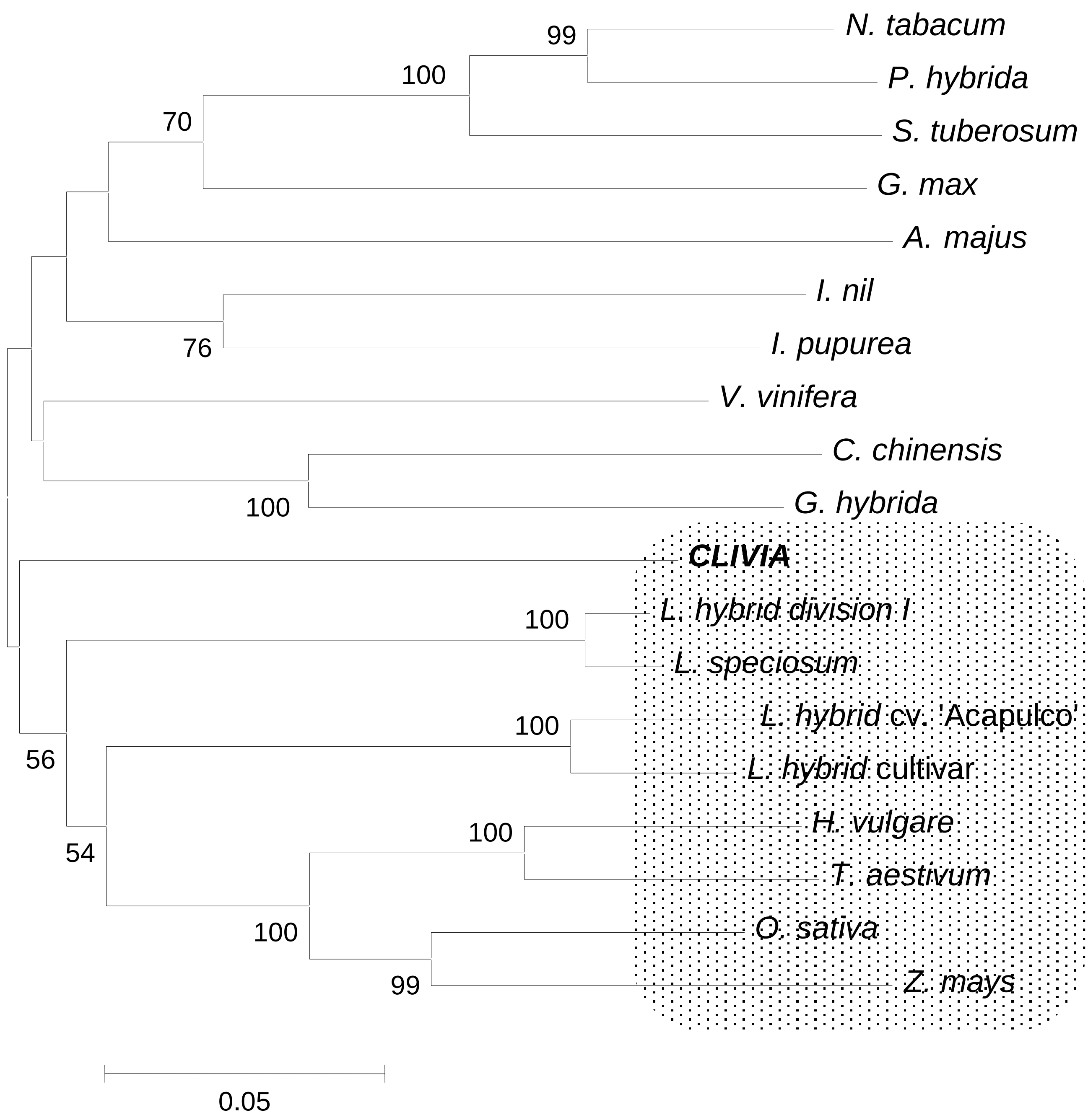


Figure 4.3: A neighbour-joining phylogenetic tree of the cDNA fragments corresponding to *CHS* consensus region in *Clivia*. Numerals adjacent to branches indicate percentage (more than 50%) of 1000 bootstrap replicates. The grey area indicates all the monocot plants grouped together. GenBank accession numbers: *Allium cepa* (AF268382), *Hordeum vulgare* (M98871), *Lilium hybrid* cv. 'Acapulco' (AAD49355), *Lilium hybrid* division I (BAB40787), *Lilium hybrid* cultivar (ABF82595), *Lilium speciosum* (BAE79201), *Oryza sativa* (BAA19186), *Triticum aestivum* (ACJ22498), *Zea mays* C2 (X60204), *Callistephus chinensis* (Z67988), *Gerbera hybrida* (Z38096), *Ipomoea nil* (AB001818), Ipomoea purpurea (AB001826), *Vitis vinifera* (X75969), *Glycine max* (FJ770471), *Solanum tuberosum* (U47739), *Nicotiana tabacum* (AF311783), *Antirrhinum majus* (X03710), *Petunia hybrida* (X14591).

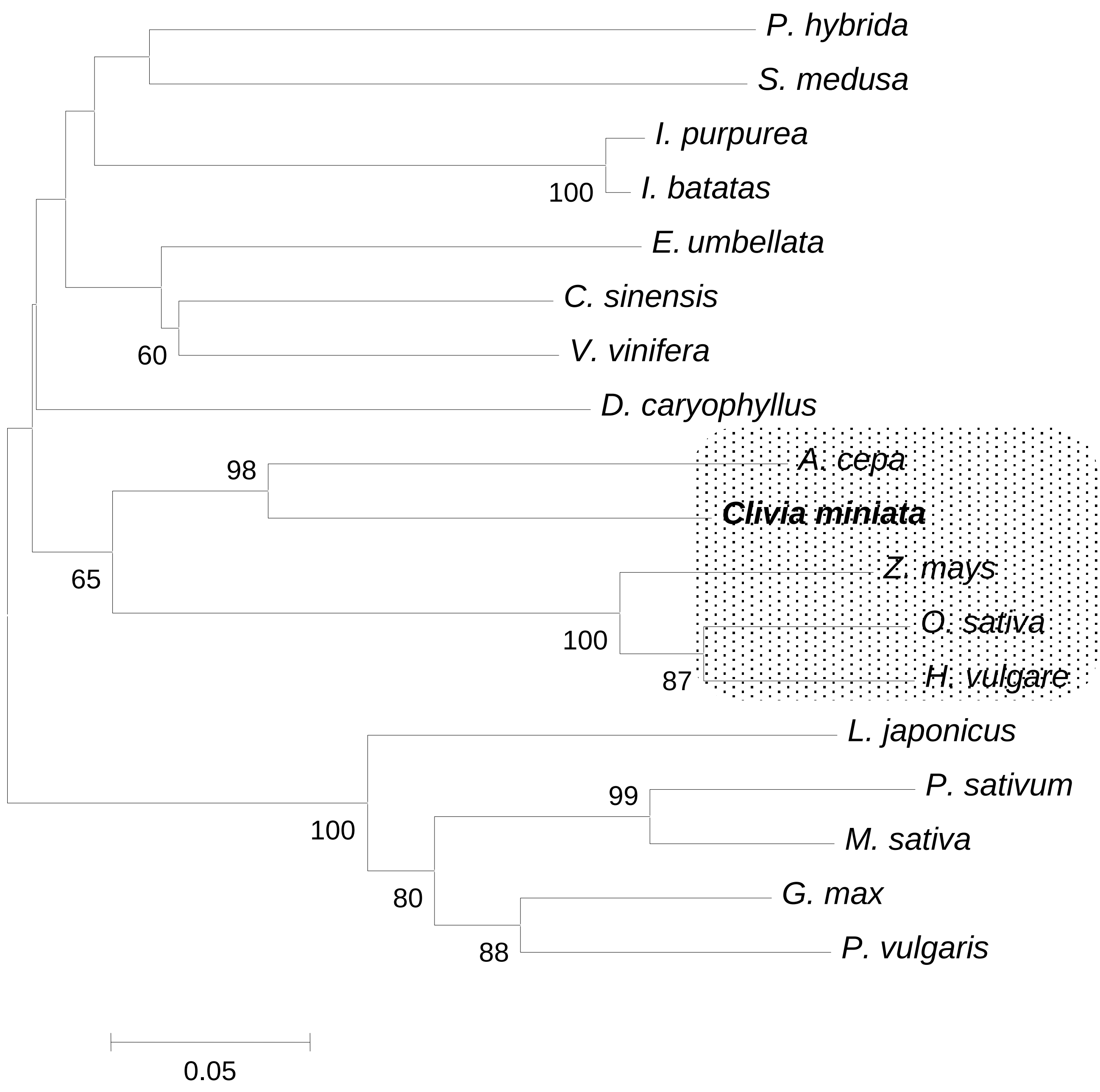


Figure 4.4: A neighbour-joining phylogenetic tree of the cDNA fragments corresponding to the *CHI* region in *Clivia miniata* var. *miniata* 'Plantation'. Numerals adjacent to branches indicate percentage (more than 50%) of 1000 bootstrap replicates. The grey area indicates all the monocot plants grouped together. GenBank accession numbers: *Elaeagnus umbellate* (AF061808), *Saussurea medusa* (AF509335), *Petunia hybrida* (Y00852), *Ipomoea purpurea* (AF028238), *Ipomoea batatas* (AB080768), *Camellia sinensis* (DQ904329), *Vitis vinifera* (X75963), *Dianthus caryophyllus* (Z67989), *Allium cepa* (AY541034), *Zea mays* (EU970806), *Oryza sativa* (AF474922), *Hordeum vulgare* (AF474923), *Lotus japonicus* (AJ548840), *Pisum sativum* (U03433), *Medicago sativa* (M91079*), Glycine max* (FJ770472), *Phaseolus vulgaris* (Z15046).

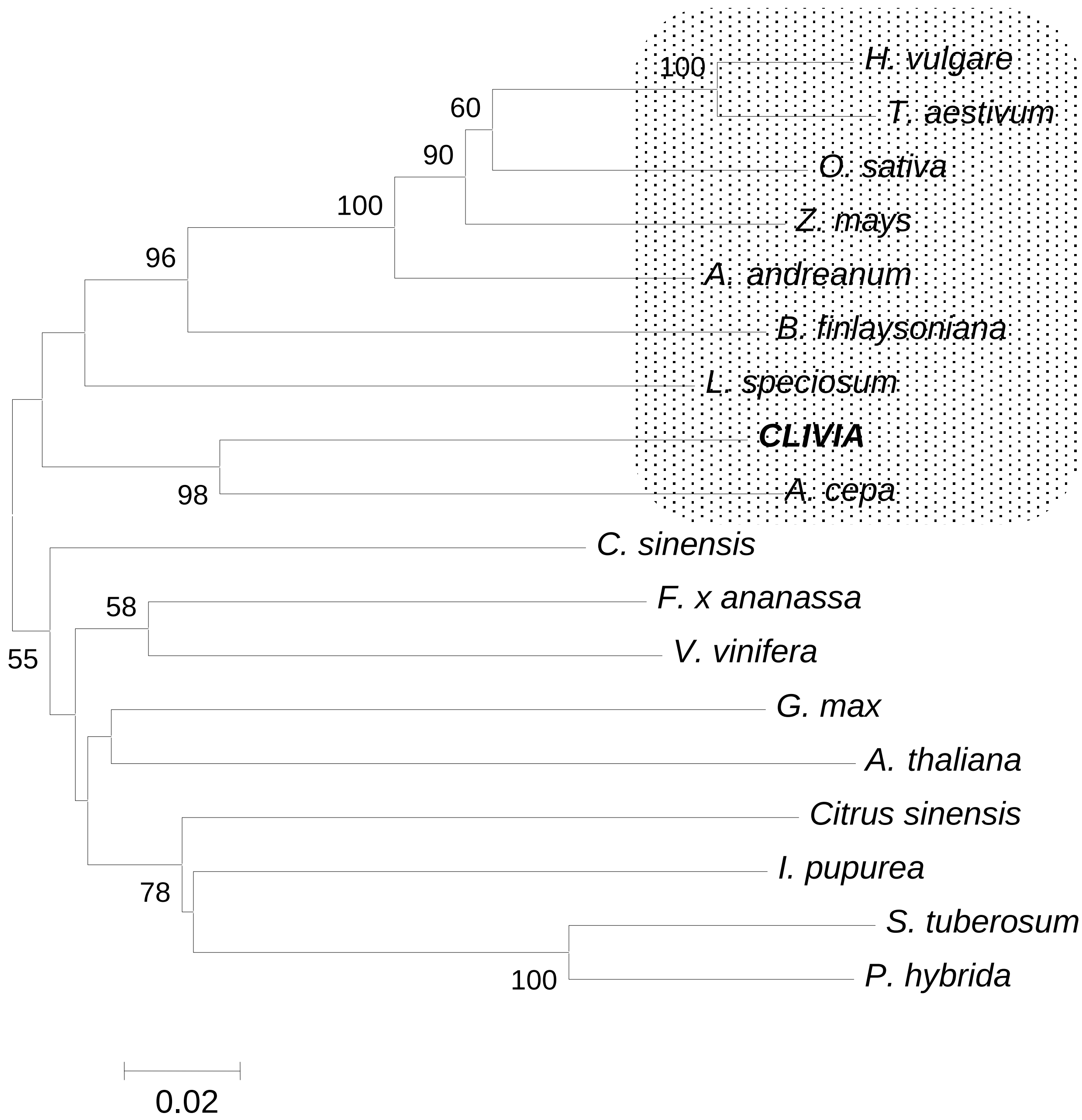


Figure 4.5: A neighbour-joining phylogenetic tree of the cDNA fragments corresponding to the *F3H* consensus region in *Clivia*. Numerals adjacent to branches indicate percentage (more than 50%) of 1000 bootstrap replicates. The grey area indicates all the monocot plants grouped together. GenBank accession numbers: *Allium cepa* (AY221246), *Lilium speciosum* (AB201532), *Bromheadia finlaysoniana* (X89199), *Hordeum vulgare* (EU921438), *Oryza sativa* (NM_001060692), *Triticum aestivum* (DQ208192), *Zea mays* (NM_001156993), *Anthurium andreanum* (DQ972935), *Ipomoea nil* (D83041), *Glycine max* (AY595420), *Gentiana triflora* (AB193311), *Fragaria x ananassa* (AY691919), *Vitis vinifera* (EF192467), *Citrus sinensis* (AB011795), *Solanum tuberosum* (AY102035), *Camellia sinensis* (AY641730), *Arabidopsis thaliana* (NM_114983), *Petunia hybrida* (AF022142).

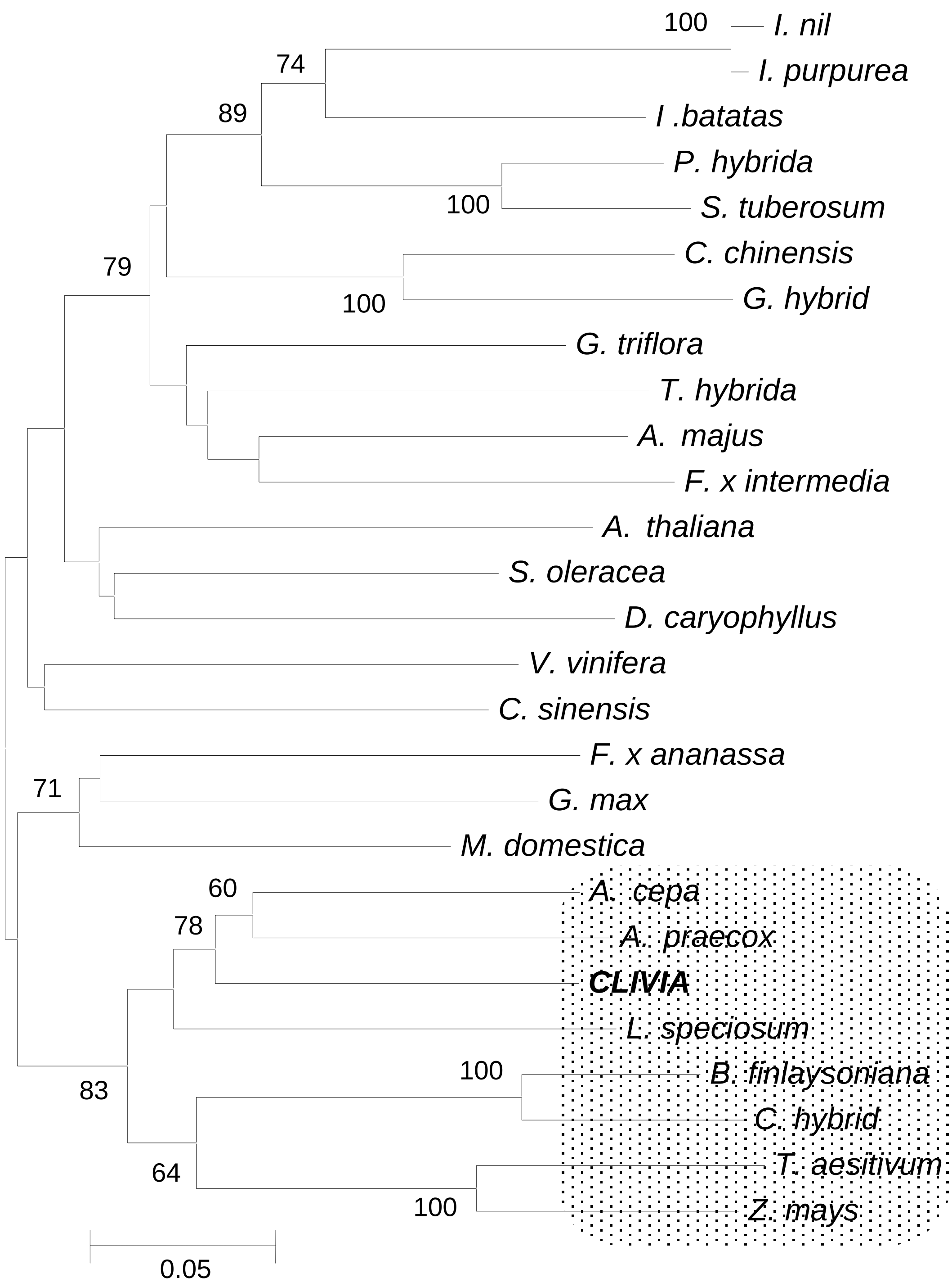


Figure 4.6: A neighbour-joining phylogenetic tree of the cDNA regions corresponding to the *DFR* consensus region in *Clivia*. Numerals adjacent to branches indicate percentage (more than 50%) of 1000 bootstrap replicates. The grey area indicates all the monocot plants grouped together. GenBank accession numbers: *Lilium speciosum* (AB201531), *Bromheadia finlaysoniana* (AF007096), *Zea mays* A1 (Y16041), *Antirrhinum majus* (X15536), *Callistephus chinensis* (Z67981), *Dianthus caryophyllus* DFRA (Z67983), *Forsythia x intermedia* (Y09127), *Gerbera hybrid* (Z17221), *Ipomoea purpurea* DFRB (AB018438), *Ipomoea batatas* (EU360845), *Ipomoea nil* DFRB (AB006792), *Malus domestica* (AF117268), *Petunia hybrida* DFRA (X15537), *Vitis vinifera* (X75964), *Solanum tuberosum* (AF449422), *Gentiana triflora* (D85185), *Torenia hybrida* (AB012924), *Fragaria x ananassa* (AY695812), *Arabidopsis thaliana* (NM_123645), *Spinacia oleracea* (AB246750), *Citrus sinensis* (AY519363), *Glycine max* (AF167556), *Allium cepa* DFR-A (AY221250), *Agapanthus praecox* (AB099529), *Cymbidium hybrid* (AF017451), *Triticum aestivum* DFR-A (AB162138).

4.2 EXPRESSION ANALYSIS OF *CHS* AND *DFR* IN *CLIVIA MINIATA* FLOWERS

4.2.1 Introduction

Tepal, stamen (male reproductive organ) and carpel (female reproductive organ) samples were collected at five different flower developmental stages of two different colour varieties of *Clivia miniata* i.e. *Clivia miniata* var. *miniata* 'Plantation' (dark orange) and *Clivia miniata* var. *citrina* 'Giddy' (group 2 yellow) (Figure 4.7). Total RNA having good purity and integrity was successfully isolated from each of the three tissue types at each developmental stage and converted to first-strand cDNA. The first-strand cDNA served as template during the subsequent real-time quantitative expression analysis.

Figure 4.7: *Clivia* flower developmental stages for *Clivia miniata* var. *miniata* 'Plantation' (Panel A), and *Clivia miniata* var. *citrina* 'Giddy' (Panel B). Each developmental stage is labelled with its corresponding number.

Relative gene expression requires an appropriate reference/"housekeeping" gene to normalise the gene expression data and adjust any sample-to-sample variation in the amount of amplifiable cDNA added to each reaction. Currently, about nine well-described reference genes are recommended for use to normalize gene expression levels (Nicot *et al*., 2005). Ideally, two or three housekeeping genes should be analysed in order to select the most appropriate gene, especially when designing a new experiment (Thellin *et al*., 1999; Bustin and Nolan, 2004). Unfortunately, limitations regarding resources and the availability of sequence data did not allow for this in the present study. Before attempting primer design for quantitative real-time PCR, an appropriate housekeeping gene had to be selected. A 100 bp cDNA fragment from the 18S rRNA of *Clivia miniata* (*Cm18S* rRNA) was selected and used as the endogenous reference. Exploitation of the *18S* rRNA gene was considered a preferred option compared to other housekeeping genes, since rRNA genes are extremely favourable in terms of steady-state expression levels (Stürzenbaum and Kille, 2001), and are considered more representative of mRNA integrity because they may remain intact in samples with degraded mRNA (Wong and Medrano, 2005). Furthermore, the *18S* rRNA gene is an ideal endogenous/internal control gene since it is constitutively expressed regardless of experimental conditions, including differences in tissue and cell types, developmental stage, and sample treatment (Stürzenbaum and Kille, 2001; Wong and Medrano, 2005). The C_t values of *Cm18S* rRNA were relatively constant throughout the real-time qPCR experiment, only varying slightly between 10 and 12 in 'Plantation' and 11 and 13 in 'Giddy (Tables 4.4 and 4.5; Appendix S). Li and Strid (2005) also determined relative transcript levels of *CHS* in *Arabidopsis* through normalisation with the *Arabidopsis 18S* rRNA gene.

The use of FastPCR Professional v5.0 enabled the determination of an *in silico* cDNA fragment size of 522 bp expected after *in vitro* PCR amplification. Since ribosomal subunits are not polyadenylated, cDNA synthesis using oligo-dT primers will not transcribe rRNA (Stürzenbaum and Kille, 2001). To ensure that total RNA included ribosomal-derived RNA, a random primer (random hexamer) was added to the cDNA synthesis mixture (See section 3.2.5). The use of random primers yields the most cDNA and is very useful for transcripts with significant secondary structure (Bustin and Nolan, 2004).

Agarose gel electrophoresis confirmed an amplified fragment similar to the predicted *in silico* length after PCR with wheat-specific *18S* rRNA primers (Figure 4.8). Direct sequencing of the PCR product resulted in a 513 bp DNA sequence, which had high sequence similarity to the corresponding fragment from *Clivia nobilis* (Appendix Q). This sequence was used to design two primers, 18SF and 18SR, that were used for quantitative expression analysis of the *Cm18S* rRNA gene (See Table 3.2).

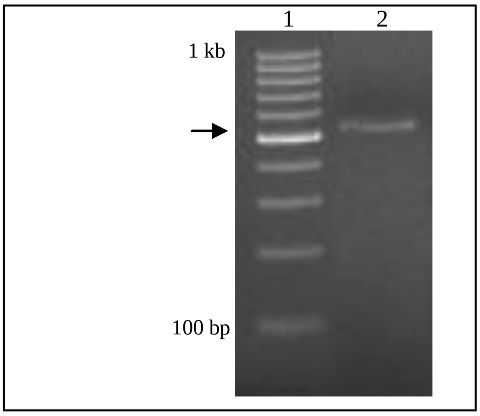


Figure 4.8: Photograph of an agarose gel showing the PCR amplified cDNA fragment of the *Cm18S* rRNA gene. Lane 1: DNA ladder; Lane 2: Amplified target fragment of 522 bp. The arrow indicates the positively amplified fragment.

This study dealt with expression of flavonoid biosynthetic genes in flower tissues, expecting high expression in favour of anthocyanin accumulation. Therefore the *18S* rRNA gene was considered a safe option for normalising expression data due to its highly abundant expression levels that may yield very small C_t values (< 15) (Dorak, 2009). This was indeed the case, since C_t values for the *Cm18S* rRNA ranged between 10 and 13.

4.2.2 Efficiency of the qPCR assay

The dilution series of a reference cDNA sample, in this case the sample for the tepal tissue at developmental stage 6 of 'Plantation' and 'Giddy', was used to construct a standard curve in order to evaluate the efficiency of the expression analysis. The C_t values were obtained from the amplification curves that were generated during real-time qPCR. According to the standard curves shown in Figure 4.9 the relationship between C_t and the logarithm of the starting copy number of the target sequence was linear up to four orders of magnitude. The integrity of the data fit to the trendline was described by the R^2-value. All R^2-values were acceptable and indicated dilution accuracy and precise pipetting ($R^2 \geq 0.990$) (Scott-Adams, 2006).

Before using the comparative C_t method for quantitation it was important to demonstrate that the efficiencies of the target genes and the reference gene were almost similar (Dorak, 2009). According to the slopes of the trendlines shown in Figure 4.9, assay efficiency for the target genes and reference gene was similar. Other factors that supported the expectance that qPCR reactions would proceed well included acceptable primer melting temperatures of 58 or 59°C, a good amplicon size range of between 80 and 114 bp, and ensuring that genomic DNA contamination was minimal by using the highly effective TRizol® RNA extraction method and performing DNase treatment of total RNA.

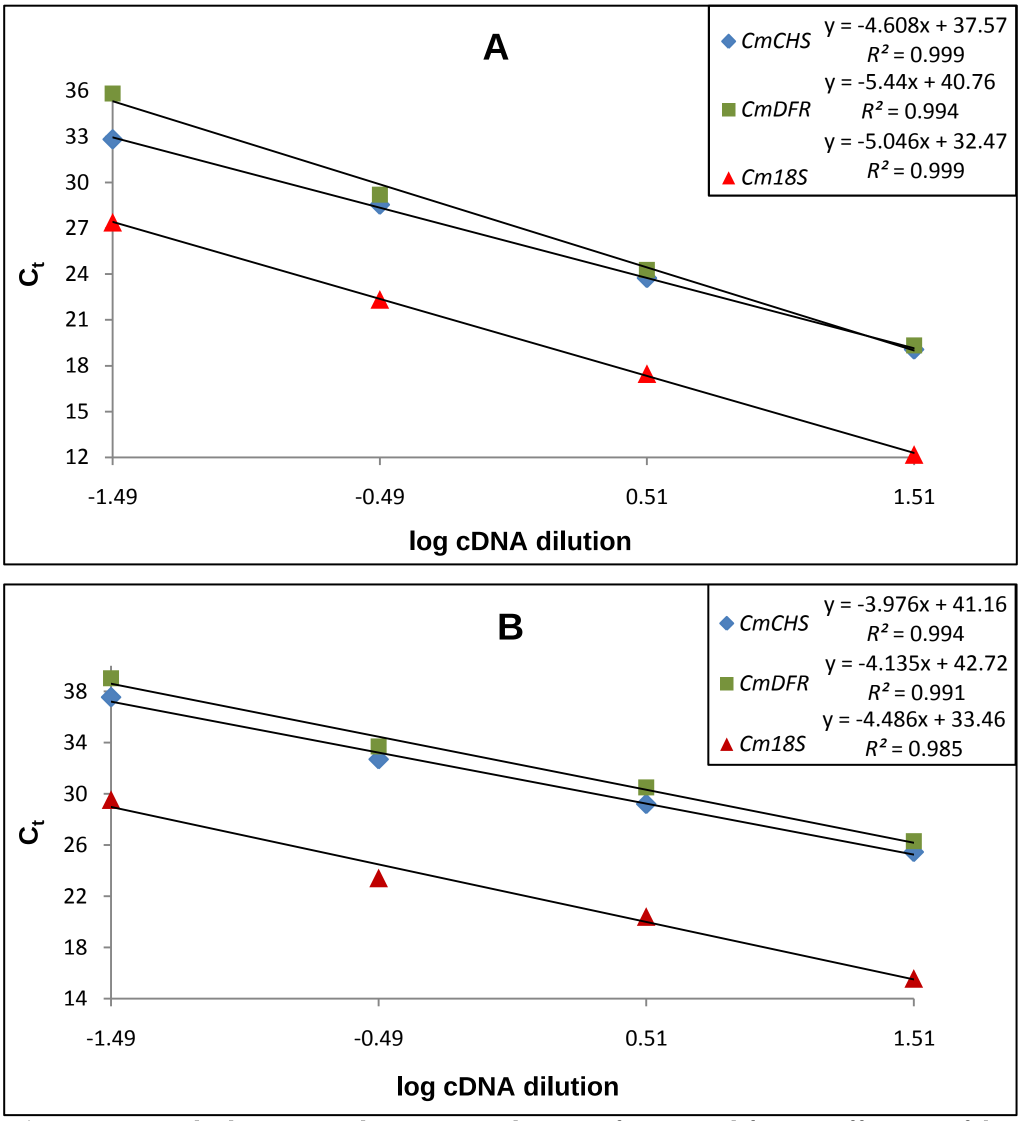


Figure 4.9: Standard curves used to give an indication of PCR amplification efficiency of the two target genes and the *18S* rRNA reference gene from *Clivia miniata* var. *miniata* 'Plantation' (Panel A) and *Clivia miniata* var. *citrina* 'Giddy' (Panel B).

4.2.3 Analysis of relative gene expression with real-time qPCR involving SYBR Green chemistry

Real-time PCR is considered an accurate and sensitive method for quantifying levels of mRNA (Peters *et al*., 2004). In the current study it allowed the detection of amplicon accumulation using the fluorescent dye SYBR® Green I. As PCR reactions progressed with a gene specific primer set, emission of a fluorescent signal was detected when free dye was incorporated into the minor groove of any newly formed double-stranded DNA (Shipley, 2006).

The fold-change of the two target genes (*CmCHS* and *CmDFR*) during temporal gene expression was calculated and results for the comparative C_t method are illustrated in Tables 4.4 and 4.5. The higher the initial copy number of a nucleic acid target in a sample, the sooner a significant increase in fluorescence is observed and the lower the C_t will be (Dorak, 2009). The C_t values obtained for samples T6 and T5 for both target genes were the lowest in ‘Plantation’ and ‘Giddy’, respectively, and provided the highest relative expression values (Tables 4.4 and 4.5).

Table 4.4: Results for the comparative C_t method used to determine the relative quantitative gene expression of the *CmCHS* (A) and *CmDFR* (B) flavonoid biosynthetic genes at each flower developmental stage of *Clivia miniata* var. *miniata* 'Plantation'. The ΔC_t value of the stamen at stage 5 was used as the calibrator (shown in bold). Single letter abbreviations: T, tepal; S, stamen; C, carpel.

A	C_t(sample)	C_t(*Cm18S* rRNA)	ΔC_t(sample)	ΔC_t(calibrator)	$\Delta\Delta C_t$	Relative expression ($2^{-\Delta\Delta Ct}$)
T2	21.65	9.93	11.72	20.46	-8.74	427.57
T3	20.95	10.36	10.59	20.46	-9.87	935.76
T4	20.54	12.42	8.12	20.46	-12.34	5184.54
T5	21.29	11.97	9.32	20.46	-11.14	2256.70
T6	19.17	12.31	6.86	20.46	-13.60	12416.75
S2	24.83	11.04	13.79	20.46	-6.67	101.83
S3	30.32	12.15	18.17	20.46	-2.29	4.89
S4	27.00	11.21	15.79	20.46	-4.67	25.46
S5	32.10	11.64	**20.46**	20.46	0.00	1.00
S6	23.04	11.59	11.45	20.46	-9.01	515.56
C2	28.12	11.47	16.65	20.46	-3.81	14.03
C3	25.80	11.61	14.19	20.46	-6.27	77.17
C4	26.87	11.28	15.59	20.46	-4.87	29.24
C5	27.53	10.37	17.16	20.46	-3.30	9.85
C6	24.85	11.15	13.70	20.46	-6.76	108.38

B	C_t(sample)	C_t(*Cm18S* rRNA)	ΔC_t(sample)	ΔC_t(calibrator)	$\Delta\Delta C_t$	Relative expression ($2^{-\Delta\Delta Ct}$)
T2	21.93	9.93	12.00	18.31	-6.31	79.34
T3	21.69	10.36	11.33	18.31	-6.98	126.24
T4	20.54	12.42	8.12	18.31	-10.20	1168.14
T5	21.18	11.97	9.21	18.31	-9.10	548.75
T6	19.92	12.31	7.61	18.31	-10.70	1663.49
S2	24.91	11.04	13.87	18.31	-4.44	21.71
S3	29.80	12.15	17.65	18.31	-0.66	1.58
S4	26.83	11.21	15.62	18.31	-2.69	6.45
S5	29.95	11.64	**18.31**	18.31	0.00	1.00
S6	23.68	11.59	12.09	18.31	-6.22	74.54
C2	26.21	11.47	14.74	18.31	-3.57	11.88
C3	23.82	11.61	12.21	18.31	-6.10	68.59
C4	24.30	11.28	13.02	18.31	-5.29	39.12
C5	25.45	10.37	15.08	18.31	-3.23	9.38
C6	25.71	11.15	14.56	18.31	-3.75	13.45

Table 4.5: Results for the comparative C_t method used to determine the relative quantitative gene expression of the *CmCHS* (A) and *CmDFR* (B) flavonoid biosynthetic genes at each flower developmental stage of *Clivia miniata* var. citrina 'Giddy'. The ΔC_t values used as calibrators are shown in bold. Single letter abbreviations: T, tepal; S, stamen; C, carpel.

A	C_t(sample)	C_t(*Cm18S* rRNA)	ΔC_t(sample)	ΔC_t(calibrator)	$\Delta\Delta C_t$	Relative expression ($2^{-\Delta\Delta Ct}$)
T2	24.92	10.98	13.94	22.44	-8.50	362.04
T3	25.35	11.60	13.75	22.44	-8.69	413.00
T4	24.66	13.12	11.54	22.44	-10.90	1910.85
T5	20.19	11.53	8.66	22.44	-13.78	14066.74
T6	22.22	11.18	11.04	22.44	-11.40	2702.35
S2	34.76	12.32	**22.44**	22.44	0.00	1.00
S3	32.99	11.72	21.27	22.44	-1.17	2.25
S4	31.77	11.53	20.24	22.44	-2.20	4.59
S5	32.03	11.67	20.36	22.44	-2.08	4.23
S6	33.79	13.26	20.53	22.44	-1.91	3.76
C2	34.81	12.78	22.03	22.44	-0.41	1.33
C3	31.40	10.82	20.58	22.44	-1.86	3.63
C4	33.49	11.34	22.15	22.44	-0.29	1.22
C5	34.12	12.84	21.28	22.44	-1.16	2.23
C6	33.32	11.45	21.87	22.44	-0.57	1.48

B	C_t(sample)	C_t(*Cm18S* rRNA)	ΔC_t(sample)	ΔC_t(calibrator)	$\Delta\Delta C_t$	Relative expression ($2^{-\Delta\Delta Ct}$)
T2	24.22	10.98	13.24	21.40	-8.16	286.03
T3	26.29	11.60	14.69	21.40	-6.71	104.69
T4	24.88	13.12	11.76	21.40	-9.64	797.86
T5	21.29	11.53	9.76	21.40	-11.60	3191.46
T6	23.34	11.18	12.16	21.40	-9.24	604.67
S2	31.75	12.32	19.43	21.40	-1.97	3.92
S3	31.79	11.72	20.07	21.40	-1.33	2.51
S4	31.39	11.53	19.86	21.40	-1.54	2.91
S5	29.52	11.67	17.85	21.40	-3.55	11.71
S6	31.09	13.26	17.83	21.40	-3.57	11.88
C2	34.18	12.78	**21.40**	21.40	0.00	1.00
C3	29.97	10.82	19.15	21.40	-2.25	4.76
C4	30.69	11.34	19.35	21.40	-2.05	4.14
C5	31.62	12.84	18.78	21.40	-2.62	6.15
C6	31.59	11.45	20.14	21.40	-1.26	2.39

4.2.4 Expression of flavonoid biosynthetic genes in Clivia miniata var. miniata 'Plantation'

The comparative threshold (C_t) method ($\Delta\Delta$Ct) was used to analyze temporal gene expression of *CmCHS* and *CmDFR* in the tepal, stamen and carpel tissues of an orange ('Plantation') and yellow ('Giddy') flower variety of *Clivia miniata*. Flower development stage 1 was not analyzed since this stage was morphologically defined by a very small, dark green bud not expected to show any significant degree of expression of the two target genes. The relative amount of each target sequence was determined by normalizing with the reference values of the *Cm18S* rRNA internal control, relative to a calibrator, by calculating $2^{-\Delta\Delta Ct}$ (Tables 4.4 and 4.5). The sample that had the lowest expression level of a target gene (or highest ΔC_t value) was designated as the calibrator (Tables 4.4 and 4.5).

In 'Plantation', transcription of *CmCHS* and *CmDFR* increased as tepals grew and peaked at stage 4 just before anthesis (Figure 4.10 A). Between stage 4 and 5 transcription of both genes decreased as the flower was opening, after which their transcription increased drastically towards the end of flower development (Figure 4.10 A). In the carpel of 'Plantation' both *CmCHS* and *CmDFR* had very similar expression levels, especially from stage 2 to stage 5. Transcription of the genes peaked during the third developmental stage and then gradually decreased through stage 4 up to stage 5 where the genes were the least expressed (Figure 4.10 B). *Further* expression of *CmCHS* increased considerably from stage 5 up to the point where the carpel was fully developed, while transcription of *CmDFR* only increased slightly (Figure 4.10 B).

The target genes in the stamens of 'Plantation' showed similarity regarding their temporal trend of expression, although *CmCHS* was expressed at much higher levels than *CmDFR*. This was depicted in separate graphs to indicate that the trend of expression was the same (Figure 4.10 C). Transcription of both genes decreased during stage 2 and was very low at stage 3, followed by a slight increase towards the middle of stage 4 with a decrease again during stage 5 to reach the equally low levels found during stage 3 (Figure 4.10 C). Afterwards the same steep up-regulation as in the tepals and carpel could be observed until flower development was completed (full bloom).

There was a strong positive correlation in expression trends, for *CmCHS* and *CmDFR* during different developmental stages in the tepal and stamen samples of 'Plantation' ($R > 0.950$, $p \leq$

0.05) (Table 4.6). When comparing stages 2 to 5 for *CmCHS* and *CmDFR* in the carpel of ‘Plantation’, a high correlation was also present (R = 0.996). High correlations may indicate the presence of certain transcription factors that regulate both *CmCHS* and *CmDFR* simultaneously.

Table 4.6: Correlations (R) between expression of *CmCHS* and *CmDFR* in different tissues during flower developmental stages 2 to 6 of orange ‘Plantation’ (o) and yellow ‘Giddy’ (y). Single-letter abbreviations: T – tepal, C – carpel, S – stamen.

	CmCHS expression					
	T-o	C-o	S-o	T-y	C-y	S-y
CmDFR expression	**0.952**	0.309	**0.996**	**0.993**	0.547	0.446

According to the abovementioned changes observed in transcription of *CmCHS* and *CmDFR* in the flower organs of ‘Plantation’, two phases of temporal expression were distinguished: (1) transcription of both genes increased as the flower bud grew, then decreased to very low levels before entering the second phase where (2) anthesis was activated and transcription of the genes increased drastically until the flower was in full bloom. The relationship between the three tissue types (i.e. carpel, stamens and tepals) concerning the temporal expression of each gene was tested with one-way ANOVA. According to the results there was no significant difference in temporal expression of *CmCHS* between the tissues ($p > 0.05$), while the stamen and carpel tissues were significantly different to the tepal tissue concerning temporal expression of *CmDFR* ($p < 0.05$). The gene expression for *CmCHS and CmDFR* among the three different tissues was not statistically significantly different. Differences in *CmDFR* expression between the tepals and the rest of the flower, however, cannot be explained up to this point.

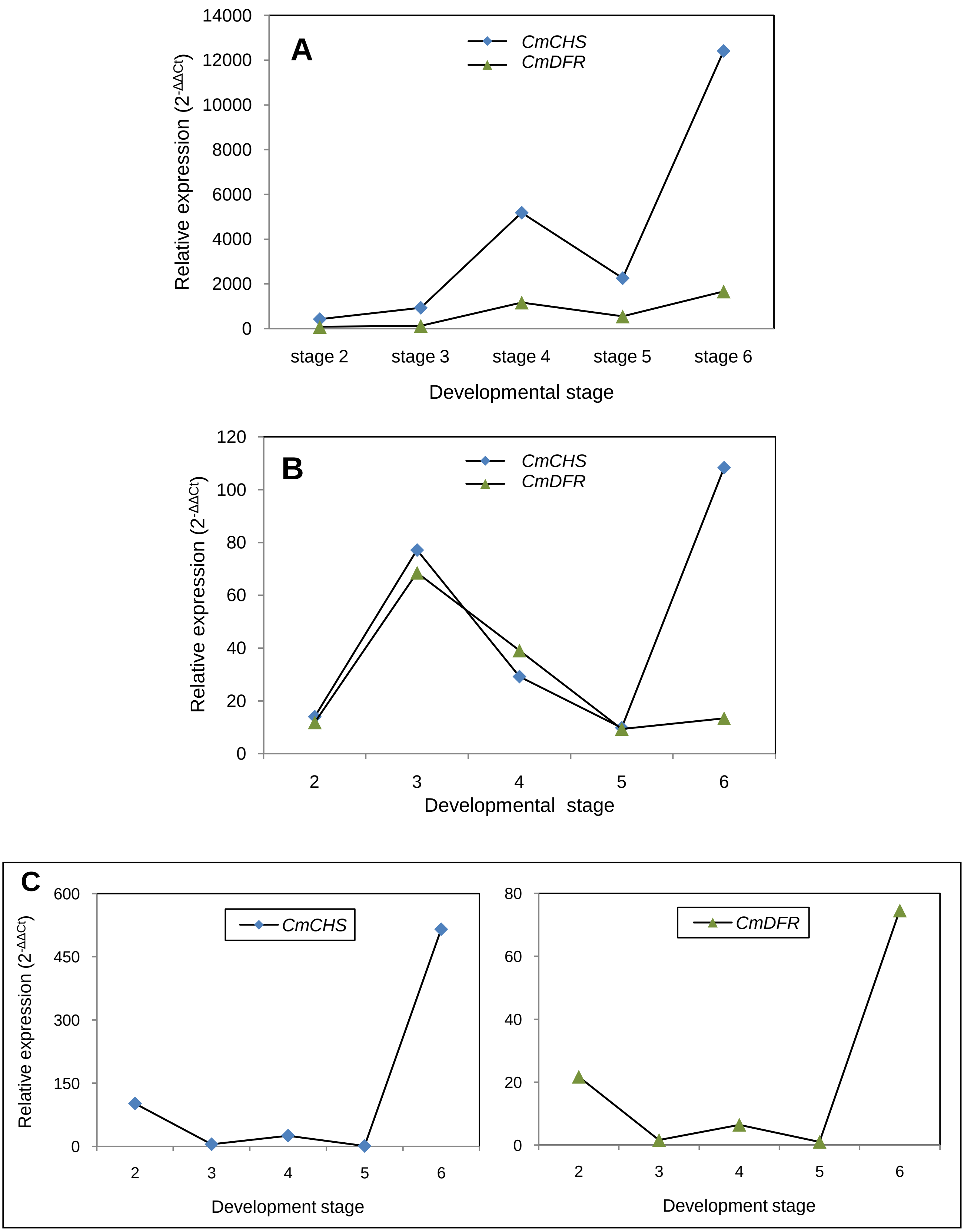


Figure 4.10: Relative expression of *CmCHS* and *CmDFR* in flower tepals (Panel A), carpel (Panel B) and stamens (Panel C) from development stages 2 to 6 of *Clivia miniata* var. *miniata* 'Plantation'. The *Cm18S* rRNA was used to normalise expression of these genes under identical conditions.

4.2.5 Expression of flavonoid biosynthetic genes in Clivia miniata var. citrina 'Giddy'

In the tepals of 'Giddy', transcription of *CmCHS* and *CmDFR* was constant from stage 2 to 3, and started to increase between stage 3 and 4 just before the flower opened (Figure 4.11 A). At stage 5 (during anthesis) both genes were highly expressed followed by down-regulation towards the end of tepal development (Figure 4.11 A). The trend in expression of the two target genes was very similar with a positive correlation ($R > 0.950$, $p \leq 0.05$) (Table 4.6).

In the carpel of 'Giddy', *CmCHS* and *CmDFR* showed a similar trend in their temporal expression with higher levels of *CmDFR* compared to *CmCHS* (Figure 4.11 B). Transcription of both genes increased from stage 2 and peaked during stage 3, followed by decreased expression until stage 4 but increased again to peak a second time during stage 5 (Figure 4.11 B). Afterwards expression of both genes was steeply down-regulated towards the end of carpel development (Figure 4.11 C). In the stamens of 'Giddy' no similarities were seen in the transcription of the two genes. *CmDFR* was mostly present at higher levels than *CmCHS* during stages 2 and 3, and especially during stages 5 and 6 (Figure 4.11 C). Based on these observations it appears that transcription of *CmCHS* and *CmDFR* tends to decrease from the onset of anthesis (during stage 5) until completion of yellow flower development (stage 6). A decrease in transcription of these genes is expected to affect the production of anthocyanin derivatives, ultimately leading to lower anthocyanin concentration in yellow *Clivia* flowers. The parallelism between anthocyanin biosynthetic gene expression and anthocyanin production in the tepals of 'Giddy' was further investigated in Section 4.3.

Low correlation values shown in Table 4.6 between expression of the two target genes in the carpel and stamen tissues of 'Giddy' could imply that a different regulatory system is present that controls anthocyanin pigmentation. The carpel tissue, however, did show a similar pattern in temporal expression of the two genes (Figure 4.11 B). Regulation of anthocyanin biosynthesis has been shown to be complicated in the stamens of other plant species (Nakatsuka *et al*., 2009). According to the one-way ANOVA results for 'Giddy', there was no significant difference between the temporal expression in the tepal, stamen and carpel tissues of both of the target genes ($p > 0.05$). This phenomenon cannot be explained and should be investigated further.

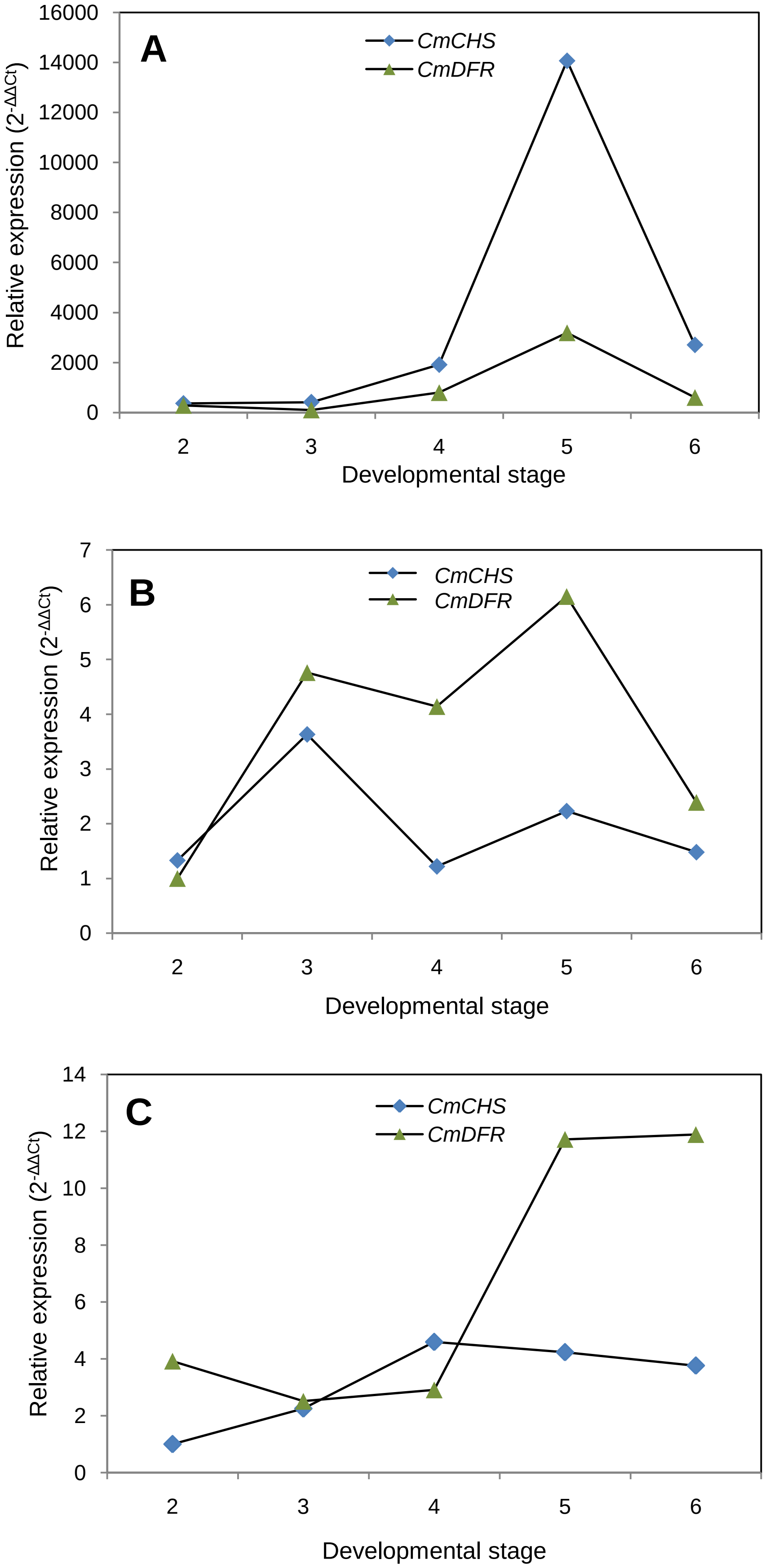


Figure 4.11: Relative expression of *CmCHS* and *CmDFR* in the tepal (Panel A), carpel (Panel B) and stamens (Panel C) of *Clivia miniata* var. *citrine* 'Giddy'. The *Cm18S* rRNA was used to normalize expression of these genes under identical conditions.

4.2.6 Future prospects concerning regulation of anthocyanin pigmentation in Clivia

The structural genes for flower pigmentation in dicot plants have been divided into separate groups according to differences in their transcriptional regulation (See section 2.4). In *Petunia* x *hybrida, Antirrhinum majus* and *Ipomoea purpurea* for example, *CHS* and *CHI* are classified into the early biosynthetic genes (EBGs) for anthocyanin and flavone and/or flavonol biosynthesis, while the late biosynthetic genes (LBGs) include *DFR*, *ANS* and modification enzymes responsible for anthocyanin biosynthesis (See Figure 2.2). The expression of these two groups is independently regulated by different regulatory proteins (Almeida *et al*., 1989; Martin *et al*., 1991; Huits *et al*., 1994; Quattrocchio *et al*., 1998; Park *et al*., 2007). Anthocyanin pigmentation in the monocots *Zea mays* and *Hordeum vulgare* appears to be co-ordinately regulated as a single module without division of EBGs and LBGs (Dooner *et al*., 1991; Meldgaard, 1992; Martin and Gerats, 1993). The regulatory pattern of *CHS*, *DFR* and *F3H* in *Pisum sativum* was demonstrated to be similar to that of *Zea mays*, and transcriptional regulation of *CHS* and *DFR* in the tepals of Asiatic hybrid lily was also shown to be similar to that in kernels of *Zea mays* and *Hordeum vulgare* (Uimari and Strommer, 1998; Nakatsuka *et al*., 2003).

In the present study, *CmCHS* (an EBG) and *CmDFR* (a LBG) were transcriptionally active throughout flower development in pigmented tissue of both the orange and yellow flower varieties, suggesting co-ordinate regulation as a single module for anthocyanin biosynthesis in *Clivia miniata*. As mentioned before, each flower organ analysed exhibited similar temporal expression for *CmCHS and CmDFR* , except in the stamens of 'Giddy' where each gene was expressed differently. These observations support the possibility of co-ordinate regulation by either the same or alternative transcription factors, depending on the tissue type.

CmCHS expression was generally higher than *CmDFR* in the flower parts of 'Plantation' and in the tepals of 'Giddy'. It has to be borne in mind that a higher mRNA level of *CmCHS* might be necessary to ensure production of both co-pigments (flavones and/or flavonols) and anthocyanins. As mentioned in section 2.6.2, co-pigments have the important role of stabilising and enhancing anthocyanin pigmentation in the cellular vacuole. This scenario best fits the orange colour of 'Plantation' that is probably produced by pelargonidin derivatives (Koopowitz *et al*., 2003).

The regulation of anthocyanin biosynthesis was discussed in Chapter 2 (see section 2.4). As mentioned, different transcriptional factors for anthocyanin biosynthesis activate the structural genes and are known to include members of protein families containing R2R3-MYB domains, bHLH domains and conserved WD40-repeat proteins. Combinations of these proteins and their interactions determine the set of genes to be expressed (Koes *et al*., 2005; Mol *et al*., 1998). Furthermore, the ratio and amounts of *bHLH* and *R2R3-MYB* transcripts also alter the amount of anthocyanins produced (de Majnik *et al*., 1998). They have been shown to either bind directly to the promoter region of the anthocyanin biosynthetic genes, or activate genes encoding bHLH proteins. Furthermore, MYB-type proteins can also act as repressors by competing for binding sites in target gene promoters or by interacting with bHLH proteins to sequester them into inactive complexes (Mato *et al*., 2000; Laitinen *et al*., 2008).

In *Clivia*, transcriptional factors such as those mentioned above are expected to be involved in the regulation of anthocyanin biosynthesis. To understand the regulatory system in *Clivia* that confers flower colouration, genes for MYB and bHLH transcription factors should be isolated and their spatial and temporal expression investigated. In monocots, the genes involved in the regulation of anthocyanin biosynthesis in flowers have only been cloned for the orchids *Oncidium* Gower Ramsey (*OgMYB1*) and *Phalaenopsis* (*PsMyb*, *PsMyc*, and *PsWd*) as well as *Lilium* hybrid cultivars (*LhbHLH1* and *LhbHLH2*). Each regulatory gene was isolated with the use of primers, designed from alignments of conserved domains of these regulatory proteins, combined with the application of RACE-PCR (Chiou and Yeh, 2008; Ma and Pooler, 2009; Nakatsuka *et al*., 2009).

From a plant breeder's perspective, another very efficient tool known as Marker-assisted selection (MAS) should also be considered here. MAS can be used to map candidate anthocyanin biosynthetic and regulatory genes in a population segregating for a desired colour phenotype. The application of molecular markers that is highly polymorphic such as restriction fragment length polymorphism (RFLP) markers, randomly amplified polymorphic DNA (RAPD) markers, microsatellites, inter-simple sequence repeat (ISSR) markers, and amplified fragment length polymorphism (AFLP) markers can be used to construct a molecular linkage map in order to identify alleles/markers associated with the gene and/or quantitative trait loci (QTL) of interest. This form of MAS has the advantage that plants with traits in demand can be selected at the seedling stage.

DNA polymorphisms located in sequences of both anthocyanin biosynthetic and regulatory genes have been reported to co-segregate with genes responsible for certain colour phenotypes. An example is the *A* locus that controls anthocyanin accumulation in the foliage, flower and young fruits of pepper that was found to co-segregate with a MYB-type transcription factor (Chagné *et al*., 2007). Another example was the identification of the *P*, *R* and *I* loci that are closely linked in potato and they encode F3'5'H, DFR and a MYB transcription factor respectively (De Jong *et al*., 2004). Abe *et al*. (2002) also identified a single dominant *LAP* (*Lilium* anthocyanin pigmentation) locus, which is responsible for anthocyanin pigmentation in the tepals of Asiatic hybrid lily, after having constructed PCR-based linkage maps during RAPD and ISSR analyses.

4.3 TOTAL ANTHOCYANIN DETERMINATION

4.3.1 Total anthocyanin determination

UV-visible spectrophotometry was used to measure anthocyanin absorbance. The extraction of anthocyanins with acidic methanol (pH < 1) as organic solvent worked effectively since anthocyanins exist primarily in the form of a stable red flavylium cation at pH below 2 (Figure 4.12) (See section 2.6.1). Absorbance measurements were taken at a wavelength of 530 nm where these stable forms of anthocyanins absorbed light maximally. Spectrophotometric measurements delivered absorbance readings for all samples. All absorbance values are shown in Table 4.7.

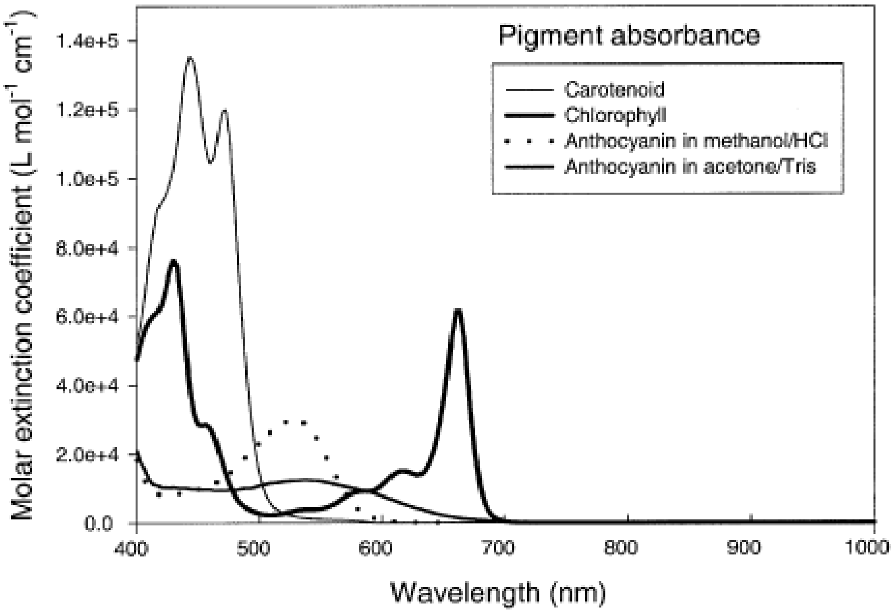


Figure 4.12: Molar extinction coefficient spectra for mixed carotenoids, chlorophyll *a* + *b* and anthocyanin. A higher value is shown for the anthocyanin in acidic solution at 530 nm (modified from: Sims and Gamon, 2002).

Table 4.7: Absorbance values at 530 nm for all samples as well as anthocyanin concentrations at each developmental stage expressed as $\bar{A}_{530nm}$/100 mg fresh weight of tepal tissue.

	Clivia miniata var. *miniata* 'Plantation'			*Clivia miniata* var. *citrina* 'Giddy'		
Sample in triplicate	**A_{530nm}**	***Adjusted A_{530nm}**	**$\bar{A}_{530nm}$/100 mg FW**	**A_{530nm}**	***Adjusted A_{530nm}**	**$\bar{A}_{530nm}$/100 mg FW**
2.1	0.0441	0.0437		0.0287	0.0289	
2.2	0.0330	0.0317	0.0374	0.0254	0.0247	0.0211
2.3	0.0385	0.0367		0.0096	0.0097	
3.1	0.3798	0.3798		0.0186	0.0190	
3.2	0.4129	0.4143	0.3809	0.0173	0.0175	0.0197
3.3	0.3207	0.3486		0.0226	0.0225	
4.1	0.9120	0.9728		0.0375	0.0379	
4.2	1.1971	1.1931	1.0619	0.0224	0.0213	0.0366
4.3	0.9790	1.0198		0.0509	0.0507	
5.1	1.2731	1.2400		0.123	0.1214	
5.2	0.9833	0.9833	1.1707	0.0477	0.0477	0.1010
5.3	1.2672	1.2887		0.1358	0.1340	
6.1	2.3742	2.2902		0.0834	0.0799	
6.2	1.2081	1.1542	1.9095	0.1511	0.1537	0.1216
6.3	2.2839	2.2839		0.1294	0.1311	

* A_{530nm} adjusted according to start-off amount of tepal tissue.

The anthocyanin concentrations at each developmental stage in both colour varieties of *Clivia* were plotted on a bar chart (Figure 4.13). At stage 6 the anthocyanin content in the orange tepals of 'Plantation' had increased by almost 16-fold compared with that in the yellow tepals of 'Giddy'. The results indicate that colour development in the orange tepals is strongly correlated with the accumulation of anthocyanins. The absence of orange colour in yellow tepals can only be caused by the very low anthocyanin concentration, overshadowed by the high concentration of carotenoids and, to a lesser extent, the presence of chlorophylls.

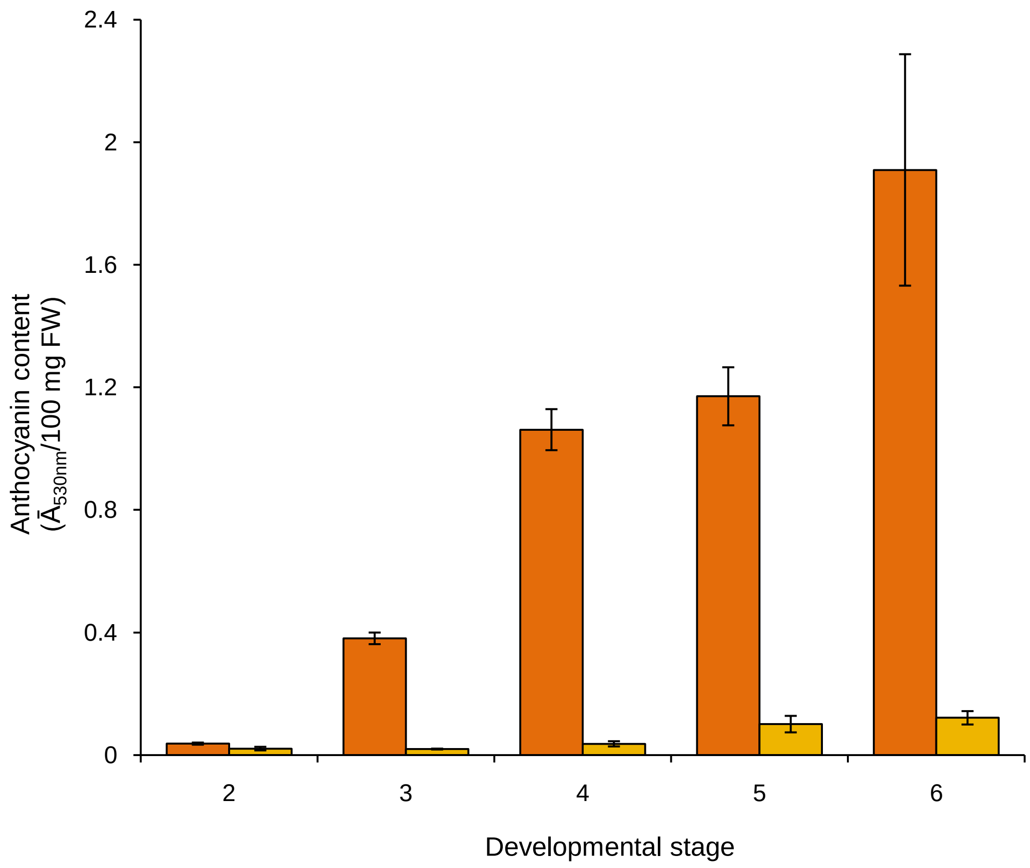


Figure 4.13: Changes in anthocyanin accumulation at five developmental stages of tepals in *C. miniata* var. *miniata* 'plantation' (orange) and *C. miniata* var. *citrina* 'giddy' (yellow). Vertical bars indicate the standard error of the mean of three absorbance readings.

4.3.2 Gene expression vs. Anthocyanin production

According to Figure 4.13 there was a semi-linear increase of anthocyanins in orange tepals, whereas the increase in yellow tepals displayed a sigmoid pattern. When the temporal expression of *CmCHS* and *CmDFR* in the tepals was compared with the anthocyanin accumulation at each stage, a clear trend became visible. In the orange tepals of 'Plantation', for example, a decrease of gene expression levels occurred between stages 4 and 5 (Figure 4.10 A), while a drastic increase of gene expression levels appeared between stages 4 and 5 in yellow tepals (Figure 4.11 B). Both cases coincided with the changes in anthocyanin content depicted in Figure 4.13. Furthermore, a slight decrease in anthocyanin content in the yellow tepals of 'Giddy' was observed after stage 5, which could be explained by the steep down-regulation of *CmCHS* and *CmDFR* expression that was observed between stages 5 and 6 (Figure 4.11 B).

As it is expected that anthocyanin concentration will fluctuate according to the extent in expression of the flavonoid biosynthetic genes, anthocyanin concentration was assigned as the dependent variable and gene expression as the independent variable. A statistical analysis was carried out to ascribe “meaning” in terms of the relation between these variables. The linear correlation between the two data sets, i.e. *CmDFR* or *CmCHS* expression versus anthocyanin concentration at each developmental stage, was assessed and the result expressed as a Pearson correlation coefficient (*R*). Results are shown in Table 4.8 and indicated a much higher correlation in *Clivia miniata* var. *miniata* 'Plantation' than in *Clivia miniata* var. *citrina* 'Giddy'.

Table 4.8: Correlations (*R*) between relative expression of *CmCHS* and *CmDFR*, and anthocyanin content in two colour varieties of *Clivia miniata*.

Code	anthocyanin content (O)	anthocyanin content (Y)
chsO	0.895	-
chsY	-	0.604
dfrO	0.918	-
dfrY	-	0.558

O: *Clivia miniata* var. *miniata* 'Plantation' (Orange variety)
Y: *Clivia miniata* var. *citrina* 'Giddy' (Yellow variety)

A clear trend was observed when comparing the pattern of flavonoid gene expression and anthocyanin accumulation. The linear correlation was tested to determine how well the expression patterns paralleled the increase in anthocyanin pigmentation in tepals. Pearson correlation coefficients (*R*), also known as product-moment correlation coefficients, for *Clivia miniata* var. *miniata* 'Plantation' were the closest to “+1” ($p \leq 0.05$), reflecting a stronger positive relationship (Figure 4.14 A and B). It must be emphasised that the outliers have a profound influence on the slope of the regression line and consequently, the correlation value. Therefore, in the case of Figures 4.15 A and B, the conclusion cannot be based on the *R* value alone because of the non-linear deviation of most of the plots. A rectangular hyperbolic model would best describe the shape and behaviour of the data.

The coefficient for *Clivia miniata* var. *citrina* 'Giddy' indicated a very narrow relationship ($p \geq 0.05$), unless we determined correlation up to stage 5 of tepal development. Up to stage 5, the relationship between the gene expression pattern and anthocyanin accumulation in *Clivia* *miniata* var. *citrina* 'Giddy' was almost perfect ($R \geq 0.990$, $p \leq 0.05$), and is shown in Figures 4.14 C and D. Furthermore, one-way ANOVA indicated that there was no statistically significant difference between temporal expression of the flavonoid biosynthetic genes and anthocyanin accumulation in neither 'Plantation' nor 'Giddy' ($p > 0.05$), further supporting that anthocyanin accumulation paralleled flavonoid biosynthesis during tepal development.

It was shown that *CmCHS* and *CmDFR* were also transcriptionally active in the carpel and stamen tissue. Therefore, as with the tepals, the expression of these genes may have contributed to anthocyanin pigmentation in these organs as well. Nakatsuka *et al*. (2003), for example, proved that these organs accumulated anthocyanin in two Asiatic hybrid lily cultivars, one orange and the other yellow, where orthologues of *CHS* and *DFR* were expressed.

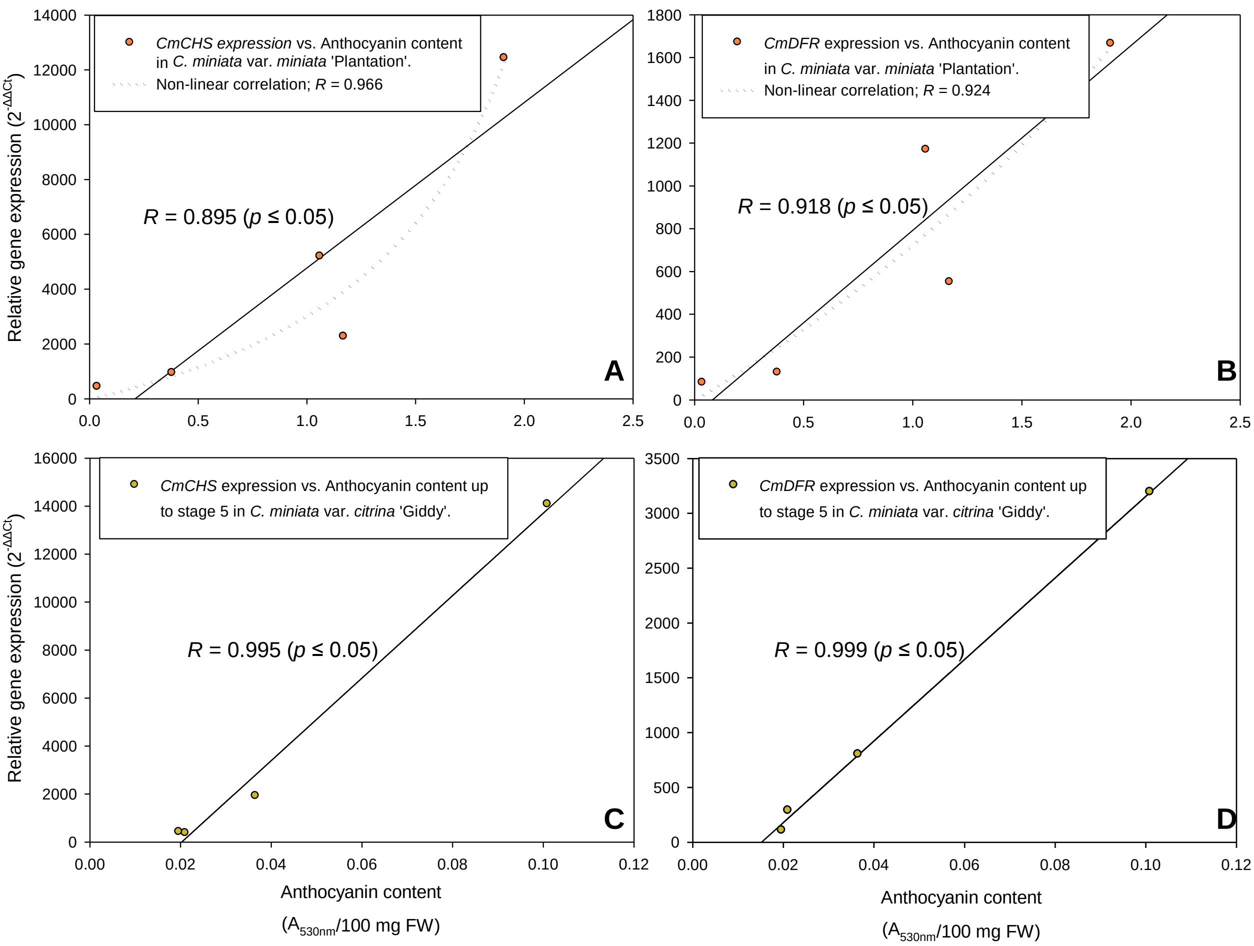


Figure 4.14: Linear correlation determined between relative gene expression of *CmCHS* and *CmDFR*, and anthocyanin content. Panels A and B: Correlation in *Clivia miniata* var. *miniata* 'Plantation'. Non-linear correlation was shown with dotted lines representing a rectangular hyperbolic curve fitting. Panels C and D: Correlation in *Clivia miniata* var. *citrina* 'Giddy' for stages 2 to 5. The *R* values in the legends indicate non-linear correlation, whereas the *R* values next to the straight lines indicate linear correlation.

4.3.3 Future considerations for total anthocyanin determination

UV-visible spectrophotometry was used to get a general idea of the fluctuation of the anthocyanin concentration during *Clivia miniata* flower development. There are more precise methods for qualitative and quantitative determination of anthocyanins. They are the pH differential method for spectrophotometry, and HPLC (high performance liquid chromatography) coupled with various types of mass spectrometers or nuclear magnetic resonance (NMR) apparatus (Guisti and Wrolstad, 2001; Durst and Wrolstad, 2001; Lee *et al*., 2008). To obtain more specific quantitative data in the future, total monomeric anthocyanins can be determined by expressing anthocyanin content as pelargonidin 3-glucoside and pelargonidin 3-rutinoside equivalents, respectively, since these are the two main anthocyanins in orange flower varieties of *Clivia miniata* (Koopowitz *et al*., 2003).

Chapter 5

CONCLUDING REMARKS

There are seven structural genes that encode certain enzymes that are sequentially involved in establishment of anthocyanin pigmentation. Four of these anthocyanin biosynthetic genes in the genus *Clivia* were targeted for identification through isolation of chosen fragments. Conventional PCR with degenerate primers and a tepal first-strand cDNA template was found to be useful in obtaining *Clivia* cDNA fragments for *CHS*, *CHI*, *F3H*, and *DFR*. It was possible to design the primers relying on either consensus data within multiple alignments through manual visualisation or with web-based programs such as CODEHOP, or by using known primer data from previous studies. All three methods proved to save time and to be more cost-effective than using peptide sequence data obtained from a purified protein.

In order to verify positive isolation of the putative *Clivia* target genes, the acquired consensus sequence data of the PCR amplified cDNA fragments was analysed in BLAST. According to these findings the identification of certain flavonoid biosynthetic genes in the genus *Clivia* was considered successful. The partial cDNA sequence data may serve as a tool for designing either homologous probes or gene-specific primers (GSPs). Homologous probes can be used for genomic and/or cDNA library screening, while GSPs can be used in methods such as conventional PCR, 3'-and 5' RACE, inverse PCR and 'genome walking'. These methods can be applied to assist in obtaining full-length DNA or cDNA sequences of each *Clivia* flavonoid biosynthetic gene, essential for further gene characterisation.

Although the purpose of this study was not a phylogenetic analysis, comparisons between the newly obtained *Clivia* consensus cDNA sequences and the corresponding cDNA fragments from other plants confirmed that Clivias are evolutionarily related to other monocots, especially *Agapanthus praecox*, *Lilium speciosum*, *Allium cepa* and *Oryza sativa*. This was evident from percentage similarity/identity and phylogenetic analyses. In order to obtain more dependable resolution in phylogenetic trees and more reliable similarity/identity matrices, the use of full-length cDNA and/or amino acid sequences of the *Clivia* flavonoid biosynthetic genes for sequence analyses is advised. Further investigation of the relationship between the genus *Clivia* and other monocots regarding cytogenetic studies, morphology, anatomy and DNA fingerprinting analysis may corroborate the findings of this study.

The theoretical amino acid alignments based on cDNA sequence data obtained from GenBank and each translated *Clivia* cDNA consensus sequence also suggested that each gene shares a common evolutionary ancestor with other homologues based on conserved structure. This was evident from sequence characteristics such as conserved motifs and amino acid residues necessary for substrate specificity and catalytic mechanisms. The existence of functional enzymes that formed part of the *Clivia* anthocyanin biosynthetic pathway was suggested accordingly. Further biochemical characterisation by investigating substrate preference and performing end-product analyses would be ideal in verifying how each enzyme affects anthocyanin biosynthesis.

The temporal expression of an early anthocyanin biosynthetic gene, *CHS*, and a late anthocyanin biosynthetic gene, *DFR*, in *Clivia miniata*, was analysed. Expression levels during five flower developmental stages in different flower tissues of an orange flowering and a yellow flowering *Clivia miniata* variety were investigated. Two-step real-time qRT-PCR based on SYBR Green I detection chemistry and DNase-treated RNA samples were used in this study. It is considered the method of choice for sensitive, reproducible, and large-scale measurements of gene expression levels, and it also minimises the accumulation of primer-dimers (Vandesompele *et al*., 2002). A *Clivia miniata 18S* rRNA fragment was targeted as reference sequence to normalise all expression data and was ideal due to its constitutive expression regardless of variation in the experimental conditions. Although the real-time quantification delivered important results, the ideal would be to at least analyse all data sets in duplicate or triplicate as a means to add support to any observations.

From the expression data it was possible to demonstrate how both target genes were transcriptionally active throughout orange and yellow-flower development. It was evident from the tepal, stamen and carpel tissues of the orange flowering variety that the expression of both genes displayed two distinguished phases, i.e. 1) transcription of both genes increased as the flower bud grew, then decreased to very low levels before entering the second phase where 2) anthesis was activated and transcription of the genes increased drastically until the flower was in full bloom. The behaviour of these genes in the yellow flower tissues, in comparison, did not show a fixed pattern, although their expression did tend to decrease from the start of anthesis until the flower was fully developed.

From these data it was also determined that *CmCHS* and *CmDFR* displayed a co-ordinate regulatory pattern in their transcription, except in the stamen tissue of the yellow *Clivia miniata* variety. Since *CmCHS* form part of the early biosynthetic genes and *CmDFR* form part of the late biosynthetic genes, it was further suggested that co-ordinate regulation of the all the structural anthocyanin biosynthetic genes as a single module may take place. This observation pointed towards a familiar mode of transcriptional regulation generally found in monocots (Dooner *et al*., 1991; Meldgaard, 1992; Martin and Gerats, 1993). Strong positive correlations between the expression values of *CmCHS CmDFR* were used to support this phenomenon during statistical analyses. We are however of the opinion that in order to understand the regulatory system conferring anthocyanin pigmentation in *Clivia*, regulatory genes that encode transcriptional factors should be isolated and their spatial and temporal expression investigated. Only after the fluctuation in the expression levels of the correct regulatory genes are compared with the expression levels of their target anthocyanin biosynthetic genes, final conclusions can be drawn.

UV-visible spectrophotometry proved to be a quick, easy and effective method for determining the total anthocyanin concentration at each developmental stage. The strong temporal increase in anthocyanin concentrations during *Clivia miniata* tepal development corresponded well with orange-colour development. Yellow tepals also contained anthocyanins but almost 16-fold less than orange tepals at the full blooming stage. Statistical analyses verified that there is a direct correlation between anthocyanin accumulation and the temporal expression of the flavonoid biosynthetic genes in the tepals and possibly other flower organs.

The findings of this study raise an important question: What is the difference between yellow and orange coloured *Clivia* flower organs at the transcriptional level? More progressive gene discovery and expression profiling together with qualitative and quantitative determination of anthocyanins (and other flavonoids) will be necessary to expand our understanding of *Clivia* flower pigmentation. This will also help to facilitate molecular breeding of novel *Clivia* flower colours in the future.

Chapter 6

SUMMARY

Anthocyanins belong to a large group of secondary plant metabolites, the flavonoids, and fulfil a range of biological functions that include the cyanic pigmentation they provide to flowers, fruits, vegetables and leaves. The anthocyanin biosynthetic pathway has been well elucidated and much effort has been made by researchers to modify some of the catalytic steps, thereby changing the colour of some ornamental and cut flower species.

The genus, *Clivia*, is an ornamental monocot indigenous to South Africa and there has been a growing interest among local and international *Clivia* breeders to introduce novel flower colour varieties into the market. Transgene technology holds new possibilities to ensure modification of *Clivia* flower colour. However, the genetics and biochemistry of the *Clivia* anthocyanin biosynthetic pathway must first be investigated before any attempts regarding biotechnology can be made.

The current study is the first to deal with the identification and expression analysis of flavonoid biosynthetic genes in the genus *Clivia*, specifically those involved in anthocyanin biosynthesis, thus identifying future prospects and motivating research in unexplored territory.

A previous study concerning an HPLC analysis of *Clivia* anthocyanin content confirmed the presence of cyanidin and pelargonidin derivatives as the main pigments in the tepals and fruits. This enabled the establishment of a putative *Clivia* anthocyanin biosynthetic pathway illustrating each enzymatic event. Conventional PCR with degenerate primers and a tepal cDNA template was used to isolate four different target sequences. Consensus cDNA fragments of 586 bp, 326 bp, 510 bp and 225 bp confirmed the existence of *Clivia* orthologues for *Chalcone synthase* (*CHS*), *Chalcone isomerase* (*CHI*), *Flavanone 3-hydroxylase* (*F3H*), and *Dihydroflavonol 4-reductase* (*DFR*), respectively. The deduced amino acid sequences of CHS, DFR and F3H harboured important conserved residues that confirmed the existence of functional enzymes. Furthermore, nucleotide sequence analyses between each new *Clivia* cDNA fragment and the corresponding fragments of other higher plants, regarding similarity/identity and phylogeny demonstrated closer homologies and evolutionary relatedness to other monocot species.

The identification of the *Clivia* flavonoid biosynthetic genes enabled the expression analyses of *CHS* and *DFR*. These structural genes encode enzymes responsible for two important controlling steps necessary to determine the nature of the final end-product(s) of the pathway. Real-time quantitative RT-PCR involving SYBR® Green chemistry was used to investigate the temporal expression of the two genes in the tepal, stamen and carpel tissues during five flower developmental stages of an orange and yellow variety of *Clivia miniata*. Statistical analyses were used to support any findings where possible. Each respective tissue type revealed its own trend in expression for both *CHS* (an early biosynthetic gene) and *DFR* (a late biosynthetic gene) throughout flower development except in the stamens of the yellow flowers. These findings suggested the co-ordinate regulation of the *Clivia miniata* anthocyanin biosynthetic genes as a single module, a model of transcriptional regulation that is often found in certain monocot species (Dooner *et al*., 1991; Meldgaard, 1992; Martin and Gerats, 1993). To understand the regulatory system that confers flowers colouration, genes that encode transcription factors should be isolated and their spatial and temporal expression investigated.

The 'parallelism' between anthocyanin biosynthetic gene expression and anthocyanin production in the tepals of the orange and yellow *Clivia miniata* varieties was also investigated. UV-visible spectrophotometry at A_{530nm} was used to quantify total anthocyanins at each developmental stage after extraction. At full bloom the orange flowers had almost 16 times more anthocyanins, which support orange colour development, than the yellow flowers. It was confirmed by the outcomes of statistical analyses that the trends in expression of *CHS* and *DFR* and anthocyanin production were similar. Methods such as HPLC are recommended for more precise qualitative and quantitative determination of total monomeric anthocyanins.

Keywords: anthocyanins, cDNA, *Clivia*, flavonoid biosynthetic genes, flowers, homology, nucleotide sequence analyses, PCR, real-time quantitative RT-PCR, spectrophotometry.

Hoofstuk 7

OPSOMMING

Antosianiene behoort aan ’n groot groep sekondêre metaboliete bekend as die flavonoïede en is verantwoordelik vir verskeie biologiese funksies wat hul rol in die pigmentasie van blomme, vrugte, groente en blare insluit. Die uitleg van die antosianien-biosintese-weg is goed bekend en navorsers het daarin geslaag om sekere ensiematiese stappe te manipuleer om sodoende die kleure van sommige ornamentele en snyblomspesies te verander.

Die genus *Clivia* is ’n ornamentele monokotiel inheems aan Suid-Afrika en daar is tans ’n groeiende belangstelling by telers om nuwe kleure in die plant se blomme tot stand te bring. In vergelyking met gewone teling, hou transgeentegnologie baie potensiaal vir nuwe moontlikhede in dié verband in, maar ’n goeie begrip van die genetika en biochemie van *Clivia*-antosianien-biosintese word vooraf vereis. Die bestaande studie is die eerste navorsingsprojek wat die identifisering en uitdrukkingsanalise van flavonoïedgene (meer spesifiek die antosianiengene) in die genus, *Clivia,* ondersoek en skep nuwe geleenthede en motiverings vir verdere diepgaande studies in hierdie veld.

’n Vorige HPLC-analise van die antosianieninhoud in Clivias het die teenwoordigheid van sianidien- en pelargonidien-verbindings in die blomblare bevestig wat tot die bekendstelling van ’n voorlopige antosianien-biosintese-weg kon lei. Gewone polimerase-kettingreaksies met priemstukmengsels en ’n blomblaar ‘cDNA’-templaat is gebruik om vier teikenvolgordes te isoleer. Hiervolgens is konsensus ‘cDNA’-fragmente van 586 bp, 326 bp, 510 bp en 225 bp onderskeidelik geïdentifiseer vir *Kalkoon-sintase*, *Kalkoon-isomerase*, *Flavanoon 3-hidroksilase* en *Dihidroflavonol 4-reduktase*. Besigtiging van die afgeleide aminosuurvolgordes vir Kalkoon-sintase, Flavanoon 3-hidroksilase en Dihidroflavonol 4-reduktase het ook tot die uitwysing van gekonserveerde residue gelei wat die funksionaliteit van hierdie ensieme in Clivias kon beklemtoon. Nukleotiedvolgorde-analises tussen elke *Clivia* ‘cDNA’-fragment en die soortgelyke ‘cDNA’-fragmente van ander plante ten opsigte van eendersheid/identiteit en filogenie is ook uitgeoefen en kon so die verwantskap aan ander monokotiele op grond van homologie en evolusionêre groepering vasstel.

Nadat die *Clivia* flavonoïedgene geïdentifiseer is, kon die volgende stap van hierdie studie onderneem word, nl. die uitdrukkingsanalise van *Kalkoon-sintase* en *Dihidroflavonol 4-reduktase*. Hierdie strukturele gene speel 'n belangrike rol in die voorkoms van die finale eindproduk(te), aangesien hulle kodeer vir ensieme wat betrokke is by belangrike kontroleringstappe in die biosintese-weg. 'Real-time quantitative RT-PCR' met 'SYBR® Green' chemie is ingespan om die uitdrukking van die twee gene oor 'n periode van vyf blomontwikkelingstadiums te verken. Blomblaar-, stuifmeeldraad-, en vrugbeginselweefsel van 'n oranje variëteit en 'n geel variëteit van *Clivia miniata* was tydens die ondersoek gebruik. Die verloop van uitdrukking van *Kalkoon-sintase* ('n vroeë biosintese-ensiem) en *Dihidroflavonol 4-reduktase* ('n laat biosintese-ensiem) tydens blomontwikkeling was meestal soortgelyk in 'n spesifieke weefseltipe, maar het verskil tussen die verskillende weefseltipes. Hierdie bevindings het die gelyke regulering van *Clivia miniata* antosianiengene tentatief as 'n enkele eenheid voorgestel. Hierdie transkripsionele reguleringsmodel word dikwels by monokotiele gevind (Dooner *et al*., 1991; Meldgaard, 1992; Martin and Gerats, 1993). Die gene wat die betrokke transkripsiefaktore enkodeer, moet eers geïsoleer word en hul uitdrukkingspatrone verken word, voordat die onderliggende reguleringsmeganismes, ten opsigte van blomkeur, verstaan kan word.

Die ewewydigheid tussen antosianiengeenuitdrukking en antosianienproduksie tydens geel en oranje blomblaarontwikkeling van *Clivia miniata* is ook ondersoek. Na ekstraksie is totale antosianiene in elke blomontwikkelingstadium deur middel van UV-visuele spektrofotometrie by A_{530nm} gekwantifiseer. Tydens die volleblomstadium het die oranje blomme, in vergelyking met die geel blomme, ongeveer 16 maal meer antosianiene bevat. Dit kon sodoende oranjekleurontwikkeling ondersteun. Ewewydigheid van die uitdrukking van *Kalkoon-sintase* én *Dihidroflavonol 4-reduktase*, aan antosianienproduksie is d.m.v. statistiese analises bevestig. Daar is ook ander metodes soos HPLC beskikbaar wat aanbeveel word vir meer akkurate kwalitatiewe en kwantitatiewe bepaling van die total monomeriese antosianiene.

Sleutelwoorde: Antosianiene, blomme, cDNA, *Clivia*, flavonoïedgene, homologie, Nukleotiedvolgorde-analises, Polimerase-kettingreaksies, 'real-time quantitative RT-PCR', spektrofotometrie.

Chapter 8

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APPENDICES

Appendix A: MOTIF algorithmic output document in which potentially conserved amino acid blocks were identified within aligned CHS amino acid sequences. GenBank accession numbers: *Hordeum vulgare* (CAA41250), *Lilium hybrid* division I chsA (BAB40787), *Lilium hybrid* division I chsB (BAB40786), *Lilium hybrid* cultivar (ABF82595), *Lilium speciosum* (BAE79201), *Oryza sativa* (BAA19186), *Triticum aestivum* (ACJ22498), *Zea mays* C2 (X60204), *Zea mays* Whp (P24824), *Secale cereal* (P53415), *Thinopyrum ponticum* (AAQ19319).

Block Maker Results

- Introduction
- Hints on saving these results for future use
- BLOCKS from MOTIF
- BLOCKS from GIBBS
- BLOCK Maps [About Block Maps]

BLOCKS from MOTIF

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**BLOCKS from MOTIF**
>CHSprimer family
11 sequences are included in 10 blocks

     CHSprimerA, width = 53
      (M98871)       6 TVEEVRNAQRAEGPATVLAIGTATPANCVYQADYPDYYFKITKSDHMADLKEK
      (P24824)       7 TVDEVRKGQRATGPATVLAIGTATPANCVYQADYPDYYFRITKSDHLTDLKEK
      (X60204)       7 TVEEVRKAQRATGPATVLAIGTATPANCVYQADYPDYYFRITKSEHLTDLKEK
      (P53415)       6 TVEEVRKAQRAEGPATVLAIGTATPANCVYQADYPDYYFKITKSDHMADLKEK
    (ABF82595)       5 TVEEVRQAQRAEGPATVLAIGTATPSNVIYQADYPDYYFRITKSEHLTSLKEK
    (BAB40787)       5 TVDEVRQAQRAQGPATVLAIGTATPSNVIYQADYPDYYFRITKSEHLTGLKEK
    (BAB40786)       4 TVEEVRKAQRAQGPATILAIGTATPSNVIYQADYPDYYFRITNSEHLTDLKQK
    (BAE79201)       5 TVEEVRQAQRAEGPATVLVIGTATPSNVIYQADYPDYYFRITKSEHLTSLKEK
    (BAA19186)       6 TVEEVRRAQRAEGPATVLAIGTATPANCVYQADYPDYYFRITKSEHMVELKEK
    (AAQ19319)       6 TVEEVRKAQRAEGPATVLAIGTATPANCVYQADYPDYYFKITKSDHMADLKEK
    (ACJ22498)       6 TVEEVRKAQRAEGPATVLAIGTATPANCVYQADYPDYYFKITKSDHMADLKEK

     CHSprimerB, width = 51
      (M98871)  (0)     59 FKRMCDKSQIRKRYMHLTEEILEENPNMCAYMAPSLDARQDIVVVEVPKLG
      (P24824)  (0)     60 FKRMCDKSMIRKRYMHLTEEFLSENPSMCAYMAPSLDARQDVVVTEVPKLG
      (X60204)  (0)     60 FKRMCDKSMIRKRYMHLTEEFLAENPSMCAYMAPSLDARQDVVVVEVPKLG
      (P53415)  (0)     59 FKRMCDKSQIRKRYMHLTEEILQDNPNMCAYMAPSLDARQDIVVVEVPKLG
    (ABF82595)  (0)     58 FKRMCEKSMIRKRYMHLNEEILTENPNVCAYMAPSLDARQDMVVVEVPKLG
    (BAB40787)  (0)     58 FKRMCEKSMIRKRYMHLNEEILAENHNVCAYMAPSLDVRQDMVVVEVPKLG
    (BAB40786)  (0)     57 FKRMCKKSMIKKRYIHLNEEILQENRNMCAYMAPSLDARQDIVVVEVPKLG
    (BAE79201)  (0)     58 FKRMCEKSMIRKRYMHLNEEILTENPNVCAYMAPSLDVRQDMVVVEVPKLG
    (BAA19186)  (0)     59 FKRMCDKSQIRKRYMHLTEEILQENPNMCAYMAPSLDARQDIVVVEVPKLG
    (AAQ19319)  (0)     59 FKRMCDKSQIRKRYMHLTEEILQDNPNMCAYMAPSLDARQDIVVVEVPKLG
    (ACJ22498)  (0)     59 FKRMCDKSQIRKRYMHLTEEILQDNPNMCAYMAPSLDARQDIVVVEVPKLG
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  CHSprimerC, width = 37
   (M98871)  (0)   110 KAAAQKAIKEWGQPRSKITHLVFCTTSGVDMPGADYQ
   (P24824)  (0)   111 KAAAQEAIKEWGQPKSRITHLVFCTTSGVDMPGADYQ
   (X60204)  (0)   111 KAAAQKAIKEWGQPKSRITHLVFCTTSGVDMPGADYQ
   (P53415)  (0)   110 KAAAQKAIKEWGQPRSKITHLVFCTTSGVDMPGADYQ
(ABF82595)  (0)   109 KEAAAKAIKEWGQPKSKITHLIFCTTSGVDMPGADYQ
(BAB40787)  (0)   109 KEAAAKAIKEWGQPKSKITHLIFCTTSGVDMPGADYQ
(BAB40786)  (0)   108 KEAASKAIKEWGQPKSKITHLIFCTTSGVDMPGADYQ
(BAE79201)  (0)   109 KEAAAKAIKEWGQPKSKITHLIFCTTSGVDMPGADYQ
(BAA19186)  (0)   110 KAAAQKAIKEWGQPRSRITHLVFCTTSGVDMPGADYQ
(AAQ19319)  (0)   110 KAAAQKAIKEWGQPRSKITHLVFCTTSGVDMPGADYQ
(ACJ22498)  (0)   110 KAAAQKAIKEWGQPRSKITHLVFCTTSGVDMPGADYQ

  CHSprimerD, width = 37
   (M98871)  (0)   147 LTKMLGLRPSVKRLMMYQQGCFAGGTVLRLAKDLAEN
   (P24824)  (0)   148 LTKALGLRVVNRLMMYQQGCFAGGTVLRVAKDVAENN
   (X60204)  (0)   148 LTKALGLRPSVNRLMMYQQGCFAGGTVLRVAKDLAEN
   (P53415)  (0)   147 LTKMLGLRPSVKRLMMYQQGCFAGGTVLRLAKDLAEN
(ABF82595)  (0)   146 LTKLLGLRPCVNRFMMYQQGCFAGGTVLRLAKDLAEN
(BAB40787)  (0)   146 LTKLLGLRPSVNRFMMYQQGCFAGGTVLRLAKDLAEN
(BAB40786)  (0)   145 LTKLLGLRPSVNRFMMYQQGCFAGGTVLRFAKDLAEN
(BAE79201)  (0)   146 LTKLLGLRPSVNRFMMYQQGCFAGGTVLRLAKDLAEN
(BAA19186)  (0)   147 LAKMLGLRPNVSRLMMYQQGCFAGGTVLRVAKDLAEN
(AAQ19319)  (0)   147 LTKMLGLRPSVKRLMMYQQGCFAGGTVLRLAEDLAEN
(ACJ22498)  (0)   147 LTKMLGLRPSVKRLMMYQQGCFAGGTVLRLAKDLAEN

  CHSprimerE, width = 39
   (M98871)  (1)   185 RGARVLVVCSEITAVTFRGPHESHLDSLVGQALFGDGAA
   (P24824)  (0)   185 RGARVMVVCSEITAVTFRGPSESHVDSLVGQALFGDGAA
   (X60204)  (1)   186 RGARVLVVCSEITAVTFRGPSESHLDSLVGQALFGDGAA
   (P53415)  (1)   185 RGARVLVVCSEITAVTFRGPHESHLDSLVGQALFGDGAA
(ABF82595)  (1)   184 RGARVLVVCSEITAVTFRGPSESHLDSLVGQALFGDGAA
(BAB40787)  (1)   184 RGARVLVVCSEITAVTFRGPSESHLDSLVGQALFGDGAA
(BAB40786)  (1)   183 CDARVLVVCSEITAVTFRGPSESHLDSLVGQALFGDGAA
(BAE79201)  (1)   184 RGARVLVVCSEITAVTFRGPSESHLDSLVGQALFGDGAA
(BAA19186)  (1)   185 RGARVLAVCSEITAVTFRGPSESHLDSMVGQALFGDGAA
(AAQ19319)  (1)   185 RGARVLVVCSEITAVTFRGPHESHLDSLVGQALFGDGAA
(ACJ22498)  (1)   185 RGARVLVVCSEITAVTFRGPHESHLDSLVGQALFGDGAA

  CHSprimerF, width = 37
   (M98871)  (1)   225 VIIGADPDLSVERPLFQLVSASQTILPDSEGAIDGHL
   (P24824)  (1)   225 GRGGADPDGRVERPLFQLVSAAQTILPDSEGAIDGHL
   (X60204)  (1)   226 VVVGADPDDRVERPLFQLVSAAQTILPDSEGAIDGHL
   (P53415)  (1)   225 VIIGADPDESIERPLFQLVSASQTILPDSEGAIDGHL
(ABF82595)  (1)   224 VIVGSDPDNAVERPLFELVSASQTILPDSEGAIDGHL
(BAB40787)  (1)   224 VIVGSDPDTAVERPLFELVSASQTILPDSEGAIDGHL
(BAB40786)  (1)   223 VIVGSDPDTSVERPLFQIVSASQTILPDSDGAIDGHL
(BAE79201)  (1)   224 VIVGSDPDTAVERPLFELVSASQTILPDSEGAIDGHL
(BAA19186)  (1)   225 VIVGSDPDEAVERPLFQMVSASQTILPDSEGAIDGHL
(AAQ19319)  (1)   225 VIIGADPDESIERPLFQLVSASQTILPDSEGAIDGHL
(ACJ22498)  (1)   225 VIIGADPDESIERPLFQLVSASQTILPDSEGAIDGHL
```

```
  CHSprimerG, width = 29
  (M98871)  (0)   262 REVGLTFHLLKDVPGLISKNIERALEEAF
  (P24824)  (0)   262 REVGLAFHLLKDVPGLISKNIERALEDAF
  (X60204)  (0)   263 REVGLTFHLLKDVPGLISKNIGRALDDAF
  (P53415)  (0)   262 REVGLTFHLLKDVPGLISKNIERALEDAF
(ABF82595)  (0)   261 REVGLTFHLLKDVPGLISKNIERSLTGAF
(BAB40787)  (0)   261 REVGLTFHLLKDVPGLISKNIEKSLTGAF
(BAB40786)  (0)   260 REVGLTFHLLKDVPGLISKNIEKSLTQAF
(BAE79201)  (0)   261 REVGLTFHLLKDVPGLISKNIERSLTGAF
(BAA19186)  (0)   262 REVGLTFHLLKDVPGLISKNIERALGDAF
(AAQ19319)  (0)   262 REVGLTFHLLKDVPGLISKNIERALEDAF
(ACJ22498)  (0)   262 REVGLTFHLLKDVPGLISKNIERALEDAF

  CHSprimerH, width = 31
  (M98871)  (0)   291 KPLGIDHWNSVFWIAHQGGPAILDMVEAKVN
  (P24824)  (0)   291 EPLGISDWNSIFWVAHPGGPAILDQVEAKVG
  (X60204)  (0)   292 KPLGISDWNSIFWVAHPGGPAILDQVEAKVG
  (P53415)  (0)   291 KPLGIDDWNSVFWIAHPGGPAILDMVEAKVN
(ABF82595)  (0)   290 APLGISDWNSLFWIAHPGGPAILDQVEAKLG
(BAB40787)  (0)   290 APLGISDWNSLFWIAHPGGPAILDQVAAKLG
(BAB40786   (0)   289 APLGITDWNSIFWIAHPGGPAILDQVELKLA
(BAE79201)  (0)   290 APLGISDWNSLFWIAHPGGPAILDQVEAKLG
(BAA19186)  (0)   291 TPLGISDWNSIFWVAHPGGPAILDQVEAKVG
(AAQ19319)  (0)   291 KPLGIDDWNSVFWIAHPGGPAILDMVEAKVN
(ACJ22498)  (0)   291 KPLGIDDWNSVFWIAHPGGPAILDMVEAKVN

  CHSprimerI, width = 38
  (M98871)  (0)   322 LNKERMRATRHVLSEYGNMSSACVLFIMDEMRKRSAED
  (P24824)  (0)   322 LDKARMRATRHVLSEYGNMSSACVLFILDEMRKRPAED
  (X60204)  (0)   323 LDKARMRATRHVLSEYGNMSSACVLFILDEMRKRSAED
  (P53415)  (0)   322 LNKERMRATRHVLSEYGNMSSACVLFIMDEMRKRSAED
(ABF82595)  (0)   321 LQKEKMRATRHVLSEYGNMSSACVLFILDEMRKTSAKM
(BAB40787)  (0)   321 LQKEKMRATRHVLSEYGNMSSACVLFILDEMRKTSAKM
(BAB40786)  (0)   320 LDKKKMQATRHVLSEYGNMSSACVLFILDEMRKASAEQ
(BAE79201)  (0)   321 LQKEKMRATRHVLSEYGNMSSACVLFILDEMRKTSAKM
(BAA19186)  (0)   322 LDKERMRATRHVLSEYGNMSSACVLFILDEMRKRSAED
(AAQ19319)  (0)   322 LNKERMRATRHVLSEYGNMSSACVLFIMDEMRKRSAED
(ACJ22498)  (0)   322 LNKERMRATRHVLSEYGNMSSACVLFIMDEMRKRSAED

  CHSprimerJ, width = 32
  (M98871)  (0)   360 GHATTGEGMDWGVLFGFGPGLTVETVVLHSVP
  (P24824)  (0)   360 GQSTTGEGLDWGVLFGFGPGLTVETVVLHSVP
  (X60204)  (0)   361 GQATTGEGLDWGVLFGFGPGLTVETVVLHSVP
  (P53415)  (0)   360 GHTTTGEGMDWGVLFGFGPGLTVETVVLHSVP
(ABF82595)  (0)   359 GKATTGEGLDWGVLFGFGPGLTVETVVLHSLP
(BAB40787)  (0)   359 GKATTGEGLDWGVLFGFGPGLTVETVVLHSLP
(BAB40786)  (0)   358 GKATTGEGLDWGVLFGFGPGLTVETVVLHSIP
(BAE79201)  (0)   359 GKATTGEGLDWGVLFGFGPGLTVETVVLHSLP
(BAA19186)  (0)   360 GHATTGEGMDWGVLFGFGPGLTVETVVLHSVP
(AAQ19319)  (0)   360 GHSTTGEGMDWGVLFGFGPGLTVETVVLHSVP
(ACJ22498)  (0)   360 GHSTTGEGMDWGVLFGFGPGLTVETVVLHSVP
```

Appendix B: CODEHOP primer map generated from conserved blocks within aligned CHS amino acid sequences. The *CHS* primers that were chosen are marked with arrows and are shown in bold.

CODEHOP Results

Oligo Summary   Not all overlapping primers are shown

```
CODEHOP Version 10/14/04.1
COPYRIGHT 1997-2004, Fred Hutchinson Cancer Research Center, Seattle, WA, USA

Parameters:
 Amino acids PSSM calculated with odds ratios normalized to 100
 and back-translated with Standard genetic code
 and codon usage table "../docs/default.codon.use"
 Maximum core degeneracy 128    Core strictness 0.10
 Clamp strictness 1.00   Target clamp temperature 60.00 C
 DNA Concentration 50.00 nM   Salt Concentration 50.00 mM
 Codon boundary 1   Most common codon 0
 Verbose 0   Output 3
 Begin 1   PolyX 5
Suggested CODEHOPS: The degenerate region (core) is printed in lower case,
the non-degenerate region (clamp) is printed in upper case.
```

Block CHSprimerA Oligos

```
  T V E E V R K A Q R A E G P A T V L A I G T A T P A N C V Y Q A D Y P D Y Y F R I T K S E H M T D L K E K

TCCGAGCACATGACCGACytnaarsaraa -3'  Core: degen=64 len=11  Clamp: score=73, len=18 temp= 61.9
AGGCCGACTACCCCgaytaytaytt -3'  Core: degen=8 len=11  Clamp: score=80, len=14 temp= 60.2
TCTACCAGGCCGACTACccngaytayta -3'  Core: degen=16 len=11  Clamp: score=83, len=17 temp= 60.1
GCGTCTACCAGGCCGACtayccngayta -3'  Core: degen=16 len=11  Clamp: score=81, len=17 temp= 61.4
TGCGTCTACCAGGCCgaytayccnga -3'  Core: degen=16 len=11  Clamp: score=80, len=15 temp= 62.6
CCAACTGCGTCTACCAGgcngaytaycc -3'  Core: degen=16 len=11  Clamp: score=80, len=17 temp= 61.8
CGCCAACTGCGTCTACcargcngayta -3'  Core: degen=16 len=11  Clamp: score=74, len=16 temp= 62.0
CCCGCCAACTGCGTCtaycargcnga -3'  Core: degen=16 len=11  Clamp: score=69, len=15 temp= 62.0
GCTGGCCATCGGCacngcnacncc -3'  Core: degen=64 len=11  Clamp: score=76, len=13 temp= 61.0
CGTGCTGGCCATCggnacngcnac -3'  Core: degen=64 len=11  Clamp: score=76, len=13 temp= 62.2
CCACCGTGCTGGCCathggnacngc -3'  Core: degen=48 len=11  Clamp: score=74, len=14 temp= 62.9
GGGCCGAGGGCccngcnacnrt -3'  Core: degen=128 len=11  Clamp: score=71, len=11 temp= 62.3
AGCGGGCCGAGggnccngcnac -3'  Core: degen=64 len=11  Clamp: score=69, len=11 temp= 60.4
GGAGGAGGTGCGGAAGgsncarmgngc -3'  Core: degen=128 len=11  Clamp: score=73, len=16 temp= 61.8
ACCGTGgangargtnmg -3'  Core: degen=64 len=11  Clamp: score=76, len=6 temp= 13.8 *** CLAMP NEEDS EXTENSION
ACCgtngangargt -3'  Core: degen=32 len=11  Clamp: score=68, len=3 temp=-30.7 *** CLAMP NEEDS EXTENSION
```

Complement of Block CHSprimerA Oligos

```
T  V  E  E  V  R  K  A  Q  R  A  E  G  P  A  T  V  L  A  I  G  T  A  T  P  A  N  C  V  Y  Q  A  D  Y  P  D  Y  Y  F  R  I  T  K  S  E  H  M  T  D  L  K  E  K
tgncanctnctyCACGCCTTCCGGGTCG -5'  Core: degen=128 len=12  Clamp: score=76, len=16 temp= 64.1
   canctnctycanGCCTTCCGGGTCGC -5'  Core: degen=128 len=12  Clamp: score=73, len=14 temp= 61.1
                                                                                        atrgtycgnctrATGGGGCTGATGATGAAGTCCTAGT -5'  Core: degen=32 len=12  Clamp: score=83, len=25 temp= 62.3
                                                                                           gtycgnctratrGGGCTGATGATGAAGTCCTAGTGG -5'  Core: degen=32 len=12  Clamp: score=82, len=24 temp= 62.4
                                                                                                 ctratrggnctrATGATGAAGTCCTAGTGGTTCAGGC -5'  Core: degen=32 len=12  Clamp: score=81, len=25 temp= 62.8
                                                                                                    atrggnctratrATGAAGTCCTAGTGGTTCAGGC -5'  Core: degen=32 len=12  Clamp: score=79, len=22 temp= 61.4
                                                                                                       ggnctratratrAAGTCCTAGTGGTTCAGGCT -5'  Core: degen=32 len=12  Clamp: score=77, len=20 temp= 61.6
                                                                                                          ctratratraarTCCTAGTGGTTCAGGCTCG -5'  Core: degen=16 len=12  Clamp: score=74, len=19 temp= 60.2
```

Block CHSprimerB Oligos

```
F  K  R  M  C  D  K  S  M  I  R  K  R  Y  M  H  L  T  E  E  I  L  Q  E  N  P  N  M  C  A  Y  M  A  P  S  L  D  A  R  Q  D  M  V  V  V  E  V  P  K  L  G
                                                                                                                                TGGTGGTGGAGGTGccnaarytngg -3'  Core: degen=64 len=11  Clamp: score=79, len=14 temp= 60.0
                                                                                                                           CATGGTGGTGGTGGAGgtnccnaaryt -3'  Core: degen=64 len=11  Clamp: score=79, len=16 temp= 60.2
                                                                                                                        GGACATGGTGGTGGTGgargtnccnaa -3'  Core: degen=32 len=11  Clamp: score=77, len=16 temp= 61.3
                                                                                                             GACGCCCGGCAGgayrtngtngt -3'  Core: degen=64 len=11  Clamp: score=77, len=12 temp= 61.6
                                                                                                           TGGACGCCCGGcargayrtngt -3'  Core: degen=32 len=11  Clamp: score=70, len=11 temp= 61.7
                                                                                                    CCCTCCCTGGACGCCmgncargayrt -3'  Core: degen=64 len=11  Clamp: score=73, len=15 temp= 61.2
                                                                                  CCCAACATGTGCGCCtayatggcncc -3'  Core: degen=8 len=11  Clamp: score=81, len=15 temp= 60.2
                                                                               ACCCCAACATGTGCgcntayatggc -3'  Core: degen=8 len=11  Clamp: score=82, len=14 temp= 60.1
                                                                     CCTGCAGGAGAACCCCaayrtgtgygc -3'  Core: degen=8 len=11  Clamp: score=72, len=16 temp= 62.2
                       CATGATCCGGAAGCGGtayatgcayyt -3'  Core: degen=8 len=11  Clamp: score=73, len=16 temp= 60.6
               GACAAGTCCATGATCCGGAAGmgntayatgca -3'  Core: degen=16 len=11  Clamp: score=74, len=21 temp= 61.8
               GACAAGTCCATGATCCGGaarmgntayat -3'  Core: degen=32 len=11  Clamp: score=71, len=18 temp= 60.9
TTCAAGCGGatgtgyranaa -3'  Core: degen=16 len=11  Clamp: score=73, len=9 temp= 46.6 *** CLAMP NEEDS EXTENSION
TTCAAGmgnatgtgyra -3'  Core: degen=32 len=11  Clamp: score=81, len=6 temp=-31.7 *** CLAMP NEEDS EXTENSION
TTCaarmgnatgtg -3'  Core: degen=16 len=11  Clamp: score=77, len=3 temp=-50.8 *** CLAMP NEEDS EXTENSION
ttyaarmgnatg -3'  Core: degen=32 len=12  Clamp: score=0, len=0 temp=-166.5 *** CLAMP NEEDS EXTENSION
```

Complement of Block CHSprimerB Oligos

```
F  K  R  M  C  D  K  S  M  I  R  K  R  Y  M  H  L  T  E  E  I  L  Q  E  N  P  N  M  C  A  Y  M  A  P  S  L  D  A  R  Q  D  M  V  V  V  E  V  P  K  L  G

aarttykcntacACGCTGTTCAGGTACTAG -5'  Core: degen=32 len=12  Clamp: score=79, len=18 temp= 61.0
   ttykcntacacrCTGTTCAGGTACTAGGCCTT -5'  Core: degen=32 len=12  Clamp: score=75, len=20 temp= 60.9
         tacacrytnttyAGGTACTAGGCCTTCGC -5'  Core: degen=32 len=12  Clamp: score=71, len=17 temp= 61.8
                                 ttykcnatrtacGTGGACTGGCTCCTCT -5'  Core: degen=32 len=12  Clamp: score=81, len=16 temp= 61.8
                                       atrtacgtrranTGGCTCCTCTAGGACGTCC -5'  Core: degen=32 len=12  Clamp: score=78, len=19 temp= 62.7
                                          tacgtrrantknCTCCTCTAGGACGTCCTCTTG -5'  Core: degen=128 len=12  Clamp: score=80, len=21 temp= 60.4
                                                                ttrgbnttryacACGCGGATGTACCGG -5'  Core: degen=96 len=12  Clamp: score=87, len=15 temp= 62.9
                                                                            acrcgnatrtacCGGGGGAGGGAC -5'  Core: degen=16 len=12  Clamp: score=77, len=12 temp= 60.4
                                                                               cgnatrtaccgnGGGAGGGACCTGCGG -5'  Core: degen=32 len=12  Clamp: score=74, len=15 temp= 61.2
                                                                                  atrtaccgnggnAGGGACCTGCGGGC -5'  Core: degen=32 len=12  Clamp: score=72, len=14 temp= 61.5
                                                                                                       gtyctryancanCACCACCTCCACGGGT -5'  Core: degen=128 len=12  Clamp: score=82, len=16 temp= 60.0
                                                                                                                   ctycanggnttyGACCCG -5'  Core: degen=64 len=12  Clamp: score=82, len=6 temp=-3.9
```

Block CHSprimerC Oligos

CHS-f

```
K  A  A  A  Q  K  A  I  K  E  W  G  Q  P  K  S  K  I  T  H  L  V  F  C  T  T  S  G  V  D  M  P  G  A  D  Y  Q
                                                                                      GACATGCCGGGCgcngaytayca -3'  Core: degen=16 len=11  Clamp: score=80, len=12 temp= 62.1
                                                                               CGGCGTGGACatgccnggngc -3'  Core: degen=16 len=11  Clamp: score=79, len=10 temp= 62.0
                                                                         CACCTCCGGCGTGgayatgccngg -3'  Core: degen=8 len=11  Clamp: score=73, len=13 temp= 61.5
                                                                      CACCACCTCCGGCgtngayatgcc -3'  Core: degen=8 len=11  Clamp: score=69, len=13 temp= 60.2
                                               CAAGATCACCCACCTGGTCttytgyacnac -3'  Core: degen=16 len=11  Clamp: score=74, len=19 temp= 61.3
                                          AAGTCCAAGATCACCCACCTGrtnttytgyac -3'  Core: degen=32 len=11  Clamp: score=76, len=21 temp= 61.3
                                  GCCAGCCCAAGTCCAAGathacncayyt -3'  Core: degen=48 len=11  Clamp: score=65, len=17 temp= 62.5
                 GGCCATCAAGGAGtggggncarcc -3'  Core: degen=8 len=11  Clamp: score=82, len=13 temp= 63.8
          CCCAGAAGGCCATCAAGgartggggnca -3'  Core: degen=8 len=11  Clamp: score=80, len=17 temp= 63.5
         GCCCAGAAGGCCATCaargartgggg -3'  Core: degen=4 len=11  Clamp: score=78, len=15 temp= 60.7
```

Complement of Block CHSprimerC Oligos

```
K  A  A  A  Q  K  A  I  K  E  W  G  Q  P  K  S  K  I  T  H  L  V  F  C  T  T  S  G  V  D  M  P  G  A  D  Y  Q
              tadttyctyaccCCGGTCGGGT -5'  Core: degen=12 len=12  Clamp: score=85, len=10 temp= 60.2
                ttyctyaccccnGTCGGGTTCAGGTTCTAGTG -5'  Core: degen=16 len=12  Clamp: score=75, len=20 temp= 60.7
                  ctyaccccngtyGGGTTCAGGTTCTAGTGGG -5'  Core: degen=16 len=12  Clamp: score=70, len=19 temp= 60.3
                                            aaracrtgntgnAGGCCGCACCTGTACG -5'  Core: degen=64 len=12  Clamp: score=75, len=16 temp= 64.2
                                                      ccncanctrtacGGGCCGCGGC -5'  Core: degen=32 len=12  Clamp: score=82, len=10 temp= 65.5
                                                        canctrtacggnCCGCGGCTGATGGTC -5'  Core: degen=32 len=12  Clamp: score=83, len=15 temp= 57.1 *** CLAMP NEEDS EXTENSION
                                                          ctrtacggnccnCGGCTGATGGTC -5'  Core: degen=32 len=12  Clamp: score=86, len=12 temp= 37.2 *** CLAMP NEEDS EXTENSION
                                                                  cgnctratrgty -5'  Core: degen=32 len=12  Clamp: score=0, len=0 temp=-294.7 *** CLAMP NEEDS EXTENSION
```

Block CHSprimerD Oligos

```
L  T  K  M  L  G  L  R  P  S  V  N  R  M  M  M  Y  Q  Q  C  C  F  A  G  G  T  V  L  R  L  A  K  D  L  A  E  N
                           TGCGTCAACCGGATGatgwwbyanca -3'  Core: degen=96 len=11  Clamp: score=62, len=15 temp= 62.8
```

Complement of Block CHSprimerD

```
L  T  K  M  L  G  L  R  P  S  V  N  R  M  M  M  Y  Q  Q  C  C  F  A  G  G  T  V  L  R  L  A  K  D  L  A  E  N
```

No suggested primers found.

Block CHSprimerE Oligos

```
R  G  A  R  V  L  V  V  C  S  E  I  T  A  V  T  F  R  G  P  H  E  S  H  L  D  S  L  V  G  Q  A  L  F  G  D  G  A  A
                                                             GGCCCTGTTCGGCgayggngcngc -3'  Core: degen=32 len=11  Clamp: score=78, len=13 temp= 63.1
                                                          GCCAGGCCCTGTTCggngayggngc -3'  Core: degen=32 len=11  Clamp: score=80, len=14 temp= 63.9
                                                         GGGCCAGGCCCTGttyggngaygg -3'  Core: degen=16 len=11  Clamp: score=79, len=13 temp= 60.6
                                               CCACCTGGACTCCCTGgtnggncargc -3'  Core: degen=32 len=11  Clamp: score=71, len=16 temp= 61.0
                      ATCACCGCCGTGACCttymgnggncc -3'  Core: degen=64 len=11  Clamp: score=78, len=15 temp= 60.1
                    CGAGATCACCGCCGTGacnttymgngg -3'  Core: degen=64 len=11  Clamp: score=79, len=16 temp= 64.0
                   CCGAGATCACCGCCgtnacnttymg -3'  Core: degen=64 len=11  Clamp: score=78, len=14 temp= 61.9
                TGCTCCGAGATCACCgcngtnacntt -3'  Core: degen=64 len=11  Clamp: score=77, len=15 temp= 62.2
             TGGTGTGCTCCGAGATCacngcngtnac -3'  Core: degen=64 len=11  Clamp: score=77, len=17 temp= 60.9
          TGGTGGTGTGCTCCGAGathacngcngt -3'  Core: degen=48 len=11  Clamp: score=75, len=17 temp= 62.8
         CTGGTGGTGTGCTCCgarathacngc -3'  Core: degen=24 len=11  Clamp: score=72, len=15 temp= 60.4
     CGGGTGCTGGTGGTGtgywsngarat -3'  Core: degen=64 len=11  Clamp: score=77, len=15 temp= 63.5
```

Complement of Block CHSprimerE Oligos

```
R  G  A  R  V  L  V  V  C  S  E  I  T  A  V  T  F  R  G  P  H  E  S  H  L  D  S  L  V  G  Q  A  L  F  G  D  G  A  A

  ctytadtgncgnCACTGGAAGGCCCCGG -5'  Core: degen=96 len=12  Clamp: score=81, len=16 temp= 62.5
                          cgncantgnaarGCCCCGGGGGTGC -5'  Core: degen=128 len=12  Clamp: score=73, len=13 temp= 64.6
                                                 canccngtycgnGACAAGCCGCTGCCG -5'  Core: degen=128 len=12  Clamp: score=83, len=15 temp= 61.2
                                                    gtycgnranaarCCGCTGCCGCGG -5'  Core: degen=128 len=12  Clamp: score=83, len=12 temp= 60.6
                                                         aarccnctrccnCGGCGG -5'  Core: degen=64 len=12  Clamp: score=86, len=6 temp= 14.6 *** CLAMP NEEDS EXTENSION
                                                           ccnctrccncgnCGG -5'  Core: degen=128 len=12  Clamp: score=90, len=3 temp=-74.3 *** CLAMP NEEDS EXTENSION
                                                             ctrccncgncgn -5'  Core: degen=128 len=12  Clamp: score=0, len=0 temp=-294.7 *** CLAMP NEEDS EXTENSION
```

Block CHSprimerF Oligos

```
V  I  V  G  A  D  P  D  E  S  V  E  R  P  L  F  Q  L  V  S  A  S  Q  T  I  L  P  D  S  E  G  A  I  D  G  H  L
                                                                                        CGAGGGCGCCATCgayggncayyt -3'  Core: degen=32 len=11  Clamp: score=78, len=13 temp= 64.4
                                                                                      TCCGAGGGCGCCathgayggnca -3'  Core: degen=24 len=11  Clamp: score=76, len=12 temp= 60.9
                                                                                  CGACTCCGAGGGCgcnathgaygg -3'  Core: degen=24 len=11  Clamp: score=74, len=13 temp= 62.9
                                GGAGCGGCCCCTGttysarhtngt -3'  Core: degen=96 len=11  Clamp: score=74, len=13 temp= 60.8
                           GCCGTGGAGCGGccnytnttysa -3'  Core: degen=128 len=11  Clamp: score=70, len=12 temp= 63.9
                     CGACGAGGCCGTGgarmgnccnyt -3'  Core: degen=128 len=11  Clamp: score=56, len=13 temp= 61.7
                GACCCCGACGAGGCCrtngarmgncc -3'  Core: degen=128 len=11  Clamp: score=56, len=15 temp= 61.2
```

Complement of Block CHSprimerF Oligos

```
V  I  V  G  A  D  P  D  E  S  V  E  R  P  L  F  Q  L  V  S  A  S  Q  T  I  L  P  D  S  E  G  A  I  D  G  H  L
                                                                                            ccncgntadctrCCGGTGGAC -5'  Core: degen=96 len=12  Clamp: score=84, len=9 temp= 27.3 *** CLAMP NEEDS EXTENSION
                                                                                               cgntadctrccnGTGGAC -5'  Core: degen=96 len=12  Clamp: score=87, len=6 temp=-26.3 *** CLAMP NEEDS EXTENSION
                                                                                                  tadctrccngtrGAC -5'  Core: degen=48 len=12  Clamp: score=82, len=3 temp=-126.1 *** CLAMP NEEDS EXTENSION
```

Block CHSprimerG Oligos

```
R  E  V  G  L  T  F  H  L  L  K  D  V  P  G  L  I  S  K  N  I  E  R  A  L  E  D  A  F
                CCTTCCACCTGCTGAAGgaygtnccngg -3'  Core: degen=32 len=11  Clamp: score=80, len=17 temp= 61.3
             TGACCTTCCACCTGCTGaargaygtncc -3'  Core: degen=16 len=11  Clamp: score=79, len=17 temp= 60.4
         GGCCTGACCTTCCACCTGytnaargaygt -3'  Core: degen=32 len=11  Clamp: score=79, len=18 temp= 61.7
CGGGAGGTGGGCCTGacnttycayyt -3'  Core: degen=32 len=11  Clamp: score=77, len=15 temp= 65.2
```

Complement of Block CHSprimerG Oligos

```
R  E  V  G  L  T  F  H  L  L  K  D  V  P  G  L  I  S  K  N  I  E  R  A  L  E  D  A  F
                              ttyctrcanggnCCGGACTAGAGGTTCTTGTAG -5'  Core: degen=64 len=12  Clamp: score=78, len=21 temp= 61.0
                                 ctrcanggnccnGACTAGAGGTTCTTGTAGCTCTC -5'  Core: degen=128 len=12  Clamp: score=78, len=23 temp= 60.4
                                                    ttyttrtadcyyTCCCGGGACCTCCTGC -5'  Core: degen=48 len=12  Clamp: score=64, len=16 temp= 62.3
```

Block CHSprimerH Oligos

```
K  P  L  G  I  S  D  W  N  S  I  F  W  I  A  H  P  G  G  P  A  I  L  D  Q  V  E  A  K  V  N
                 CGACTGGAACTCCATCTTCtggrtngcnca -3'  Core: degen=32 len=11  Clamp: score=73, len=19 temp= 63.3
              CGACGACTGGAACTCCATCttytggrtngc -3'  Core: degen=16 len=11  Clamp: score=70, len=19 temp= 61.6
          GCATCGACGACTGGAACTCCvtnttytggrt -3'  Core: degen=48 len=11  Clamp: score=73, len=20 temp= 63.2
AAGccnytnggnat -3'  Core: degen=128 len=11  Clamp: score=48, len=3 temp=-25.9 *** CLAMP NEEDS EXTENSION
```

Complement of Block CHSprimerH Oligos

```
K  P  L  G  I  S  D  W  N  S  I  F  W  I  A  H  P  G  G  P  A  I  L  D  Q  V  E  A  K  V  N
                                 aaraccyancgnGTGGGGCCGCCG -5'  Core: degen=64 len=12  Clamp: score=83, len=12 temp= 60.2
                                    accyancgngtrGGGCCGCCGGG -5'  Core: degen=64 len=12  Clamp: score=79, len=11 temp= 60.2
                                                                  ctrkwycanctyCGGTTCCACCTG -5'  Core: degen=128 len=12  Clamp: score=77, len=12 temp= 40.4 *** CLAMP NEEDS EXTENSION
                                                                        canctycrnttyCACCTG -5'  Core: degen=128 len=12  Clamp: score=67, len=6 temp=-31.7 *** CLAMP NEEDS EXTENSION
```

Block CHSprimerI Oligos

```
L  N  K  E  R  M  R  A  T  R  H  V  L  S  E  Y  G  N  M  S  S  A  C  V  L  F  I  M  D  E  M  R  K  R  S  A  E  M
                                                                     GTGCTGTTCATCATGGACgaratgmgnaa -3'  Core: degen=16 len=11  Clamp: score=82, len=18 temp= 60.7
                                                                   GCGTGCTGTTCATCATGgaygaratgmg -3'  Core: degen=8 len=11  Clamp: score=81, len=17 temp= 61.9
                                                               GCCTGCGTGCTGTTCATChtggaygaratg -3'  Core: degen=12 len=12  Clamp: score=80, len=18 temp= 61.6
                            GGCACGTGCTGTCCGAGtayggnaayatg -3'  Core: degen=16 len=12  Clamp: score=75, len=17 temp= 63.5
                            GGCACGTGCTGTCCgartayggnaa -3'  Core: degen=16 len=11  Clamp: score=70, len=14 temp= 61.7
                         CCCGGCACGTGCTGwsngartaygg -3'  Core: degen=64 len=11  Clamp: score=77, len=14 temp= 60.6
             GGATGCGGGCCACCmgncaygtnyt -3'  Core: degen=128 len=11  Clamp: score=77, len=14 temp= 60.6
          AGAGGATGCGGGCCacnmgncaygt -3'  Core: degen=64 len=11  Clamp: score=76, len=14 temp= 60.8
      AAGGAGAGGATGCGGgcnacnmgnca -3'  Core: degen=128 len=11  Clamp: score=74, len=15 temp= 61.3
CTGAACAAGGAGAGGatgmrngcnac -3'  Core: degen=64 len=11  Clamp: score=68, len=15 temp= 51.0 *** CLAMP NEEDS EXTENSION
```

Complement of Block CHSprimerI Oligos

```
                                             <----- CHS-r -----
L  N  K  E  R  M  R  A  T  R  H  V  L  S  E  Y  G  N  M  S  S  A  C  V  L  F  I  M  D  E  M  R  K  R  S  A  E  M
                                          ctyatrccnttrTACAGGAGGCGGACGC -5'  Core: degen=32 len=12  Clamp: score=77, len=16 temp= 62.1
                                             atrccnttrtacAGGAGGCGGACGC -5'  Core: degen=16 len=12  Clamp: score=66, len=13 temp= 62.1
                                                ccnttrtacwsnAGGCGGACGCACGAC -5'  Core: degen=128 len=12  Clamp: score=74, len=15 temp= 63.2
                                                                    aartaddacctrCTCTACGCCTTCTCCAGGC -5'  Core: degen=36 len=12  Clamp: score=81, len=19 temp= 63.2
                                                                       taddacctrctyTACGCCTTCTCCAGGCG -5'  Core: degen=36 len=12  Clamp: score=79, len=17 temp= 62.4
                                                                             ctrctytackcnTTCTCCAGGCGGCTCTTC -5'  Core: degen=32 len=12  Clamp: score=69, len=18 temp= 60.5
                                                                                ctytackcnttyTCCAGGCGGCTCTTC -5'  Core: degen=32 len=12  Clamp: score=61, len=15 temp= 55.4 *** CLAMP NEEDS EXTENSION
```

Block CHSprimerJ Oligos

```
G  H  A  T  T  G  E  G  M  D  W  G  V  L  F  G  F  G  P  G  L  T  V  E  T  V  V  L  H  S  V  P
                           GACTGGGGCGTGCTGttyggnttygg -3'  Core: degen=16 len=11  Clamp: score=80, len=15 temp= 60.9
             CGGCGAGGGCATGgaytggggngt -3'  Core: degen=8 len=11  Clamp: score=81, len=13 temp= 64.3
          CCACCGGCGAGGGCmtggaytgggg -3'  Core: degen=4 len=11  Clamp: score=78, len=14 temp= 63.7
         ACCACCGGCGAGggnmtggaytgg -3'  Core: degen=16 len=12  Clamp: score=79, len=12 temp= 60.1
      GCCACCACCGGCgarggnmtgga -3'  Core: degen=16 len=11  Clamp: score=74, len=12 temp= 62.7
```

Complement of Block CHSprimerJ Oligos

```
G  H  A  T  T  G  E  G  M  D  W  G  V  L  F  G  F  G  P  G  L  T  V  E  T  V  V  L  H  S  V  P
                  ctyccnkacctrACCCCGCACGACAAGC -5'  Core: degen=32 len=12  Clamp: score=88, len=16 temp= 64.3
                     ccnkacctraccCCGCACGACAAGC -5'  Core: degen=16 len=12  Clamp: score=82, len=13 temp= 64.3
                           ctraccccncanGACAAGCCGAAGCCGG -5'  Core: degen=32 len=12  Clamp: score=82, len=16 temp= 61.5
                                          aarccnaarccnGGGCCGGACTGGCA -5'  Core: degen=64 len=12  Clamp: score=81, len=14 temp= 60.1
                                             ccnaarccnggnCCGGACTGGCACCTCTG -5'  Core: degen=128 len=12  Clamp: score=81, len=17 temp= 60.2
                                                aarccnggnccnGACTGGCACCTCTGGCAC -5'  Core: degen=128 len=12  Clamp: score=82, len=18 temp= 61.4
                                                            tgncanctytgnCACCACGACGTGAGGCAC -5'  Core: degen=128 len=12  Clamp: score=80, len=18 temp= 62.7
                                                               canctytgncanCACGACGTGAGGCACGG -5'  Core: degen=128 len=12  Clamp: score=79, len=17 temp= 62.8
                                                                  ctytgncancanGACGTGAGGCACGGG -5'  Core: degen=128 len=12  Clamp: score=76, len=15 temp= 58.2 *** CLAMP NEEDS EXTENSION
```

Appendix C1: Multiple alignment of amino acid sequences of different monocot DFR proteins. GenBank accession numbers: *Hordeum vulgare* (AAB20555), *Oryza sativa* (japonica cultivar-group) (AAN74830), *Bromheadia finlaysoniana* (AAB62873), *Agapanthus praecox* (BAE78769), *Triticum aestivum* (BAD11019), *Zea mays* (NP_001152467), *Lilium speciosum* (BAE79202), *Allium cepa* (AAO63026). The highlighted areas represent the conserved peptide motifs corresponding to the deduced primer sequences used for amplification of a *Clivia DFR* cDNA fragment. Nucleotides that are identical in all sequences are marked with an asterisk.

```
L.speciosum          -------MEN-AKGPVVVTGASGYVGSWLVMKLLQYGYTIRATVRDPRDLRKTKPLLDIP 52
B.finlaysoniana      -------MENEKKGPVVVTGASGYVGSWLVMKLLQKGYDVRATIRDPTNLEKVKPLLDLP 53
A.praecox            MSVAIARDSGEMKGPVVVTGAGGYIGSWLVMKLLQHGYTVRATLRNPSNMKKTKPLLDLP 60
A.cepa               ----MMKEIGAAGGAVVVTGAGGYVGSWLVMKLLHYGYTVRATLRDSSDEAKTKPLLELP 56
T.aestivum           --------MDGNKGPVVVTGASGFVGSWLVMKLLQVGYTVRATVRDPANVEKNKPLLELP 52
H.vulgare            --------MDGNKGPVVVTGASGFVGSWLVMKLLQAGYTVRATVRDPANVEKTKPLLELP 52
O.sativa             -------MGEAVKGPVVVTGASGFVGSWLVMKLLQAGYTVRATVRDPSNVGKTKPLLELA 53
Z.mays               ---MERGAGASEKGTVLVTGASGFVGSWLVMKLLQAGYTVRATVRDPANVGKTKPLMDLP 57
                                  *.*:****.*::*********: ** :***:*:. :  * ***:::.

L.speciosum          GADERLTIWKADLS-EDASFDEAINGCTGVYHVATPMDFDSKDPENEVIQPTINGVLGIM 111
B.finlaysoniana      RSNELLSIWKADLNDIEGSFDEVIRGCVGVFHVATPMNFQSKDPENEVIKPAINGLLGIL 113
A.praecox            GAEKRLTIWKANLN-DEGSFDEAINGSTGVFHVATPMDFDSKDPENEVIKPTIEGMLGIM 119
A.cepa               GADTRLSLWEADLL-QDGSFDHVISGSIAVFHVATPMDFDSIDPENEVIKPAVNGMLSIM 115
T.aestivum           GAMERLSIWKADLS-EEGSFDDAIAGCTGVFHVATPMDFDSKDPENEVIKPTVEGMLSIM 111
H.vulgare            GAKERLSIWKADLS-EDGSFNEAIAGCTGVFHVATPMDFDSQDPENEVIKPTVEGMLSIM 111
O.sativa             GSKERLTLWKADLG-EEGSFDAAIRGCTGVFHVATPMDFESEDPENEVVKPTVEGMLSIM 112
Z.mays               GATERLSIWKADLA-EEGSFHDAIRGCTGVFHVATPMDFLSKDPENEVIKPTVEGMISIM 116
                      :   *::*:*:*   :.**. .* *. .*:******:* * ******::*:::*::.*:

L.speciosum          KSCKKAGTVKRVIFTSSAGTVNVQENQMPEYDESSWSDVDFCRRVKMTGWMYFVSKTLAE 171
B.finlaysoniana      TSCKKAGSVKRVIFTSSAGTVNVEEHQAAVYDENSWSDLHFVTRVKMTGWMYFVSKTLAE 173
A.praecox            KSCKKAGTVKRVIYTSSAGTVNVEEHQKPEYNEDSWSDLEFCRRVKMTGWMYFVSKSLAE 179
A.cepa               KSCKKAGTVKRVIFTSSAGTVNVEEHQKPEYDENSWSDIDFCRRVKMTGWMYFVSKSLAE 175
T.aestivum           RACKEAGTVKRIVFTSSAGSVNIEERQRPAYDQDNWSDIDFCRRAKMTGWMYFVSKSLAE 171
H.vulgare            RACKEAGTVKRIVFTSSAGSVNIEERPRPAYDQDNWSDIDYCRRVKMTGWMYFVSKALAE 171
O.sativa             RACRDAGTVKRIVFTSSAGTVNIEERQRPSYDHDDWSDIDFCRRVKMTGWMYFVSKSLAE 172
Z.mays               RACKEAGTVRRIVFTSSAGTVNLEERQRPVYDEESWTDVDFCRRVKMTGWMYFVSKTLAE 176
                      :*:.**:*:*:::*****:**::*.  . *:...*:*:.:  *.***********:***

L.speciosum          KAAWEFAKENDIQLISIIPTLVVGPFITSTMPPSMLTALSLITGNEAHYSILKQIQLVHL 231
B.finlaysoniana      KAAWEFVKENAIHFIAIIPTLVVGSFITNEMPPSLITALSLISGNEAHYSILKQAQFVHL 233
A.praecox            KAAWDFARENGLDLTTIIPTLVVGPFITSTMPPSMITALSLITGNKAHYSIIKQAQLVHL 239
A.cepa               KAAWEFAKANGIDLVTIIPTLVVGAFITTAMPPSMITALSLITGNEAHYSIIKQAQLVHL 235
T.aestivum           KAAMEYASENGLDFISIIPTLVVGPFLSAGMPPSLVTALALITGNEAHYSILKQVQLVHL 231
H.vulgare            KAAMEYASENGLDFISIIPTLVVGPFLSAGMPPSLVTALALITGNEAHYSILKQVQLVHL 231
O.sativa             KAAMEYAREHGLDLISVIPTLVVGPFISNGMPPSHVTALALLTGNEAHYSILKQVQFVHL 232
Z.mays               KAALAYAAEHGLDLVTIIPTLVVGPFISASMPPSLITALALITGNAPHYSILKQVQLIHL 236
                     ***  :.  : :.: ::*******.*::  **** :***:*::** .****:** *::**

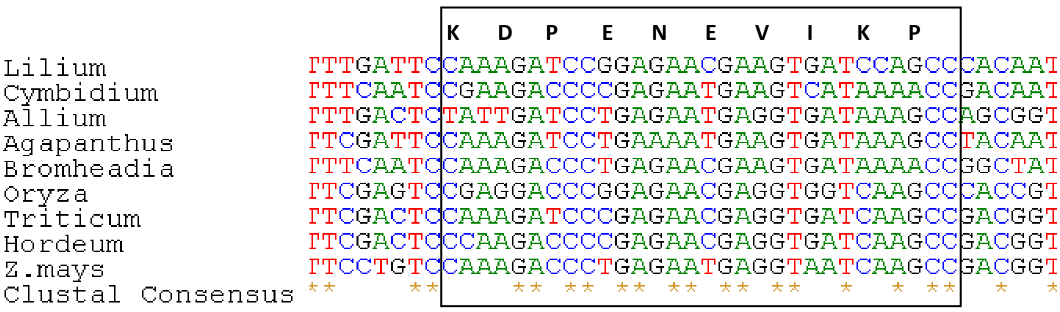
L.speciosum          DDVCKAHIFLFENPEASGRYICSSYDATIYDLARKIKDRYPQYAIPQKFEGID-DQIKPV 290
B.finlaysoniana      DDLCDAHIFVYEHPEANGRYICSSHDSTIYDLANMLKNRYATYAIPQKFKEID-PNIKSV 292
A.praecox            GDLCDAHILLLNHPKAKGGYICSSNDPTIYDIAKMLREKYPQYDIPQKFKGID-EKIPPV 298
A.cepa               DDLCEAHILLLNHPKAEGRYICSSHDVTIYDMAKMIRQNYPQYYIPQQFEGID-KGIQPV 294
T.aestivum           DDLCDAMTFLFEHPEANGRYICSSHDATIHGLARMLRDRFPEYSIPQKFAGVD-DDLQPI 290
H.vulgare            DDLCDAMTFLFEHPEANGRYICSSHDATIHGLARMLQDRFPEYDIPQKFAGVD-DNLQPI 290
O.sativa             DDLCDAEIFLFESPEARGRYVCSSHDATIHGLATMLADMFPEYDVPRSFPGIDADHLQPV 292
Z.mays               DDLCDAEIFLFENPAAAGRYVCSSHDVTIHGLAAMLRDRYPEYDVPQRFPGIQ-DDLQPV 295
                     .*:*.*  :: : * * * *:*** * **:.:*  : : :. * :*: *  ::   : .:
```

```
L.speciosum        HFSSKKLMDLGFKYQY-TFEEMFDEGIRSCIEKKLIPHQTQERYYV--HDELDLGCSKMT 347
B.finlaysoniana    SFSSKKLMDLGFKYKY-TIEEMFDDAIKTCRDKNLMPLNTEE------------------ 333
A.praecox          HFSSKKLLQLGFRFKY-SMEEMFDEAIKSCIEKKLIPLKTAEEVP----ELVEEQT---- 349
A.cepa             RFSSKKLVDLGFRYKY-SMESMFDEAIKTCVERKFIPLQTAVELQLKPYELLEHNNKNGV 353
T.aestivum         HFSSKKLLDHGFSFRY-TAEDMFDAAIRTCREKGLIS---------------------L 327
H.vulgare          HFSSKKLLDHGFSFRY-TTEDMFDAAIHTCRDKGLIP---------------------L 327
O.sativa           HFSSWKLLAHGFRFRY-TLEDMFEAAVRTCREKGLLPPLPPPPTTA-----------VAG 340
Z.mays             RFSSKKLQDLGFTFRYKTLEDMFDAAIRTCQEKGLIP----------------------- 332
                    *** **    ** ::* : *.**: .:::* :: ::.

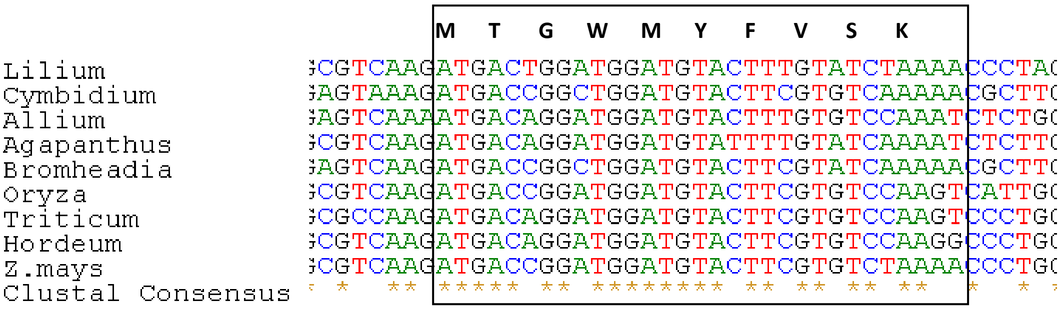
L.speciosum        NDKLDLGGSKLNSMDEMVRGHNERVSVALQ-- 377
B.finlaysoniana    ---LVLAAEKYDEVKEQIAVK----------- 351
A.praecox          -AVAKIIVEQAIVTKVNRDGSEERVPIATH-- 378
A.cepa             VTNTIKIVGQMVNTKAMITEHEENEPIATH-- 383
T.aestivum         GDAPPPAAGGKLGALAAGKGQAIGAET----- 354
H.vulgare          GDVPAPAAGGKLGALAAGEGQAIGAET----- 354
O.sativa           GDGSAGVAGEKEPILGRGTGTAVGAETEALVK 372
Z.mays             ---LATAAGGDGFASVRAPGETEATIGA---- 357
```

Appendix C2: Sections of the monocot *DFR* cDNA alignment in BioEdit 7.0. Boxed areas indicate conserved regions with high cross-species identity used as reference during *DFR* primer design. The corresponding peptides are also shown above each alignment. Nucleotides that are identical in all sequences are marked with asterisks. GenBank accession number: *Hordeum vulgare* (S69616), *Oryza sativa* (japonica cultivar-group) (Y07956), *Bromheadia finlaysoniana* (AF007096), *Agapanthus praecox* (AB099529), *Triticum aestivum* (AY373831), *Zea mays* (NP_001158995), *Lilium speciosum* (AB201531), *Allium cepa* (AY221250).

DFR-f



DFR-r



Appendix D: Multiple alignment of monocot *CHI* cDNA sequences. GenBank accession numbers: *Hordeum vulgare* (AF474923) *Oryza sativa* (japonica cultivar-group) (AF474922), *Zea mays* (Z22760), *Allium cepa* (AY541034). The highlighted areas show the conserved areas exhibiting a high degree of cross-species identity and were therefore used for primer designing. Nucleotides that are identical in all sequences are marked with an asterisk.

```
O.sativa        ATGGCGGCCGTGTCGGAGGTGGAGGTCGACGGC-GTCGTGTTCCCGCCGGTGGCCCGCCC 59
H.vulgare       ATG---GCCGTGTCGGAGCTGGAGGTCGACGGC-GTCGTCTTCCCGCCGCTCGCCCGCCC 56
Z.mays          ATG---GC-GTGCCGGAGGTGGTGGTCGACGGCCGTCGTCTTCCCGCCGGTGGCCCGCCC 56
A.cepa          ATGGAAGCAGTGACAAAGTTGGACGTAGAAGGA-ACTGCCTTTGATTCAGTCATCATCCC 59
                ***   ** *** *  ** ***  ** ** **     *  **     *  *   *  ***

O.sativa        GCCGGGCTCCGGCCACGCCCACTTCCTCGCCGGCGCAGGTGTGAGGGGAGTGGAGATCGC 119
H.vulgare       GCCGGGCTCCGCCCACGCCCACTTCCTCGCCGGCGCAGGCGTGCGCGGGATGGAGATCGG 116
Z.mays          GCCGGGCTCCGCCGGCTCGCACTTCCTCGGCGGCGCAGGCGTGCGAGGCGTCGAGATCGG 116
A.cepa          TCCCGGTTCATCCAAAACGCACTTTCTCGGCGGTGCAGGTGTAAGGGGTTTGGAAATAGG 119
                 ** ** **   *    * ***** **** *** ***** **  * **  * ** ** *

O.sativa        CGGCAACTTCATCAAGTTCACGGCCATCGGCGTGTACCTGGAGGAGGGCGCGGCCGTGCC 179
H.vulgare       CGGCCACTTCATCAAGTTCACGGCCATCGGCGTGTACCTGCAGGCCGACGCCGCCGTCTC 176
Z.mays          AGGCAACTTCATCAAGTTCACGGCCATCGGCGTGTACCTGGAG---GACGCGGCCGTGCC 173
A.cepa          AGGTAAATTTATAGCGTTTACCGCAATCGGCATCTACTTGGAAACAGATTCAA---TTCC 176
                 **  * ** **   *** ** ** ****** * *** ** *    *   *     *  *

O.sativa        GGCGCTGGCCAAGAAGTGGGCCGGCAAGTCCGCCGACGAGCTCGCCGCCGACGCCGCCTT 239
H.vulgare       CGCGCTCGCCGCCAAGTGGGCCGGCAAGCCCGCCGCCGATCTCGCCTCCGACGCCGCCTT 236
Z.mays          CGCGCTGGCCAAGAAGTGGGGCGGCAAGACGGCCGACGAGCTCGCCTCCGACGCCGCCTT 233
A.cepa          GTTTCTTGCTGATAAATGGAAAGGAAAAACAGGCGAGGAACTCGCTGGTTCACTCGACTT 236
                    ** **    ** ***   ** **  * * **  ** *****         ** ***

O.sativa        CTTCCGCGACGTCGTCACCGGCGATTTCGAGAAGTTCACGAGGGTGACGATGATCCTGCC 299
H.vulgare       CTTCCGCGACGTCGTCACCGGCGAGTTCGAGAAGTTCACGAGGGTGACAATGATCCTGCC 296
Z.mays          CTTCCGCGACGTCGTCACGGGCGACTTTGAGAAGTTCACGAGGGTGACGATGATCCTCCC 293
A.cepa          TTTTCGAGATATATGCACAGGACCCTTTGAGAAATTTACTAATGTAACAATGATTCTCCC 296
                 ** ** **  *   *** **    ** ***** ** ** *  ** ** ***** ** **

O.sativa        GCTCACCGGCGAGCAGTACTCGGACAAGGTGACGGAGAACTGCGTCGCGGCGTGGAAGGC 359
H.vulgare       GCTGACGGGCGCGCAGTACTCGGACAAGGTGACGGAGAACTGTGTCGCGTACTGGAAGGC 356
Z.mays          GCTGACGGGCGAGCAGTACGCGGAGAAAGTGACGGAGAACTGCGTGGCGTTCTGGAAGGC 353
A.cepa          TCTAACGGGAGAACAGTACTCCGAAAAAGTGACAGAAAATTGTGTAGCTTATTGGAAAGC 356
                 ** ** ** *  ****** * ** ** ***** ** ** ** ** **    ***** **

O.sativa        CGCCGGCGTGTACACGGACGCCGAGGGCGCGGCCGCGGACAAGTTCAAGGAGGCCTTCAA 419
H.vulgare       CGCCGGCGTGTACACGGACGCTGAGGCCGCCGCCGTCGACAAGTTCAAGGAGGCCTTCGG 416
Z.mays          CGCCGGCCTGTACACGGACGCCGAGGGCGTCGCCGTGGAGAAGTTCAGGGAGGTGTTCAA 413
A.cepa          AATTGGAATCTACACGGATGCAGAAGCGTCGGCTGTTGATAAGTTTAAACAAGCTTTTAA 416
                    **  * ******** ** ** *     ** *  ** ***** *   * *  **

O.sativa        GCCCCACAGCTTCCCTCCGGGCGCGTCCATCCTCTTCACCCACTCCCCGCCCGGCGTCCT 479
H.vulgare       GCCCCACAGCTTCGCCCCCGGCGCCTCCATACTCTTCACGCACTCCCCCGCCGGCGTGCT 476
Z.mays          GCCAGAGACTTTCGCGCCGGGCCGCTCCATCCTCTTCACGCACTCGCCCGCCGGAGTCCT 473
A.cepa          ACCTGAGAGTTTCCCTCCTGGTTCATCCATTCTCTTCACTCATACCCCTAGCGGAACACT 476
                 **  * *  *** * ** **    ***** ******** **  * **   ***    **

O.sativa        CACCGTCGCGTTCTCCAAGGACTCGTCGGTGCCAGAGGGCGCCGTGGCGGCGGCGGCGAT 539
H.vulgare       CACCGTCGCCTTCTCCAAGGACTCGTCGGTGCCGGAGTCCG-----GCGGCG-TGGCCAT 530
Z.mays          CACCGTCGCCTTCTCCAAGGACTCGTCGGTGCCAGCGGCCG-----GCGGCG-TGGCGAT 527
A.cepa          AAAGATTGCATTTTCGAAAGACGGTTTGGTTCCTAAAGATG-----AAGGCG-TACTCAT 530
                 *   * ** ** ** ** ***   * *** **       *       ****      **
```

```
O.sativa        CGAGAACAGGGCGCTCTGCGAGGCGGTGCTGGATTCCATCATCGGCGAGCACGGGGTCTC 599
H.vulgare       CGAGAACGCCAGGCTCTGCGAGGCCGTGCTGGAGTCCATCATCGGCGAGCACGGGGTGTC 590
Z.mays          CGAGAACAAGCGCCTCTGCGAGGCCGTGCTGGAGTCCATCATCGGGGAGCGCGGCGTGTC 587
A.cepa          TGAGAATAAGGCGCTGACACAAGCCGTATTGGAGTCGATTATTGGGGAGCATGGTGTATC 590
                 *****       **     * ** **  **** ** ** ** ** ****  ** ** **

O.sativa        GCCGGCGGCGAAGCGGAGCATAGCGGCCCGCGTCTCGCAGCTCCTG---AAGGCGGA--- 653
H.vulgare       GCCGGCGGCCAAGCTCAGCCTGGCCAACAGGGTCGCTGAGCTGCTG---AAGGGGGCCGC 647
Z.mays          GCCGGCCGCGAAGCTGAGCCTCGCCGCGAGGGTGTCGGAGCTCCTCGCCAAGGAGACCGC 647
A.cepa          ACCTGCTGCTAAGCTAAGCATAGCATCGAGATTGTCAGAGATTATG---AACAAAGTTGG 647
                 ** ** ** ****  *** * **     *  *  *  ** *  *    **

O.sativa        ATCCACCGGCGACGTGGCGGCGGCGGAGCCCGCGCCGGTGTCCGCGTGA 702
H.vulgare       ACACGCCGGCGGCGAGCCGGCCGCGGAGCCCGTGCCGGTTTCGGTGTGA 696
Z.mays          CGCGGCCGCCGACGCGCCGCAGGCGGAGCCCGTCTCCATCACCGCCTGA 696
A.cepa          AAACGTGGAAGAAAAGTTACCGGTGCT-TTCATGA-------------- 681
                       *  *    *      * *     *
```

Appendix E: Multiple alignment of monocot *F3H* cDNA sequences. GenBank accession numbers: *Hordeum vulgare* (P28038), *Oryza sativa* (japonica cultivar-group) (AAN74830), *Zea mays* (NP_001105695), *Anthurium andreanum* (ABI50233), *Lilium speciosum* (AB201532), *Allium cepa* cultivar H6 (doubled haploid line) (AY221246). The highlighted areas exhibit a high degree of cross-species identity used for primer designing. Nucleotides that are identical in all sequences are marked with an asterisk.

```
H.vulgare         ATGGCGCCGGTGAGCAACGAGAC------GTTCCTCCCGACGGAGGCGTGGGGGGAGGCC 54
O.satvia          ATGGCGCCGGTGGCCAC---GAC------GTTCCTCCCGACGG---CGTCGAACGAGGCG 48
Z.maize           ATGGCTCCCGTGAGCATCAGCGCTGTTCCGTTCCTCCCGACGGCGGCGGAGGGGGAGACG 60
A.andraeanum      ATGGTTCCCGCGGCAAC----AC-----CCTTCCTGCCGACGA---CCGCGGAGGAGGCG 48
L.speciosum       ATGGCTCCTGTTGCGACT---AC------CTTCCTCCCAACAA---TCTCCGACGAAAAG 48
A.cepa            ATGGCACCGGCACAAACT---CC------ATTTCTACCCACAA---TCTCAGATGAAAAA 48
                  ****  ** *     *      *       ** ** ** **             **

H.vulgare         ACGCTGCGCCCGTCCTTCGTGCGGGACGAGGACGAGAGGCCCAAGGTGGCGCACGACCGC 114
O.satvia          ACGCTGCGGCCGTCGTTCGTGCGCGACGAGGACGAGCGCCCCAGGGTGGCGTACAACCAG 108
Z.maize           AACGTGCGCGCGTCGTTCGTGCGCGAGGAGGACGAGCGCCCCAAGGTGCCGCACGACCGC 120
A.andraeanum      ACGCTGCGCCCCAGCTTCATCCGCGACGAGGACGAGCGCCCCAAGGTGCCCCACAACCAG 108
L.speciosum       ACCTTGAGGGCGAGCTTTGTGCGCGATGAAGATGAGCGCCCGAAGGTCGCCTACAACAAC 108
A.cepa            ACTCTACGTTCCAGCTTCGTGGGGGACGAAGACGAGCGTCCCAAGATTGCATACAACGTG 108
                  *   *  *  *    **  *  * ** ** ** *** * ** * * *  *  ** **

H.vulgare         TTCAGCGACGCGGTGCCGCTGATCTCGCTCCACGGCATCGACG---------GCGCGCG- 164
O.satvia          TTCAGCGACGCGGTCCCGGTGATCTCGCTCCAGGGGATCGACG---------AAGCGGC- 158
Z.maize           TTCAGCGACGAGGTGCCGGTGGTGTCGCTCGAGGGCATCGACG---------GGCCGCGC 171
A.andraeanum      TTCAGCGACGCCATCCCCGTCCTCTCCCTCGCCGGCATCGACGACTCCGACGGGTCGCCC 168
L.speciosum       TTCAGCGATGACATCCCGATCATCTCCCTGGCGGGGATGGACGAG---GATGGACCGAT- 164
A.cepa            TTTAGCGATGAAATTCCAATAATATCCCAGGATGGCATCGACGATGC-----AAATGGA- 162
                  ** ***** *   * **  *  * **  *     ** ** ****             *

H.vulgare         --CCGGGCCCAGATCCGGGACCGCGTGGCCGCCGCCTGCGAGGACTGGGGCATCTTCCAG 222
O.satvia          --GCGGGCGGAGATCCGTGCCCGCGTGGCCGGCGCGTGCGAGGAGTGGGGCATCTTCCAG 216
Z.maize           AGGCGGGCCGAGATCCGCGGCCGCGTGGCCGCGGCCTGCGAGGACTGGGGCATCTTCCAG 231
A.andraeanum      CGCAGGGCGGAGCTGTGCCGCGAGATCGTGGAGGCCTGCGAAGGGTGGGGCATCTTCCAG 228
L.speciosum       --TAGGTCTGAAATATGCGGCAAGATCGTCGCCGCCTGCGAGGACTGGGGTATTTTTCAG 222
A.cepa            AAGAGAGGGGAAATATGTAGGAAGATAGTAGAAGCACGCGAGGACTGGGGGGTGTTTCAG 222
                      *     *  *  *        * *  *  **   **** *  *****  * ** ***

H.vulgare         GTGATCGACCACGGCGTGGATGCGGACCTCATCGCCGACATGACGCGCCTGGCTCGCGAG 282
O.satvia          GTGGTGGACCACGGCGTGGACGCGGGGCTCGTCGCCGACATGGCGCGCCTCGCCCGCGAC 276
Z.maize           GTGGTGGACCACGGCGTGGACGCCGCGCTCGTGGCCGACATGGCGCGCCTCGCCCGAGAC 291
A.andraeanum      GTGGTGGACCACGGCGTCGACCCGGGCCTGGTCGCCGACATGACGCGCCTCGCCACGGAG 288
L.speciosum       GTGGTGGACCATGGAGTCGATGCCGGTCTGGTGACGGAGATGAACCGACTGGCGCGTGAG 282
A.cepa            GTAATTGACCATGGCGTTGAGCAGGAGGTGATTAAGGACATGACAAAAATGGCTAGAGAG 282
                  **  * ***** ** ** **    *  *   *    ** ***       *  **    **

H.vulgare         TTCTTCGCGCTGCCCGCCGAGGACAAGCTCCGGTACGACATGTCCGGCGGCAAGAAGGGC 342
O.satvia          TTCTTCGCGCTGCCGCCGGAGGACAAGCTCCGGTTCGACATGTCCGGCGGCAAGAAGGGC 336
Z.maize           TTCTTCGCGCTCCCGCCCGAGGACAAGCTCCGCTTCGACATGTCCGGCGGGAAGAAGGGC 351
A.andraeanum      TTCTTCGCCCTCCCGCCCGAGGACAAGCTCCGCTACGACATGTCCGGGGGCAAGAAGGGC 348
L.speciosum       TTTTTCGCACTGCCGCCGGAGGACAAGCTGAGATTTGATATGACAGGTGGGAAGAAGGGT 342
A.cepa            TTCTTCGCATTGCCGCCTGAAGAGAAGTTGAGGTTCGATATGTCAGGTGGAAAGAAGGGC 342
                  ** *****  * **   * ** ** *** *  * * *  ** *** * ** ** ********
```

```
H.vulgare       GGCTTCATCGTCTCCAGCCACCTACAGGGTGAGGCGGTGCAGGACTGGAGGGAGATAGTG 402
O.satvia        GGATTCATCGTCTCCAGCCACCTCCAGGGTGAAGCGGTGAAAGACTGGCGGGAGATCGTG 396
Z.maize         GGCTTCATCGTCTCCAGCCACCTCCAGGGGGAGGCGGTGCAGGACTGGCGTGAGATCGTG 411
A.andraeanum    GGCTTCATCGTCTCCAGCCACCTCCAGGGGGAGGCCGTGCAGGACTGGAGGGAGATCGTG 408
L.speciosum     GGCTTCATCGTTTCCAGTCATCTCCAGGGTGAAGCAGTGCAAGATTGGAGGGAGATAGTG 402
A.cepa          GGTTTCATTGTTTCCAGTCATCTTCAGGGAGAAGCGGTTCAAGACTGGAGAGAGATAGTA 402
                ** ***** ** ***** ** ** ***** ** ** **  * ** *** * ***** **

H.vulgare       ACCTACTTCTCGTACCCGGTGAAGGCGCGGGACTACGGGCGGTGGCCGGAGAAGCCGGCG 462
O.satvia        ACCTACTTCTCGTACCCGGTGAAGTCCCGCGACTACTCGCGGTGGCCCGACAAGCCGGCG 456
Z.maize         ACCTACTTCTCGTACCCGGTGAAGGCCCGCGACTACTCCCGGTGGCCGGACAAGCCGGCG 471
A.andraeanum    ACCTACTTCTCGTACCCAGTACGGGCGCGGGACTACACGAGGTGGCCCGACAAGCCGGAG 468
L.speciosum     ACATACTTCTCATACCCGATCCGGGTCAGGGACTACTCGAGGTGGCCAGACAAGCCCGAG 462
A.cepa          ACATACTTCTCATACCCGATCAGAGCCAGAGACTACTCCCGCTGGCCCGATAAGCCCGAA 462
                ** ******** *****  *        * ******    * ***** ** ***** *

H.vulgare       GGGTGGTGCGCGGTGGTGGAGCGGTACAGCGAGCGGCTCATGGGGCTGTCGTGCAATCTG 522
O.satvia        GGGTGGCGCGCAGTGGTGGAGCAGTACAGCGAGCGGCTCATGGGCCTCGCCTGCAAGCTG 516
Z.maize         GCGTGGCGGGCGGTGGTGGAGCGGTACAGCGAGCAGCTGATGGCGCTGGCGTGCAGGCTC 531
A.andraeanum    GGGTGGCGGGCGGTGGTGGAGGCGTACAGCGAGGGGTTGATGGGGCTCGCCTGCAAGCTG 528
L.speciosum     GGTTGGAGGGCCGTCGTCGAGGCCTATAGCGAGCAGTTGATGGGCCTGGCCTGCAAGCTC 522
A.cepa          GGTTGGATTTCCGTTGCTGAAAAATACAGCGAAAAACTCATGGACTTGGCCTGTAAATTA 522
                *  ***    * ** *  **    ** *****     * ****   *  * ** *   *

H.vulgare       ATGGGCGTGCTGTCGGAGGCCATGGGCCTGGAGACGGAGGCGCTGGCCAAGGCGTGCGTC 582
O.satvia        CTGGGCGTGCTCTCCGAGGCCATGGGCCTCGACACCAACGCGCTGGCCGATGCCTGCGTC 576
Z.maize         CTGGGCGTGCTCTCCGAGGCCATGGGCCTGGACACGGAGGCGCTGGCCAGGGCCTGCGTG 591
A.andraeanum    CTGGGGGTGCTGTCCGAGGCCATGGGGCTGGACAAGGAGGCGCTCGCCAAGGCCTGCGTC 588
L.speciosum     CTAGGGGTCTTGTCCGAGGCCATGGGCCTTGACAAGGAGGCCCTGACGAAGGCATGTGTA 582
A.cepa          CTGGGCATCCCTTCAGAAGCCATGGGCTTGGACACAGAGGCCTTAACTAAGGCCTGCATC 582
                 * **  *    ** ** ********  * ** *   * **  *  *    ** **  *

H.vulgare       GACATGGACCAGAAAGTGGTGGTCAACTTCTACCCGCGGTGCCCGCAGCCCGACCTCACC 642
O.satvia        GACATGGACCAGAAGGTTGTCGTCAACTTCTACCCCAAGTGCCCCCAGCCCGACCTCACC 636
Z.maize         GACATGGACCAGAAGGTGGTGGTCAACTTCTACCCGAGGTGCCCGCAGCCGGACCTCACG 651
A.andraeanum    GACATGGACCAGAAGGTGGTGGTGAACTTCTACCCCAGGTGCCCCCAGCCCGACCTCACC 648
L.speciosum     GACATGGACCAAAAGATTGTGGTCAACTTCTATCCGAAGTGTCCTCAGCCCGACCTGACC 642
A.cepa          GATATGGACCAGAAGATGGTGGTGAACTTCTACCCAAAATGCCCTCAACCTGATCTCACT 642
                ** ******** **  * ** ** ******** **    ** ** ** ** ** ** **

H.vulgare       CTGGGCCTCAAGCGCCACACCGACCCCGGCACCATAACGCTCCTCCTGCAGGACCTCGTC 702
O.satvia        CTTGGCCTCAAGCGCCACACCGACCCCGGTACCATCACGCTCCTCCTCCAGGACCTCGTC 696
Z.maize         CTGGGGCTCAAGCGCCACACCGACCCCGGCACCATCACGCTGCTGCTGCAGGACCTGGTC 711
A.andraeanum    CTCGGGGTCAAGCGCCACACCGACCCCGGCACCATCACCCTCCTCCTCCAGGACCAGGTC 708
L.speciosum     CTCGGGCTTAAGCGCCACACTGACCCAGGCACCATCACCCTCCTTCTCCAGGACCAGGTC 702
A.cepa          CTAGGCTTGAAGCGTCATACCGATCCTGGTACCATCACTCTGCTGCTTCAGGACCAGGTC 702
                ** **  * ***** ** ** ** ** ** ***** ** ** ** ** *******  ***

H.vulgare       GGCGGCCTCCAGGCCACCCGCGACGGCGGCAAGAACTGGATCACCGTCCAGCCCATCTCC 762
O.satvia        GGCGGCCTCCAGGCCACCCGCGACGCCGGCAAGACGTGGATCACCGTCCAGCCCATCCCC 756
Z.maize         GGCGGCCTCCAGGCCACGCGCGACGGCGGCCGGACCTGGATCACCGTGCAGCCCGTGGAG 771
A.andraeanum    GGCGGCCTCCAGGCCACCAGAGACGGCGGCAAGACCTGGATCACCGTCCAGCCCGTCGAG 768
L.speciosum     GGTGGCCTCCAAGCTACTAAGGATGGTGGTAACACCTGGATTACCGTCAAGCCGATTGAG 762
A.cepa          GGCGGTCTGCAAGCGACTAAAGATGGTGGAAAGACTTGGATCACTGTTCAACCCGTTGAA 762
                ** ** ** ** ** **    ** *  **    *  ***** ** **  * **  *

H.vulgare       GGCGCATTCGTCGTCAACCTCGGCGACCACGGCCACTTCATGAGCAACGGCAGGTTCAAG 822
O.satvia        GGCTCCTTCGTCGTCAACCTCGGTGACCATGCTCACTATCTGAGCAATGGGAGGTTCAAG 816
Z.maize         GGCGCCTTCGTCGTCAACCTCGGCGACCACGGCCACCTCCTGAGCAACGGCAGGTTCAAG 831
A.andraeanum    GGCGCCTTCGTCGTCAACCTCGGCGACCACGGTCACCTCCTGAGCAACGGGCGGTTCAAG 828
L.speciosum     GGTGCCTTTGTTGTCAATCTCGGAGATCATGGACATTTTTTGAGCAATGGGAGATTCAAA 822
A.cepa          GGCGCATTTGTAGTTAACCTTGGTGACCATGGCCATTATTTGAGCAATGGTAGGTTCAAG 822
                **  * ** ** ** ** ** ** ** ** *  **     ******* **  * *****
```

```
H.vulgare       AACGCGGACCACCAGGCGGTGGTGAACGGGGAGAGCAGCAGGCTGTCGATCGCGACGTTC 882
O.satvia        AACGCGGATCACCAGGCGGTGGTGAACTCCGACTGCTGCCGGCTGTCGATCGCGACGTTC 876
Z.maize         AACGCGGACCACCAGGCGGTGGTGAACTCGGAGTGCAGCCGCCTGTCCATCGCCACGTTC 891
A.andraeanum    AACGCGGACCACCAGGCGGTGGTGAACTCGGAGCGCAGCCGGCTGTCGATCGCGACGTTC 888
L.speciosum     AACGCGGACCACCAGGCGGTGGTGAACTCGAATTCTAGTCGTTTGTCGATAGCGACATTT 882
A.cepa          AATGCAGACCACCAGGCAGTGGTGAATTCAAACTACAGCAGGCTGTCGATTGCCACGTTT 882
                ** ** ** ******** ********     *     *  *  **** ** ** ** **

H.vulgare       CAGAACCCGGCGCCGGACGCGAGGGTGTGGCCGCTGGCGGTGAGGGAGGGGGAGGAGCCC 942
O.satvia        CAGAACCCGGCGCCGGACGCGATGGTGTACCCGCTGGCGGTGCGCGACGGGGAGGAGCCC 936
Z.maize         CAGAACCCGGCGCCCGACGCGACGGTGTACCCGCTGGCCGTGCGCGACGGGGAGGCGCCC 951
A.andraeanum    CAGAACCCGGCGCCGGAGGGGGTCGTGTACCCGCTGGCCGTCCGGGAGGGGGAGAAGCCG 948
L.speciosum     CAGAACCCAGCGCCAGAGGCGACGGTTTACCCGTTGGCAATCAGGGACGGAGAGAAGCCA 942
A.cepa          CAGAATCCGGCACCGGAGGTAATTGTATATCCTCTGGCAATCAGGGAAGGAGAAAAACCG 942
                ***** ** ** ** ** *     ** *  **  ****  *  * ** ** **    **

H.vulgare       ATACTGGAGGAGCCCATCACCTTCACCGAGATGTACCGCCGCAAGATGGAGCGCGACCTC 1002
O.satvia        ATACTGGAGGAGCCGATCACGTTCGCGGAGATGTACCGGCGCAAGATGGCACGCGACCTC 996
Z.maize         ATACTGGACCATCCCATCACCTTCGCCGAGATGTACCGCCGCAAGATGGCCCGCGACATC 1011
A.andraeanum    GTGCTCGACGAGCCCATCTCCTTCTCCGAGATGTACCGGAGGAAGATGAGCCGAGACCTG 1008
L.speciosum     GTTTTGGATGCCCCGATGACATTCAGCGAGATGTATAGAAAGAAAATGAGTAAAGATATC 1002
A.cepa          ATATTAGAGGAGCCGATTACTTTTGCAGAGATGTATAGAAGGAAGATGAGCAAAGACATT 1002
                 *  * **    ** **  * **    ********  *    ** ***      **  *

H.vulgare       GACCTCGCCAAGCGCAAGAAGCAGGCCAAGGACCAGCTGATGCAGCAGCAG------CTG 1056
O.satvia        GAGCTCGCCAAGCTCAAGAAGAAGGCCAAGGAGCAGCGGCAGCTGCAGCAGGCGGCGCTG 1056
Z.maize         GAGCTCGCCAGGCTCAAGAAGCAGGCCAAGGCCGACAAGAAGCAGCAGCAG--------- 1062
A.andraeanum    GAGCTCGCCAGGCTCAAGAAACAGGCCAAG------CTGCAGCAGCAGGAG--------- 1053
L.speciosum     GAGCTTGCGAAACTCAAGAAGTTGGCTAACA-----CGGAAGC-GCAGGAGG-------- 1048
A.cepa          GAGCTTGCAAAGCTCAAGAAACTGGCAAAGAAAAACAAGGAAATTTCGGAGAAGACACAA 1062
                ** ** ** *  * ******   *** **         *        * **

H.vulgare       CAGCTCCAGCAGCAGCAGGCGGTCGCCGCGGCGCCCATGCCCACCGCCACCAAGCCCCT- 1115
O.satvia        CCGCCGCCGCCGCCGACGCAGGTCGCCGCGGAGTTGGCAGCACAGAAGCCCAAGTCTCT- 1115
Z.maize         ---------------CAGAGTGCCAACAAGGAGTTCGC------CGACTCCAAGCCTCT- 1100
A.andraeanum    ---------------GTGCTGGCCAAGGCCGAGGATGC---CAACAAACACAAAGCCCT- 1094
L.speciosum     -------------TTGCGAAGAAACCCACCGAAGTTGCG---GTTCAACCGGGAAAACT- 1091
A.cepa          ATTGCAGCTCTGCCCAAGGCCATTGATGAGATACTAGCCTAGTTTGTATTAAGAACAATG 1122
                                 *                                        *

H.vulgare       ---CAACGAAATTCTTGCCTAGATCCTTCCGGCGGCGGGCGGCGATTAAATACTTCAAAT 1172
O.satvia        ---CGACGAGATTCTTGCCTAG-------------------------------------- 1134
Z.maize         ---CGACGCCATTTTTGCCTGA-------------------------------------- 1119
A.andraeanum    ---CGATGAGATTCTGGCCTGA-------------------------------------- 1113
L.speciosum     ---AGATCAAATCCTTGCTTAG-------------------------------------- 1110
A.cepa          ATATACTAGAATAATCACTTTGTCATTGTATGTTATATCCATTGCTTTTCTACTATTGCT 1182
                          **  *  * *

H.vulgare       TGATGGATGCGTGGGATTGATTCTCCTAAGTACTAGTACGATATAAATTATTGCATGCAT 1232
O.satvia        ------------------------------------------------------------
Z.maize         ------------------------------------------------------------
A.andraeanum    ------------------------------------------------------------
L.speciosum     ------------------------------------------------------------
A.cepa          GCAGTATGCAATAAACTAAATTCCACAATTTGATTGGAAAAAA----------------- 1225

H.vulgare       ATATCCGTACGTGTGTAGCAGGGAGGAGCTCGGCCTGTAATAACGTCGTGCGTGGAACTG 1292
O.satvia        ------------------------------------------------------------
Z.maize         ------------------------------------------------------------
A.andraeanum    ------------------------------------------------------------
L.speciosum     ------------------------------------------------------------
A.cepa          ------------------------------------------------------------
```

Appendix F: CAP3 alignment of the forward (F) and reverse (R) sequencing results of a *CHS* cDNA fragment in *Clivia miniata* var. *miniata* 'Plantation' (12), *Clivia miniata* var. *citrina* 'Kirstenbosch Yellow' (ky), *Clivia miniata* var. *miniata* 'Teleurstelling' (tel), *Clivia miniata* var. *citrina* 'Giddy' (g) and *Clivia caulescens* (cc), which produced a consensus sequence of 586 bp. "rc" refers to the reverse compliment of a sequence. IUPAC symbols for degeneracy are shown in bold and include W (A or T), M (A or C), Y (C or T), R (A or G), S (C or G), and K (G or T).

```
                        .    :    .    :    .    :    .    :    .    :    .    :
12R_rc             TATCAGCTCACCAAACTCCTCGGCCTCCGCCCTTCTGTCAAGCGCCTCATGATGTATCAG
kyR_rc             TATCAGCTCACCAAACTCCTCGGCCTCCGCCCTTCTGTCAAGCGCCTCATGATGTATCAG
telR_rc            TAYCAGCTCACCAAACTCCTCGGCCTCCGCCCTTCTGTCAAGCGCCTCATGATGTATCAG
ccR_rc             TATCAGCTCACCAAACTCCTCGGCCTSCGCCCTTCTGTCAAGCGCCTCATGATGTACCAG
gR_rc                         CAAACTCCTCGGCCTCCGCCCTTCTGTCAAGCGCCTCAKGATGTATCAG
gF                                                         AGCGCCTCATGATGTATCAG
12F                                                        AGCGCCTCATGATGTATCAG
                   ____________________________________________________________
consensus          TATCAGCTCACCAAACTCCTCGGCCTCCGCCCTTCTGTCAAGCGCCTCATGATGTATCAG 60

                        .    :    .    :    .    :    .    :    .    :    .    :
12R_rc             CAAGGCTGCTTTGCCGGWGGCACGGTCCTCCGCCTMGCCAAAGATCTCGCTGAGAACAAC
kyR_rc             CAAGGCTGCTTTGCCGGWGGCACGGTCCTCCGCCTAGCCAAAGATCTCGCTGAGAACAAC
telR_rc            CAAGGCTGCTTTGCCGGWGGCACGGTCCTCCGCCTMGCCAAAGATCTCGCTGAGAACAAC
ccR_rc             CAAGGCTGCTTYGCCGGAGGCACGGTCCTCCGCCTGGCCAAAGATCTCGCYGAGAACAAC
gR_rc              CAAGGCTGCTTTGCCGGAGGCACGGTCCTCCGCCTMGCCAAAGATCTCGCTGAGAACAAC
gF                 CAAGGCTGCTTTGCCGGWGGCACSGTYCTCCGCCTMGCCAAAGATCTCGCTGAGAACAAC
12F                CAAGGCTGCTTTGCCGGWGGCACSGTCCTCCGCCTMGCCAAAGATCTCGCTGAGAACAAC
kyF                   GGCTGCTTTGCCGGWGGCACGGTCCTCCGCCTAGCCAAAGATCTCGCTGARAACAAC
telF                  GGCTGCTTTGCCGGAGGCACGGTCCTCCGMCTAGCCRAARATCTCGCTGAGAACAAC
ccF                   GGYTGCTTKGCCGGAGGAACSKTCCTCCGCCTGGCCAAAGATCTSGCYGAGAACAAC
                   ____________________________________________________________
consensus          CAAGGCTGCTTTGCCGGWGGCACGGTCCTCCGCCTMGCCAAAGATCTCGCTGAGAACAAC 120

                        .    :    .    :    .    :    .    :    .    :    .    :
12R_rc             CGTGGYGCACGGGTTCTCGTCGTCTGCTCGGAGATCACGGCTGTCACRTTCCGCGGCCCM
kyR_rc             CGYGGCGCACGGGTTCTCGTCGTCTGCTCGGAGATCACGGCTGTCACRTTCCGCGGCCCC
telR_rc            CGTGGCGCACGGGTTCTCGTCGTCTGCTCGGAGATCACGGCTGTCACRTTCCGCGGCCCC
ccR_rc             CGTGGCGCSSGRGTTCTYGTCGTCTGCTCSGAAATCACSGCCGTCACGTTCCGTGGCCCC
gR_rc              CGTGGYGCACGGGTTCTCGTCGTCTGCTCGAAAAACACGGCTGTCACRTTCCGCGGCCCC
gF                 CGTGGYGCACGRGTTCTCGTCGTCTGCTCGGAGATCACGGCTGTCACRTTCCGCGGCCCC
12F                CGTGGYGCACGGGTTCTCGTCGTCTGCTCGGAGATCACGGCTGTCACRTTCCGCGGCCCC
kyF                CGYGGCGCACGGGTTCTCGTCGTCTGCTCGGARATCACGGCTGTCACRTTCCGCGGCCCC
telF               CGTGGCGCACGGGTTCTCGTCRTCTGCTCSGARATCACGGCTGTCACATTCCGCGGCCCC
                   ____________________________________________________________
consensus          CGTGGYGCACGGGTTCTCGTCGTCTGCTCGGARATCACGGCTGTCACRTTCCGCGGCCCC 180

                        .    :    .    :    .    :    .    :    .    :    .    :
12R_rc             TCCGACACTCACCTCGACAGTCTCGTSGGGCARGCCTTGTTCGGCGACGGTGCGGCYGCC
kyR_rc             TCCGACACTCACCTCGACAGTCTCGTSGGGCARGCCTTGTTCGGCGACGGTGCGGCYGCC
telR_rc            TCCGACACTCACCTCGACAGTCTCGTSGGGCARGCCTTGTTCGGCGACGGTGCGGCYGCC
ccR_rc             TCCGACACYCACCTCGACAGTCTCGTSGGGCAGGCCTTKTTCGGCGACGGKGCGGCYGCY
gR_rc              TCCGACACTCACCTCGACAGTCTCGTSGGGCARGCCTTGTTCGGCGACGGTKCRGCYGCC
gF                 TCCGACACTCACCTCGACAGTCTCGTSGGGCARGCCTTGTTCGGCGACGGTGCGGCYGCC
12F                TCCGACACTCACCTCGACAGTCTCGTSGGGCARGCCTTGTTCGGCGACGGTGCGGCYGCC
kyF                TCCGACACTCACCTCGACAGTCTCGTSGGGCARGCCTTGTTCGGCGACGGTGCGGCYGCC
telF               TCCRACACTCACCTCGACAGTCTCGTSGGGCARGCCTTGTTCGGCGACGGTGCGGCYGCC
ccF                TCCGACACYCACCTCGACAGTCTCGTSGGGCAGGCCTTKTTCGGCGACGGKGCGGCYGCY
                   ____________________________________________________________
consensus          TCCGACACTCACCTCGACAGTCTCGTSGGGCARGCCTTGTTCGGCGACGGTGCGGCYGCC 240
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12R_rc ATGATCATTGGAGCAGACCCTGTCGAGAAYGTCGAGMGGCCAATCTTCGAGCTCRTMTCT
kyR_rc ATGATCATTGGMGCAGMCCYTKTSRAGAAYGTCGAGCGGCCAWTCTTCGAGCTCRTMTCT
telR_rc ATGATCATTGGAKCAGACCCTGTCGAGAAYGTCGAGCGGCCAATCTTCGAGCTCRTCTCT
ccR_rc ATGATCATYGGKGCMGAYCCYGTCGAGATCRTCGARCGGCCAATYTTYGAGCTCGTCTCY
gR_rc ATGATCATTGGAKCAGACCCTGTCGAGAAYGTCGAGCGGCCAATCTTCGAGCTCRTCTCT
gF ATGATCATTGGAGCAGACCCTGTCGAGAAYGTCGAGCGGCCAATCTTCGAGCTCRTCTCT
12F ATGATCATTGGAGCAGACCCTGTCGAGAAYGTCGAGCGGCCAATCTTCGAGCTCRTCTCT
kyF ATGATCATTGGAGCAGACCCTGTCGAGAAYGTCGAGCGGCCAATCTTCGAGCTCRTCTCT
telF ATGATCATTGGAGCAGACCCTGTCGAGAAYGTCGAGCGGCCAATCTTCRAGCTCRTCTCT
ccF MTGATCATCGGKGCWGAYCCYGTCKAGAKSRTCGARCGGCCAATYTTYGAGMTSGTCTCY

consensus ATGATCATTGGAGCAGACCCTGTCGAGAA**Y**GTCGAGCGGCCAATCTTCGAGCTC**R**TCTCT 300

. : . : . : . : . : . :
12R_rc GCAGCWCAGACTCTYTGYCCRGACAGTGAAGGTGCGATCGATGGGCATTTACGGGAAGTG
kyR_rc GCAGCACAGACTCTCTGCCCRGACAGTGAAGGTGCGATCGATGGGCATTTACGGGAAGTG
telR_rc GCAGCACAGACTCTCTGCCCRGACAGTGAAGGTGCGATCGATGGGCATTTACGGGAAGTG
ccR_rc GCSGCTCAGACTCTWTKCCCKRACAKYGAAGGWGCSATCGACGGGCATTTARGGGARGTG
gR_rc GCAGCACAGACTCTCTGCCCRGACAGTGAAGGTGCGATMGATGGGCATTTACGGGAAGTG
gF GCAGCWCAGACTCTCTGCCCRGACAGTGAAGGTGCGATMGATGGGCATTTACGGGAAGTG
12F GCAGCACAGACTCTCTGCCCRGACAGTGAAGGTGCGATCGATGGGCATTTACGGGAAGTG
kyF GCAGCACARACTCTCTGCCCGGACAGTGAAGGTGCGATCGATGGGCATTTACGGGAAGTG
telF GCAGCACARACTCTCTGCCCGGACAGTGAAGGTGCGATCGATGGGCATTTACGGGAAGTG
ccF GCRGCTCAGACTCTWTGCCCTGACWKSGAAGGWGCGATCGAYGGGCATYTACGGGARGTG

consensus GCAGCACAGACTCTCTGCCC**R**GACAGTGAAGGTGCGATCGATGGGCATTTACGGGAAGTG 360

. : . : . : . : . : . :
12R_rc GGGCTCACATTCCACCTGCTGAAGGATGTTCCGGGGATCATATCCAAGAACATCGAGAAG
kyR_rc GGGCTCACATTCCACCTGCTGAAGGATGTTCCGGGGATCATATCCAAGAACATCGAGAAG
telR_rc GGGCTCACATTCCACCTGCTGAAGGATGTTCCGGGGATCATATCCAAGAACATCGAGAAG
ccR_rc GGGCTCACMTTCCACYTRYTSAAGGATGTTCCRGGGATCATATCCAAGAACATCGAGAAG
gR_rc GGGCTCACATTCCACCTGCTGAAGGATGTTCCGGGGATCATATCCAAGAACATCGAGAAG
gF GGGCTCACATTCCACCTGCTGARGGATGTTCCGGGGATCATATCCAAGAACATCGAGAAG
12F GGGCTCACATTCCACCTGCTGAAGGATGTTCCGGGGATCATATCCAAGAACATCGAGAAG
kyF GGGMTCACATTCCACCTGCTGAAGGATGTTCCGGGGATCATATCCAAGAACATCGAGAAG
telF GGGCTCACATTCCACCTGCTGAAGGATGTTCCGGGGATCATATCCAAGAACATCGAGAAG
ccF GGGYTCMCWTTCCMCYTRCTSAAGGATGTYCCRGGGATCATATCCAARAACATCRAGAAG

consensus GGGCTCACATTCCACCTGCTGAAGGATGTTCCGGGGATCATATCCAAGAACATCGAGAAG 420

. : . : . : . : . : . :
12R_rc TGCCTTGACGACGCRTTCAAGYCATTGGATATATCAGATTGGAACTCGTTGTTCTGGATC
kyR_rc TGCCTTGACGACGCRTTCAAGYCATTGGATATATCAGATTGGAACTCGTTGTTCTGGATC
telR_rc TGCCTTGACGACGCRTTCAAGYCATTGGATATATCAGATTGGAACTCGTTGTTCTGGATC
ccR_rc WGCYTMRATGAKGCGTTCRGGCCSTTGGGKATATMRGATTGGAACTCGKTGTTYTGGATC
gR_rc TGCCTTGACGACGCRTTCAAGYCATTGGATATATCAGATTGGAACTCGTTGTTCTGGATC
gF TGCCTTGACGACGCRTTCAAGYCATTGGATATATCAGATTGGAACTCGTTGTTCTGGATC
12F TGCCTTGACGACGCRTTCAAGYCATTGGATATATCAGATTGGAACTCGTTGTTCTGGATC
kyF TGCCTTGACGACGCRTTCAAGYCATTGGATATATCAGATTGGAACTCGTTGTTCTGGATC
telF TGCCTTGACGACGCRTTCAAGYCATTGGATATATCAGATTGGAACTCGKTGTTCTGGATC
ccF TGCCTMGAYGAYGCGTTCRRGCCSTTGGGWATATCRRATKGGAACTCGTTGTTYTGGATC

consensus TGCCTTGACGACGC**R**TTCAAG**Y**CATTGGATATATCAGATTGGAACTCGTTGTTCTGGATC 480

. : . : . : . : . : . :
12R_rc GCKCATCCTGGGGGGCCRGSGATACTGGAYCAGGTGGAGGAGAAGCTGAAGCTGAAGG
kyR_rc GCKCATCCTGGKGGKCCAGCGATACTGGATCAGGTGGTGRMGAASCTGAAGCTGAAGG
telR_rc GCKCATCCTGGGGGGCCRGSGATACTGGATCAGGTGGAGGAGAAGCTGAAGCTGAAGGGG
ccR_rc GCGCATCCTGGTGGSCCAGSGATACTGGACCAGGTRGAGGRGAAGCTGAAGCTGAAGGAG
gR_rc GCKCATCCTGGTTT-CCTTC-ATACTGGATCAGGTGGAGGAGAAGCTGAAGCTGAAGGGG
gF GCKCATCCTGGGGGGCCRGCGATACTGGATCAGGTGGAGGAGAAGCTGAAGCTGAAGGGG

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12F              GCKCATCCTGGGGGGCCRGCGATACTGGATCAGGTGGAGGAGAAGCTGAAGCTGAAGGGG
kyF              GCKCATCCTGGGGGGCCRGCGATACTGGATCAGGTGGAGGAGAAGCTGAAGCTGAAGGGG
telF             GCKCATCCTGGGGGGCCRGCGATACTGGATCAGGTGGAGGAGAAGCTGAAGCTGAAGGGG
ccF              GCGCATCCTGGKGGSCCRGCGATACTGGACCAGGTRGAGGRGAAGCTGARSCTGAAGGYG
                 ____________________________________________________________
consensus        GCKCATCCTGGGGGGCCRGCGATACTGGATCAGGTGGAGGAGAAGCTGAAGCTGAAGGGG 540
                      .    :    .    :    .    :    .    :    .
gR_rc            GAGAAGATGAGG
gF               GAGAAGATGAGGGCGACGAGACAAGTGCTGAGCGAGWACGGGAACM
12F              GAGAAGATGAGGGCGACGAGACAAGTGCTRAGCGAGTAYGGMAAYA
kyF              GAGAAGATGAGGGCGACGAGACAAGTGCTRAGCGAGTACGGMAACA
telF             GAGAAGATGAGGGCGACGAGACAAGTGCTRAGCGAGTAYGGMAACA
ccF              GARAARATGAGGGCGACRAGAMARGKGCTWAGSGAGTAKGGAAACA
                 ______________________________________________
consensus        GAGAAGATGAGGGCGACGAGACAAGTGCTGAGCGAGTACGGMAACA 586
```

Appendix G: CAP3 alignment of the forward (F) and reverse (R) sequencing results after amplification of a *CHI* cDNA fragment in *Clivia miniata* var. *miniata* ‘Plantation’ (12), which produced a consensus sequence of 326 bp. “rc” refers to the reverse compliment.

```
                        .    :    .    :    .    :    .    :    .    :    .    :
12R_rc              GTTCACGGCCATCGGAGTGTACTTGGAGAGTGATGCTGTTAAGATACTTGYTGATAAATG
12F                                                             ACTTGCTGATAAATG
                    ____________________________________________________________
consensus           GTTCACGGCCATCGGAGTGTACTTGGAGAGTGATGCTGTTAAGATACTTGCTGATAAATG 60

                        .    :    .    :    .    :    .    :    .    :    .    :
12R_rc              GAGAGGGAAAGGAGCTGAAGAACTTGCTGATTCAATTGATTTCTTTAGAGATATCTACAC
12F                 GAGAGGGAAAGGAGCTGAAGAACTTGCTGATTCAATTGATTTCTTTAGAGATATCTACAC
                    ____________________________________________________________
consensus           GAGAGGGAAAGGAGCTGAAGAACTTGCTGATTCAATTGATTTCTTTAGAGATATCTACAC 120

                        .    :    .    :    .    :    .    :    .    :    .    :
12R_rc              AGGACCCTTTGAGAAGTTCACCAAAGTGMCAATGATTATCCYTYTAACTGGCGCMCAATA
12F                 AGGACCCTTTGAGAAGTTCACCAAAGTGACAATGATTATCCCTCTAACTGGCGCACAATA
                    ____________________________________________________________
consensus           AGGACCCTTTGAGAAGTTCACCAAAGTGACAATGATTATCCCTCTAACTGGCGCACAATA 180

                        .    :    .    :    .    :    .    :    .    :    .    :
12R_rc              CACCGAGAAGGTATCCGAGAACTGTGTTGCATACTGGAAAGYTATTGGTATTTMCACSGA
12F                 CACCGAGAAGGTATCCRARAACKGKGTTGCATACKGGAAAGCTATKGGTATYTACACCGA
                    ____________________________________________________________
consensus           CACCGAGAAGGTATCCGAGAACTGTGTTGCATACTGGAAAGCTATTGGTATTTACACCGA 240

                        .    :    .    :    .    :    .    :    .    :    .    :
12R_rc              AGCKGAAGASGCAGCCATCGAGAAATTCAAAGAAGTCTTCAGAAC
12F                 AGCTGAARACGCAGCCATCGAGAAATTCAAAGAAGTCTTCAGAACCGAGAACTTCCCTCC
                    ____________________________________________________________
consensus           AGCTGAAGACGCAGCCATCGAGAAATTCAAAGAAGTCTTCAGAACCGAGAACTTCCCTCC 300

                        .    :    .    :    .
12F                 GGGCGCCTCCATTCTCTTCACTCAAA
                    __________________________
consensus           GGGCGCCTCCATTCTCTTCACTCAAA 326
```

Appendix H: CAP3 alignment of the forward (F) and reverse (R) sequencing results of *F3H* cDNA fragments in *Clivia miniata* var. *miniata* ‘Plantation’ (12), *Clivia miniata* var. *citrina* ‘Kirstenbosch Yellow’ (ky), *Clivia miniata* var . *miniata* ‘Teleurstelling’ (tel), which produced a consensus sequence of 510 bp. “rc” refers to the reverse compliment of a sequence.

```
                       .    :    .    :    .    :    .    :    .    :    .    :
kyR_rc             GGAGATGACGAGGATGGCAAGAGAGTTTTTCGCGTKGCCGCCAGAGGACAAGTTGAGGTT
12R_rc             GGAGATGACGAGGATGGCAAGAGAGTTTTTCGCGTTGCCGCCAGAGGCCAAGTTGAGGTT
12F                               GGCAAGAGAGTTCTTCGCGTTGCCGCCAGAGGACAAGTTGAGGTT
kyF                                   AGAGAGTTCTTCGCGTTGCCGCCAGAGGACAAGTTGAGGTT
telF                                          CGCGTTGCCGCCAGAGGACAARTTGAGGTT
telR_rc            GGAGATGACGAGGATGGCAAGAGAGTTCTTCGCGTTGCCGCCAGAGGCCAARTTGAGGTT
                   ____________________________________________________________
consensus          GGAGATGACGAGGATGGCAAGAGAGTTCTTCGCGTTGCCGCCAGAGGACAAGTTGAGGTT 60

                       .    :    .    :    .    :    .    :    .    :    .    :
kyR_rc             TGATATGTCTGGTGGAAAGAAGGGTGGATTCATCGTGTCTAGCCACTYCCAGGGTGAAGC
12R_rc             TGATATGTCTGGTGGAAAGAAGGGTGGATTCATCGTGTCTAGCCACCTCCAGGGTGAAGC
12F                TGATAKGTMTGGTGGAAAGAAGGGTGGATTCATCGTGTCTAGCCACCTCCAGGGTGAAGC
kyF                TGATATGTCTGGTGGAAAGAAGGGTGGATTCATCGTGTCTAGCCACCTCCAGGGTGAAGC
telF               TKATATGTCTGGKGGAAAGAAGGGTGGATTSRTCGTGTCTAGSCACCTCCAGGGTGRAGC
telR_rc            TGATATGTCTGGTGGAAAGAAGGGTGGATTCATCGTGTCTAGCCACCTCCAGGGKGAAGC
                   ____________________________________________________________
consensus          TGATATGTCTGGTGGAAAGAAGGGTGGATTCATCGTGTCTAGCCACCTCCAGGGTGAAGC 120

                       .    :    .    :    .    :    .    :    .    :    .    :
kyR_rc             AGTCCAAGACTGGAGGGAGATTGTGACATTYTTTTCYTACCCAATAAAGGCCCGTGACTA
12R_rc             AGTCCAAGMCTGGAGGGAGATTGTGACATTCTTCTCCTACCCAATAAAGGCCCGTGACTA
12F                AGTACAAGACTGGAGGGAGATTGTGACATTCTTCTCCTACCCAATAAAGGCCCGTGACTA
kyF                AGTACAAGACTGGAGGGAGATTGTGACATTCTTCTCCTACCCAATAAAGGCCCGTGACTA
telF               AGTACAAGACTGGRGGGAGATTGKGACATTCTTSTCSTASCCAATAAAGGCCCGTGACTA
telR_rc            AGTCCAAGACTGGAGGGAGATTGTGACATTCTTCTCCTACCCAATAAAGGCCCGTGACTA
                   ____________________________________________________________
consensus          AGTACAAGACTGGAGGGAGATTGTGACATTCTTCTCCTACCCAATAAAGGCCCGTGACTA 180

                       .    :    .    :    .    :    .    :    .    :    .    :
kyR_rc             TTCAAGGTGSCCAGACAAGCCCGACGGTTGGATATCCGGTGCAGAAAAATACAGCGGAAA
12R_rc             TTCAAGGTGGCCAGACAAGCCCGACGGTTGGATTTCCGGTGCAGAAAAATMCAGCGGAAA
12F                TTCAAGGTGGCCAGACAAGCCCGACGGTTGGATATCCGGTGCAGAAAAATACAGCGGAAA
kyF                TTCAAGGTGGCCAGACAAGCCCGACGGTTGGATATCCGGTGCAGAAAAATACAGCGGAAA
telF               TTCAAGGTGGCCAGACAAGCCCGACGGTTGGATATCCGGYGSAGRAAAATACAGCGRAAA
telR_rc            TTCAAGGTGGCCAGACAAGCCCGACGGTTGGATTTCCGGTGCAGAAAAATACAGCGAAAA
                   ____________________________________________________________
consensus          TTCAAGGTGGCCAGACAAGCCCGACGGTTGGATATCCGGTGCAGAAAAATACAGCGGAAA 240

                       .    :    .    :    .    :    .    :    .    :    .    :
kyR_rc             AYTAATGGGATKGGCATGCAAAYTCTTGGGGGTCCTTTCSGAAGCCATGGGACTSGACCA
12R_rc             ACTAATGGGATTGGCATGCAAACTCTTGGGGGTCCTTTCSGAAGCCATGGGACTCGACCA
12F                ACTAATGGGATTGGCATGCAAACTCTTGGGGGTCCTTTCCGAAGCCATGGGACTCGACCA
kyF                ACTAATGGGATTGGCATGCAAACTCTTGGGGGTCCTTTCCGAAGCCATGGGACTCGACCA
telF               ACTAATGGGATTGGCATGCAAACTCTTGGGGGTCCTTTCCGAAGCCATGGGACTCGACCA
telR_rc            ACTAATGGGATTGGCATGCAAACTCTTGGGGGTCCTTTCSGAAGCCATGGGACTCGACCA
                   ____________________________________________________________
consensus          ACTAATGGGATTGGCATGCAAACTCTTGGGGGTCCTTTCCGAAGCCATGGGACTCGACCA 300
```

```
                             .         :         .         :         .         :
kyR_rc              CGAGGCCTTGACCAAGGCYTGSGTCGACAKGGACCAAAAGATGGTSGTCAATTTYTACCC
12R_rc              CGAGGCCTTGACCAAGGCCTGCGTCGACATGGACCAAAAGATGGTCGTCAATTTCTACCC
12F                 CGAGGCCTTGACCAAGGCCTGCGTCGACATGGACCAAAAGATGGTCGTCAATTTCTACCC
kyF                 CGAGGCCTTGACCAAGGCCTGCGTCGACATGGACCAAAAGATGGTCGTCAATTTCTACCC
telF                CGAGGCCTTGACCAAGGCCTGCGTCGACATGGACCAAAAGATGGTCGTCAATTTCTACCC
telR_rc             CGAGGCCTTGACCAAGGCCTGCGTCGACATGGACCAAAAGATGGTCGTCAATTTCTACCC
                    ____________________________________________________________
consensus           CGAGGCCTTGACCAAGGCCTGCGTCGACATGGACCAAAAGATGGTCGTCAATTTCTACCC 360

                             .         :         .         :         .         :
kyR_rc              AAAGTGTCCGCAGCYTGATYTCACTYTCGGTYGGAAGCGTCATACCGATCCKGGCACCAT
12R_rc              AAAGTGTCCGCACCCTGATCTCACTCTCGKTCGGAAGCGTCATACCGATCCKGGCACCAT
12F                 AAAGTGTCCGCAGCCTGATCTCACTCTCGGTCTGAAGCGTCATACCGATCCTGGCACCAT
kyF                 AAAGTGTCCGCAGCCTGATCTCACTCTCGGTCTGAAGCGTCATACCGATCCTGGCACCAT
telF                AAAGTGTCCGCAGCCTGATCTCACTCTCGGTCTGAAGCGTCATACCGATCCTGGCACCAT
telR_rc             AAAGTGTCCGCACCCTGATCTCACTCTCGKTCGGAAGCGTCATACCGATCCKGGCACCAT
                    ____________________________________________________________
consensus           AAAGTGTCCGCAGCCTGATCTCACTCTCGGTCGGAAGCGTCATACCGATCCTGGCACCAT 420

                             .         :         .         :         .         :
kyR_rc              CATTYKGYTTYTTCAGGATCAGGTKGGTGGCYTCCAGGCCACCAAGGACGGKGGAAAGAC
12R_rc              CATTYKGYTTYTTCAGGATCAGTTTGGGGGCYTCCAGSCCMCCAAGGMCGGTGGAAAGAC
12F                 CA
kyF                 CACTCTGCTTCTTCAGGATCAGGTTGGTGGCCTACAGGCCACCAAGGACGGTGGAAAGAC
telF                CACTCTGCTTCTTCAGGATCAGGTTGGTGGCCTACAGGCCACCAAGGACGGTGGAAAGAC
telR_rc             CATTYKGYTTYTTCAGGATCAGTTTGGGGGCYTCCAGSCCCCCAAGGNCCGTGGAAAGAC
                    ____________________________________________________________
consensus           CATTCTGCTTCTTCAGGATCAGGTTGGTGGCCTCCAGGCCACCAAGGACGGTGGAAAGAC 480

                             .         :         .         :         .         :
kyR_rc              TTGGATTACCGTTCAACCAG
12R_rc              TTG-ATTACCGTTCAACCAGTGGAGGGCGC
kyF                 TTGGATTACCGTTCAACCAGTGGAGGG
telF                TTGGATTACCGTTCAACCAGTGGAGGG
telR_rc             TTG-ATTACCYTTCAACCAGTGGAGGG
                    ______________________________
consensus           TTGGATTACCGTTCAACCAGTGGAGGGCGC 510
```

Appendix I: CAP3 alignment of the forward (F) and reverse (R) sequencing results of a *DFR* cDNA fragment in *Clivia miniata* var. *miniata* ‘plantation’ (12), *Clivia miniata* var. *citrina* ‘Kirstenbosch Yellow’ (ky), *Clivia miniata* var . *miniata* ‘Teleurstelling’ (tel), *Clivia miniata* var. *citrina* ‘Giddy’ (g) and *Clivia caulescens* (cc), which produced a consensus sequence of 227 bp. “rc” refers to the reverse compliment of a sequence.

```
                          .    :    .    :    .    :    .    :    .    :    .    :
gR_rc                 CAAAGATCCGGAGAACGAAGTGATCAARCCAGCAATAGTCGGAGTGTTGAGCATCATGAG
kyR_rc                              ACGAAGKGATCAAGCCAGCAATAGTCGGAGTGTTGAGCATCATGAG
telR_rc                             ACGAAGTGATCAARCCAGCAATAGTCGGAGTGTTGAGCATCATGAG
ccR_rc                                GAAGTGATCAARCCAGCAATAGTCGGAGTGTTGAGCATCATGAG
12R_rc                              ACGAAGTGATMAARCCAGCAATAGTCGGAGTGTTGAGCATCATGAG
kyF                                                               TGTTGAGCATCATGAG
telF                                                               GTTGAGCATCATGAG
12F                                                                    AGCATCATGAG
                      ____________________________________________________________
consensus             CAAAGATCCGGAGAACGAAGTGATCAAGCCAGCAATAGTCGGAGTGTTGAGCATCATGAG 60

                          .    :    .    :    .    :    .    :    .    :    .    :
gR_rc                 ATCATGTAAGAAAGCAAGGTCAGTCCAACGAGTTATTTTCACATCATCTGCAGGAACTGT
kyR_rc                ATCATGTAAGAAAGCAAGGTCAGTCCAACGRGTTATTTTCACATCATCTGCAGGAACTGT
telR_rc               ATCATGTAAGAAAGCAAGGTCAGTCCAACGAGTTATTTTCACATCATCTGCAGGAACTGT
ccR_rc                ATCATGTAAGAAAGCAAGGTCAGTCCAACGAGTTATTTTCACATCATCTGCAGGAACTGT
12R_rc                ATCATGTAAGAAAGCAAGGTCAGTCCAACGAGTTATTTTCACATCATCTGCAGGAACTGT
kyF                   ATCATGTAAGAA-GCAAGGTCAGTCCAACGAGTTATTTYYWCWTCAWCTGCAGGAACTGT
telF                  ATCATGTAAGAAAGCAAGGKCAGTCCMACGAGTTATTTTCACWTCATCTGCAGGAACTGT
gF                                 GCAAGGTCAGTCCAACGAGTTATTTTCACWTCATCTGCAGGAACTGT
ccF                                     GTCAGTCCAACGAGTTATTTTYACWTCATCTGCAGGAACTGK
12F                   ATCATGTAAGAAAGCAAGGKCAGTCCAACGAGTTATTTTCACATCATCTGCAGGAACTGT
                      ____________________________________________________________
consensus             ATCATGTAAGAAAGCAAGGTCAGTCCAACGAGTTATTTTCACATCATCTGCAGGAACTGT 120

                          .    :    .    :    .    :    .    :    .    :    .    :
gR_rc                 GAATATGGAGGAACGTCAAAAGCCTGAATACGATGAAAACTCATGGAGTGATATCGAGT
kyR_rc                GAWTAWGRAGGAACGTCAAAAGCCTGAATACGATGAAAACTCATGGAGTGATATCGAGT
telR_rc               GAATATGGAGGAACGTCAAAAGCCTGAATACGATGAAAACTCATGGAGT
ccR_rc                GAATATGGAGGAACGTCAAAAGCCTGAATACGATGAAAACTCATGGAGTGATATCGAGT
12R_rc                GAATATGGAGGAACGTCAAAAGCCTGAATACGATGAAAACTCATGGAGTGATATCGAGT
kyF                   GAATATGGAGGAACGTCAAAAGCCTGAATACGATGAAAACTCATGGAGTGATATCGAGTT
telF                  GAATWTGGAGGAACSTCAAAAGCCTGAATACGATGAAAACTCATGGAGTGATATCGAGTT
gF                    GAATATGGAGGAACGTCMAAAGCCTGAATACSATGAAAACTCATGGAGTGATATCGAGTT
ccF                   GAATATGGAGGAACGTCMAAAGCCTGAATACGATGAAAACTCATGGAGTGATATCGAGTT
12F                   GAATATGGAGGAACSYCMAAASCCTGAATACRATRAAAACTCATGGAGTGATATCGAGTT
                      ____________________________________________________________
consensus             GAATATGGAGGAACGTCAAAAGCCTGAATACGATGAAAACTCATGGAGTGATATCGAGTT 180

                          .    :    .    :    .    :    .    :    .
kyF                   CTGCATGCGCATAAAAATGACSGGATGGATGTACTTTGTATCCAA
telF                  CTGCATGCGCATAAAAATGACCGGATGGATGTACTTTGTATCCAA
gF                    CTGCATGCGCATAAAAATGACCGGATGGATGTAC
ccF                   CTGCATGCGCATAAAAATGACCGGATGGATGTA
12F                   CTGCATGCGCATAAAAATGACCGGATGGATGTACTTTGTATCCAA
                      _____________________________________________
consensus             CTGCATGCGCATAAAAATGACCGGATGGATGTACTTTGTATCCAA 225
```

Appendix J: Percentage matrix for similarity and identity analysis between a new *Clivia* consensus *CHS* cDNA sequence and the corresponding region in other plant *CHS* cDNA sequences. Identity values are in italics. GenBank accession numbers: *Allium cepa* (AF268382), *Hordeum vulgare* (X58339), *Lilium hybrid* cv. 'Acapulco' (AAD49355), *Lilium hybrid* division I (BAB40787), *Lilium hybrid* cultivar (ABF82595), *Lilium speciosum* (BAE79201), *Oryza sativa* (BAA19186), *Triticum aestivum* (ACJ22498), *Zea mays* C2 (X60204), *Callistephus chinensis* (Z67988), *Gerbera hybrida* (Z38096), *Ipomoea nil* (AB001818), *Vitis vinifera* (X75969), *Petroselinum crispum* (V01538), *Camellia sinensis* (D26594), *Glycine max* (FJ770471), *Solanum tuberosum* (U47739), *Nicotiana tabacum* (AF311783), *Antirrhinum majus* (X03710), *Petunia hybrida* (X14591).

	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**	**21**
1. CLIVIA		*72.9*	*72.0*	*70.8*	*72.1*	*75.3*	*75.7*	*73.7*	*73.5*	*71.7*	*72.5*	*72.3*	*72.4*	*73.1*	*69.4*	*72.2*	*70.6*	*70.4*	*70.8*	*71.5*	*69.6*
2. A. cepa	74.3		*75.9*	*75.9*	*76.3*	*75.1*	*75.7*	*76.1*	*74.5*	*72.9*	*69.8*	*73.5*	*73.5*	*71.0*	*72.4*	*68.8*	*70.8*	*68.0*	*69.8*	*74.5*	*68.6*
3. H. vulgare	73.5	75.1		*76.1*	*76.7*	*74.7*	*74.3*	*84.9*	*89.2*	*81.1*	*67.8*	*73.1*	*74.3*	*73.5*	*70.2*	*68.9*	*68.8*	*66.8*	*65.9*	*67.3*	*64.7*
4. Lilium 'Acapulco'	72.2	75.1	75.3		*93.9*	*78.2*	*77.1*	*78.2*	*74.3*	*73.3*	*70.2*	*73.7*	*73.9*	*74.5*	*72.0*	*72.4*	*68.8*	*68.8*	*70.6*	*69.4*	*69.2*
5. L. hybrid div 1	73.7	75.5	75.9	93.1		*79.0*	*78.0*	*78.2*	*74.3*	*73.3*	*70.8*	*72.9*	*74.3*	*75.3*	*72.9*	*72.0*	*69.0*	*69.2*	*70.4*	*69.4*	*68.8*
6. L. hybrid	76.5	74.3	73.9	77.3	78.2		*96.5*	*77.4*	*75.3*	*73.0*	*74.7*	*74.7*	*72.4*	*74.7*	*72.7*	*74.1*	*71.4*	*71.6*	*70.8*	*70.4*	*71.0*
7. L. speciosum	76.9	74.9	73.5	76.3	77.1	95.7		*76.8*	*75.3*	*73.0*	*74.7*	*74.3*	*71.2*	*73.9*	*73.1*	*73.5*	*71.4*	*71.6*	*71.2*	*71.0*	*70.6*
8. O. sativa	75.3	75.3	84.1	77.3	77.3	76.7	76.1		*80.8*	*85.9*	*69.6*	*76.4*	*75.7*	*70.8*	*68.6*	*69.2*	*68.8*	*67.1*	*68.0*	*69.2*	*65.7*
9. T. aestivum	74.9	73.7	88.4	73.5	73.5	74.5	74.5	80.0		*79.0*	*70.0*	*71.4*	*70.0*	*73.1*	*70.2*	*70.0*	*70.7*	*66.5*	*68.8*	*67.1*	*68.5*
10. Z. mays	72.9	72.2	80.4	72.7	72.7	72.4	72.4	85.3	78.4		*67.8*	*71.5*	*72.6*	*71.2*	*68.2*	*67.3*	*67.4*	*65.9*	*66.2*	*69.8*	*65.9*
11. C. chinensis	73.7	69.0	66.9	69.4	70.0	73.9	73.9	68.8	69.2	67.1		*81.0*	*67.8*	*73.1*	*73.9*	*71.4*	*72.7*	*72.7*	*72.5*	*69.6*	*71.5*
12. G. hybrida	73.5	72.9	72.2	72.9	72.0	73.9	73.5	75.7	70.6	70.8	80.2		*73.9*	*74.7*	*69.4*	*72.2*	*69.2*	*69.7*	*70.0*	*70.4*	*69.4*
13. I. nil	73.5	72.7	73.5	73.1	73.5	71.6	70.4	74.9	69.2	72.2	66.9	73.1		*72.9*	*70.0*	*72.2*	*72.0*	*71.6*	*71.8*	*69.4*	*71.2*
14. V. vinifera	74.1	70.2	72.7	73.7	74.5	73.9	73.1	70.0	72.2	70.4	72.2	74.1	72.0		*75.7*	*73.3*	*71.9*	*71.6*	*72.2*	*72.7*	*70.2*
15. P. crispum	70.0	71.6	69.4	71.2	72.0	71.8	72.2	67.8	69.4	67.3	73.1	68.6	69.2	74.9		*72.2*	*70.2*	*72.4*	*74.2*	*73.3*	*72.0*
16. C. sinensis	73.3	68.0	68.4	71.6	71.2	73.3	72.7	68.4	69.2	66.5	70.6	71.6	71.4	72.4	71.4		*77.1*	*77.6*	*76.9*	*72.2*	*78.6*
17. G. max	71.6	70.0	68.4	68.0	68.2	70.6	70.6	68.0	70.2	66.9	72.0	68.8	70.6	71.2	69.4	76.3		*73.3*	*77.1*	*71.8*	*76.3*
18. S. tuberosum	71.4	67.1	66.1	68.0	68.4	70.8	70.8	66.3	65.7	65.1	71.8	69.0	70.8	70.8	71.6	76.7	72.4		*85.7*	*69.8*	*85.3*
19. N. tabacum	71.8	69.0	65.1	69.8	69.6	70.0	70.4	67.1	68.6	65.5	71.8	69.6	71.0	71.4	73.7	76.1	76.3	84.9		*73.1*	*89.8*
20. A. majus	72.4	73.7	66.7	68.6	68.6	69.6	70.2	68.4	66.3	69.0	68.8	69.6	68.6	71.8	72.4	71.4	71.0	69.0	72.2		*74.7*
21. P. hybrida	71.0	67.8	63.9	68.4	68.0	70.2	69.8	64.9	68.0	65.7	70.8	69.0	70.4	69.4	71.2	77.8	75.5	84.5	89.0	73.9	

Appendix K: Percentage matrix for similarity and identity analysis between a new *Clivia miniata* var. *miniata* 'Plantation' consensus *CHI* cDNA sequence and the corresponding region in other plant *CHI* cDNA sequences. Identity values are in italics. GenBank accession numbers for protein sequences: *Oryza sativa* (AAO65886), *Hordeum vulgare* (AAM13449), *Allium cepa* (AAS48418), *Zea mays* (Q08704), *Medicago sativa* (1EYPA), *Phaseolus vulgaris* (CAA78763), *Pisum sativum* (AAA50174), *Dianthus caryophyllus* (CAA91931), *Ipomoea purpurea* (ABW69677), *Raphanus sativus* (AAB87071), *Saussurea medusa* (AAM48130), *Lotus japonicas* (BAC54038), *Glycine max* (AAK69432).

	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**
1. Clivia miniata		*65.2*	*65.8*	*74.8*	*66.1*	*63.9*	*63.9*	*60.0*	*66.7*	*66.4*	*66.1*	*64.2*	*64.2*	*64.8*
2. O. sativa	64.8		*88.5*	*62.7*	*87.6*	*54.8*	*57.6*	*51.5*	*66.1*	*67.3*	*65.5*	*59.7*	*56.6*	*59.1*
3. H. vulgare	65.5	88.2		*61.5*	*85.2*	*57.4*	*58.2*	*53.2*	*66.7*	*66.4*	*64.2*	*59.4*	*59.1*	*60.3*
4. A. cepa	73.6	62.4	61.2		*64.7*	*61.2*	*57.9*	*57.4*	*65.0*	*65.2*	*64.5*	*65.8*	*58.0*	*60.3*
5. Z. mays	64.8	87.3	84.8	63.6		*58.0*	*59.7*	*53.5*	*68.5*	*68.2*	*67.3*	*61.2*	*58.1*	*61.5*
6. M. sativa	62.7	54.5	57.3	60.0	57.0		*81.2*	*83.0*	*65.8*	*63.6*	*60.0*	*62.7*	*78.5*	*80.6*
7. P. vulgaris	62.7	57.3	57.9	56.7	58.5	80.0		*76.7*	*64.5*	*65.5*	*62.0*	*62.7*	*77.0*	*86.7*
8. P. sativum	59.1	51.5	53.3	57.0	52.7	82.1	75.8		*60.7*	*60.9*	*57.0*	*58.0*	*71.5*	*74.8*
9. D. caryophyllus	65.5	65.8	66.4	64.5	67.3	64.5	63.3	60.0		*70.3*	*68.5*	*68.2*	*61.8*	*65.8*
10. I. purpurea	65.2	67.0	66.1	63.9	67.0	62.4	64.2	60.0	69.1		*66.4*	*70.6*	*63.0*	*66.1*
11. R. sativus	64.8	65.2	63.9	63.3	66.1	58.8	61.2	56.1	67.3	65.2		*64.5*	*57.9*	*63.6*
12. S. medusa	63.0	59.4	59.1	64.5	60.0	61.5	61.5	57.3	67.0	69.4	63.3		*62.1*	*66.4*
13. L. japonicus	63.3	56.7	59.7	57.3	57.3	77.3	75.8	70.6	60.6	61.8	56.7	60.9		*78.8*
14. G. max	63.6	58.8	60.0	59.1	60.3	79.4	85.5	73.9	64.5	64.8	62.4	65.2	77.6	

Appendix L: Percentage matrix for similarity and identity analysis between a new *Clivia* consensus *F3H* cDNA sequence and the corresponding region in other plant *F3H* cDNA sequences. Identity values are in italics. GenBank accession numbers: *Allium cepa* (AY221246), *Lilium speciosum* (AB201532), *Bromheadia finlaysoniana* (X89199), *Hordeum vulgare* (EU921438), *Oryza sativa* (NM_001060692), *Triticum aestivum* (DQ208192), *Zea mays* (NM_001156993), *Anthurium andreanum* (DQ972935), *Ipomoea nil* (D83041), *Glycine max* (AY595420), *Gentiana triflora* (AB193311), *Fragaria x ananassa* (AY691919), *Vitis vinifera* (EF192467), *Citrus sinensis* (AB011795), *Solanum tuberosum* (AY102035), *Camellia sinensis* (AY641730), *Arabidopsis thaliana* (NM_114983), *Petunia hybrida* (AF022142).

	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**
1. CLIVIA		*81.2*	*78.2*	*75.1*	*74.5*	*72.4*	*74.9*	*72.9*	*75.7*	*74.5*	*75.3*	*75.1*	*75.5*	*76.9*	*74.9*	*72.9*	*74.9*	*71.8*	*74.1*
2. A. cepa	81.2		*75.7*	*75.1*	*72.4*	*72.0*	*72.9*	*71.6*	*72.9*	*74.1*	*73.5*	*77.1*	*73.5*	*76.5*	*72.8*	*76.7*	*76.9*	*72.7*	*74.9*
3. L. speciosum	78.2	75.7		*77.1*	*76.9*	*76.1*	*77.3*	*76.3*	*80.4*	*74.5*	*73.9*	*72.4*	*77.3*	*77.6*	*75.3*	*72.7*	*80.8*	*73.7*	*72.7*
4. B. finlaysoniana	75.1	75.1	77.1		*81.0*	*77.5*	*80.0*	*78.8*	*80.8*	*75.7*	*73.3*	*71.4*	*75.7*	*77.8*	*75.1*	*71.8*	*76.5*	*71.0*	*71.8*
5. O. sativa	74.5	72.4	76.9	81.0		*87.5*	*88.2*	*88.8*	*86.9*	*74.7*	*72.0*	*69.8*	*76.7*	*78.2*	*71.6*	*67.8*	*75.9*	*73.3*	*68.6*
6. T. aestivum	72.4	72.0	76.1	77.5	87.5		*87.3*	*94.9*	*87.1*	*72.9*	*72.5*	*70.4*	*75.9*	*75.7*	*70.3*	*67.8*	*75.5*	*72.0*	*68.0*
7. Z. mays	74.9	72.9	77.3	80.0	88.2	87.3		*88.6*	*88.2*	*74.5*	*72.9*	*70.2*	*78.6*	*77.8*	*70.8*	*68.0*	*77.5*	*72.7*	*69.0*
8. H. vulgare	72.9	71.6	76.3	78.8	88.8	94.9	88.6		*87.5*	*73.3*	*71.6*	*70.8*	*76.5*	*76.7*	*69.8*	*67.8*	*75.5*	*71.6*	*68.4*
9. A. andreanum	75.7	72.9	80.4	80.8	86.9	87.1	88.2	87.5		*76.9*	*76.1*	*70.8*	*79.2*	*80.6*	*71.6*	*69.8*	*79.6*	*73.5*	*70.4*
10. I. nil	74.5	74.1	74.5	75.7	74.7	72.9	74.5	73.3	76.9		*76.7*	*75.5*	*79.2*	*77.8*	*79.2*	*78.8*	*78.6*	*73.9*	*78.2*
11. G. max	75.3	73.5	73.9	73.3	72.0	72.5	72.9	71.6	76.1	76.7		*74.7*	*78.2*	*77.1*	*75.9*	*75.7*	*79.0*	*75.9*	*76.9*
12. G. triflora	75.1	77.1	72.4	71.4	69.8	70.4	70.2	70.8	70.8	75.5	74.7		*77.1*	*74.9*	*72.9*	*78.2*	*75.7*	*72.5*	*77.5*
13. F. ananassa	75.5	73.5	77.5	75.7	76.7	75.9	78.6	76.5	79.2	79.2	78.2	77.1		*82.5*	*78.8*	*74.9*	*78.8*	*77.6*	*76.1*
14. V. vinifera	76.9	76.5	77.6	77.8	78.2	75.7	77.8	76.7	80.6	77.8	77.1	74.9	82.5		*77.1*	*75.1*	*81.4*	*76.3*	*74.9*
15. C. sinensis	74.9	72.9	75.3	75.1	71.6	70.6	70.8	69.8	71.6	79.2	75.9	72.9	78.8	77.1		*77.5*	*77.3*	*74.7*	*78.5*
16. S. tuberosum	72.9	76.7	72.7	71.8	67.8	67.8	68.0	67.8	69.8	78.8	75.7	78.2	74.9	75.1	77.5		*76.9*	*71.8*	*89.8*
17. Camellia sinensis	74.9	76.9	80.8	76.5	75.9	75.5	77.5	75.5	79.6	78.6	79.0	75.7	78.8	81.4	77.3	76.9		*76.9*	*75.9*
18. A. thaliana	71.8	72.7	73.7	71.0	73.3	72.0	72.7	71.6	73.5	73.9	75.9	72.5	77.6	76.3	74.7	71.8	76.9		*74.1*
19. P. hybrida	74.1	74.9	72.7	71.8	68.6	68.0	69.0	68.4	70.4	78.2	76.9	77.5	76.1	74.9	78.8	89.8	75.9	74.1	

Appendix M: Percentage matrix for similarity and identity analysis between a new *Clivia* consensus *DFR* cDNA sequence and the corresponding region in other plant *DFR* cDNA sequences. Identity values are in italics. GenBank accession numbers: *Lilium speciosum* (AB201531), *Bromheadia finlaysoniana* (AF007096), *Zea mays* A1 (Y16041), *Antirrhinum majus* (X15536), *Callistephus chinensis* (Z67981), *Dianthus caryophyllus* DFRA (Z67983), *Forsythia x intermedia* (Y09127), *Gerbera hybrida* (ZZ17221), *Ipomoea purpurea* DFRB (AB018438), *Ipomoea batatas* (EU360845), *Ipomoea nil* DFRB (AB006792), *Malus domestica* (AF117268), *Petunia hybrid* DFRA (X15537), *Vitis vinifera* (X75964), *Solanum tuberosum* (AF449422), *Gentiana triflora* (D85185), *Torenia hybrida* (AB012924), *Fragaria x ananassa* (AY695812), *Arabidopsis thaliana* (NM_123645), *Spinacia oleracea* (AB246750), *Citrus sinensis* (AY519363), *Allium cepa* DFR-A (AY221250), *Agapanthus praecox* (AB099529), *Cymbidium hybrid* (AF017451), *Triticum aestivum* DFR-A (AB162138).

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26
1. CLIVIA		*81.2*	*78.6*	*77.7*	*73.4*	*72.5*	*72.1*	*72.1*	*72.3*	*70.3*	*69.4*	*69.9*	*69.9*	*66.4*	*65.5*	*66.7*	*70.0*	*66.5*	*66.1*	*68.6*	*69.9*	*69.6*	*69.4*	*69.6*	*67.8*	*72.9*
2. A. cepa	80.3		*81.2*	*76.4*	*71.6*	*71.2*	*71.2*	*70.3*	*74.2*	*70.3*	*70.3*	*70.7*	*70.3*	*70.3*	*64.2*	*63.9*	*70.9*	*67.8*	*67.0*	*69.4*	*69.9*	*68.7*	*68.1*	*68.1*	*69.6*	*71.2*
3. A. praecox	77.7	80.3		*75.5*	*71.2*	*69.4*	*72.5*	*71.2*	*72.1*	*69.0*	*69.9*	*67.2*	*68.1*	*66.4*	*67.4*	*64.1*	*66.5*	*61.7*	*61.3*	*67.2*	*69.0*	*66.5*	*66.4*	*68.3*	*64.3*	*71.2*
4. L. speciosum	76.9	75.5	74.7		*69.9*	*68.1*	*68.1*	*71.0*	*68.6*	*74.2*	*66.5*	*69.9*	*68.1*	*67.2*	*65.5*	*69.3*	*71.1*	*66.1*	*66.5*	*72.4*	*64.6*	*68.7*	*73.4*	*67.7*	*68.7*	*71.6*
5. V. vinifera	72.5	70.7	70.3	69.0		*72.1*	*72.1*	*75.5*	*76.0*	*68.1*	*70.3*	*67.2*	*72.1*	*66.8*	*69.9*	*70.7*	*72.1*	*69.9*	*70.7*	*65.1*	*70.3*	*69.4*	*65.8*	*69.0*	*69.0*	*73.8*
6. G. triflora	71.6	70.3	68.6	67.2	71.2		*73.9*	*72.3*	*74.2*	*66.8*	*76.9*	*65.1*	*68.0*	*70.7*	*74.7*	*71.2*	*76.4*	*74.7*	*74.7*	*64.2*	*77.3*	*76.0*	*69.1*	*76.4*	*73.8*	*72.1*
7. A. thaliana	71.2	70.3	71.6	67.2	71.2	73.4		*74.7*	*71.6*	*65.1*	*70.7*	*65.1*	*72.5*	*72.5*	*68.6*	*69.9*	*68.6*	*64.3*	*64.3*	*66.4*	*73.8*	*69.4*	*64.6*	*72.9*	*69.4*	*70.3*
8. S. oleracea	71.2	69.4	70.3	70.7	74.7	72.1	73.8		*73.4*	*67.2*	*73.8*	*62.9*	*71.2*	*76.4*	*71.2*	*67.7*	*71.6*	*68.0*	*68.8*	*66.4*	*73.4*	*69.4*	*64.6*	*70.7*	*69.0*	*73.8*
9. C. sinensis	72.1	73.4	71.2	67.7	75.1	73.4	70.7	72.5		*67.2*	*72.5*	*66.4*	*73.8*	*71.2*	*68.6*	*69.4*	*69.4*	*67.2*	*67.7*	*66.4*	*71.2*	*72.5*	*66.8*	*71.2*	*71.6*	*75.1*
10. B. finlaysoniana	69.4	69.4	68.1	73.4	69.0	65.9	64.2	66.4	66.4		*65.5*	*71.6*	*65.9*	*66.4*	*62.9*	*63.9*	*68.0*	*66.5*	*65.9*	*72.5*	*62.0*	*64.9*	*88.6*	*67.5*	*62.3*	*72.8*
11. T. hybrida	68.6	69.4	69.0	65.9	69.4	76.0	69.9	72.9	71.6	64.6		*65.5*	*64.9*	*69.0*	*74.2*	*69.0*	*72.9*	*71.2*	*71.6*	*65.8*	*79.0*	*72.1*	*67.0*	*73.8*	*71.6*	*70.4*
12. T. aestivum	69.0	69.9	66.4	69.0	66.4	64.2	64.2	62.0	65.5	70.7	64.6		*63.3*	*61.6*	*64.6*	*63.3*	*62.0*	*64.3*	*64.3*	*85.2*	*62.9*	*64.6*	*69.0*	*62.4*	*61.6*	*72.3*
13. F. x. ananassa	69.0	69.4	67.2	67.2	71.2	67.7	71.6	70.3	72.9	65.1	64.6	62.4		*67.2*	*67.5*	*67.2*	*67.5*	*66.2*	*66.2*	*65.9*	*65.8*	*68.8*	*64.2*	*63.0*	*68.0*	*76.9*
14. D. caryophyllus	66.4	69.4	65.5	66.4	65.9	69.9	71.6	74.2	70.3	65.5	68.1	60.7	66.4		*70.3*	*66.4*	*71.6*	*65.5*	*65.9*	*63.3*	*70.3*	*68.1*	*66.8*	*66.8*	*71.6*	*72.5*
15. C. chinensis	64.6	63.3	66.8	64.6	69.0	73.8	67.7	70.3	67.7	62.0	73.4	63.8	67.2	69.4		*83.8*	*71.2*	*73.4*	*74.2*	*65.9*	*75.1*	*72.9*	*64.6*	*72.5*	*72.9*	*68.6*
16. G. hybrida	66.4	63.3	63.8	69.0	69.9	70.3	69.0	66.8	68.6	63.3	68.1	62.4	66.4	65.5	83.0		*72.5*	*69.0*	*69.9*	*64.2*	*72.9*	*70.7*	*63.0*	*69.0*	*71.2*	*67.7*
17. I. batatas	69.4	70.3	65.9	71.2	71.2	75.5	67.7	70.7	68.6	67.7	72.1	61.1	67.2	70.7	70.3	71.6		*79.5*	*80.3*	*63.3*	*71.6*	*77.3*	*64.8*	*73.4*	*76.9*	*68.6*
18. I .nil	65.9	67.2	61.1	65.5	69.0	73.8	63.8	67.7	66.4	65.9	70.3	63.8	65.9	64.6	72.5	68.1	78.6		*98.7*	*62.4*	*68.6*	*76.9*	*65.2*	*72.5*	*76.4*	*65.9*
19. I. purpurea	65.5	66.4	60.7	65.9	69.9	73.8	63.8	68.6	66.8	65.1	70.7	63.8	65.9	65.1	73.4	69.0	79.5	97.8		*63.3*	*69.4*	*76.9*	*65.2*	*72.5*	*76.4*	*66.8*
20. Z. mays	67.7	68.6	66.4	72.5	64.2	63.3	65.5	65.5	65.5	71.6	65.5	84.3	65.1	62.4	65.1	63.3	62.4	61.6	62.4		*63.8*	*65.9*	*70.3*	*64.6*	*62.0*	*71.6*
21. A. majus	69.0	69.0	68.1	63.8	69.4	76.4	72.9	72.5	70.3	61.1	78.2	62.0	65.5	69.4	74.2	72.1	70.7	67.7	68.6	62.9		*70.7*	*63.8*	*79.0*	*69.0*	*69.0*
22. P. hybrida	69.0	68.1	65.9	68.1	68.6	75.1	68.6	68.6	71.6	64.6	71.2	63.8	68.6	67.2	72.1	69.9	76.4	76.0	76.0	65.1	69.9		*63.3*	*76.4*	*90.4*	*71.0*
23. Cymbidium	68.6	67.2	65.5	72.5	65.5	68.6	63.8	63.8	65.9	87.8	66.4	68.1	63.3	65.9	63.8	62.4	64.2	64.6	64.6	69.4	62.9	62.4		*67.8*	*61.6*	*71.1*
24. Forsythia	69.0	67.2	67.7	66.8	68.1	75.5	72.1	69.9	70.3	67.2	72.9	61.6	63.8	65.9	71.6	68.1	72.5	71.6	71.6	63.8	78.2	75.5	67.2		*72.1*	*68.1*
25. S.tuberosum	67.2	69.0	63.8	68.1	68.1	72.9	68.6	68.1	70.7	62.0	70.7	60.7	67.7	70.7	72.1	70.3	76.0	75.5	75.5	61.1	68.1	89.5	60.7	71.2		*69.3*
26. M.domestica	72.1	70.3	70.3	71.6	72.9	71.2	69.4	72.9	74.2	72.9	69.9	72.1	76.0	71.6	67.7	66.8	67.7	65.1	65.9	70.7	68.1	70.7	71.2	67.2	69.0	

Appendix N: Multiple alignment of CHS amino acid sequences corresponding to the deduced amino acid region in *Clivia*. Asterisks over the C, F, H and N residues that are also highlighted in grey indicate the catalytic residues (Cys163, Phe215, His303 and Asn336, respectively) of CHS. Conserved areas are highlighted in black. GenBank accession numbers: *Hordeum vulgare* (M98871), *Lilium hybrid* cv. 'Acapulco' (AAD49355), *Lilium hybrid* division I (BAB40787), *Lilium hybrid* cultivar (ABF82595), *Lilium speciosum* (BAE79201), *Oryza sativa* (BAA19186), *Triticum aestivum* (ACJ22498), *Zea mays* C2 (X60204), *Bromheadia finlaysoniana* CHS3 (AAB62876), *Callistephus chinensis* (Z67988), *Gerbera hybrida* (Z38096), *Ipomoea* purpurea (AB001826), *Perilla frutescens* (BAA19656), *Vitis vinifera* (X75969), *Camellia sinensis* (D26594), *Glycine max* (FJ770471), *Solanum tuberosum* (U47739), *Solanum pinnatisectum* (AAX63402), *Nicotiana tabacum* (AF311783), *Antirrhinum majus* (X03710), *Petunia hybrida* (X14591).

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CLIVIA              YQLTKLLGLRPSVKRLMMYQQGCFAXGTVLRXAKDLAENNRXARVLVVCSXITAVXFRGP
Lil.hybrid          YQLTKLLGLRPSVNRFMMYQQGCFAGGTVLRFAKDLAENNCDARVLVVCSEITAVTFRGP
L.hybrid div I      YQLTKLLGLRPSVNRFMMYQQGCFAGGTVLRLAKDLAENNRGARVLVVCSEITAVTFRGP
L.speciosum         YQLTKLLGLRPSVNRFMMYQQGCFAGGTVLRLAKDLAENNRGARVLVVCSEITAVTFRGP
O.sativa            YQLAKMLGLRPNVNRLMMYQQGCFAGGTVLRVAKDLAENNRGARVLAVCSEITAVTFRGP
H.vulgare           YQLTKMLGLRPSVKRLMMYQQGCFAGGTVLRLAKDLAENNRGARVLVVCSEITAVTFRGP
Lilium              YQLTKLPGLRPSVNRFMMYQQGCFAGGSVLLLSKDLAENNRGARVLVVCSEITAVTFRGP
T.aestivum          YQLTKMLGLCPSVKRLMMYQQGCFAGGTVLRLAKDLVENNRGARVLVVCSEITAVTFRGP
Z.mays              YQLTKALGLXXVVNRLMMYQQGCFAGGTVLRVAKDVAENNRGARVMVVCSEITAVTFRGP
B.finlaysoniana     YQLTRLLGLRPSVNRFMLYQQGCFAGGTVLRLAKDLAENNAGARVLVVCSEITAVTFRGP
C.chinensis         YQLTKLLGLRPSVKRFMMYQQGCFAGGTVLRLAKDLAENNKGARVLVVCSEITAVTFRGP
G.hybrida           YQLTKLLGLRPSVKRFMMYQQGCFAGGTVLRLAKDLAENNKGARVLVVCSEITAVTFRGP
P.frutescens        YQLTKLLGLRPSVKRFMMYQQGCYAGGTVLRMAKDLAENNAGARVLVVCSEITAVTFRGP
V.vinifera          YQLTKLLGLKPSVKRLMMYQQGCFAGGTVLRLAKDLAENNAGSRVLVVCSEITAVTFRGP
C.sinensis          YQLTKLLGLRPSVKRLMMYQQGCFAGGTVLRLAKDLAENNKGARVLVVCSEITAVTFRGP
S.tuberosum         YQLAKLLGLRPSVKRLMMYQQGCFVGGTVLRLAKDLAENNKGARVLVVCSEITAVTFRGP
S.pinnatisectum     YQLTKLLGLRPSVKRLMMYQQGCFAGGTVLRLAKDLAENNKGARVLVVCSEITAVTFRGP
N.tabacum           YQLTKLLGLRPSVKRFMMYQQGCFAGGTVLRMAKDLAENNKGARVLVVCSEITAVTFRGP
A.majus             YQLTKLLGLRPSVKRFMMYQQGCFAGGTVLRMAKDLAENNAGARVLVVCSEITAVTFRGP
I.purpurea          YQLTKLLGLQPSVKRFMMYQQGCFAGGTVIRLAKDLAENNKGARVLVVCSEITAVTFRGP
P. hybrida          YQLTKLLGLRPSVKRLMMYQQGCFAGGTVLRLAKDLAENNKGARVLVVCSEITAVTFRGP

                                 *
CLIVIA              SDTHLDSLXGXALFGDGAXAMIIGADPVEXVERPIFELXSAAQTLCXDSEGAIDGHLREV
Lil.hybrid          SESHLDSLVGQALFGDGAAAVIVGSDPDTSVERPLFQIVSASQTILPDSDGAIDGHLREV
L.hybrid div I      SESHLDSLVGQALFGDGAAAVIVGSDPDTAVERPLFELVSASQTILPDSEGAIDGHLREV
L.speciosum         SESHLDSLVGQALFGDGAAAVIVGSDPDTAVERPLFELVSASQTILPDSEGAIDGHLREV
O.sativa            SESHLDSMVGQALFGDGAAAVIVGSDPDEAVERPLFQMVSASQTILPDSEGAIDGHLREV
H.vulgare           HESHLDSLVGQALFGDGAAAVIIGADPDLSVERPLFQLVSASQTILPDSEGAIDGHLREV
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Lilium              SESHLDSLVGQALFGDGAAAVIVGSDPEPSVERSLFQIVSASQTILPDSEGAIDGHLREV
T.aestivum          HESHLDSLVGQALFGDGAAAVIIGADPDESIERPLFQLVSASQTILPDSEGAIDGHLREV
Z.mays              SESHVDSLVGQALFGDGAAAGRGGADPDGRVERPLFQLVSAAQTILPDSEGAIDGHLREV
B.finlaysoniana     SESHLDSLVGQALFGDGAAAIIVGSDPDSATERPLFQLVSASQTILPESEGAIDGHLREI
C.chinensis         NDTHLDSLVGQALFGDGAAAVIVGADPDLTTERPLFEMISAAQTILPDSEGAIDGHLREV
G.hybrida           NDTHLDSLVGQALFGDGAAAVIVGSDPDLTTERPLFEMVSAAQTILPDSEGAIDGHLREV
P.frutescens        SESHLDSLVGQALFGDGAAAVIVGSDPVVGVERPLFQLVSAAQTILPDSDGAIDGHLREV
V.vinifera          SDTHLDSLVGQALFGDGAAAVIIGADPDTKIELPLFELVSAAQTILPDSEGAIDGHLREV
C.sinensis          SDAHLDSLVGQALFGDGAAAIIVGSDPIPEVEKPLFELVSAAQTILPDSDGAIDGHLREV
S.tuberosum         SESHLDSLVGQALFGDGAAAIIMGSDPIIGVERPLFELVSAAQTLVPDSEGAIDGHLREV
S.pinnatisectum     NESHLDSLVGQALFGDGAAAIIIGSDPIISVERPLFELVSAAQALVPDSEGAIDGHLREV
N.tabacum           NDTHLDSLVGQALFGDGAAAVIIGSDPIPEVERPLFELVSAAQTLLPDSEGAIDGHLREV
A.majus             ADTHLDSLVGQALFGDGAAAVIVGSDPVVGVERPLFQIVTAAQTLLPDSHGAIDGHLREV
I.purpurea          SDAHLDSLVGQALFGDGAAALIIGSDPDPDLERPLFQLVSAAQTILPDSGGAIDGHLREV
P. hybrida          NDTHLDSLVGQALFGDGAGAIIIGSDPIPGVERPLFELVSAAQTLLPDSHGAIDGHLREV
                                                                         *
CLIVIA              GLTFHLLKDVPGIISKNIEKCLDDXFKXLDISDWNSLFWIXHPGGXAILDQVEEKLKLKG
Lil.hybrid          GLTFHLLKDVPGLISKNIEKSLTQAFAPLGITDWNSIFWIAHPGGPAILDQVELKLALDK
L.hybrid div I      GLTFHLLKDVPGLISKNIEKSLTGAFAPLGISDWNSLFWIAHPGGPAILDQVAAKLGLQK
L.speciosum         GLTFHLLKDVPGLISKNIERSLTGAFAPLGISDWNSLFWIAHPGGPAILDQVEAKLGLQK
O.sativa            GLTFHLLKDVPGLISKNIERALGDAFTPLGISDWNSIFWVAHPGGPAILDQVEAKVGLDK
H.vulgare           GLTFHLLKDVPGLISKNIERALEEAFKPLGIDHWNSVFWIAHQGGPAILDMVEAKVNLNK
Lilium              GLTFHLLKDVPGLISKNIEKSLVQAFAPLGITDWNSIFWIAHPGGPAILDQVELKLALEK
T.aestivum          GLTFHLLKDVPGLISKNIERALEDAFKPLGINDWNSVFWIAHPGGPAILDMVEAKVNLNK
Z.mays              GLAFHLLKDVPGLISKNIERALEDAFEPLGISDWNSIFWVAHPGGPAILDQVEAKVGLDK
B.finlaysoniana     GLTFHLLKDVPGLISKNIQKCLLDAFKPLGVHDWNSIFWIAHPGGPAILDQVEIKLGLKA
C.chinensis         GLTFHLLKDVPGLISKNIEKALTQAFSPLGITDWNSIFWIAHPGGPAILDQVELKLGLKE
G.hybrida           GLTFHLLKDVPGLISKNIEKALTTAFSPLGINDWNSIFWIAHPGGPAILDQVELKLGLKE
P.frutescens        GLTFHLLKDVPGLISKNIEKSLKEAFGPLGISDWNSVFWIAHPGGPAILDQVEAKLGLKP
V.vinifera          GLTFHLLKDVPGLISKNIEKSLVEAFTPIGISDWNSLFWIAHPGGPAILDQVELKLGLKE
C.sinensis          GLTFHLLKDVPGLISKNIEKSLNEAFQPLNITDWNSLFWIAHPGGPAILDQVELKLALKP
S.tuberosum         GLTFHLLKDVPGLISKNIEKSLLEAFQPLGISDWNSLFWIAHPGGPAILDQVELKLGLKQ
S.pinnatisectum     GLTFHLLKDVPGLISKNIEKSLVEAFQPIGISDWNSLFWIAHPGGPAILDQVELKLGLKP
N.tabacum           GLTFHLLKDVPGLISKNIEKSLVEAFQPLGISDWNSLFWIAHPGGPAILDQVELKLGLKQ
A.majus             GLTFHLLKDVPGLISKNIEKSLKEAFDPLGISDWNSVFWIAHPGGPAILDQVEEKLGLKP
I.purpurea          GLTFHLLKDVPGLISKHIEKSLNEAFQPLGIRDWNSLFWIAHPGGPAILDQVEEKLELKP
P. hybrida          GLTFHLLKDVPGLISKNIEKSLEEAFRPLSISDWNSLFWIAHPGGPAILDQVEIKLGLKP
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                                   *
CLIVIA                EKMRATRQVLSEYXN
Lil.hybrid            KKMQATRHVLSEYGN
L.hybrid div I        EKMRATRHVLSEYGN
L.speciosum           EKMRATRHVLSEYGN
O.sativa              ERMRATRHVLSEYGN
H.vulgare             ERMRATRHVLSEYGN
Lilium                KKMRATRHVLSEYGN
T.aestivum            ERMRATRHVLSEYGN
Z.mays                ARMRATRHVLSEYGN
B.finlaysoniana       EKLAASRNVLAEYGN
C.chinensis           EKMRATRHVLSEYGN
G.hybrida             EKLRATRHVLSEYGN
P.frutescens          EKLRSTRHVLGEYGN
V.vinifera            EKLRATRHVLSEYGN
C.sinensis            EKLRATRHVLSEYGN
S.tuberosum           EKLRATREVLSNYGN
S.pinnatisectum       EKLRATREVLSNYGN
N.tabacum             EKLKATRKVLSNYGN
A.majus               EKLRSTRQVLSEYGN
I.purpurea            EKLRATRHVLSEYGN
P. hybrida            EKLKATRNVLSNYGN
```

Appendix O: Multiple alignment of F3H amino acid sequences corresponding to the deduced amino acid region in *Clivia*. GenBank accession numbers: *Allium cepa* (AY221246), *Lilium speciosum* (AB201532), *Bromheadia finlaysoniana* (X89199), *Hordeum vulgare* (EU921438), *Oryza sativa* (NM_001060692), *Triticum aestivum* (DQ208192), *Zea mays* (NM_001156993), *Anthurium andreanum* (DQ972935), *Ipomoea nil* (D83041), *Glycine max* (AY595420), *Gentiana triflora* (AB193311), *Fragaria x ananassa* (AY691919), *Vitis vinifera* (EF192467), *Citrus sinensis* (AB011795), *Solanum tuberosum* (AY102035), *Camellia sinensis* (AY641730), *Arabidopsis thaliana* (NM_114983), *Petunia hybrida* (AF022142). The positions of three of the five conserved 2-ODD-type motifs are labeled with bold lines. Asterisks indicate conserved amino acid residues necessary for ligating ferrous iron. Conserved areas are highlighted in black.

```
CLIVIA              EMTRMAREFFALPPEDKLRFDMSGGKKGGFIVSSHLQGEAVQDWREIVTFFSYPIKARD
A. cepa             DMTKMAREFFALPPEEKLRFDMSGGKKGGFIVSSHLQGEAVQDWREIVTYFSYPIRARD
L. speciosum        EMNRLAREFFALPPEDKLRFDMTGGKKGGFIVSSHLQGEAVQDWREIVTYFSYPIRVRD
B. finlaysoniana    DMTRLAREFFELPPEEKLRFDMSGGKKGGFIVSSHLQGEVVQDWREIVTYFSYPIRTRD
O. sativa           DMARLARDFFALPPEDKLRFDMSGGKKGGFIVSSHLQGEAVKDWREIVTYFSYPVKSRD
T. aestivum         DMTRLSREFFALPAEDKLRYDMSGGKKGGFIVSSHLQGEAVQDWREIVTYFSYPVKARD
Z. mays             DMARLARDFFALPPEDKLRFDMSGGKKGGFIVSSHLQGEAVQDWREIVTYFSYPVKARD
H. vulgare          DMTRLAREFFALPAEDKLRYDMSGGKKGGFIVSSHLQGEAVQDWREIVTYFSYPVKARD
A. andreanum        DMTRLATEFFALPPEDKLRYDMSGGKKGGFIVSSHLQGEAVQDWREIVTYFSYPVRARD
I. nil              EMTRLSKDFFSLPPEEKLLFDMSGGKKGGFIVSSHLQGEAVKDWREIVTYFSYPVRARD
G. max              EMTRLAKEFFALPPDEKLRFDMSGAKKGGFIVSSHLQGESVQDWREIVTYFSYPKRERD
G. triflora         EMTRLAREFFDLPPEEKLRFDMSGGKKGGFIVSSHLQGEAVRDWREIVTYFSYPIKSRD
F. x ananassa       EMTRLAREFFALPPEEKLRFDMSGGKKGGFIVSSHLQGEAVQDWREIVTYFSYPVRHRD
V. vinifera         EMTRLAREFFALPPEEKLRFDMSGGKKGGFIVSSHLQGEAVQDWREIVTYFSYPLRTRD
C. sinensis         DMTRLATEFFALPPEEKLKFDMSGGKKGGFIVSSHLQGEVVKDWREIVTYFSFPKQSRD
S. tuberosum        EMTKLAKEFFELPPDEKLRFDMSGGKKGGFIVSSHLQGEVVQDWREIVTYFSYPIRARD
Camellia sinensis   EMTRLAREFFALPPEEKLRFDMSGGKKGGFIVSSHLQGEAVQDWREIVTYFSYPIRARD
A. thaliana         DMTRLARDFFALPPEDKLRFDMSGGKKGGFIVSSHLQGEAVQDWREIVTYFSYPVRNRD
P.hybrida           QMTTFAKEFFALPPEEKLRFDMSGGKKGGFIVSSHLQGEVVQDWREIVTYFSYPTRARD
```

Motif 2

```
CLIVIA              YSRWPDKPDGWISGAEKYSGKLMGLACKLLGVLSEAMGLDHEALTKACVDMDQKMVVNF
A. cepa             YSRWPDKPEGWISVAEKYSEKLMDLACKLLGIPSEAMGLDTEALTKACIDMDQKMVVNF
L. speciosum        YSRWPDKPEGWRAVVEAYSEQLMGLACKLLGVLSEAMGLDKEALTKACVDMDQKIVVNF
B. finlaysoniana    YSRWPDKPEGWRAVVEEYSAKLMELACNLLGVLSEAMGLDREALAQACVDMDQKLVVNF
O. sativa           YSRWPDKPAGWRAVVEQYSERLMGLACKLLGVLSEAMGLDTNALADACVDMDQKVVVNF
T. aestivum         YGRWPEKPAGWRAVVERYSERLMGLSCKLLGVLSEAMGLESEALAKACVDMDQKVVVNF
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```
Z. mays             YSRWPDKPAAWRAVVERYSEQLMALACRLLGVLSEAMGLDTEALARACVDMDQKVVVNF
H. vulgare          YGRWPEKPAGWCAVVERYSERLMGLSCNLMGVLSEAMGLETEALAKACVDMDQKVVVNF
A. andreanum        YTRWPDKPEGWRAVVEAYSEGLMGLACKLLGVLSEAMGLDKEALAKACVDMDQKVVVNF
I. nil              YSRWPDKPEGWRAVTEKYSEKLMDLACKLLEVLSEAMGLEKEALSKACVELDQKLVVNF
G. max              YSRWPDTPEGWRSVTEEYSDKVMGLACKLMEVLSEAMGLEKEGLSKACVDMDQKVVVNY
G. triflora         YSRWPDKPEGWKSVTEKYSEQLMNLACKLLEVLSESMRLEKEALTKACVDMDQKIVVNF
F. x ananassa       YSRWPDKPEGWRDVTTQYSDELMGLACKLLEVLSEAMGLEKEALTKACVDMDQKVVVNF
V. vinifera         YSRWPDKPEGWRSVTQEYSEKLMGLACKLLEVLSEAMDLDKDALTNACVDMDQKVVVNF
C. sinensis         YSRWPDKPEGWMEVTKEYSDKLMGVACKLLEVLSEAMGLEKEALTKACVDMDQKIVVNY
S. tuberosum        YSRWPDKPQGWIAVTEKYSEKLMDLACKLLEVLSEAMGLEKEALTKACVDMDQKVVVNF
Camellia sinensis   YSRWPDKPEGWRAVTETYSEKLMDLACKLLEVLSEAMGLEKEALTKACVDMDQKVVINF
A. thaliana         YSRWPNKPEGWVKVTEEYSERLMSLACKLLEVLSEAMGLEKESLTNACVDMDQKIVVNY
P.hybrida           YSRWPDKPEGWIAVTQKYSEKLMELACKLLDVLSEAMGLEKEALTKACVDMDQKVVVNF
```

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                    Motif 3         * *    Motif 4
CLIVIA              YPKCPQPDLTLGRKRHTDPGTIILLLQDQVGGLQATKDGGKTWITVQPVEG
A. cepa             YPKCPQPDLTLGLKRHTDPGTITLLLQDQVGGLQATKDGGKTWITVQPVEG
L. speciosum        YPKCPQPDLTLGLKRHTDPGTITLLLQDQVGGLQATKDGGNTWITVKPIEG
B. finlaysoniana    YPKCPQPDLTLGLKRHTDPGTITLLLQDQVGGLQATKDGGETWITVQPVQN
O. sativa           YPKCPQPDLTLGLKRHTDPGTITLLLQDLVGGLQATRDAGKTWITVQPIPG
T. aestivum         YPRCPQPDLTLGLKRHTDPGTITLLLQDLVGGLQATRDGGKTWITVQPISG
Z. mays             YPRCPQPDLTLGLKRHTDPGTITLLLQDLVGGLQATRDGGRTWITVQPVEG
H. vulgare          YPRCPQPDLTLGLKRHTDPGTITLLLQDLVGGLQATRDGGKNWITVQPISG
A. andreanum        YPRCPQPDLTLGVKRHTDPGTITLLLQDQVGGLQATRDGGKTWITVQPVEG
I. nil              YPKCPEPDLTLGLKRHTDPGTITLLLQDQVGGLQATKDGGKTWITVQPVDG
G. max              YPKCPQPDLTLGLKRHTDPGTITLLLQDQVGGLQATRDNGKTWITVQPVEA
G. triflora         YPKCPQPDLTLGLKRHTDPGTITLLLQDQVGGLQATRDGGKSWITVQPVDG
F. x ananassa       YPKCPQPDLTLGLKRHTDPGTITLLLQDQVGGLQATRDGGKTWITVQPVEG
V. vinifera         YPQCPQPDLTLGLKRHTDPGTITLLLQDQVGGLQATRDGGKTWITVQPVEG
C. sinensis         YPKCPQPDLTLGLKRHTDPGTITLLLQDQVGGLQATKDNGKTWITVQPIEG
S. tuberosum        YPKCPEPDLTLGLKRHTDPGTITLLLQDQVGGLQATKDNGKTWITVQPVEG
Camellia sinensis   YPKCPQPDLTLGLKRHTDPGTITLLLQDQVGGLQATRDGGKTWITVQPVEG
A. thaliana         YPKCPQPDLTLGLKRHTDPGTITLLLQDQVGGLQATRDNGKTWITVQPVEG
P.hybrida           YPKCPEPDLTLGLKRHTDPGTITLLLQDQVGGLQATKDNGKTWITVQPVEG
```

Appendix P: Multiple alignment of DFR amino acid sequences corresponding to the deduced amino acid region in *Clivia*. The region marked by the dotted lines has been postulated to control substrate specificity of DFR (Beld *et al*., 1989), and the amino acid residue (indicated by the asterisk) is especially important for substrate specificity (Johnson *et al*., 2001). Conserved areas are highlighted in black. GenBank accession numbers: *Lilium speciosum* (BAE79202), *Bromheadia finlaysoniana* (AAB62873), *Zea mays* A1 (AAD10505), *Antirrhinum majus* (P14721), *Callistephus chinensis* (P51103), *Forsythia x intermedia* (CAA63703), *Gerbera hybrida* (P51105), *Ipomoea purpurea* (BAA36407), *Ipomoea batatas* (BAA34637), *Ipomoea nil* (BAA22076), *Malus domestica* (AAO39817), *Petunia hybrida* DFRA (CAA56160), *Vitis vinifera* (CAA53578), *Solanum tuberosum* (AAM73809), *Gentiana triflora* (BAA12736), *Torenia hybrida* (BAB20075), *Fragaria x ananassa* (AAS89833), *Arabidopsis thaliana* (P51102), *Citrus sinensis* (AAY87036), *Allium cepa* (AAO63026), *Agapanthus praecox* (BAE78769), *Cymbidium hybrid* (AAC17843), *Triticum aestivum* (BAE16365).

```
                                                          *
CLIVIA            KDPENEVIKPAIVGVLSIMRSCKKARSVQRVIFTSSAGTVNMEERQKPEYDENSWSDIEFCMRIKMTGWMYFVS
A. cepa           IDPENEVIKPAVNGMLSIMKSCKKAGTVKRVIFTSSAGTVNVEEHQKPEYDENSWSDIDFCRRVKMTGWMYFVS
A. praecox        KY--NEVIKPTIEGMLGIMKSCKKAGTVKRVIYTSSAGTVNVEEHQKPEYNEDSWSDLEFCRRVKMTGWMYFVS
L. speciosum      KDPENEVIQPTINGVLGIMKSCKKAGTVKRVIFTSSAGTVNVQENQMPEYDESSWSDVDFCRRVKMTGWMYFVS
V. vinifera       KDPENEVIKPTIEGMLGIMKSCAAAKTVRRLVFTSSAGTVNIQEHQLPVYDESCWSDMEFCRAKKMTAWMYFVS
G. triflora       KDPKNEVIKPTIDGFLSIIRSCVKAKTVKKLVFTSSAGTVDVQEQQKPVYDENDWSDLDFINSTKMTGWMYFVS
A. thaliana       KDPENEVIKPTVNGMLGIMKACVKAKTVRRFVFTSSAGTVNVEEHQKNVYDENDWSDLEFIMSKKMTGWMYFVS
C. sinensis       KDPENEVIRPTINGMVSIMRACKNAKTVRRLVFTSSAGTLDVEEHRKPVYDETSWSDLDFVRSVKMTGWMYFVS
B. finlaysoniana  KDPENEVIKPAINGLLGILTSCKKAGSVKRVIFTSSAGTVNVEEHQAAVYDENSWSDLHFVTRVKMTGWMYFVS
T. hybrida        DDPENEVIKPTVDGMLSIIRSCAKAQTVKRLIFTNSAGTLNVEEHQKPVYDESNWSDLDFIYSTKMTGWMYFVS
T. aestivum       KDPENEVIKPTVEGMLSIMRACKEAGTVKRIVFTSSAGSVNIEERQRPAYDQDNWSDIDFCRRAKMTGWMYFVS
F. x ananassa     EDPENEVIKPTINGMLDIMKACLKAKTVRRVVFTSSAGAVAIEEHRKEVYSENNWSDVVFCRKVKMTGWMYFVS
C. chinensis      KDPENEIIKPTIEGILSIIRSCAKAKTVKKLVYTSSAGTVNVQETQLPVYDESHWSDLDFIYSKKMTAWMYFVS
G. hybrida        KDPENEIIKPTIEGVLSIIRSCVKAKTVKKLVFTSSAGTVNGQEKQLHVYDESHWSDLDFIYSKKMTAWMYFVS
I. batatas        KDPENEVIKPAINGVLNIINSCVKAKTVKRLVFTSSAGTLNVQPQQKPVYDESCWSDLDFIYAKKMTGWMYFAS
I. nil            KDPENEVIKPAVKGILSIINSCAKAKTVKKLVFTSSTAAVHIKETQQLEYDESSWSDLDFIYANKMGGWMYFAS
I. purpurea       KDPENEVIKPAVKGILSIINSCAKAKTVKKLVFTSSTAAVHIKETQQLVYDESSWSDLDFIYANKMGGWMYFAS
Z. mays           KDPENEVIKPTVEGMISIMRACKEAGTVRRIVFTSSAGTVNLEERQRPVYDEESWTDVDFCRRVKMTGWMYFVS
A. majus          VDPENEVIKPTIDGMLNIIKSCVQAKTVKKFIFTTSGGTVNVEEHQKPVYDETDSSDMDFINSKKMTGWMYFVS
P. hybrida        KDPENEVIKPTVRGMLSIIESCAKANTVKRLVFTSSAGTLDVQEQQKLFYDQTSWSDLDFIYAKKMTGWMYFAS
C. hybrid         EDPENEVIKPTISGLLGILRSCKRVGTVKRVIFTSSAGTVNVEEHQATVYDESSWSDLDFVTRVKMTGWMYFVS
F. x intermedia   KDPENEVIKPTIEGFLSLIRSCTKAKTVKRIVFTSSAGTVNVEEHQKSVYDETDYSDLNFIYSKKMTGWMYFAS
S. tuberosum      KDPENEVIQPTVRGMLSIIESCAKAKTVKRLVFTSSAGTLDVQEDQKLFYDETSWSDLDFIYAKKMTGWMYFVS
M. domestica      KDPENEVIKPTINGLLDILKACQKAKTVRKLVFTSSAGTVNVEEHQKPVYDESNWSDVEFCRSVKMTGWMYFVS
G. max            KDPENEVIKPTINGVLDIMKACLKAKTVRRLIFTSSAGTLNVIERQKPVFDDTCWSDVEFCRRVKMTGWMYFVS
```

Appendix Q: *Cm18S* rRNA partial cDNA sequence obtained after sequencing of a newly obtained PCR fragment amplified with wheat-specific primers. The sequence showed 99% nucleotide identity to the *18S* rRNA cDNA sequence from *Clivia nobilis* (GenBank accession number: AF206889), as indicated by the asterisks.

>DNA sequence of *C. miniata* var. *miniata* 'Plantation' 18S rRNA fragment

```
TTCAAGAACGAAAGTTGGGGGCTCGAAGACGATCAGATACCGTCCTAGTCTCAACCATAAACGATGCC
GACTAGGGATCGGCGGATGTTGCTTTTAGGACTCCGCCGGCACCTTATGAGAAATCAAAGTTTTTGGG
TTCCGGGGGGAGTATGGTCGCAAGGCTGAAACTTAAAGGAATTGACGGAAGGGCACCACCAGGAGTGG
AGCCTGCGGCTTAATTTGACTCAACACGGGGAAACTTACCAGGTCCAGACATAGTAAGGATTGACAGA
TTGAGAGCTCTTTCTTGATTCTATGGGTGGTGGTGCATGGCCGTTCTTAGTTGGTGGAGCGATTTGTC
TGGTTAATTCCGTTAACGAACGAGACCTCAGCCTGCTAACTAGCTACGCGGAGGCATCCCTCCGCGGC
CAGCTTCTTAGAGGGACTATGGCCGCTTAGGCCACGGAAGTTTGAGGCAATAACAGGTCTGTGATGCC
CTTAGATGTTCTGGGCCGCACGCGCGCTACACTGATG (513 bp)
```

ClustalX v2.0 alignment

```
C.miniata       TTCAAGAACGAAAGTTGGGGGCTCGAAGACGATCAGATACCGTCCTAGTCTCAACCATAA
C.nobilis       ATCAAGAACGAAAGTTGGGGGCTCGAAGACGATCAGATACCGTCCTAGTCTCAACCATAA
                 ***********************************************************

C.miniata       ACGATGCCGACTAGGGATCGGCGGATGTTGCTTTTAGGACTCCGCCGGCACCTTATGAGA
C.nobilis       ACGATGCCGACCAGGGATCGGCGGATGTTGCTTTTAGGACTCCGCCGGCACCTTATGAGA
                *********** ************************************************

C.miniata       AATCAAAGTTTTTGGGTTCCGGGGGGAGTATGGTCGCAAGGCTGAAACTTAAAGGAATTG
C.nobilis       AATCAAAGTTTTTGGGTTCCGGGGGGAGTATGGTCGCAAGGCTGAAACTTAAAGGAATTG
                ************************************************************

C.miniata       ACGGAAGGGCACCACCAGGAGTGGAGCCTGCGGCTTAATTTGACTCAACACGGGGAAACT
C.nobilis       ACGGAAGGGCACCACCAGGAGTGGAGCCTGCGGCTTAATTTGACTCAACACGGGGAAACT
                ************************************************************

C.miniata       TACCAGGTCCAGACATAGTAAGGATTGACAGATTGAGAGCTCTTTCTTGATTCTATGGGT
C.nobilis       TACCAGGTCCAGACATAGTAAGGATTGACAGACTGAGAGCTCTTTCTTGATTCTATGGGT
                ******************************** ***************************

C.miniata       GGTGGTGCATGGCCGTTCTTAGTTGGTGGAGCGATTTGTCTGGTTAATTCCGTTAACGAA
C.nobilis       GGTGGTGCATGGCCGTTCTTAGTTGGTGGAGCGATTTGTCTGGTTAATTCCGTTAACGAA
                ************************************************************

C.miniata       CGAGACCTCAGCCTGCTAACTAGCTACGCGGAGGCATCCCTCCGCGGCCAGCTTCTTAGA
C.nobilis       CGAGACCTCAGCCTGCTAACTAGCTACGCGGAGGCATCCTTCCGCGGCCAGCTTCTTAGA
                *************************************** ********************

C.miniata       GGGACTATGGCCGCTTAGGCCACGGAAGTTTGAGGCAATAACAGGTCTGTGATGCCCTTA
C.nobilis       GGGACTATGGCCGCTTAGGCCACGGAAGTTTGAGGCAATAACAGGTCTGTGATGCCCTTA
                ************************************************************

C.miniata       GATGTTCTGGGCCGCACGCGCGCTACACTGATG
C.nobilis       GATGTTCTGGGCCGCACGCGCGCTACACTGATG
                *********************************
```