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Light-Induced Anthocyanin Pigmentation in Transgenic *Lc* Petunia

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Abstract

Introduction of *Leaf colour (Lc)*, a bHLH transcription factor from maize, under the control of the CaMV35S promoter into petunia (cv. Mitchell) plants resulted in enhanced anthocyanin pigmentation in vegetative tissues. Anthocyanin biosynthesis was observed to be dependent on the level of light the plants were grown under: plants grown in a plastic greenhouse remained green, while plants exposed to high-light were dark purple. The nature of this response to light and the associated molecular mechanisms were the focus of this investigation.

Molecular analysis of gene expression in Mitchell petunia showed that light induced the expression of the early flavonoid structural genes, as well as flavonol synthase (*FLS*) required for flavonol production. Light induced both the early and late structural genes required for anthocyanin biosynthesis in the transgenic *Lc* Mitchell petunia plants, but reduced the expression of *FLS*. Light-induced flavonoid gene expression was examined under three light treatments: shade ($50 - 350 \mu\text{mol m}^{-2} \text{sec}^{-1}$); ambient-greenhouse ($300 - 750 \mu\text{mol m}^{-2} \text{sec}^{-1}$) and high-light ($750 \mu\text{mol m}^{-2} \text{sec}^{-1}$). The level of flavonoid gene expression was dependent upon light intensity. High-light was required to maximally activate anthocyanin pigmentation in *Lc* petunia. Expression of the *Lc* transgene remained unchanged irrespective of light intensity, indicating that the light-induced changes in anthocyanin synthesis were not due to variable expression of the transgene.

Anthocyanin regulation occurs primarily at the transcriptional level, and two classes of transcription factors, Myb and bHLH, are generally involved. Transient expression studies using several exogenous Myb transcription were carried out using shade-grown (non-induced) *Lc* petunia material. The induction of coloured cells in the treated tissue supports the idea that the bHLH transgene (LC) is interacting with an endogenous Myb under high-light conditions, resulting in the activation of the flavonoid biosynthetic pathway and accumulation of anthocyanin pigments. A partial sequence of a candidate endogenous Myb transcription factor from petunia was cloned. It was light-induced and shares structural features with other anthocyanin-regulating Myb transcription factors, particularly *An2* from petunia. This Myb in combination with LC may be responsible for the light-induced anthocyanin pigmentation observed in *Lc* petunia.

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List of Abbreviations

CaMV35S	35S promoter, from the cauliflower mosaic virus (CaMV)
4CL	4-coumarate:CoA ligase
A _{xxx}	absorbance, at the wavelength indicated by the numerical value (xxx)
Amp	ampicillin
ANS	anthocyanidin synthase
ARE	anthocyanin regulatory element
bHLH	basic helix-loop-helix; class of transcription factor
bp	base pairs
bZIP	basic region leucine zipper; class of transcription factor
C4H	cinnamate 4-hydroxylase
cDNA	complementary deoxyribonucleic acid
CHI	chalcone isomerase. Syn. Chalcone flavanone isomerase (CFI)
CHS	chalcone synthase
cm	centimetre
cpm	counts per minute
CTAB	cetyltrimethyl ammonium bromide
°C	degrees Celsius
d	days
DFR	dihydroflavonol 4-reductase
DNA	deoxyribonucleic acid

dNTP	deoxy-nucleotide-triphosphate
DW	dry-weight
EDTA	ethylenediaminetetraacetate
F3H	flavanone 3-hydroxylase
F3' H	flavonoid 3'-hydroxylase
F3'5'H	flavonoid 3'5'-hydroxylase
FNS	flavone synthase
FLS	flavanol synthase
FW	fresh-weight
g	gram
g	gravity, or g-force
GFP	green fluorescent protein, originally from <i>Aequorea victoria</i>
GMO	genetically modified organism
GT	glucosyl-transferase
h	hours
HPLC	high performance liquid chromatography
Kan	kanamycin
kb	kilobases
kPa	kiloPascal
LC	LEAF COLOUR; bHLH transcription factor from <i>Zea mays</i>
LB	Luria-Bertani; bacterial growth media
LRU	light regulatory unit. Syn. Light regulatory element (LRE)
M	molar; moles per litre
min	minute
milliQ	water which has been purified using Milli-Q Ultrapure system
µg	microgram
µM	micromolar
µL	microlitre
mg	milligram
mJ	millijoules
mL	millilitre
mM	millimolar
MOPS	3-(N-morpholino) propanesulfonic acid
MRE	myb recognition element

ms	millisecond
MS	Murashige and Skoog: tissue culture media
N	normal
NaB	sodium borate buffer
NaAc	sodium acetate
ng	nanograms
nm	nanometre
ORF	open reading frame
PAL	phenylalanine ammonia lyase
PCR	polymerase chain reaction
psi	pounds per square inch
PVP	polyvinyl pyrrolidone
RACE	rapid amplification of cDNA ends
RNA	ribonucleic acid
RNase	ribose nuclease
rpm	revolutions per minute
RT	reverse-transcription
RRE	'R' response element
rRNA	ribosomal ribonucleic acid
SDS	sodium dodecyl sulphate
sec	second
SSC	sodium chloride-sodium citrate buffer
Spec	spectinomycin
Strep	streptomycin
TAE	tris acetate EDTA buffer
TBE	tris borate EDTA buffer
tDNA	transfer DNA; DNA transferred from <i>Agrobacterium tumefaciens</i> into the host genome
Tris	tris(hydroxymethyl) aminomethane
U	enzyme units
UV	ultra violet
V	volt
v/v	volume/volume
w/v	weight/volume

WT	wild type
YM	yeast-mannitol; bacterial growth media

- Gene names (and loci) are italicised e.g. *Leaf colour*, e.g. *CHS*.
- Proteins are capitalised e.g. LEAF COLOUR, e.g. CHS.
- ‘Light’ refers to photosynthetically active radiation (400 - 700 nm).
Photosynthetic photon flux density was measured in $\mu\text{mol m}^{-2} \text{sec}^{-1}$.

Chapter 1

Introduction

1.1 Background

Cut flowers and ornamental plants represent important markets both internationally and for New Zealand. The New Zealand export market for cut flowers, bulbs and live plants is worth \$79.4 million dollars, while the domestic market for cut flowers is worth approximately \$70 million (Kerr et al., 2005). The colours and pigment patterns of flowers and plants are often the key attribute consumers look for when purchasing cut flowers or ornamental plants for the garden. To succeed in these highly competitive markets, it is imperative to develop plants and flowers with novel colours, plant forms and pigmentation patterns.

Traditional breeding and hybridisation has resulted in an extensive array of colours and plant forms within many species. However, in some species the colour palette is narrow (e.g. lacking blue or yellow) and traditional breeding has been unable to produce some of the highly desired colours. Carnation is one of the world's most important cut flowers and while a large range of colours is available, blue and true purple colours are lacking (de Vetten et al., 1999). Genetic engineering has enabled the production of delphinidin, a type of pigment lacking in carnations, resulting in a range of plants with mauve to purple flowers (Fukui et al., 2003), but not with blue. The understanding of the regulation of pigmentation which contributes to the colours and patterns in plants is critical if further advances are to be made in developing plants and flowers with novel colours and pigmentation patterns.

In addition to genetic factors, environmental factors can have an impact upon pigmentation in flowers and ornamental species. Flowers or ornamental plants bred in regions where light intensity is strong (e.g. New Zealand), may fail to produce vibrant colours when grown under weaker light conditions (e.g. Northern hemisphere) (pers.

comm. Mr Peter Moffat, Kiwi Orchids). An understanding of how such factors may impact on pigmentation may help direct breeding programs to reduce the effects of detrimental environmental conditions.

In an attempt to alter anthocyanin pigmentation in petunia, plants were transformed with *Leaf colour (Lc)*, a maize anthocyanin regulatory gene (Bradley et al., 1998). The initial results were promising, as the transgenic *Lc* petunia plants displayed altered anthocyanin pigmentation throughout the vegetative tissues, as shown in Figure 1.1. The leaves and stems were dark purple with anthocyanins, while flowers of the *Lc* petunias were acyanic (white) due to a regulatory mutation present in the Mitchell petunia genetic background, which *Lc* was not able to overcome. However, subsequent experiments, including an open field trial, revealed that the pigmentation was variable depending on the growth conditions of the plants, especially light levels. This raised a number of questions regarding the influence of light¹ on anthocyanin accumulation in *Lc* petunia.

This thesis investigated the light-induced anthocyanin pigmentation observed in *Lc* petunia, characterising this response at the biochemical and molecular levels, and investigating the interactions between transgenic and endogenous transcription factors.

1.2 Petunia

The genus *Petunia* belongs to the Solanaceae family, which consists of many species indigenous to South America. *Petunia hybrida* is the most widely grown species (from which a vast array of cultivars are available), and is believed to be derived from *Petunia axillaris* and *Petunia integriflora*, although no wild hybrids of these two species have been identified (Ando et al., 2001).

¹ 'Light' refers to photosynthetically active radiation (400 - 700 nm). Photosynthetic photon flux density was measured in $\mu\text{mol m}^{-2} \text{sec}^{-1}$.

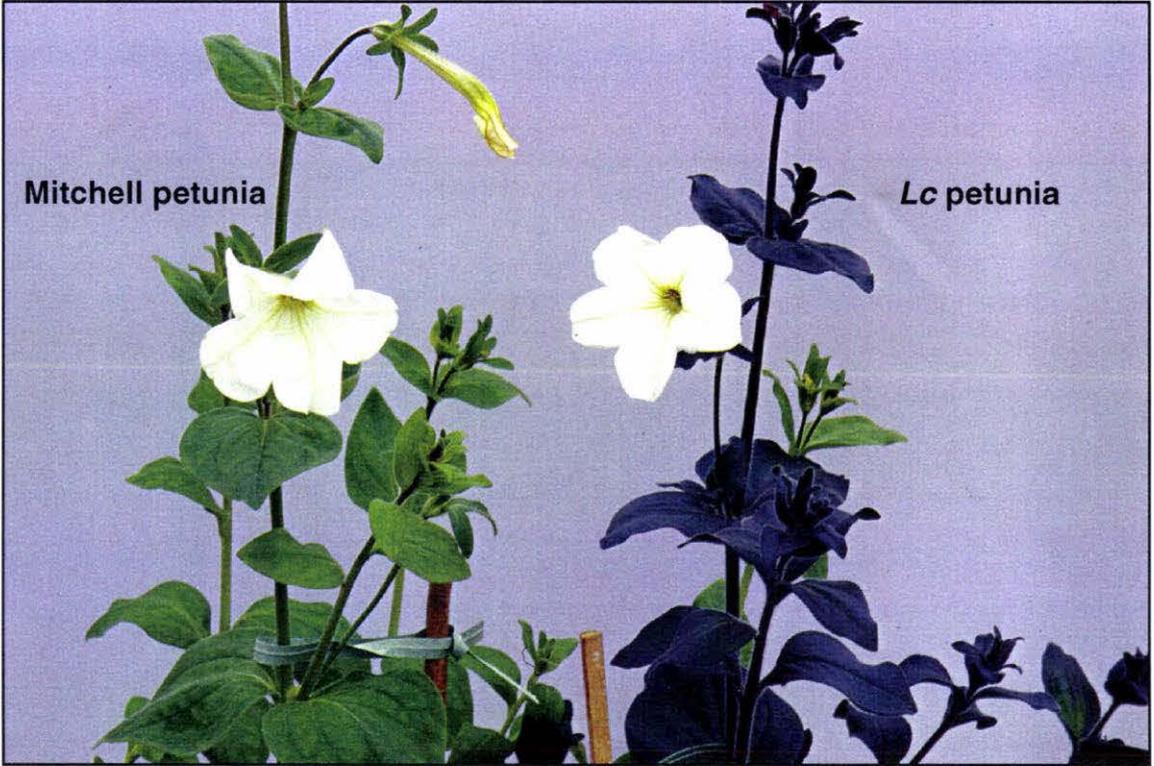


Figure 1.1: Pigmentation phenotypes of Mitchell and *Lc* petunia grown under high light.

Petunia (*Petunia hybrida*) is an ornamental species grown widely as an annual bedding plant for its brightly coloured flowers. They are facultative long day, annual plants that undergo a juvenile period before becoming competent to flower. The plant architecture is derived from an alternate (spiral) pattern of leaf initiation, and plant sizes range from 25 - 60 cm depending on the species or cultivar (Armitage, 1985).

Petunia has been used as a model species for investigations into many aspects of plant development, including meristem identity, floral development, self-incompatibility, senescence, transposons, floral scent (volatiles) and pigmentation. Petunia is a favoured model plant species because of its short generation time, ease of growing, large leaves and flowers (ready source of tissue), mutant collections and transformation efficiency (Gerats and Vandenbussche, 2005). The range of flower colours and readily available collections of mutants has made petunia a particularly useful model species for studying anthocyanin and flavonoid biosynthesis and regulation.

The petunia cultivar used in this study is Mitchell petunia (*Petunia axillaris* × [*Petunia axillaris* × *Petunia hybrida* cv. 'Rose of Heaven']). It is a particularly useful cultivar due to its high fertility, robust growth habit, rapid growth in tissue culture (Ausubel et al., 1980) and superior transformation efficiency (Gerats and Vandenbussche, 2005). Mitchell petunia has mutations in two anthocyanin regulatory loci, *an2* and *an4*, resulting in acyanic petals and anthers respectively (Cornu and Farcy, 1981).

1.3 Plant pigments

Plant pigments include a wide variety of chromogenic compounds which give rise to the various colours and pigmentation patterns observed in plants. The major plant pigment classes are chlorophylls, carotenoids and anthocyanins and they generally give green, yellow/orange and red/pink/blue colours respectively.

Chlorophylls and carotenoids are required for the light harvesting reactions of photosynthesis, and are located in the chloroplasts within cells. Carotenoids are also produced in non-photosynthetic tissues, accumulating in plastids and providing

colouration to flowers and fruits to attract pollinators and seed distributors respectively (Davies, 2000).

The main pigments focused on in this study are the anthocyanin pigments, formed via the flavonoid biosynthetic pathway. They range in colour from orange/red through to pink, purple and blue, depending on the level of hydroxylation and sidechain substitution of the anthocyanin molecule, as well as factors such as cellular pH and inter- and intra-molecular interactions with other anthocyanins, co-pigments (such as flavones and flavonols) and metal ions (Mol et al., 1998; Aida et al., 2000; Yoshida et al., 2000; Yoshida et al., 2002). Anthocyanins are generally considered to be synthesised in the cytoplasm but are subsequently transported and stored in the vacuole, where they may be found as soluble vacuolar contents (Davies and Schwinn, 1997) or aggregated into anthocyanic vacuolar inclusions (Markham et al., 2000).

Anthocyanins are found in flowers, fruits and vegetative tissues. Their roles in flowers and fruits are to attract pollinators and seed distributors respectively (Mol et al., 1998), but their role in vegetative tissues is somewhat more contentious. It has been shown that anthocyanins can help protect photosynthetic tissues from photoinhibition (damage to the photosynthetic apparatus due to excess quanta) by absorbing blue-green light (Feild et al., 2001; Neill and Gould, 2003). The location of these pigments in upper epidermal tissues observed in some species, suggests anthocyanins may act to screen chloroplasts from this extra energy (Steyn et al., 2002). It has been proposed that the *de novo* synthesis of anthocyanins in the leaves of deciduous species during autumn provides a photoprotective role, by screening tissues from excess light while nutrients are actively being retrieved from the senescing leaves (Feild et al., 2001; Hoch et al., 2003). In addition to possible screening roles, anthocyanins (and other flavonoids) have antioxidant properties, and this suggests a role for coping with reactive oxygen species (ROS) generated during photosynthesis, particularly under conditions of photoinhibition (typically high light and low temperature). However, the cellular localisation of anthocyanins in the vacuoles does not correlate well with the site of free radical/ROS production i.e. chloroplasts. However, Neill and Gould (2003) suggest that colourless cytosolic anthocyanins could scavenge ROS, while coloured vacuolar anthocyanins could act to reduce photoinhibition through screening excess quanta.

Anthocyanins may also contribute to UV photoprotection. While standard anthocyanins have relatively poor UV absorptive properties (compared to other polyphenols and flavonoids) (Steyn et al., 2002), acylated anthocyanins have enhanced UV absorption compared to simple anthocyanin-glycosides (Giusti et al., 1999). Recently, a UV protective role for polyacylated anthocyanins from floral tissues has been demonstrated (Mori et al., 2005). However, UV protection is primarily provided by colourless polyphenols (hydroxy-cinnamic acid derivatives) and flavonoids (particularly flavonols) (Li et al., 1993; Middleton and Teramura, 1993; Jin et al., 2000).

1.4 Flavonoid biosynthetic pathway

The flavonoid biosynthetic pathway (Figure 1.2), which branches off the greater phenylpropanoid pathway, is responsible for producing a range of secondary metabolites, including flavones, flavanones, flavonols, proanthocyanidins (condensed tannins) and anthocyanins.

The basic flavonoid structure is composed of a 15-carbon backbone consisting of two aromatic rings (A and B rings) separated by a three-carbon region which may also form a ring (C ring) (Davies and Schwinn, 1997). Figure 1.3 A and B show the basic flavonol and anthocyanidin structures respectively, positions of the A, B and C rings, and the numbering system for the carbon atoms. The degree of hydroxylation of the B ring at the positions R1 (3') and R2 (5') is a key difference between individual flavonoids, essentially establishing three distinct groups of flavonoids that progress through the biosynthetic steps of the flavonoid pathway.

The first committed step towards flavonoid biosynthesis is catalysed by chalcone synthase (CHS), for which a multigene family has been identified in petunia. Although 12 *CHS* genes have been identified in petunia, only four are known to be expressed. This contrasts to *Antirrhinum* which has only one *CHS* gene (Holton and Cornish, 1995). The isomerisation of chalcones to flavanones is catalysed by chalcone isomerase (CHI), although this isomerisation reaction occurs spontaneously (with reduced efficiency) without enzymatic intervention

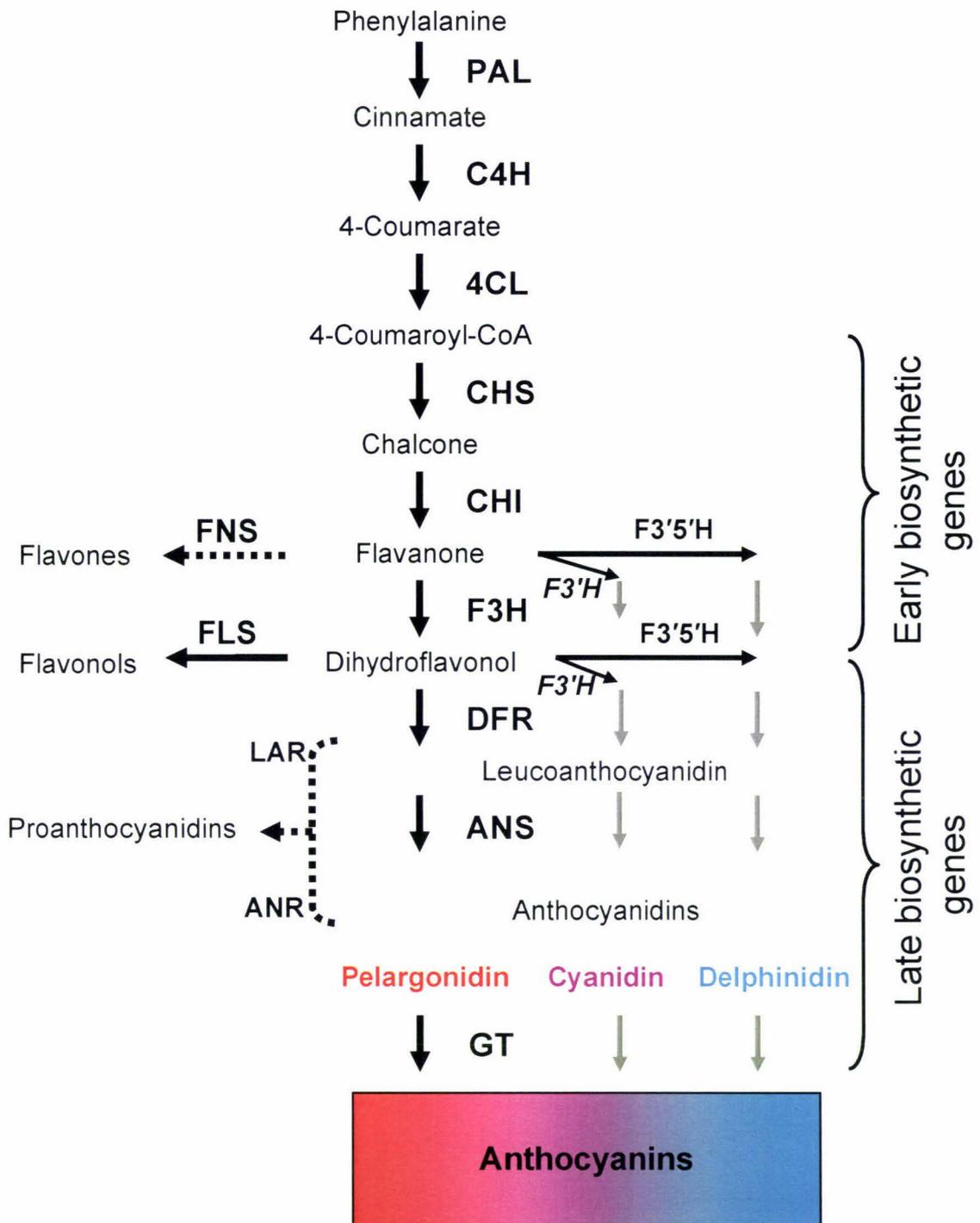
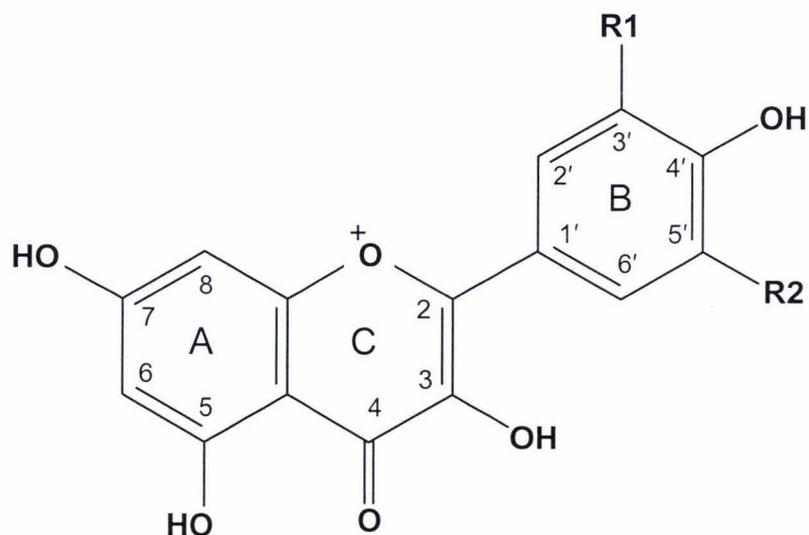
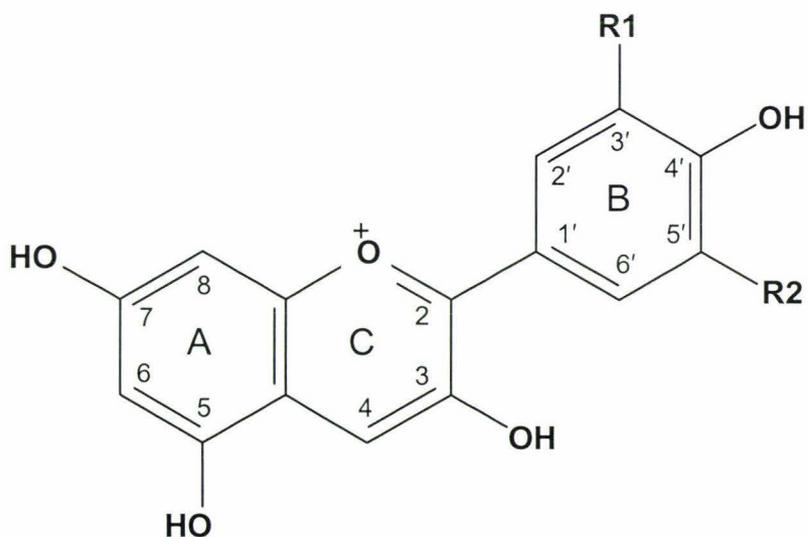


Figure 1.2: The flavonoid biosynthetic pathway, leading to the production of the major flavonoid types; flavones, flavonols, proanthocyanidins (condensed tannins) and anthocyanins. Dotted arrows represent flavonoids not produced appreciably in petunia.

PAL, Phenylalanine ammonia lyase; C4H, cinnamate 4-hydroxylase; 4CL, 4-coumarate:CoA ligase; CHS, chalcone synthase; CHI, chalcone isomerase; F3H, flavanone 3-hydroxylase; F3'H, flavonoid 3'-hydroxylase; F3'5'H, flavonoid 3'5'-hydroxylase; FNS, flavone synthase; FLS, flavonol synthase; DFR, dihydroflavonol 4-reductase; ANS, anthocyanidin synthase; GT, glucosyltransferases; LAR, leucoanthocyanidin reductase; ANR, anthocyanidin reductase.

A

Flavonol

B

Anthocyanidin

Figure 1.3: Structures of flavonols **A** and anthocyanidins **B**. The A, B and C rings are indicated, and carbon atoms labelled. The flavonol backbone, **A**, is hydroxylated at the R1 and R2 positions, determining the various flavonols: R1, R2 = H, kaempferol; R1 = OH, R2 = H, quercetin; R1, R2 = OH, myricetin. The anthocyanidin backbone, **B**, is substituted at the R1 and R2 positions, determining the various anthocyanidins: R1, R2 = H, pelargonidin; R1 = OH, R2 = H, cyanidin; R1, R2 = OH, delphinidin; R1 = OCH₃, R2 = H, peonidin; R1 = OH, R2 = OCH₃, petunidin; R1, R2 = OCH₃, malvidin.

(Martin and Gerats, 1993; Holton and Cornish, 1995). Flavanone 3-hydroxylase (F3H) hydroxylates the 3 position of the flavonoid backbone to produce the dihydroflavonols (Martin and Gerats, 1993; Holton and Cornish, 1995).

The basic flavonoid structure (4'-OH) can be further hydroxylated at the 3' and 5' positions by flavonoid 3'-hydroxylase (F3'H) and flavonoid 3'5'-hydroxylase (F3'5'H) respectively. These three differently hydroxylated flavonoid backbones (4'-OH, 3'4'-OH, 3'4'5'-OH, Figure 1.3) can progress through the flavonoid pathway to produce mono-, di- and tri-hydroxylated flavonols and anthocyanidins (as indicated in Figure 1.2).

Flavone synthase (FNS) catalyses flavone synthesis. In petunia, however, the primary non-anthocyanin flavonoids produced are flavonols, and flavonol synthase (FLS) catalyses flavonol production. They are glycosylated, forming the common flavonols kaempferol (4'-OH), quercetin (3'4'-OH) and myricetin (3'4'5'-OH).

The first committed step towards anthocyanin production is catalysed by dihydroflavonol-4-reductase (DFR), followed by a dioxygenase reaction performed by anthocyanidin synthase (ANS) (Martin and Gerats, 1993), producing the basic anthocyanidin structures. The anthocyanidins are glycosylated by various glucosyltransferases, forming the common anthocyanins pelargonidin (4'-OH), cyanidin (3'4'-OH) and delphinidin (3'4'5'-OH). Glycosylation is generally required to stabilise the pigment molecules and increase their solubility. These common anthocyanins can then be further modified by acylation and may form complex inter- and intra-molecular pigment complexes. The level of substitution and hydroxylation results in a wide range of anthocyanin pigments with different optical and chemical properties.

Proanthocyanidins (condensed tannins) are polymers produced from flavan-3-ol precursors, catechins and epicatechins, by the enzymes leucoanthocyanidin reductase (LAR) and anthocyanidin reductase (ANR) respectively. While the biosynthetic steps required for the production of proanthocyanidin precursors have been determined in several species, the polymerisation steps have not been established (Dixon et al., 2005).

1.4.1 Induction of the flavonoid pathway

Anthocyanin pigments and other flavonoids are produced in response to a wide range of developmental and environmental signals. Perception of these signals results in the temporal and spatial expression of flavonoid biosynthetic genes, resulting in the accumulation of anthocyanins and other flavonoids throughout a range of vegetative and reproductive tissues. Floral pigmentation patterning is a good example of strict developmental control, both spatially and temporally, producing striking pigmentation patterns which are recognised by pollinators. Many tissues produce anthocyanins and other flavonoids as part of a developmental sequence, but may further be influenced by environmental stimuli such as light and temperature (Davies and Schwinn, 2003).

Pathogen infection is linked to the biosynthesis of flavonols, isoflavonoids and 3'-deoxyanthocyanins, while various stress factors e.g. nutrient deficiency (particularly phosphate) and dehydration may induce anthocyanin production. This is further reviewed by Dixon and Paiva (1995) and Chalker-Scott (1999).

The response of the flavonoid pathway to both light intensity and quality has been well documented. For apple skin to gain red anthocyanin pigmentation, exposure to light, particularly strong light, is required (Arakawa, 1988). Similarly, adequate lighting is required to produce vibrant, intensely coloured flowers in lilies, stock (Kawabata et al., 2002), roses (Biran and Halevy, 1974), lisianthus (*Eustoma grandiflorum*) (Griesbach, 1992) and many other important floriculture crops. A combination of gibberellin (produced in anthers) (Weiss and Halevy, 1989), light and sugar are required for anthocyanin pigmentation in the corolla of developing petunia flowers (Katz and Weiss, 1998; Katz and Weiss, 1999; Weiss, 2000).

Another class of flavonoids, the flavonols, have been shown to be induced by light in many species. Flavonols provide UV protection and, not surprisingly, are induced by UV containing spectra in many species, including parsley (*Petroselinum crispum*) (Lozoya et al., 1991), *Arabidopsis* (Hemm et al., 2004; Mehrrens et al., 2005), bilberry (*Vaccinium myrtillus* L.) (Jaakola et al., 2004), apple (Merzlyak et al., 2002) and petunia (Ryan et al., 1998). In addition to their UV protective qualities, flavonols also act as co-

pigments in flowers, capable of forming intermolecular complexes with anthocyanins (Mol et al., 1998; Aida et al., 2000), and may also be under developmental control in addition to light regulation. Flavonols themselves impart an ivory appearance to acyanic (white) flowers which, although inconspicuous to the human eye, are visible to insects due to their UV absorption (Mol et al., 1998).

The induction of the flavonoid pathway by various environmental and developmental signals, infers that changes to the regulation of the biosynthetic genes must be occurring.

1.5 Regulation of the flavonoid biosynthetic pathway

The model plant species for studying anthocyanin regulation traditionally have been *Zea mays* (maize), *Petunia* sp (petunia) and *Antirrhinum majus* (snapdragon) and it is these species from which most of the information about anthocyanin regulation has been derived. Several regulatory genes required for flavonoid and anthocyanin metabolism have been identified, cloned and characterised in these and other plant species. The regulation of flavonoid metabolism is achieved primarily through transcriptional regulation of the biosynthetic genes mediated by bHLH and Myb transcription factors, and a WD40 repeat protein.

Higher level factors involved in the regulation of the flavonoid-regulating transcription factors are largely unknown. Some mutants with altered flavonoid regulation have been identified, which are likely to be involved in upstream signal transduction pathways. These include the *constitutive photomorphogenic (COP)/de-etiolated (DET)* mutants involved in light signal transduction. Light signalling converges on the COP signalosome and, in the absence of light, results in the degradation of LONG HYPOCOTYL5 (HY5), a bZIP transcription factor which activates light-regulated gene expression (Serino and Deng, 2003). While HY5 has been shown to directly activate *CHS* expression (Ang et al., 1998), it probably also results in the light-induced expression (directly or indirectly) of flavonoid-specific transcription factors. Recently a new mutant, *increased chalcone synthase expression 1 (icx1)* has been identified in

Arabidopsis, which appears to act as a negative regulator on *CHS* expression in epidermal tissues. It may regulate the expression or activity of a common component to light, cytokinin, sucrose and low temperature signal transduction pathways for the regulation of flavonoid genes upstream of *HY5* (Wade et al., 2003). It seems likely that as for the floral induction model (Koorneef et al., 1998), there will be a complex series of interactions ultimately resulting in flavonoid production.

In recent years, the focus for research on flavonoid gene regulation has moved towards investigating the hierarchical nature of the regulatory systems: ‘what regulates the regulators’. This would not only provide new approaches to engineering flavonoid metabolism, but also serve as a model for the developmental regulation which gives rise to pigment patterning observed in plants (particularly flowers). The focus on flavonoid regulation for the purposes of this study, however, has been on the transcription factors acting directly on the biosynthetic genes.

1.5.1 bHLH transcription factors

The first major class of flavonoid regulators are bHLH or *myc*-like transcription factors. These proteins are homologous to *myc* transcription factors in animals, and are characterised by a basic helix-loop-helix (bHLH) domain (Ludwig et al., 1989). The *R* gene family of bHLH transcription factors in maize include *Red (R)* (Chandler et al., 1989), *Leaf colour (Lc)* (Ludwig et al., 1989) *Booster (B)* (Chandler et al., 1989) and *Sienna (Sn)* (Tonelli et al., 1991), and their spatial and temporal expression patterns are responsible for regulating pigmentation in different tissues throughout the maize plant. The sequence information of R proteins indicates that they act as transcription factors, and agrees with the observation that functional alleles were required to regulate genes required for flavonoid metabolism (Ludwig et al., 1989). Early functional experiments, where *Lc* was transiently introduced into maize tissues by particle bombardment (biolistics) provided evidence that a maize R gene could induce pigmentation in many tissues (Ludwig et al., 1990).

BHLH anthocyanin regulatory transcription factors have also been identified in petunia, snapdragon and *Arabidopsis*. In snapdragon *Delila (Del)* regulates anthocyanin

production in the corolla tube of the flower and encodes a bHLH transcription factor. It has been shown to regulate *DFR* (see Figure 1.2), the first committed step to anthocyanin biosynthesis (Goodrich et al., 1992). *Mutabilis* (*Mut*) regulates anthocyanin pigmentation in the corolla lobes and also encodes a bHLH transcription factor (Schwinn et al., 2006). *Anthocyanin-1* (*An1*) and *Jaf13* are both bHLH transcription factors which have been isolated from petunia (Spelt et al., 2000), and are thought to have roles in regulating anthocyanin biosynthesis. AN1 is required for anthocyanin pigmentation in petunia petals, and both AN1 and JAF13 have been shown to be able to directly activate *DFR* (Quattrocchio et al., 1998; Spelt et al., 2000), the committing step to anthocyanin biosynthesis. Similarly, in *Arabidopsis*, bHLH transcription factors have been identified for flavonoid regulation, including *Transparent testa 8* (*TT8*) (Nesi et al., 2000).

The bHLH class of transcription factors clearly have an important role in the regulation of anthocyanin and flavonoid metabolism.

1.5.2 Myb transcription factors

The second major class of flavonoid regulators are Myb transcription factors. These proteins act as transcription factors and are identified by the 'Myb' DNA binding domain, which was first identified in *myb*-oncoproteins in animals (Klempnauer et al., 1982; Weston, 1998). The Myb DNA binding domain is characterised by a series of three imperfect repeats: R1, R2, and R3. These repeats each form a helix-turn-helix motif and have three evenly spaced tryptophan residues, which are characteristic of a Myb repeat. The R3 repeat is involved in sequence specific recognition and binding to DNA, while the R2 repeat may contribute to less specific DNA binding (Ogata et al., 1992). Mybs containing only the second and third repeats, the R2R3 Mybs, represent a large subfamily of Myb transcription factors in plants (125 have been identified in *Arabidopsis*) (Martin and Paz-Ares, 1997; Stracke et al., 2001; Petroni et al., 2002). Their diverse functions are still being investigated, but their involvement in regulating phenylpropanoids, flavonoids and anthocyanins is well documented (Martin and Paz-Ares, 1997; Kranz et al., 1998; Romero et al., 1998; Stracke et al., 2001; Petroni et al., 2002).

The earliest identification of an R2R3 Myb transcription factor which has a role in anthocyanin regulation was the characterisation of *Colourless1 (C1)* in maize (Cone et al., 1986; Paz-Ares et al., 1986). A *C1* paralogue *Purple leaf (Pl)* is also present in maize, which regulates anthocyanin metabolism in different tissues to *C1* (Cone et al., 1993a). *C1* and *PL* have been shown to be functionally interchangeable (Cone et al., 1993b), suggesting a gene duplication event (Cone et al., 1993a), allowing for differential tissue-specific regulation of anthocyanins. In petunia, the *Anthocyanin2 (An2)* locus, which regulates anthocyanin pigmentation in the corolla of the flower, has been cloned and encodes an R2R3 Myb transcription factor homologous to *C1/Pl* (Quattrocchio et al., 1998; Quattrocchio et al., 1999), while it appears the *Anthocyanin4 (An4)* regulatory locus also encodes a Myb protein, linked to flavonoid biosynthesis in anthers (Quattrocchio et al., 1998; Quattrocchio et al., 1999; Koes et al., 2005).

Similarly in snapdragon, Myb genes which regulate anthocyanin biosynthesis have been identified, namely *Rosea1*, *Rosea2*, and *Venosa* (Martin et al., 2001). These are required for pigmentation, and their spatial expression allows for pigmentation patterning. *ROSEA1* and *ROSEA2* regulate anthocyanin production in both lobes of the flower, while *VENOSA* is responsible for a striking pigmentation pattern, which is limited to the epidermal cells overlaying the veins (Schwinn et al., 2006). *Arabidopsis* has two anthocyanin regulatory Myb genes, *Producer of Anthocyanin Pigment1 (PAP1, AtMyb75)* and *Producer of Anthocyanin Pigment2 (PAP2, AtMyb90)* (Borevitz et al., 2000), as well as others such as *Transparent testa2 (TT2)* which regulate proanthocyanidin biosynthesis (Nesi et al., 2001; Debeaujon et al., 2003).

The R2R3 Mybs discussed thus far act as transcriptional activators, but another class of R2R3 Myb transcription factors appear to have repressive functions. This subgroup of Myb transcription factors clusters to 'subgroup 4' characterised by the consensus amino acid motif pdLNL[D/E]LXi[G/S] (Kranz et al., 1998). In snapdragon *AmMyb308* and *AmMyb330* are believed to encode repressive Myb transcription factors which repress lignin and hydroxycinnamic acid production (Tamagnone et al., 1998). The *Arabidopsis* homologue *AtMYB4* also represses hydroxycinnamic acid production. In the absence of UVB irradiation, cinnamate 4-hydroxylase (*C4H*) is repressed by *AtMYB4*, but once irradiated with UVB, *AtMyb4* is down-regulated, de-repressing *C4H*, enabling the production of sinapate esters, which are effective UVB protectants

(Jin et al., 2000). The possible mechanisms for repression by these Myb transcription factors include competition for co-regulators (such as bHLH transcription factors) with strong activating Mybs (Tamagnone et al., 1998), a type of repression called 'squenching' (Gill and Ptashne, 1988) or, alternatively, direct active repressive functions, which may include binding to promoter elements of target genes (Jin et al., 2000). AtMYB4 has been shown to actively repress its target genes. It has been shown to bind DNA elements, and a motif within the C terminal domain has been identified as being required for its repressive action (Jin et al., 2000).

It is becoming clear, from the work in maize, snapdragon, petunia and *Arabidopsis*, that families of functionally similar Myb proteins exist in many species, giving rise to various induced and developmental pigment patterns.

1.5.3 WD40 regulator

The third class of regulatory factor involved in flavonoid and anthocyanin regulation are the WD40 repeat proteins. They are not transcription factors, have no intrinsic enzymatic activity and do not bind directly to the promoters of flavonoid structural genes (Ramsay and Glover, 2005). These proteins contain structural features which facilitate multiple protein-protein interactions (i.e. docking sites) (Smith et al., 1999). These proteins are involved in a wide variety of processes, from signal transduction to regulating developmental processes (Smith et al., 1999; Ramsay and Glover, 2005).

WD40 repeat proteins involved in flavonoid regulation have been identified in several of the model species: *Pale aleurone colour1 (PAC1)* from maize (Carey et al., 2004); *Transparent testa glabra1 (TTG1)* from *Arabidopsis* (Walker et al., 1999); *Anthocyanin11 (An11)* from petunia (de Vetten et al., 1997); *Perilla frutescens* WD (*PFWD*) from *Perilla frutescens* (Sompornpailin et al., 2002). The WD40 proteins are generally regarded as being expressed ubiquitously throughout the plant, even in non-pigmented tissues (de Vetten et al., 1997; Walker et al., 1999; Sompornpailin et al., 2002; Carey et al., 2004). The involvement of the *An11*, *PAC1* and *TTG1* loci in flavonoid regulation were all initially recognised by mutant analysis, where anthocyanins failed to be produced (or were produced at reduced levels). In addition to

the defects in flavonoid metabolism, *ttg1* mutants also displayed defects in trichome development and seed mucilage formation, demonstrating that TTG1 appears to have overlapping functions with other processes, something also observed for the bHLH and Myb classes of transcription factors.

WD40 proteins have been implicated in the nuclear transport of transcription factors. The cellular location of AN11 and PFWD has been shown to be primarily cytosolic, despite the presence of a putative nuclear localisation signal (de Vetten et al., 1997; Sompornpailin et al., 2002). However, yeast two-hybrid experiments have shown that PFWD interacts with a bHLH transcription factor, MYC-RP, and coexpression of these factors *in planta* resulted in an increase in nuclear localisation (Sompornpailin et al., 2002), which has raised the suggestion that the WD40 protein may be involved in transporting the flavonoid transcription factors into the nucleus (Broun, 2005).

1.5.4 Myb-bHLH-WD40 regulatory complex

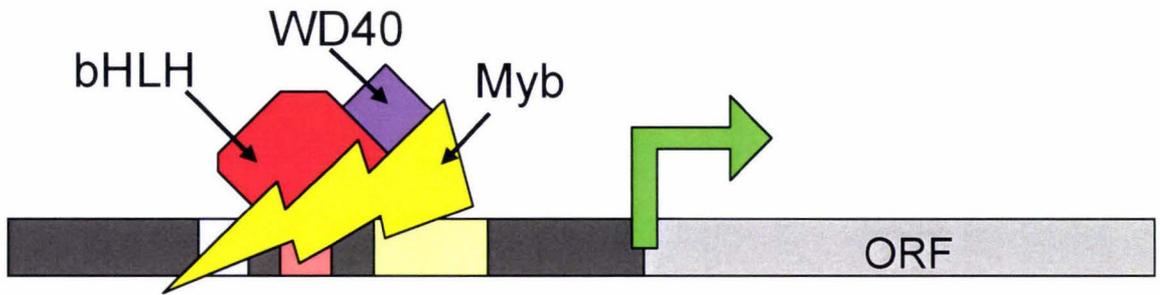
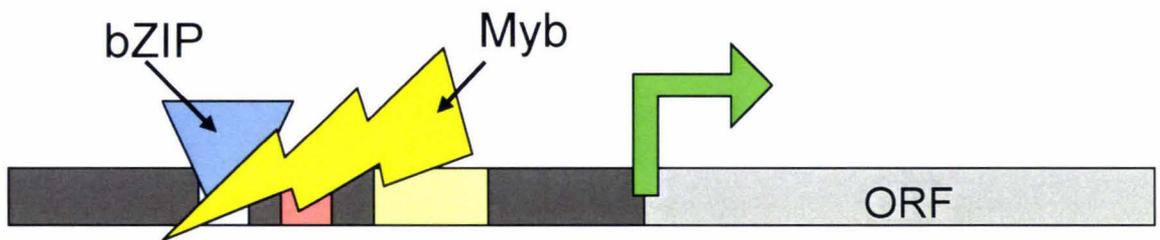
It has been recognised for some time, that introduction of a single transcription factor (bHLH, or Myb) was often insufficient to drive anthocyanin production, especially in tissues which were not normally pigmented (Ludwig et al., 1990; Lloyd et al., 1992). However, co-introduction of bHLH and Myb transcription factors could induce anthocyanin formation in colourless tissues (Goff et al., 1990; Lloyd et al., 1992), and subsequent promoter activation assays showed that co-introduction of a bHLH and Myb transcription factor resulted in a significantly higher activation than either transcription factor alone (Goff et al., 1992). Similar results have been observed in petunia, particularly through the use of transient assays, which have shown that co-expression of a bHLH (*Jaf13*, *An1*, *Lc*) and Myb (*An2*, *C1*) transcription factors could activate a DFR-reporter construct, and induce anthocyanin production (Quattrocchio et al., 1998). This suggested that a Myb-bHLH regulatory complex was required for the regulation of the flavonoid structural genes.

In petunia, the *An1* (bHLH), *An2* (Myb) and *An11* (WD40) loci are required for anthocyanin pigmentation in the corolla (Quattrocchio et al., 1993), and in *Arabidopsis* the *TT8* (bHLH), *TT2* (Myb) and *TTG1* (WD40) loci are required for proanthocyanidin

accumulation in the seed coat (Nesi et al., 2001), suggesting that these three components may interact cooperatively to regulate the structural genes. Recently, a paper by Baudry *et al.* (2004) showed that a ternary complex consisting of a Myb-bHLH-WD40 existed, which activates the structural genes required for proanthocyanidin production in the seed coat of *Arabidopsis*. A combination of yeast two- and three-hybrid experiments showed that TT8, TT2 and TTG1 interacted together, in a complex. TTG1 (WD40) interacted most strongly with TT8 (bHLH), although some interaction with TT2 (Myb) was observed. The TT2-TT8 (Myb-bHLH) complex could activate the *Banyuls* promoter (BANYULS = ANR, shown in Figure 1.2) but this activation was enhanced with the introduction of TTG1, suggesting it may play a role in stabilising the bHLH-Myb interaction. When this study was extended to a whole plant system this interaction appeared to occur *in planta* (Baudry et al., 2004).

Such a (regulatory) complex may regulate anthocyanin structural genes in the manner shown in the model in Figure 1.4A. Similar models to the one shown in Figure 1.4A have been published in Baudry *et al.* (2004), Broun (2005) and Ramsay *et al.* (2005). The Myb, bHLH and WD40 proteins form a complex and the transcription factors bind to elements within the target promoters and activate transcription. Hartmann and coworkers (2005) have identified *cis*-elements in the promoters of the early flavonoid biosynthetic genes of *Arabidopsis* which are recognised by Myb (Myb recognition element, MRE) and bHLH (R response element, RRE) transcription factors. These *cis*-elements lie adjacent to each other, and probably facilitate the binding of the Myb-bHLH-WD40 complex to the target promoters. It is assumed that similar *cis*-elements are present in the late biosynthetic genes required for anthocyanin biosynthesis based upon the requirement for the Myb-bHLH-WD40 complex to regulate these genes. Supporting this, Myb and bHLH binding elements were identified in the *Bronze2* gene required for anthocyanin synthesis (a late biosynthetic gene), and were both found to be required for activation by R (bHLH) and C1 (Myb) (Bodeau and Walbot, 1996).

The target structural genes for anthocyanin biosynthesis regulated by the Myb-bHLH-WD40 complex differ between species. In maize, C1-R-[PAC1] co-ordinately regulate both the early and late biosynthetic genes (Quattrocchio et al., 1998). In contrast, in petunia, AN2-AN1-AN11 regulates the late biosynthetic genes, but not the early biosynthetic genes, *CHI* and *F3H* (Quattrocchio et al., 1993; Quattrocchio et al., 1998)

A**B**

ACE	= ACGT containing element (recognised by bZIP transcription factors)
RRE	= 'R' response element (recognised by bHLH transcription factors)
MRE	= Myb recognition element (recognised by Myb transcription factors)

Figure 1.4: Models of transcription factor complexes involved in regulating **A** anthocyanins and **B** the early biosynthetic genes and *FLS*. ACE, RRE and MRE elements are recognised and bound by bZIP, bHLH and Myb transcription factors respectively.

and in snapdragon, *CHS* and *CHI* escape regulation by ROSEA-DELILA-[AmWD40?] (Schwinn et al., 2006). These differences in gene targets lie in the promoters, not the regulators (Quattrocchio et al., 1993; Quattrocchio et al., 1998), and probably occurs so that other flavonoids such as flavones or flavonols can be independently regulated from anthocyanins (Davies and Schwinn, 2003). This is particularly important to recognise for petunia, as the early biosynthetic genes must be activated before metabolites will be supplied for anthocyanin production. This may account for the requirement of light (i.e. activating the early biosynthetic genes) for pigmentation in developing petunia flowers. Similarly, light activation of the early biosynthetic genes may be required for the light-induced anthocyanin pigmentation in *Lc* petunia.

It is interesting to note that the Myb-bHLH-WD40 regulatory complex is shared with other developmental programs, such as trichome development, seed mucilage production (Broun, 2005; Ramsay and Glover, 2005) and vacuolar acidification (Koes et al., 2005). Specific Myb and bHLH transcription factors have been identified for these processes, although some appear to have overlapping functions. AN1 (bHLH) and AN2 (Myb) regulate anthocyanin production in the corolla of petunia flowers, but AN1 also regulates vacuolar acidification (Spelt et al., 2002) with a newly identified Myb, PhPH4 (Quattrocchio et al., 2006).

1.5.5 Light regulation of the early flavonoid biosynthetic genes

The early biosynthetic genes of the phenylpropanoid and flavonoid pathway (including *PAL*, *CAH*, *4CL*, and *CHS*) have been shown to be induced by light, particularly short wavelengths (UVA/blue and UVB). The UV light responsiveness of these genes is consistent with the role of polyphenols (cinnamic acid derivatives), and flavonoids (particularly flavonols) as photoprotectants. Investigations of *cis*-elements within the promoters of these genes have provided insight into their regulation.

The 'light regulatory unit' (LRU), which consists of a Myb recognition element (MRE) and ACGT containing element (ACE), has been found to be necessary and sufficient to confer UV responsiveness to a core-promoter (Hartmann et al., 1998). The ACE has

been shown to bind basic region/leucine zipper (bZIP) transcription factors (Feldbrügge et al., 1994), and as discussed in Section 1.5.4 Myb transcription factors bind to the MRE. The common plant regulatory factor (CPRF) bZIP transcription factors from parsley, and G-box binding factors (GBF) bZIP transcription factors from *Arabidopsis* have been shown to recognise and bind the ACE elements within the LRU of *CHS* promoters (Feldbrügge et al., 1994; Menkens et al., 1995; Jakoby et al., 2002). *Long hypocotyl5 (Hy5)* is a positive regulator of photomorphogenesis (Ang and Deng, 1994), and encodes a bZIP transcription factor (Ang et al., 1998). Light perception and signalling pathways converge on the COP signalosome complex, which in the absence of light signalling results in COP1 targeting HY5 for degradation (Serino and Deng, 2003). HY5 has been shown to bind to ACE elements within light responsive promoters, and is involved in light induction of these target genes (Ang et al., 1998; Chattopadhyay et al., 1998).

Recently, it was demonstrated that the LRU present in the promoters of *CHS*, *CHI*, *F3H* and *FLS*, mediates the coordinated expression of these genes in response to UV-containing light which is required for flavonol production (Hartmann et al., 2005). The LRU consists of an MRE and ACE, and each was found to be required for light induced activation of the genes. In addition to this, the promoters of the *CHS*, *CHI* and *F3H* contain an RRE (bHLH) adjacent to the MRE (Myb) and ACE (bZIP) elements, which were shown to confer Myb/bHLH responsiveness. It was suggested that a common set of *cis*-elements could be utilised for light induced regulation (to produce flavonols) via the MRE and ACE, or for developmentally controlled regulation via the MRE and RRE (anthocyanins or other flavonoids) (Hartmann et al., 2005).

A newly characterised R2R3 Myb, AtMYB12, is believed to be the flavonol-specific regulator (Mehrtens et al., 2005). AtMYB12 has been shown to require a functional MRE, and works independently from a bHLH partner. The evidence suggests that it may cooperate with a bZIP partner to regulate the early biosynthetic genes and *FLS* to give light-induced expression (Mehrtens et al., 2005). Figure 1.4B shows a model for light-regulated activation of the early biosynthetic genes (for flavonol production), by bZIP and Myb transcription factors, which is based on the combined results of Hartmann *et al.* (2005) and Mehrtens *et al.* (2005).

The models presented in Figure 1.4 show that the location of the RRE and ACEs *cis*-elements adjacent to the MRE may allow for combinatorial control of the structural genes for light-induced or developmentally regulated expression. This is important for petunia, as flavonols are the primary flavonoid produced in leaves, and the current understanding of the regulation of flavonols and the early biosynthetic genes suggest that their regulation is mediated by a different transcription factor complex (Myb-bZIP, Figure 1.4B) compared to anthocyanin regulation (Myb-bHLH-WD40, Figure 1.4A).

1.5.6 Light regulation of flavonoid transcription factors

The recognition that light can regulate anthocyanin biosynthesis led to the analysis of the expression of flavonoid transcription factors. The flavonoid and anthocyanin structural genes are transcriptionally regulated by the Myb and bHLH transcription factors, and there is evidence that these transcription factors are themselves transcriptionally regulated in response to environmental signals, including light.

The light-inducibility of the regulatory loci, *R* (bHLH), *B* (bHLH) and *P1* (Myb, the dominant allele) in maize, were investigated and compared with the induction of flavonoid/anthocyanin structural genes (Taylor and Briggs, 1990). The expression of *R* was shown to be induced by intense light, and *R* expression (but not *B* or *P1*) matched the accumulation of anthocyanins (Taylor and Briggs, 1990). Similarly, the bHLH transcription factor *Sn* from maize has also been shown to be light-induced (Tonelli et al., 1991; Procissi et al., 1997).

The Myb transcription factors C1 and pl from maize (recessive but not a null mutation) both show light inducibility (Cone et al., 1993b; Kao et al., 1996; Procissi et al., 1997; Piazza et al., 2002), and the light-induced expression of Myb anthocyanin regulatory genes has been found to be the factor limiting anthocyanin biosynthesis in different tissues (Procissi et al., 1997; Gong et al., 1999a; Piazza et al., 2002). MYB-P1 from *P. frutescens*, the determining factor for anthocyanin production in leaves, is differentially expressed in red compared to green forms of *P. frutescens*, and exhibits light inducibility (Gong et al., 1999a).

The *Arabidopsis* flavonol regulator, AtMYB12, and the putative snapdragon orthologue, AmMYB305, are probably light regulated. It was shown in *Arabidopsis* that the early biosynthetic genes and *FLS* were co-ordinately expressed in response to UV-containing light, and the promoters of each of these genes contained binding sites for Myb and bZIP transcription factors, which were required for conferring light responsiveness (Hartmann et al., 2005). Since then, AtMYB12 has been identified as a regulator of flavonol production (Mehrtens et al., 2005), which infers that it is probably also induced by light, so that it can co-ordinately regulate the structural genes.

1.6 The transcription factor LEAF COLOUR (LC)

LEAF COLOUR (LC), as stated earlier, is a bHLH transcription factor, which regulates anthocyanin biosynthesis in the leaves of maize (Ludwig et al., 1989). It has been used widely for studying the regulatory mechanisms involved in anthocyanin regulation (Ludwig et al., 1990; Cone et al., 1993b; Quattrocchio et al., 1993), and has also been used as a means to try to alter flavonoid metabolism in heterologous plant species (Goldsbrough et al., 1996; Boase et al., 1998; Bradley et al., 1998; Bradley et al., 1999; Bovy et al., 2002; Ray et al., 2003).

Early experiments where *Lc* was introduced into maize tissue resulted in pigmentation in most tissues. However, an important observation was that *Lc* never induced pigmentation in maize endosperm cells – a tissue that has never been reported to produce anthocyanins. Ludwig *et al.* (1990) suggested that there was a factor (or factors) that were suppressing anthocyanin biosynthesis in these cells, and that the lack of an *R* gene in this tissue was not the limiting factor preventing the tissue from becoming pigmented. Another interpretation is that, in the endosperm tissue, regulatory factors required for induction of anthocyanin production were absent, and *R* could not complement this. From our current understanding of the interactions that occur between the bHLH and Myb transcription factors (for model, see Figure 1.4A) the lack of pigmentation observed in endosperm tissue transformed with *Lc* could be due to the absence of an endogenous Myb transcription factor.

Lc failed to upregulate flavonoid biosynthesis in pelargonium (*Pelargonium × domesticum* Dubonnet) or lisianthus (*Eustoma grandiflorum*), despite detectable *Lc* expression by northern analysis. This may be because there is no Myb partner to which LC can functionally bind (the result of divergent evolution from maize). Alternatively, promoters for flavonoid biosynthetic genes may not contain *cis*-elements recognisable by LC (Bradley et al., 1999). *Lc* expression was not detected in stable chrysanthemum (*Dendranthema grandiflorum*) transformants, despite expression of kanamycin resistance, and presence of the *Lc* transgene construct (Boase et al., 1998). One explanation for this result is that some form of gene silencing may have been occurring. Interestingly, gene silencing has been reported to occur between bHLH *R* genes, and methylation was implicated (Tonelli et al., 1994). Alternatively post-transcriptional gene silencing due to interactions with endogenous bHLH factors may be acting, as has been observed when flavonoid structural genes were overexpressed (Napoli et al., 1990). Silencing may prevent *Lc* accumulation and account for the results in chrysanthemum.

Tomato plants (*Lycopersicon esculentum*), transformed with CaMV35S::*Lc* produced anthocyanins throughout the plant. However, pigmentation only occurred upon exposure to high light, and shaded leaves remained green until light exposure (Goldsbrough et al., 1996). This finding was also observed when CaMV35S::*Lc* was introduced into Mitchell petunia. The *Lc* petunia plants were highly pigmented when grown under high irradiation, but under conditions of lower light intensities, tissues remained green (Davies and Schwinn, 2003). This requirement for strong light was also observed in transgenic CaMV35S::*Lc* alfalfa (*Medicago sativa*) which turned dark red when exposed to strong light intensities, or when cold-stressed (Ray et al., 2003). These results were unexpected as the *Lc* transgene expression driven from a CaMV35S promoter is generally regarded as being strong, constitutive and light-insensitive (Benfey and Chua, 1989). Ray *et al.* (2003) suggested that *Lc* may be interacting with suppressive Myc (bHLH) or Myb proteins. While there is some evidence for repressive anthocyanin regulatory Myb proteins (e.g. FaMYB1 (Aharoni et al., 2001)), there is more experimental support for a bHLH (LC) interacting with strong activating Myb transcription factors, as discussed in Section 1.5.4. It seems more likely that the conditions of high light or cold stress induce the expression of a Myb protein capable of

forming an active complex with LC. Genes corresponding to Myb proteins have been reported to be induced by light (Cone et al., 1993b; Procissi et al., 1997; Gong et al., 1999a; Piazza et al., 2002), nitrogen stress (Miyake et al., 2003) and dehydration (Ithal and Reddy, 2004), stresses known to induce anthocyanin accumulation in many plants. Therefore, it is plausible that the light-induced pigmentation observed in *Lc* tomato, petunia, and alfalfa is due to the induction of a Myb protein, enabling anthocyanin activation.

1.7 Project hypothesis and aims

This thesis investigated light-induced anthocyanin pigmentation in the petunia plants originally described in Bradley *et al.* (1998) which had been transformed with the maize *Leaf colour* cDNA (*Lc* petunia). These *Lc* petunia plants had been grown in the first field trial of a genetically modified ornamental species in New Zealand. When grown under field conditions, with high light and environmental stresses, the plants became intensely pigmented with anthocyanins throughout the vegetative tissues. However, when grown under weaker light considerably less anthocyanin pigment was produced. Since *Lc* was under the control of the strong, constitutive, light-insensitive CaMV35S promoter, the light-induced pigmentation phenotype of *Lc* petunia was unexpected. This raised several questions about the regulation of the anthocyanin biosynthetic genes in response to light, and in conjunction with LC.

To address this question, the following hypothesis was proposed:

- Light induces an anthocyanin-regulatory Myb transcription factor with which LC is able to partner leading to activation of the anthocyanin structural genes and pigmentation.

There is a significant amount of evidence supporting light induction of Myb anthocyanin regulators (Section 1.5.6), and the requirement of both bHLH (such as LC) and Myb transcription factors to regulate anthocyanin structural genes (Section 1.5.4), as presented in the model in Figure 1.4A.

As previously described, (Section 1.2) the petunia cultivar Mitchell has null alleles for the two known anthocyanin Mybs, AN2 and AN4. Therefore, if an endogenous petunia Myb regulator is identified, it should be independent of these loci.

Alongside the experimental hypothesis, the project had a number of specific aims:

- To re-establish a hemizygous seed line of *Lc* petunia from those developed by Bradley *et al.*, (1998), to gain a standard source of plant material for investigating light-induced anthocyanin pigmentation (Chapter 3).
- To determine the effect of lighting intensity on *Lc* transgene expression, in order to confirm that the expression of *Lc* from the CaMV35S promoter was not responsive to light intensity (Chapter 3).
- To measure the differences in expression of flavonoid structural genes and flavonoid and anthocyanin levels in Mitchell and *Lc* petunia grown under different light intensities, in order to characterise the response at the biochemical and molecular levels (Chapter 4).
- To examine whether supplying heterologous Myb transcription factors could complement the requirement for light for pigmentation in *Lc* petunia (Chapter 5).

Consequently, the overall aim of this research project was to further characterise the transgenic *Lc* petunia, and to provide deeper insight into the requirement for high light levels to promote anthocyanin biosynthesis in these plants.

Chapter 2

General materials and methods

2.1 Chemicals

Unless otherwise stated, chemicals and reagents were supplied by BDH, Sigma, Merck and Roche. Solvents were sourced from Merck.

2.2 Media

Bacterial and tissue culture media were prepared with milliQ water, and sterilised by autoclaving (120°C and 15 psi, for 20 min). Solid media was cooled to 50°C before the addition of antibiotics, while liquid media was allowed to cool to room temperature before the addition of antibiotics. Recipes for media and antibiotic stocks in are included in Appendix 1.

2.3 Buffers and solutions

At all times, milliQ water was used to make up solutions. In cases where a solution was not autoclaved before use, autoclaved milliQ water was used. Recipes for buffers and solutions are included in Appendix 1.

2.4 Bacterial manipulation

2.4.1 General bacterial growth conditions

The *Escherichia coli* strain used for general lab manipulations was NovaBlue (Novagen). Unless otherwise stated, *E.coli* was grown on LB-agar plates or LB-broth (Appendix 1.1) under appropriate antibiotic selection at 37°C. Liquid cultures were grown at 37°C in a shaking incubator at 250 rpm.

The *Agrobacterium tumefaciens* strain used was LBA4404 (Invitrogen). Unless otherwise stated, *A.tumefaciens* was grown on YM-agar plates and YM-broth (Appendix 1.1), under appropriate antibiotic selection at 28°C. Liquid cultures were grown at 28°C in a shaking incubator at 250 rpm.

2.4.2 Chemically competent *E.coli* cells

This method is based on that of Inoue *et al.* (1990), except that cells were grown more slowly at 18°C until the required optical density was reached. The antibiotic selection for Novablue cells was 10 mg L⁻¹ tetracycline.

2.4.3 Heat-shock transformation of *E.coli*

Heat-shock transformation using chemically competent NovaBlue *E.coli* cells (Section 2.4.2) was performed for basic manipulation of plasmid stocks and ligated PCR products. Freshly thawed competent cells (50 µL) were incubated with approximately 1 µg plasmid DNA (or 2 µL of a ligation reaction) on ice for 20 min. After 20 min, cells were heat-shocked in a 42°C water bath for 45 sec, and then transferred back onto ice for 5 min. Cells were allowed to recover in LB-broth for 1 h at 37°C following transformation, before being plated out onto LB-agar plates with the appropriate antibiotic selection.

2.5 Plasmid DNA preparations

2.5.1 *E.coli* plasmid preparation using a standard alkaline lysis method

The following plasmid preparation method was routinely used when plasmid DNA was required for diagnostic purposes: 1 mL of overnight *E.coli* culture was used for each preparation. Cells were pelleted by centrifugation in a bench-top centrifuge and liquid media removed. Cells were re-suspended in 250 μL solution I, lysed with 250 μL solution II, and the solution was neutralised with 250 μL of solution III (Appendix 1.2). The protein and carbohydrate precipitate was removed by centrifugation at 18 000 g . DNA was precipitated from the cleared lysate with 1 volume isopropanol. DNA was pelleted by centrifugation, washed with 70% (v/v) ethanol, and air-dried for 15 min. DNA pellets were dissolved in 20 μL RNase water (20 μL of 10 mg mL^{-1} RNase A added to 980 μL sterile water).

2.5.2 *E.coli* plasmid preparations – large volume

The QIAGEN midiprep plasmid kit was used to prepare large volumes of high concentration, high purity plasmid preparations. DNA from this system was used for biolistic-mediated transformation, and providing a clean source of plasmid for gene inserts required for use as radioisotope labelled probes.

Plasmid DNA was isolated from 50 mL of overnight *E.coli* culture following the manufacturer's instructions. This method is based on a modified alkaline lysis method, but incorporates the use of an anion exchange column that binds plasmid DNA and purifies it from the cellular lysate. DNA was then eluted from the column, and precipitated with isopropanol. The DNA pellet was dissolved in 100 - 300 μL of 10 mM tris (pH 7.0).

2.5.3 *E.coli* plasmid preparation – small volume

The Sigma GenElute plasmid miniprep kit was used when small volumes of pure plasmid were required for DNA sequencing. Plasmid DNA was isolated from 1.5 mL of overnight *E.coli* culture following the manufacturer's instructions. This preparation kit is based on a modified alkaline lysis system which incorporates a silica column that binds plasmid DNA from the cleared cellular lysate. The plasmid DNA was then eluted from the column in 50 μ L of water.

2.5.4 *A.tumefaciens* plasmid preparation using a standard alkaline lysis method

This method was used only for diagnostic purposes to determine whether the *A.tumefaciens* culture contained the correct binary vector construct. DNA was isolated as described in Section 2.5.1 except that 3 mL of a 48 h liquid culture grown in YM-broth was used.

2.6 DNA manipulations

2.6.1 Restriction-endonuclease digestion

Restriction-endonuclease digests of plasmid DNA were performed routinely for diagnostic purposes and for isolation of plasmid inserts for use as radioactively labelled probes for northern and Southern blots. Typically, 1 - 2 μ g of plasmid DNA was digested with 5 U restriction-endonuclease enzyme(s) (Roche), in a total volume of 15 μ L in 1 \times commercial restriction enzyme buffer (Roche), matched to the enzyme, or enzyme combination. The digest reaction mixture was left to incubate at 37°C for 1 - 2 h. Digested DNA was separated by agarose-gel electrophoresis (Section 2.6.2).

2.6.2 Agarose-gel electrophoresis

Agarose-gel electrophoresis was routinely used to separate DNA following extraction, PCR or restriction-endonuclease digestion. The appropriate amount of agarose was added to 1 × electrophoresis buffer to give a 1% (w/v) gel, and heated until the agarose had completely melted. The gel solution was allowed to cool to 50°C, before the addition of 5 µL of ethidium bromide (10 mg mL⁻¹) to 250 mL of agarose gel solution. Gels were poured into the gel apparatus and once set, covered in 1 × electrophoresis buffer. 10 × loading dye (Appendix 1.3.2) was added to DNA samples to give a final 1 × concentration before loading onto the gel. The electrophoresis buffers routinely used were TAE, TBE and NaB (Appendices 1.3.1), and gels were run at 50 - 80 V, 80 - 120 V or 100 - 200 V, respectively, until adequate separation was achieved. DNA was visualised by ethidium bromide fluorescence using a short wavelength UV transilluminator (Alpha Innotech), and digital photographs obtained using the Alphaimager 2000 Documentation and Analysis System (Alpha Innotech). For publication quality gel images, fluorescent images of ethidium bromide stained gels were captured using the FLA-5100 imaging system (Fujifilm). DNA size estimations were made by comparing the mobility of the DNA samples against a DNA ladder marker, when run on agarose gels (e.g. PCR products or restriction digests). The ladder used was the 1 kb Plus ladder (Invitrogen): 0.5 µL of ladder was mixed with 8.5 µL of water and 1 µL of 10 × loading dye (Appendix 1.3.2) and loaded next to DNA samples.

2.6.3 DNA quantification

DNA concentration was assessed by gel quantification and spectrophotometric methods. The spectrophotometric method enabled assessment of the purity of the sample in addition to the concentration of DNA.

2.6.3.1 Gel quantification

Sample DNA (1 µL) was made up to a final volume of 10 µL with water and 1 µL of

10 × loading dye, and electrophoresed on an agarose-gel next to 2 μL of high mass or low mass DNA quantification ladders (Invitrogen), also made up to 10 μL with water and 1 μL of 10 × loading dye. The amount of DNA in the unknown samples was estimated by the fluorescent intensity of ethidium bromide staining compared to the mass ladders.

2.6.3.2 Spectrophotometric method

An aliquot of DNA sample (typically 1 – 5 μL) was diluted with milliQ water, to give a final volume of 500 μL. The absorbance of the diluted DNA was determined at 260 and 280 nm using a Shimadzu UV-visible recording spectrophotometer, using quartz cuvettes and blanked with water.

The absorption at 260 nm × 50* × dilution factor = DNA concentration μg mL⁻¹

* assumes that 50 μg mL⁻¹ of double stranded DNA has an $A_{260} = 1.0$. The ratio of absorbance values of the sample, A_{260}/A_{280} , was used to assess the purity of the DNA sample, where a pure sample has an $A_{260}/A_{280} = 1.8 - 2.0$.

2.7 DNA purification

2.7.1 Gel extraction and purification

PCR products, or inserts generated by restriction digest of plasmid DNA, were separated by gel electrophoresis and bands corresponding to the inserts/PCR products were excised from the gel. DNA was purified from the gel slices using the QIAGEN MinElute Gel Extraction kit following the manufacturer's instructions. The basis of the extraction is that the DNA, solubilised in a high salt buffer, will bind to a silica membrane and allow gel contaminants to pass through. Salt was removed with an ethanol wash, and DNA was eluted from the membrane in 10 μL of elution buffer.

2.7.2 PCR reaction clean-up

PCR reactions were purified using the QIAGEN MinElute Reaction cleanup kit, following the manufacturer's instructions. This purification removes enzymes, unused dNTPs and oligonucleotide-primers from the PCR products. The purified PCR products were then suitable for cloning.

2.8 Hybridisation and probing of northern and Southern blots

2.8.1 Pre-hybridisation

Before northern or Southern blot membranes were probed, they were pre-hybridised in Church and Gilbert buffer (Appendix 1.6.3) at 65°C for 2 h. This was to prime the membrane and nucleic acids for probing, and to reduce non-specific binding of radioactive probe to the membranes.

2.8.2 Probing

To make probes for gene expression analysis for northern blots, or to detect transgene presence and copy number for Southern blots, ³²P radioactively labelled probes were made. The HighPrime labelling kit (Roche) was used, following the manufacturer's instructions. Approximately 50 ng of template DNA, corresponding to cDNA inserts of the genes of interest, were used for making the probe. The basis of the probe labelling system is randomly primed PCR incorporation of ³²P cytosine residues into the product. Unincorporated radioactive-nucleotides were removed with ProbeQuant G50 micro-columns (Pharmacia Biotech Inc.). The radioactive probe was added to fresh Church and Gilbert pre-hybridisation buffer and membranes were incubated overnight on a shaking-platform incubator at 65°C to allow hybridisation.

2.8.3 Stringency washes

Membranes for both northern and Southern blots were washed following incubation with a radioactive probe. This was done to remove excess probe, bound non-specifically to the membranes or nucleic acids. The standard washing protocol was to incubate membranes in a series of SSC washes for 30 min at 65°C with shaking, each wash containing 0.1% (w/v) SDS. The salt stringency washes ranged from $3 \times \text{SSC}$ to $0.1 \times \text{SSC}$ (Appendix 1.6.2), and washes were performed to the most stringent salt conditions possible to remove non-specifically bound probe, without losing the entire radioactive signal (determined using a Geiger counter). Washes were stopped when no background signal was present, or if the radioactivity dropped below 5 cpm.

2.8.4 Autoradiography

Once northern and Southern blot membranes had been probed and washed, they were sealed in plastic, and exposed to MS film (Kodak) in autoradiography cassettes with intensifying screens. The cassettes were stored at -80°C during exposure so the intensifying screens could work at maximum efficiency. The length of exposure depended on the strength of the radioactive signal; typically they were exposed for 72 h. Autoradiographs were developed in developer for 2 min and fixative for 2 min, following manufacturer's instructions (Kodak).

2.8.5 Stripping membranes

Membranes were stripped of radioactive probe in boiling SDS. Sterile water was brought to boiling point, and SDS solution added to the water to a final concentration of 0.1% (w/v). Membranes were covered in the boiling SDS and left until cold. The cold SDS was removed, and membranes were checked for radioactive signal. If no signal remained, membranes were sealed in plastic and stored at -20°C or re-probed. If a radioactive signal was still detected, the stripping procedure was repeated until no signal remained.

2.9 Plant material and growing conditions

Petunia Mitchell (*Petunia axillaris* × [*Petunia axillaris* × *Petunia hybrida* cv. ‘Rose of Heaven’]) (Ausubel et al., 1980) was originally obtained by Dr Simon Deroles from the School of Biological Sciences, University of Auckland, New Zealand. The *Lc* transgenic petunia plants were generated by transforming wild type Mitchell petunia with *Leaf colour (Lc)*, a bHLH transcription factor that regulates anthocyanin synthesis in *Zea mays*, under the control of the CaMV35S promoter (Bradley 1998). Appendix 3.1 shows the binary vector originally used to transform the petunia plants via *Agrobacterium*-mediated transformation.

The two transgenic *Lc* petunia seed lines used in this study, 118B and 118C, were derived from transformant 118, as originally described in Bradley *et al.* (1998). The exact genealogy of the 118B and 118C lines is unknown, although the 118C line is believed to be derived from back-crossing a homozygous *Lc* parent to wild type Mitchell petunia. The seeds were in long-term storage at 4°C for approximately 5 years prior to this study.

Plants were grown in a potting mix of 60% (v/v) bark, 40% (v/v) 4 – 7 mm pumice and supplemented with Dolomite (5000 g m⁻³), superphosphate (200 g m⁻³), calcium ammonium nitrate (100 g m⁻³), potassium sulphate (500 g m⁻³), iron (5000 g m⁻³), Osomcote slow release fertiliser (4300 g m⁻³), Micromax trace elements (50 g m⁻³) and Terrazole fungicide (100 g m⁻³).

The plants were grown within the PC2 containment greenhouse facility at Crop & Food Research, Palmerston North. The greenhouse was constructed from double-skin plastic.

Chapter 3

Phenotypic and genotypic assessment of *Lc* petunia

3.1 Introduction

Transgenic petunia carrying the bHLH transcription factor *Leaf colour (Lc)* under the control of the CaMV35S promoter became intensely pigmented with anthocyanins when grown under field conditions. However, a range of pigmentation phenotypes were observed when these plants were grown within a plastic greenhouse. Subsequent experiments have shown that the differences in pigmentation observed between field- and greenhouse-grown *Lc* petunia may be due to light intensity (Davies and Schwinn, 2003). The focus of this project was to investigate the response of these plants to light. Firstly, a consistent seedling population had to be established, and it was necessary to establish that the transgene was being expressed constitutively.

The two lines of *Lc* petunia available, each carrying CaMV35S::*Lc*, were initially assessed phenotypically and genetically, for their suitability for investigating the light-induced pigmentation phenotypes observed in these plant lines. Both lines were derived from the same primary transformant '118' (Bradley et al., 1998). It had been recognised that plants of the 118B line produced two or more pigmentation phenotypes when grown under high-light, whereas plants of the 118C line displayed only one pigmentation phenotype when grown under the same conditions (Boase et al., 2000).

It is some years since the *Lc* petunia had been used for the field trial. The initial phase of this project therefore involved reassessing the transgenic plants for their pigmentation phenotypes when grown under high- and low-light; confirming the presence of the

transgene by assessing their ability to regenerate shoots in the presence of kanamycin, and determining both transgene copy number and zygosity. This characterisation would then allow a decision as to which plant line was most suitable for further investigation.

A separate factor under examination was the expression of the transgene. The CaMV35S promoter is considered to be constitutive, however, in some cases this has been shown not to be the case (Benfey and Chua, 1989). It was also important to establish that the observed light-induced pigmentation phenotype was not due to variable *Lc* expression. Northern analysis was performed to determine *Lc* expression in plants grown under shade, ambient-greenhouse and high-light treatments.

3.2 Materials and methods

3.2.1 Plant material and growth conditions

The two transgenic *Lc* petunia seed lines, 118B and 118C, (Section 2.9) were grown and assessed for their suitability for investigating the light induced pigmentation. Seeds of wild type Mitchell and *Lc* (line 118C) petunia (Section 2.9) were germinated under ambient-greenhouse conditions. Seedlings were individually potted before being transferred to ambient-greenhouse growth condition (see below), to prevent induction of pigmentation. Petunia plants were grown under two treatments, ambient-greenhouse and high-light, within the PC2 containment glasshouse facility at Crop & Food Research, Palmerston North. The 'high-light' growth treatment was performed within a Contherm Cat 640 controlled environment growth cabinet and plants were arranged randomly within the cabinet and spaced to prevent shading. Plants were grown under a 12 h photoperiod, at constant 22°C and 65% humidity. Light levels were recorded throughout the growth cabinet, and averaged 750 $\mu\text{mol m}^{-2} \text{sec}^{-1}$ at pot height. Light in the growth cabinet was provided by 12 HPI-T metal halide bulbs (Philips). All light measurements were determined using a LI-COR Li-250 light meter, using the LI-190SA quantum sensor.

Plants grown under the 'ambient-greenhouse' growth treatment were grown within the plastic-house without additional shading or lighting. Light levels ranged from 100 - 300 $\mu\text{mol m}^{-2} \text{sec}^{-1}$. Light levels were determined at pot height from repetitive measurements taken at midday throughout the course of the experiment. Light levels varied due to the angle of the sun and weather conditions during the course of the day. The experiment was run during Autumn (April-May, 2004): the photoperiod was approximately 12 h and the greenhouse was heated at 16°C, and vented at 21°C. Plants were photographed and assessed for pigmentation after two weeks of treatment.

A second experiment repeating and extending this experiment was performed in January 2005, and it was this plant material that was used to monitor expression of *Lc* by northern analysis, under the following light treatments: high-light, ambient-greenhouse and shade. Plant material was maintained under these conditions for 12 days before collection.

The high-light treatment was the same as described above, except that the photoperiod was changed to 14 h to match the greenhouse conditions. The ambient-greenhouse treatment was the same as described above, except that as it was Summer the photoperiod was approximately 14 h and light levels ranged from 300 - 750 $\mu\text{mol m}^{-2} \text{sec}^{-1}$. The shade treatment was a tent of 50 % black polypropylene shade cloth within the GMO plastic-house facility. Light levels varied from 50 - 350 $\mu\text{mol m}^{-2} \text{sec}^{-1}$. Leaf material was collected and immediately frozen in liquid nitrogen for RNA extraction. Material from each plant was collected separately, each representing a separate replicate.

3.2.2 Genomic DNA extraction from petunia

Genomic DNA was extracted from petunia leaves and buds using a CTAB-based nucleic acid extraction protocol (Zhang, 1998). The plant tissue was immediately

frozen in liquid nitrogen upon collection, and ground to a fine powder with a mortar and pestle in liquid nitrogen at a later date.

Frozen ground plant tissue (1 – 2 g FW) was added to 15 mL of extraction buffer (Appendix 1.6.1), pre-warmed to 55°C. Samples were mixed well before the addition of 1 volume of chloroform:isoamyl alcohol (24:1). Samples were then mixed by vortexing for 1 min, and incubated at 55°C for 15 min. The cell lysate mixture was centrifuged at 10 000 g for 15 min, until the supernatant was clear. The aqueous supernatant was carefully collected and 20 µL of RNase A (10 mg mL⁻¹) was added. Samples were incubated at 37°C for 30 min.

After RNase digestion, 1 volume of chloroform was added to the supernatant, vortexed for 1 min, and centrifuged at 10 000 g. The supernatant was collected, and this process was repeated, as necessary, until a clear supernatant with no protein or carbohydrate interface was achieved. Typically two chloroform washes were performed.

One third volume of 8 M LiCl was added to the supernatant and gently mixed. DNA was precipitated by adding 2½ volumes of cold 100% ethanol to a known volume of DNA solution. DNA was pelleted by centrifugation at 10 000 g for 20 min at 4°C. The supernatant was removed, and the pellet washed in 70% (v/v) ethanol. A second centrifugation at 10 000 g for 10 min was performed. The supernatant was removed and DNA pellets left to air dry. The DNA pellet was dissolved in 400 µL 10 mM tris (pH 7.0).

3.2.3 Junction-fragment Southern blots

3.2.3.1 Genomic DNA restriction digest

Genomic DNA (20 µg) (Section 3.2.2) was digested with 200 U *Hind*III restriction endonuclease, in a total volume of 400 µL 1 × buffer B (Roche Ltd). The digest was

incubated at 37°C overnight, and a 5 µL sample was taken, and run on a 1% (w/v) NaB gel (Section 2.6.2) to ensure digestion was complete.

The remaining digest was re-precipitated with $\frac{1}{10}$ volume 3 M NaAc (pH 5.5) and 0.7 volumes of isopropanol. DNA was pelleted by centrifugation at 18 000 × g for 10 min. The supernatant was removed, and the pellet washed in 70% (v/v) ethanol. A second centrifugation at 18 000 g for 5 min was performed. The supernatant was removed and DNA pellets left to air dry. The DNA pellet was dissolved in 30 µL 10 mM tris (pH 7.0).

3.2.3.2 Southern blotting

Loading dye (20 µL of 10 × dye) (Appendix 1.3.2) was added to the 30 µL of digested DNA samples (Section 3.2.3.1) (the higher concentration of loading dye was used to ensure the sample sank) into a 0.8% (w/v) agarose TBE gel containing ethidium bromide. The gel was loaded dry, with buffer reaching only the sides of the gel, but not covering it. This was to prevent the genomic DNA floating out. The DNA was run slowly into the gel, and once inside, the gel was covered with buffer, and the electrophoresis continued (20 V overnight). The size marker used was 5 µL of *Hind*III λ phage fragments (Gibco BRL Ltd). The gel was visualised under UV light to ensure good separation of DNA, and assess loading accuracy.

The blot was assembled using 0.4 M sodium hydroxide as transfer buffer, as described in Brown (1999), except that denaturation of the gel was not performed, as alkaline transfer negates this requirement (membrane manufacturer's instruction). The membrane used was Hybond N+ Membrane (Amersham). After transfer, the blot was disassembled, and the membrane rinsed in 2 × SSC buffer. The membrane was then subjected to UV-C cross-linking (Hoefer) for 1 min at 70 000 mJ cm⁻² and washed again in 2 × SSC. The membrane was visualised under UV light, ensuring complete transfer to the membrane, and the location of the molecular size standards was marked.

3.2.3.3 Probing

Junction-fragment Southern blots were probed with ^{32}P radioactively labelled probes as described in Section 2.8.2. The blot was pre-hybridised (Section 2.8.1), to prepare the blot for probing. The template for the *Lc* probe was obtained by excising the *Lc* cDNA from the plasmid pLC349 by *Xba*I restriction digest (Section 2.6.1), followed by gel electrophoresis (Section 2.6.2) and gel-purification of the insert (Section 2.7.1). Stringency washes were performed (Section 2.8.3) and membranes were exposed to film (Section 2.8.4).

3.2.4 Kanamycin sensitivity

Young petunia leaves, of both wild type Mitchell and transgenic 118B and 118C *Lc* plants were collected and sterilised in 15% (v/v) bleach with Tween 20 for 15 min. Explants were made and grown on shoot regeneration media containing 100 mg L^{-1} kanamycin (Appendix 1.5.1). Shoot regeneration media lacking kanamycin was used as a control to assess the ability of the explants to form shoots. Explants were cultured in sealed petri dishes, under a 16 h photoperiod, supplied by cool fluorescent tubes ($25 \mu\text{mol m}^{-2} \text{sec}^{-1}$), for four weeks. After this time, plant lines were assessed for their ability to form shoots, indicating kanamycin resistance, or failure to produce shoots and onset of senescence as kanamycin sensitive.

3.2.5 Self-cross of 118C line

To determine the zygosity of the 118C line, plants were selfed. Flowers were self-pollinated, and labelled. Seed was collected from mature pods, and seeds were stratified by storing them at 4°C for 6 weeks. Seeds were sown and transferred to high-light conditions (Section 3.2.1) and assessed for their pigmentation phenotypes.

3.2.6 Northern blots

3.2.6.1 RNA extraction from petunia leaves

The plant material used for northern analysis is described in Section 3.2.1. RNA was extracted from frozen leaf tissue using a modified ‘hot borate’ protocol (Hunter et al., 2002). Frozen, ground leaf tissue (1 g FW) was used per extraction. The RNA pellets were dissolved in 100 μL of sterile water.

3.2.6.2 RNA quantification

RNA was quantified by spectrophotometry as described in Section 2.6.3.2, except that, 40 $\mu\text{g mL}^{-1}$ of RNA has an $A_{260} = 1.0$ (50 $\mu\text{g mL}^{-1}$ DNA: $A_{260} = 1.0$) therefore:

$$A_{260} \times 40 \times \text{dilution factor} = \text{RNA concentration } \mu\text{g mL}^{-1}$$

RNA purity was also assessed, as described in Section 2.6.3.2. A small RNA sample (1 μL) was run on a 1% (w/v) TBE gel (Section 2.6.2) to check RNA quality and integrity. RNA was stored at -80°C .

3.2.6.3 Northern blotting

Total RNA (15 μg) was diluted to a final volume of 15 μL . One volume of 2 \times RNA denaturing solution (Appendix 1.3.2) was added to each diluted RNA sample, and then samples were denatured at 65°C for 10 min, removing RNA secondary structure. Tubes were placed on ice, and then 10 μL of 10 \times loading buffer (Appendix 1.3.2) was added. RNA size standards (5 μL) (Invitrogen), were also prepared in the same manner as the RNA samples.

Gel tank and apparatus were soaked in 2% (v/v) Absolve (PerkinElmer Life Sciences, Inc.), a solution which removes RNase contamination, to ensure the integrity of the RNA samples is maintained. Gloves were worn at all times when handling RNA

samples or equipment used for RNA work, and filter-tips were always used when pipetting.

RNA samples and size marker were loaded onto a 1.2% (w/v) denaturing agarose gel (1 × MOPS, 0.66 M formaldehyde) and covered with 1 × MOPS electrophoresis buffer. RNA was electrophoresed slowly (typically 30 V) overnight until good separation was achieved. After electrophoresis, a gel photo was taken (using a UV transilluminator) as a record, as the ethidium bromide in the RNA denaturing buffer enables fluorescent detection of RNA. The gel photo of the ethidium bromide stained RNA also provides an assessment of the RNA loading of each sample. The blot was assembled as described in Brown *et al.* (2004), a capillary action based transfer system using 10 × SSC transfer buffer (Appendix 1.6.2). The membrane used was Hybond XL Membrane (Amersham). After transfer, the blot was disassembled, and the membrane rinsed in 2 × SSC buffer. The membrane was then subjected to UV-C cross-linking (Hoefer) for 1 min at 70 000 mJ cm⁻² and washed again in 2 × SSC. The membrane was visualised under UV light, ensuring complete transfer to the membrane, and the location of the molecular size standards marked.

3.2.6.4 Probing

The blot was pre-hybridised (Section 2.8.1), to prepare the blot for probing. The *Lc* probe is described in Section 3.2.3.3. Stringency washes were performed (Section 2.8.3) and membranes were exposed to film (Section 2.8.4). Membranes were stripped of radioactive signal (Section 2.8.5) and re-probed for 26S rRNA as a loading control. The template for the 26S rRNA was 26S rRNA from *Asparagus*, in the plasmid pTIP6: the cDNA insert was excised by restriction digest (*EcoRI*) (Section 2.6.1), separated by gel electrophoresis (Section 2.6.2), gel purified (Section 2.7.1) and used as a template for probe making (Section 2.8.2).

3.3 Results

3.3.1 Leaf colour phenotypes

All plants from line 118C displayed dark purple anthocyanin pigmentation in leaf tissue when grown under high-light conditions. A comparison of plants grown under high-light or ambient-greenhouse treatments is shown in Figure 3.1A. New shoots which were partially shaded by older shoots showed less pigment accumulation. A close-up of an individual plant exposed to high-light is shown in Figure 3.1B. The leaves have an intensely purple colour due to the presence of anthocyanin pigments. There was no apparent segregation of phenotype in the 118C line.

Line 118B plants displayed two phenotypes: purple and green. The differences in pigmentation became apparent when plants were grown under high-light in the growth cabinet. Similar to the 118C line, 118B plants grown under high-light and ambient-greenhouse treatments showed contrasting leaf colours (Figure 3.2A). A close up the two phenotypes observed after high light treatment of the 118B plants is shown in Figure 3.2B. The number of purple and green plants presented in Figure 3.2A is not representative of the 118B population. The frequency of purple:green is approximately 3:1 (data not shown), raising the possibility that this is a segregating population, and that the 'green' plants may not contain the tDNA fragment. The pigmentation data from individual plants of both 118B and 118C lines is included in Table 3.1 (refer to page 48).

The older leaves of the ambient-greenhouse grown *Lc* petunia (both 118B and C) were purple with anthocyanins from a previous high-light exposure of seedlings during a pilot experiment (indicated in Figures 3.1 and 3.2). Once transferred to ambient-greenhouse conditions, new leaves were green and did not accumulate anthocyanins.

An interesting observation of the purple plants from both *Lc* lines was that it was not uncommon for them to contain leaves with green sectors, which can be seen in Figure



Figure 3.1: A *CaMV35S::Lc 118C* line grown under ambient-greenhouse and high-light. Plants on the left were exposed to high-light ($750 \mu\text{mol m}^{-2} \text{sec}^{-1}$) in a growth cabinet, while plants on the right were grown under ambient-greenhouse conditions ($100 - 300 \mu\text{mol m}^{-2} \text{sec}^{-1}$). Older leaves are purple due to previous high-light exposure, as indicated by the arrow. **B** Close up of a typical 118C plant grown under high-light. This plant like some of the other *Lc* plants displays a leaf sector which failed to produce anthocyanins.



Figure 3.2: **A** CaMV35S::*Lc* 118B line grown under ambient-greenhouse and high-light. Plants on the right were exposed to high-light ($750 \mu\text{mol m}^{-2} \text{sec}^{-1}$) in a growth cabinet, while plants on the left were grown under ambient-greenhouse conditions ($100 - 300 \mu\text{mol m}^{-2} \text{sec}^{-1}$). Older leaves are purple due to previous high-light exposure, as indicated by the arrow. **B** Close up of the two phenotypes of 118B plants grown under high-light: dark purple and green.

3.1B. These sectors contained no anthocyanin pigments at all, and the margins of the sectors were very distinct.

3.3.2 Junction-fragment Southern blots – copy number of *Lc* tDNA

Junction-fragment Southern blots were performed to determine the transgene copy number of the transgenic petunia lines. *Hind*III was selected as a suitable restriction enzyme, as it cleaved the tDNA sequence once (see Appendix 3.1 for map) generating one band per copy of the *Lc* cDNA.

Figure 3.3 shows the junction-fragment Southern blots. All plants from the 118C line have a single band. Some individuals from the 118B line do not contain a band corresponding to the *Lc* cDNA. The bands present in all the 118C individuals and some of the 118B individuals appeared at the same molecular weight (approximately 6 kb). There is some non-specific hybridisation to the blots, but there was only one band present at ~ 6 kb. The *Hind*III junction-fragment generates one band per copy of the *Lc* transgene cassette.

Small differences in band intensity are due to variable loading of DNA. Distinguishing between hemizygous and homozygous individuals cannot be determined unequivocally based on the intensity of the bands because an internal control for loading was not performed. However, the brightest bands observed in the 118B line (#42, #45 and #46) probably do have twice as much signal, as comparison with the ethidium bromide stained gel revealed that loading was essentially even (data not shown).

3.3.3 Kanamycin sensitivity

The two transgenic plant lines, 118B and 118C, and wild type Mitchell controls, were assessed for their ability to regenerate shoots in the presence of kanamycin, as plants

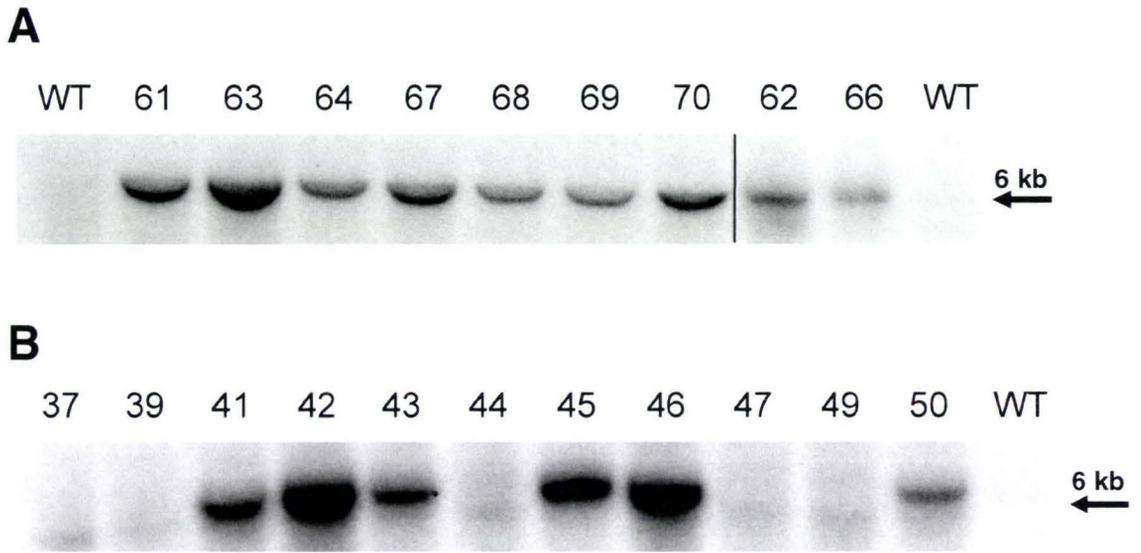


Figure 3.3: *Hind*III junction-fragment Southern blots. **A** *Lc* 118C line; **B** *Lc* 118B line. The numbers refer to the GMO plant identification number, each representing an individual plant from each seed line. WT = wild type Mitchell petunia. There was only one band present on the blots, located at approximately 6 kb as indicated.

Table 3.1: Combined results for kanamycin sensitivity, presence of *Leaf colour* transgene as determined by junction-fragment Southern blots and pigmentation phenotype of transgenic *Lc* petunia plants grown under high-light.

GMO ID number	Pigmentation phenotype	Kanamycin sensitivity	Transgene presence
118B line			
37	Green	Sensitive	No
39	Green	Sensitive	No
41	Purple	Resistant	Yes
42	Purple	Resistant	Yes
43	Purple	Resistant	Yes
44	Green	Sensitive	No
45	Purple	Resistant	Yes
46	Purple	Resistant	Yes
47	Green	Sensitive	No
49	Green	Sensitive	No
50	Purple	Resistant	Yes
118C line			
61	Purple	Resistant	Yes
62	Purple	Resistant	Yes
63	Purple	Resistant	Yes
64	Purple	Resistant	Yes
67	Purple	Resistant	Yes
68	Purple	Resistant	Yes
69	Purple	Resistant	Yes
70	Purple	Resistant	Yes
WT 'Mitchell'			
	Green	Sensitive	No

expressing the *NptII* selectable marker from the tDNA construct they were transformed with (Appendix 3.1) should be kanamycin resistant. Leaf tissue from Mitchell petunia plants was included as a negative control.

Wild type Mitchell tissue did not proliferate shoots when grown in the presence of kanamycin (Figure 3.4A) whereas in the absence of kanamycin, wild type tissue was competent to proliferate shoots (Figure 3.4B). Explants from a plant of the 118B line that was kanamycin sensitive are shown in Figure 3.4C and D, while kanamycin resistant explants from an individual from the same seed line are shown in Figure 3.4F. Figure 3.4E shows kanamycin resistant explants from a plant of the 118C line. The

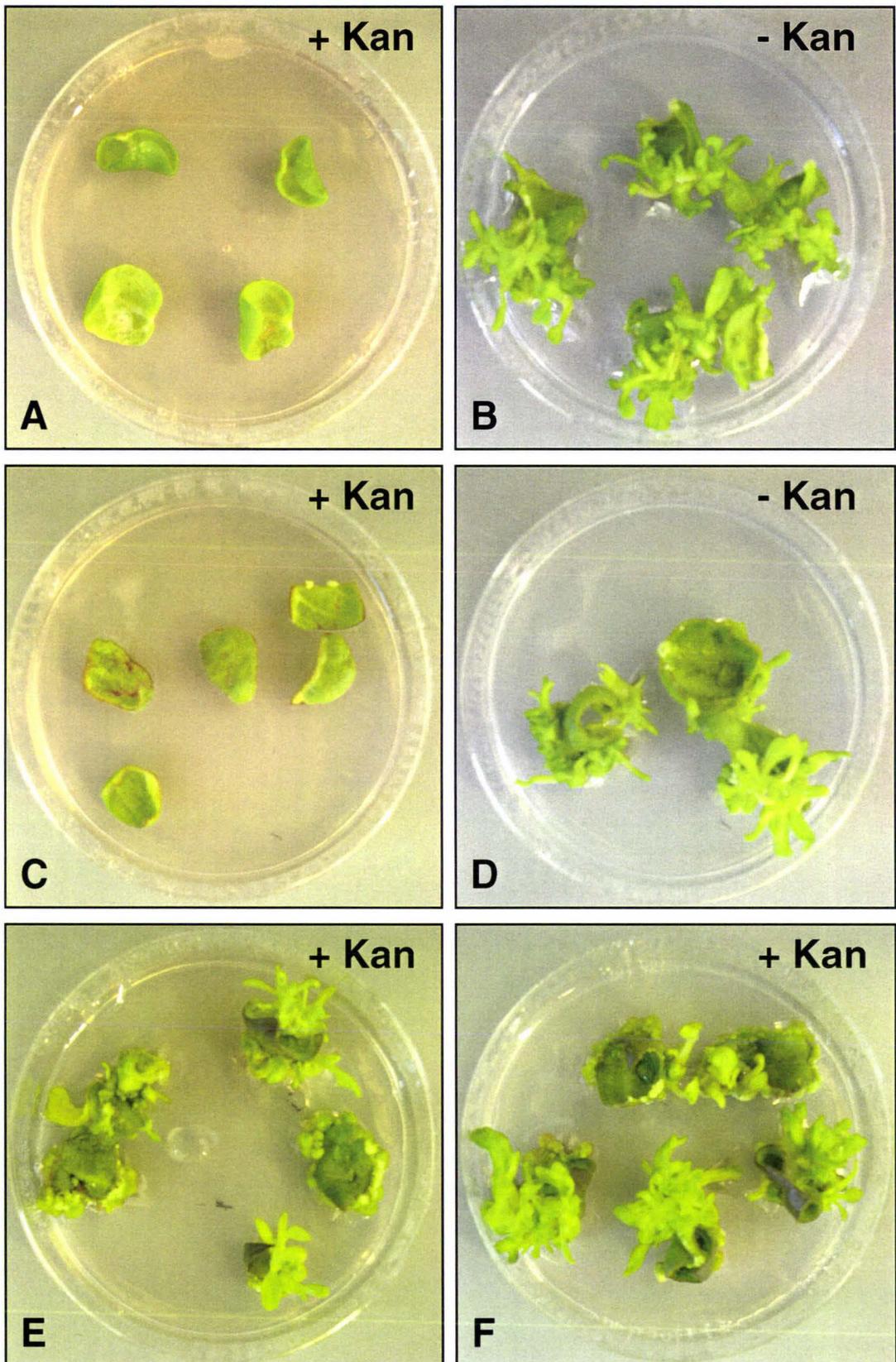


Figure 3.4: Kanamycin sensitivity assay. Leaf discs from wild type Mitchell plants and plants from the 118B and 118C *Lc* petunia seed lines were grown on shoot regeneration media with (+ Kan) or without (- Kan) 100 mg L⁻¹ kanamycin. **A/B:** Mitchell explants **C/D:** *Lc* 118B plant #39 explants. **E:** *Lc* 118C plant #62 explants. **F:** *Lc* 118B plant #41 explants.

results of the kanamycin sensitivity experiments are summarised in Table 3.1. All the plants from the 118C plants were kanamycin resistant. The 118B line contained a mixture of both kanamycin resistant and sensitive plants. The wild type explants, as expected, were kanamycin sensitive.

Table 3.1 summarises the data from the pigmentation phenotypes, kanamycin sensitivity and junction-fragment Southern blots. The combined data shows that those plants which were purple (from either 118B or 118C) were also kanamycin resistant and had a transgene present, while green plants were kanamycin sensitive and did not contain the transgene.

3.3.4 Zygoty of the 118C line

Lc 118C plants were selfed to determine the zygoty of the 118C line and the resulting seedlings were assessed for the segregation of the pigmentation phenotype. Segregation of the pigmentation phenotype in the offspring would show that the parent line is hemizygous. These seedlings were grown under high-light, and their pigmentation phenotypes were assessed. The seedlings were a mixture of purple and green plants (data not shown), suggesting a segregating population, and that the parent 118C line is hemizygous for the transgene.

Note: A homozygous line of *Lc* petunia (also derived from the same transformant '118') is part of the seed collection at Crop & Food Research. To maintain a hemizygous seed line (equivalent to 118C) for future studies, this homozygous line was back-crossed to wild type Mitchell, regenerating a fresh hemizygous seed line.

3.3.5 Expression of *Leaf colour (Lc)* in transgenic lines

Expression of *Lc* was examined to determine whether the light-induced pigmentation observed in the *Lc* petunia lines was due to variable expression of *Leaf colour*.

Northern blots were performed on RNA extracted from 118C *Lc* petunia plants grown under shade ($50 - 350 \mu\text{mol m}^{-2} \text{sec}^{-1}$), ambient-greenhouse ($300 - 750 \mu\text{mol m}^{-2} \text{sec}^{-1}$) and high-light ($750 \mu\text{mol m}^{-2} \text{sec}^{-1}$) lighting conditions. The results are presented in Figure 3.5. Expression of *Lc* from the CaMV35S promoter was constitutive, and levels remained unchanged between lighting treatments. There was no *Lc* signal detected in any of the wild type Mitchell samples. The 26S *rRNA* blot shows that small differences in *Lc* levels were due to slight differences in RNA loading.

3.4 Discussion

The two *Lc* petunia lines, 118B and 118C, both displayed light-induced anthocyanin pigmentation. All 118C plants were purple when grown under high-light, whereas the 118B line had plants that were purple and green. This raised the possibility, from a phenotypic perspective, that the 118B line was a segregating population, consisting of a mixture of homozygous, hemizygous and wild type Mitchell plants. The 118C line, however, displayed a stable pigmentation phenotype, indicating it is either homozygous, or hemizygous. Previous work at Crop & Food Research had revealed three phenotypes in the 118B line during an open field trial; dark purple, purple and green. The 118C line had a single pigmentation phenotype which corresponded to the ‘purple’ phenotype observed in the 118B plants (Boase et al., 2000). These field results indicated that the 118B line was a segregating population, and that the dark purple, purple and green phenotypes corresponded to plants homozygous and hemizygous for the *Lc* transgene, as well as plants which had lost the *Lc* transgene (effectively wild type Mitchell). Under the growth conditions used in this study, however, a distinction between the dark purple and purple phenotypes could not clearly be made. A second field trial growing *Lc* petunia in under full sunlight was not possible due to the costs and other restrictions associated with regulatory approval.

Genotypically, as both 118B and 118C lines are derived from the same original explant ‘118’ the *Lc* tDNA if present should be at the same locus (Figure 3.3). While there was

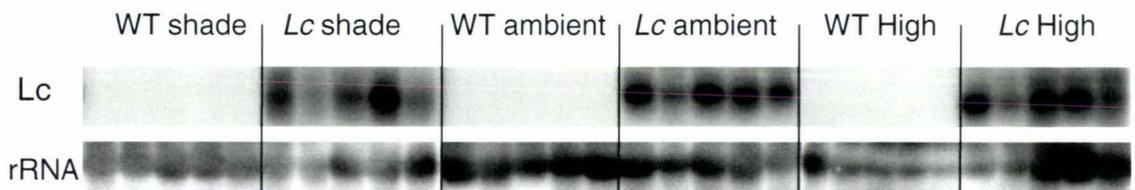


Figure 3.5: Northern blot of *Lc* transgene expression from the CaMV35S promoter in 118C *Lc* transgenic petunia, grown under different lighting regimes: shade ($50 - 350 \mu\text{mol m}^{-2} \text{sec}^{-1}$), ambient-greenhouse (ambient) ($350 - 750 \mu\text{mol m}^{-2} \text{sec}^{-1}$), and high-light (high) ($750 \mu\text{mol m}^{-2} \text{sec}^{-1}$) conditions. Five individual plants were analysed per treatment. Wild type Mitchell samples from plants grown under the same growth conditions were run as negative controls. Approximately $15 \mu\text{g}$ total RNA was loaded per lane. As a loading control, 26S rRNA was also probed.

only one insertion event, and one copy at that locus, however, some of the 118B individuals did not contain an *Lc* transgene (Table 3.1). This supported the phenotypic observations that the 118B population may be segregating, and represent a mixture of homozygous, hemizygous and wild type Mitchell plants. The variable *Lc* signal strength observed in Southern blots of the 118B line (Figure 3.3) also suggests that there was a mixture of hemizygous and homozygous individuals for plants which did contain the *Lc* transgene. Bradley *et al.* (1998) performed junction fragment Southern blots on the F₂ generation of the 118 *Lc* transformant from which both the 118B and 118C seed lines were derived. Two of the plants examined had reduced transgene signal with equal DNA loading, and those plants also had slightly reduced *Lc* transcript levels, and reduced anthocyanin levels (Bradley *et al.*, 1998). These results are consistent with the idea that the F₂ generation of *Lc* petunia, was a segregating population.

It is clear from the combined data that those plants which contained an *Lc* transgene were also kanamycin resistant (transgenic selectable marker) and displayed a purple anthocyanin pigment phenotype after exposure to high-light. The stable pigmentation phenotype, Southern analysis, and results of the 118C self cross show that the 118C line is hemizygous for LC, while the 118B line is a segregating population. It was decided from this characterisation of the transgenic plant lines that 118C line was stable and hemizygous for the *Lc* construct and would be used for further investigation into the light-inducible phenotype of the plants.

An observation made for some individual plants from both the *Lc* petunia seed lines, was that it was relatively common for the purple leaves to contain green sectors (Figure 3.1B). It is likely that these sectors represent regions where some form of gene silencing is acting. Post-transcriptional gene silencing was first reported when transgenic petunia plants expressing structural genes of the flavonoid pathway, displayed non-pigmented sectors in flowers (Napoli *et al.*, 1990; van der Krol *et al.*, 1990). Post-transcriptional gene silencing is a common problem encountered with transgenic organisms, especially when strong promoters are used. Such promoters include the CaMV35S promoter, which result in unnaturally high transcript concentrations. This is of particular importance to transcription factors, since they are

usually expressed at very low levels when performing their natural functions (Czechowski et al., 2004; Latchman, 2004). However, it has not been confirmed that the green sectors in the *Lc* petunia leaves are due to post-transcriptional gene silencing. This phenomenon will not impact on the project, as typically only one leaf was affected in any one plant, and only about 25% of the plants were affected. As a precaution, leaves which contained green sectors were excluded from all subsequent experiments.

The results from the northern analysis indicated that expression of CaMV35S::*Lc* did not change under different lighting intensities (Figure 3.4) and, therefore, was not likely to contribute to the light-induced phenotype. This result was not unexpected as the CaMV35S promoter is generally considered to be a strong and constitutive promoter (Odell et al., 1985; Benfey and Chua, 1989). However, there are examples of tissue-specific and developmental expression patterns from CaMV35S driven transgenes (Benfey et al., 1989), so it was important to establish that the expression of *Lc* in *Lc* petunia was not affected by lighting intensity. Similar results were recorded for transgenic CaMV35S::*Lc* alfalfa (*Medicago sativa*), where *Lc* expression was consistent between non-stressed greenhouse conditions, cold-stress and high-light conditions, yet light- and cold-stress-induced anthocyanin pigmentation was observed (Ray et al., 2003).

The expression levels of the *Lc* transgene appeared to be quite low, as long exposure times for the autoradiographs were required. Although only a semi-quantitative, this result is consistent with the results observed in Bradley *et al.* (1998), where the 118 transformant had considerably lower transgene expression signal than other *Lc* transformants analysed. The strong expressors of the *Lc* transgene appeared to have developmental defects, as they were stunted and failed to regenerate roots (Bradley et al., 1998). Very high concentrations of transcription factors may interfere with endogenous developmental gene regulation. High expression of a bHLH transcription factor, as in the case of *Lc*, may result in hetero-dimer formation with other endogenous regulatory factors which would not normally occur. Even higher concentrations of the introduced transcription factor may allow low-affinity interactions to take place, which can lead to *silencing*, a form of repression which occurs through the sequestration of

endogenous transcription factors (Gill and Ptashne, 1988). Competition for promoter element binding sites by new, inactive, transcription factor complexes may also have deleterious effects for the regulation of developmental processes. Myb and bHLH transcription factors regulate a multitude of developmental processes; these include trichome development (e.g. *GLABROUS1*; Myb (Oppenheimer et al., 1991) and *GLABRA3*; bHLH (Bernhardt et al., 2003; Ramsay et al., 2003)), cell fate and patterning during leaf ontogeny (e.g. *PHANTASTICA*; Myb (Waites et al., 1998), e.g. *MIXTA*; Myb (Glover et al., 1998)), and mediating gibberellin responses (e.g. *GAMYB* (Gubler et al., 1995)). Expression of the *Lc* transgene could therefore interfere with a number of developmental processes.

The petunia line 118C was selected as most suited to an investigation of light-induced anthocyanin pigmentation in *Lc* petunia, as it is a stable, non-segregating hemizygous population. The expression of *Lc* was constant throughout the light treatments, and therefore *Lc* expression was not responsible for the light-induced pigmentation.

Chapter 4

Light-induced anthocyanin pigmentation in

Lc petunia

4.1 Introduction

Petunia plants transformed with the anthocyanin regulator *Leaf colour* (*Lc*) displayed enhanced anthocyanin pigmentation throughout their vegetative tissue. However, the intensity of the enhanced pigmentation was variable and appeared dependent upon the lighting conditions that plants were grown under. Initial characterisation of these plants (Chapter 3) revealed that the expression of *Lc* from the CaMV35S promoter was constitutive and not affected by light intensity.

The effect of *Lc* expression on pigment accumulation and flavonoid structural gene expression was investigated, by monitoring pigment levels and performing northern blots of the structural genes required for anthocyanin and flavonol biosynthesis in both Mitchell and *Lc* (118C, herein referred to as just '*Lc*') petunia plants grown under shade, ambient-greenhouse and high-light treatments. Previous work had shown an upregulation of the late structural genes required for anthocyanin biosynthesis (Bradley et al., 1998), but the influence of light had not been considered. The effect of *Lc* on the overall regulation of the flavonoid pathway was also under question, as different responses in the target genes regulated have been reported for petunia compared to maize (Quattrocchio et al., 1998). The two early biosynthetic genes *CHS* and *CHI*, the flavonol-specific gene *FLS*, and the late biosynthetic gene *ANS* were monitored (see Figure 1.2).

Analysis of total flavonoid and anthocyanin levels and profiles was performed to determine if any changes observed in structural gene expression correlated with changes

to the levels and types of flavonoids and anthocyanins present. One point of interest was whether or not the accumulation of anthocyanin pigments also influenced the accumulation of other pigments. Chlorophyll and carotenoid levels in Mitchell and *Lc* petunia plants, grown under the various lighting treatments, were also measured.

4.2 Materials and methods

4.2.1 Plant material and growth conditions

Seeds of wild type Mitchell and *Lc* (line 118C) petunia (Section 2.9) were germinated under ambient-greenhouse conditions. Seedlings were individually potted before being transferred to the 'shade' growth condition (see below), to prevent induction of pigmentation. Plants were grown until they had approximately 10 nodes, and axillary buds were removed to prevent a branched architecture which would result in shading. Once they had grown to approximately 10 nodes, five Mitchell and five *Lc* plants were transferred to each growth condition; high-light, ambient-greenhouse and shade.

The high-light, ambient-greenhouse and shade light treatments are described in Section 3.2.1. The experiment was performed in January 2005, the natural photoperiod at this time was approximately 14 h. The light levels were: shade 50 - 350 $\mu\text{mol m}^{-2} \text{sec}^{-1}$; ambient-greenhouse 300 - 750 $\mu\text{mol m}^{-2} \text{sec}^{-1}$; high-light 750 $\mu\text{mol m}^{-2} \text{sec}^{-1}$. Light levels in ambient-greenhouse and shade treatments varied due to the angle of the sun and weather conditions during the course of the day.

Plants were maintained under these conditions for 12 days before tissue was collected for RNA and pigment analysis. Leaves at nodes 7 - 15 were collected during the illuminated part of the day, and frozen immediately in liquid nitrogen. At a later date, the tissue was ground to a fine powder in liquid nitrogen. The tissue was stored at -80°C. It was decided that plants would be maintained under the light treatments for 12 days, as this provided sufficient time for the *Lc* plants to become deeply pigmented before the plants flowered. Each of the plants was treated as a separate replicate, and all tissue from each plant was collected and stored separately.

4.2.2 Flavonoid extraction

Frozen, ground leaf tissue (1.5 - 4 g FW) (Section 4.2.1) was freeze-dried and stored at -20°C. Anthocyanins and other flavonoids were extracted from 50 mg (DW) of ground petunia leaves, in 2 mL of acidified 70% (v/v) methanol for 72 h, followed by an overnight extraction in 2 mL of acidified 90% (v/v) methanol (Appendix 1.7). The methanol extracts were pooled and concentrated in a Savant SC210 Speedvac, and adjusted to a final volume of 1 mL with 80% (v/v) acidified methanol (Appendix 1.7). Anthocyanin and other flavonoid concentrations were determined spectrophotometrically (at 530 and 350 nm respectively), and samples of the extracts were used for HPLC analysis.

4.2.2.1 HPLC analysis of anthocyanins and other flavonoids

The flavonoids were analysed by high performance liquid chromatography (HPLC), using a Waters 600 solvent delivery system with a Phenomenex Prodigy (5 µm, 250 × 4.6 mm) RP-18 end-capped column (column temperature 30°C) and a Waters 996 PDA detector. Elution (0.8 mL min⁻¹) was performed using a solvent system comprising solvent A [HOAc:CH₃CN:H₃PO₄:H₂O (20:24:1.5:54.5)] and 1.5% H₃PO₄ (solvent B) and a linear gradient starting with 35% A, increasing to 67% A at 20 mins, 90% A at 23 mins and 100% A at 29.3 mins, remaining at 100% A for a further 10 min.

Anthocyanins were detected at 530 nm and other flavonoids at 350 nm. Anthocyanin levels were determined as cyanidin 3-*O*-glucoside (Extrasynthese, Genay, France) equivalents and other flavonoids as quercetin-3-*O*-rutinoside (Apin Chemicals, Abingdon, Oxon, UK) equivalents.

4.2.3 Chlorophyll/carotenoid extraction

Chlorophylls and carotenoids were extracted from 20 mg (DW) of ground petunia leaves (Section 4.2.1) in 1 mL of acetone:methanol (7:3) with 200 mg CaCO₃ and vortexed. Samples were centrifuged for 2 min (bench top centrifuge), and the supernatant

collected into a foil covered tube. One mL of acetone:methanol (7:3) was added to the tissue/CaCO₃ pellet, vortexed and centrifuged, and the supernatant collected. This process was repeated until the plant tissue was colourless (typically four times). The supernatants from each sample were pooled. One volume of diethyl ether and one volume of water was added to the supernatants, and mixed by inversion. The phases were allowed to separate, and the top ether phase containing the pigments was collected into a foil covered glass vial. A further 2 mL of diethyl ether was added to the remaining bottom phase, mixed, and the ether phase collected and added to the previous collection. The ether phases were dried under a stream of nitrogen, and the carotenoids and chlorophylls were dissolved in 1 mL ethyl acetate. The pigment extract was then analysed spectrophotometrically to determine chlorophyll content, chlorophyll *a/b* ratio, and carotenoid concentration.

4.2.3.1 Spectrophotometric determination of total carotenoid and chlorophyll concentration

To determine the concentration of total carotenoids, chlorophylls and the ratio of chlorophyll *a*/chlorophyll *b* present in an extract, a known aliquot of the pigment extract (typically 50 µL) was taken, and made up to 1 mL with chloroform. The absorbance values were determined at 480 nm, 648 nm and 666 nm in a Jasco V-530 UV/Vis spectrophotometer (Jasco, Tokyo, Japan) and the pigment concentrations calculated using the equation described by Wellburn (1994) (Appendix 2).

4.2.4 Northern blotting

Northern analysis was performed on RNA from Mitchell and *Lc* petunia to monitor changes in the expression of the flavonoid biosynthetic genes between Mitchell and *Lc* petunia under the different light treatments. RNA was extracted from the plant material (Section 4.2.1) as described in Section 3.2.6.1 and northern blotting as described in

Section 3.2.6.3. Each of the plants grown under the various light treatments was analysed by northern analysis, giving five individual plants per treatment.

4.2.4.1 Probing

Northern blots were probed with ^{32}P radioactively labelled probes as described in Section 2.8.2. The petunia flavonoid structural genes *CHS*, *CHI*, *FLS* and *ANS* were probed by excising the cDNA inserts from the plasmids pPN297 (*NotI*), pPN300 (*EcoRI/BamHI*), pPN302 (*EcoRI/XhoI*), and pPN299 (*EcoRI/XhoI*) respectively, by restriction digest (Section 2.6.1) followed by gel purification (Section 2.7.1). As a loading control, 26S rRNA was probed with pTIP6 as described in Section 3.2.6.4. Stringency washes were performed (Section 2.8.3), and membranes exposed to film (Section 2.8.4). Membranes were stripped of radioactive signal (Section 2.8.5) prior to the next gene being probed.

4.3 Results

4.3.1 Anthocyanin pigmentation phenotypes

The anthocyanin pigmentation phenotypes for Mitchell and *Lc* petunia plants grown under the three light treatments, shade, ambient-greenhouse and high-light are shown in Figure 4.1 A/B, C/D and E/F respectively. Mitchell petunia plants grown under shaded conditions (Figure 4.1A), did not produce detectable levels of anthocyanins, and *Lc* petunia appeared largely non-pigmented (Figure 4.1B). Upon close inspection, shade-grown *Lc* petunia had a border 1 - 2 cells wide at the leaf margins that were pigmented with anthocyanins. Both Mitchell and *Lc* petunia grown under the shade conditions were spindly and tall with very elongated internodes as a result of being grown under light limited conditions.

Mitchell petunia plants grown under ambient-greenhouse conditions displayed no detectable anthocyanin pigmentation (Figure 4.1C). *Lc* petunia had a bronzed

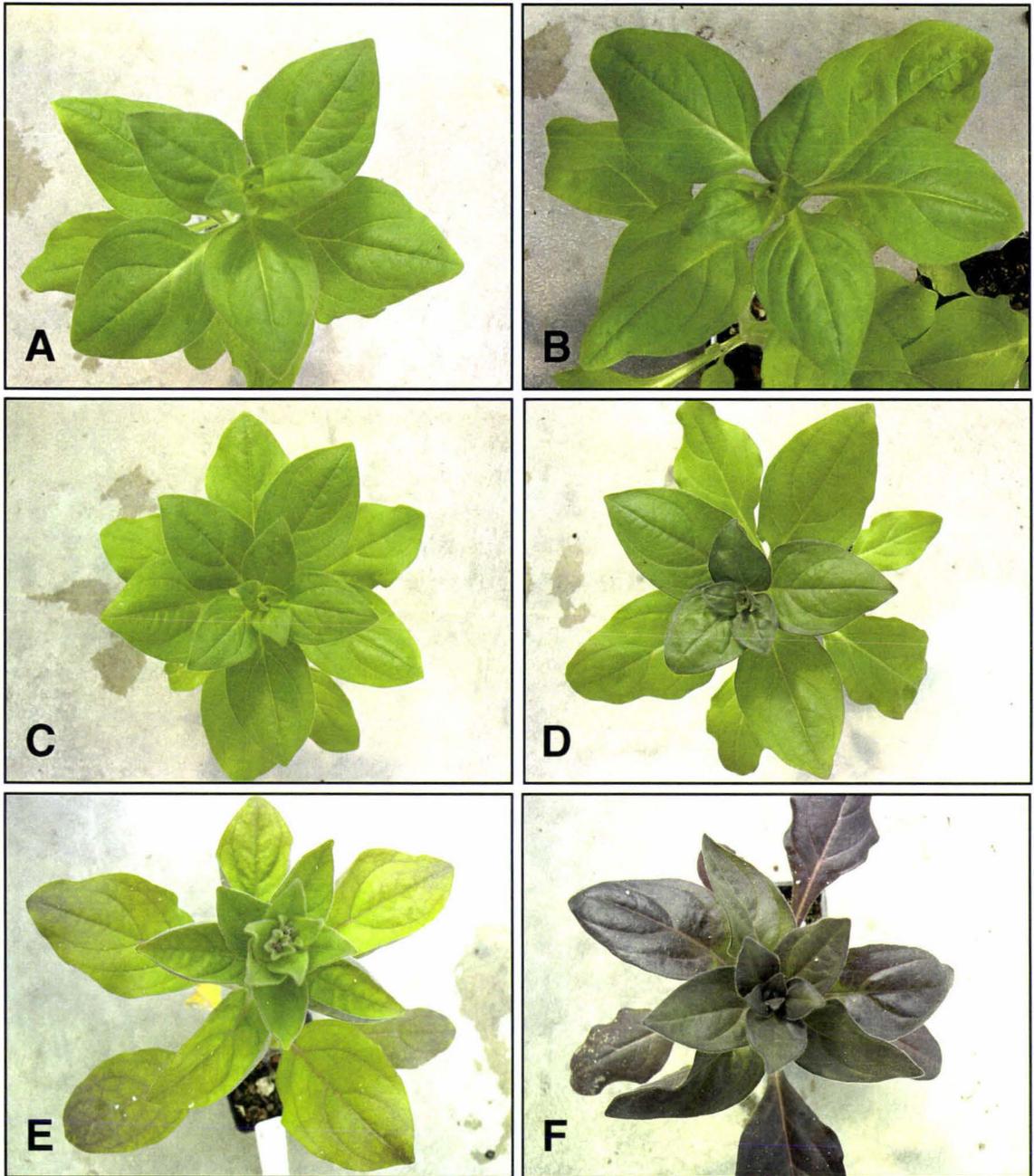


Figure 4.1: Anthocyanin pigmentation phenotypes of wild type Mitchell, and *Lc* petunia grown under the lighting treatments; shade, ambient-greenhouse and high-light. **A** and **B** are Mitchell and *Lc* petunia (respectively) grown under shade conditions ($50 - 350 \mu\text{mol m}^{-2} \text{sec}^{-1}$). **C** and **D** are Mitchell and *Lc* petunia (respectively) grown under ambient-greenhouse conditions ($300 - 750 \mu\text{mol m}^{-2} \text{sec}^{-1}$). **E** and **F** are Mitchell and *Lc* petunia (respectively) grown under high-light conditions ($750 \mu\text{mol m}^{-2} \text{sec}^{-1}$). The individual plants shown are representative of the five plants grown per treatment.

appearance, especially on the new leaves at the apex, in addition to the border around the leaf margins (Figure 4.1D).

When Mitchell plants were grown under high-light conditions, anthocyanin pigmentation was observed. Figure 4.1E shows that purple anthocyanin pigmentation is visible on the leaves, especially the veins. The stems of the plant, unable to be seen in Figure 4.1E, were pale red with anthocyanin pigments. The modest amount of anthocyanin developed slowly over the light-treatment period. The *Lc* plants were dark purple with anthocyanin pigments throughout the leaves and stems exposed to the light (Figure 4.1F). Anthocyanin pigmentation was visible within 12 h of exposure of the *Lc* plants to high-light. The intensity of the pigmentation increased throughout the light treatment and, therefore, the older leaves appeared more intensely purple than newly exposed leaves at the apex.

4.3.2 Pigment analysis

HPLC analysis of methanolic leaf extracts enabled quantification and classification of anthocyanins and other flavonoids. Mitchell and *Lc* petunia plants had equivalent levels of other flavonoids (found to be flavonols) in all light treatments, as shown in Figure 4.2A. The concentration of flavonols increased with increased lighting intensity, from approximately $7 \text{ mg g}^{-1}\text{DW}$ for shade grown plants, to $25 \text{ mg g}^{-1}\text{DW}$ for high-light grown plants. Anthocyanin pigments (Figure 4.2B) were not detected in Mitchell and *Lc* plants grown under shade conditions, or in Mitchell plants grown under ambient-greenhouse conditions. *Lc* petunia grown under ambient-greenhouse conditions had trace amounts of anthocyanins, as did Mitchell plants grown under high-light conditions ($< 0.5 \text{ mg g}^{-1}\text{DW}$). *Lc* petunia grown under high-light conditions, however, had a large increase in anthocyanin concentration to almost $3 \text{ mg g}^{-1}\text{DW}$.

Representative HPLC traces of leaf extracts from Mitchell and *Lc* petunia, grown under high-light, for other flavonoids (350 nm) and anthocyanins (530 nm) are shown in Figures 4.3 and 4.4 respectively. Both Mitchell and *Lc* petunia accumulate similar flavonoid compounds, and spectral data suggests that the major peaks in Figure 4.3A

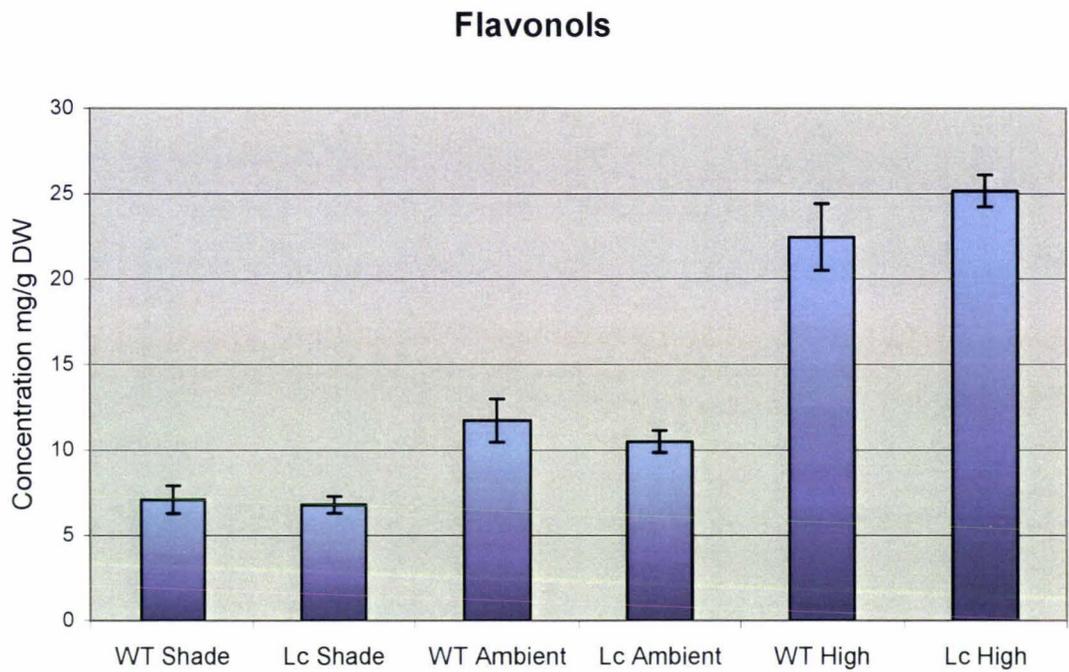
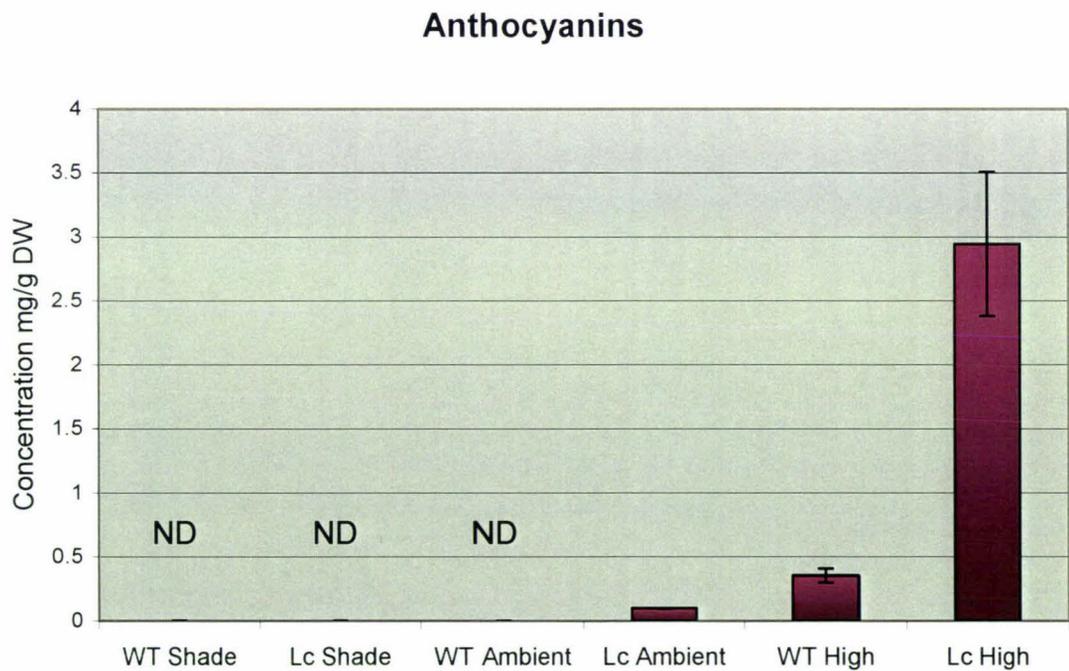
A**B**

Figure 4.2: Concentration of flavonols, **A**, and anthocyanins, **B**, in wild type Mitchell (WT) and *Lc* petunia leaf tissue grown under three lighting treatments: high-light, ambient-greenhouse (ambient) and shade. (Mean \pm SEM, $n = 5$). ND = not detected.

A

Mitchell Petunia

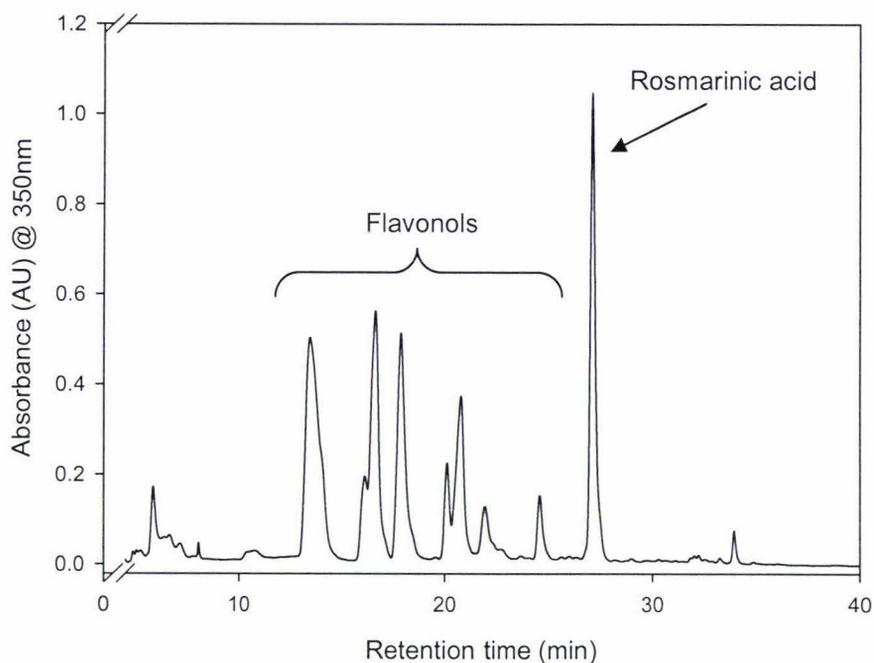
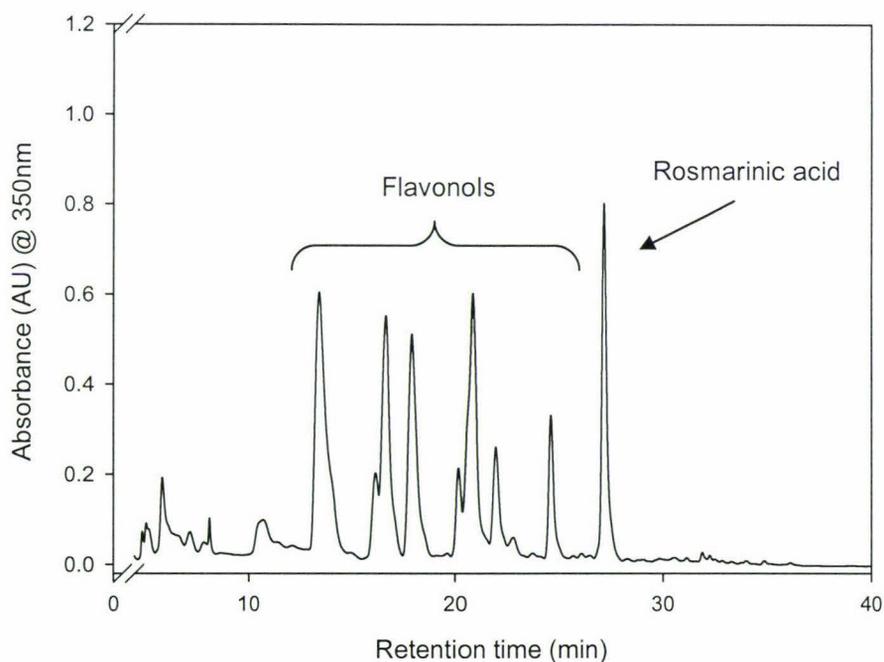
**B***Lc* Petunia

Figure 4.3: Representative HPLC chromatograms of methanolic leaf extracts from Mitchell, **A**, and *Lc*, **B**, petunia grown under high-light, monitored at 350 nm. The major flavonoids, the flavonols, and a non-flavonoid compound, rosmarinic acid, are indicated.

A

Mitchell Petunia

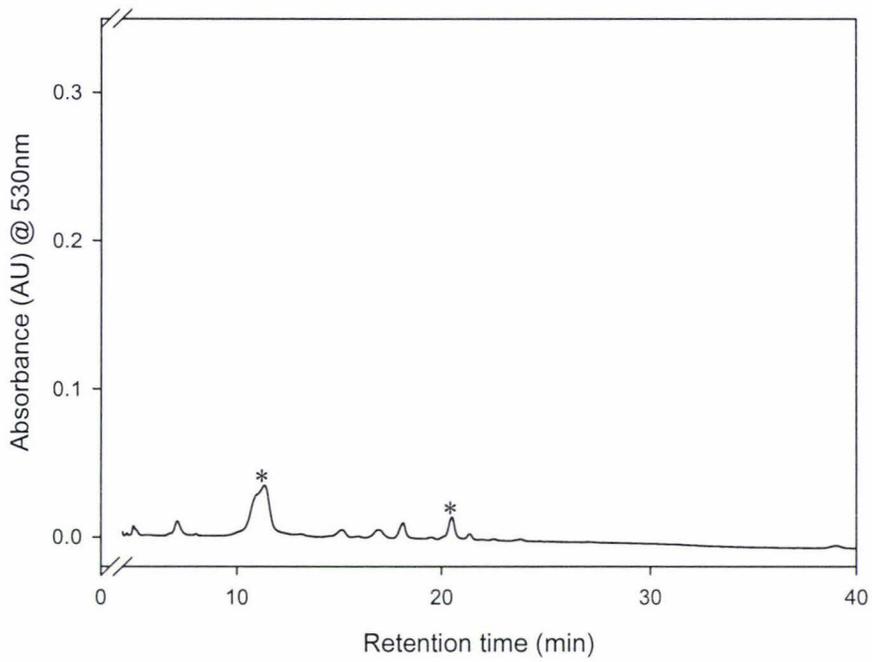
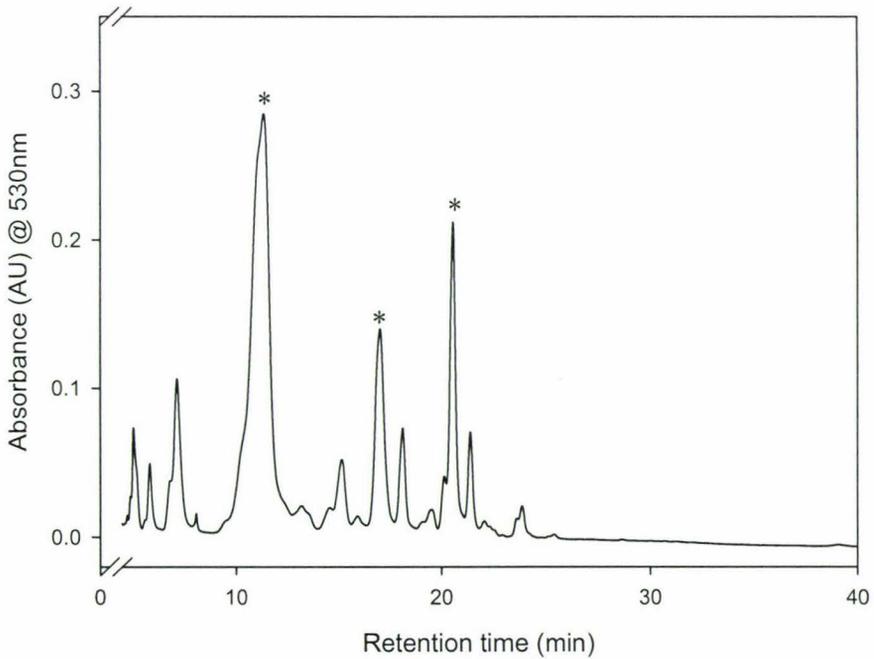
**B***Lc* Petunia

Figure 4.4: Representative HPLC chromatograms of methanolic leaf extracts from Mitchell, **A**, and *Lc*, **B**, petunia grown under high-light. The absorbance was monitored at 530 nm to detect anthocyanins. The major anthocyanin peaks are marked (*).

and B correspond to various flavonols. In addition, a non-flavonoid compound was also present. This compound was identified as rosmarinic acid, based on previous analysis and the spectral data (Bloor et al., 1998; Troncoso et al., 2005). Despite being the major peak detected at 350 nm, rosmarinic acid was excluded from 'other flavonoid' (flavonol) concentration (Figure 4.2) as it is not a flavonoid. There were three major anthocyanin peaks detected in Mitchell and *Lc* petunia extracts (Figure 4.4) and, based upon their spectral properties, these were tentatively identified as tri-hydroxylated anthocyanins. The same anthocyanin peaks were detected in *Lc* and wild type Mitchell petunia, albeit at different levels.

The most striking effect noted for the other pigments was that under the high-light treatment, *Lc* plants showed a large reduction in carotenoid concentration compared to wild type Mitchell plants (Figure 4.5B). The trend for carotenoid levels in other treatments was not clear, although there appears to be a slight reduction with increasing light intensity, which follows the trend for chlorophylls. The general trend for chlorophyll levels was that they decreased with increasing light intensity (Figure 4.5A). The chlorophyll *a/b* ratios increased between shade to ambient-greenhouse conditions, and were equivalent in Mitchell and *Lc* petunia. However, under high-light conditions, the *a/b* ratios were reduced, and there was a clear difference between Mitchell and *Lc* petunia.

4.3.3 Northern analysis of flavonoid structural gene expression

The pattern of expression for different flavonoid biosynthetic genes in leaf tissue from wild type Mitchell and *Lc* plants grown under different light treatments is shown in Figure 4.6. *CHS*, *CHI* and *FLS* are all expressed at very low levels in both Mitchell and *Lc* petunia plants grown under shade (Figure 4.6A). A faint signal for *ANS* was detected in *Lc* petunia, but this was absent in Mitchell petunia. The *rRNA* control shows RNA loading to be approximately equal.

Flavonoid biosynthetic gene expression from Mitchell and *Lc* plants grown under ambient-greenhouse conditions is shown in Figure 4.6B. *CHS*, *CHI* and *FLS* were all

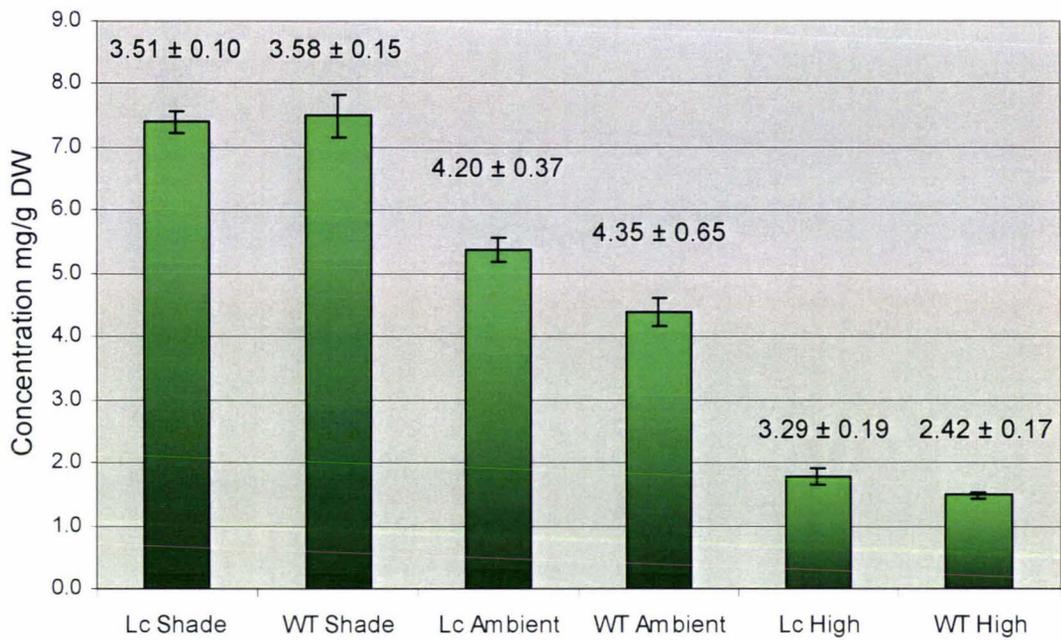
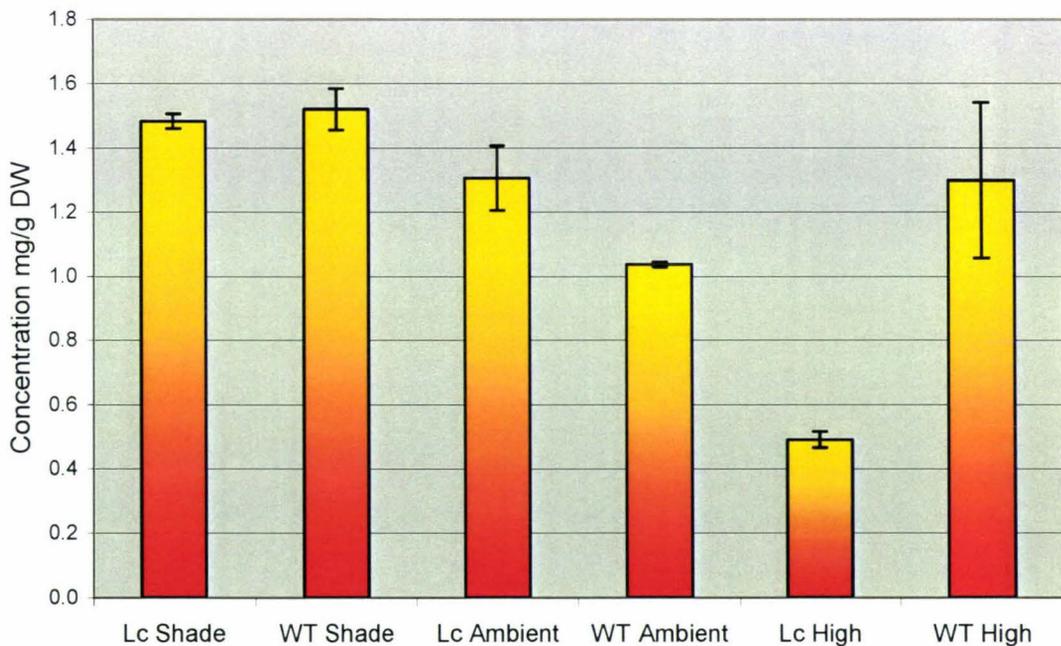
A**Total Chlorophylls****B****Total Carotenoids**

Figure 4.5: Total chlorophyll, **A**, and carotenoid, **B**, concentration in wild type Mitchell (WT) and *Lc* petunia leaf tissue grown under three lighting treatments: high-light, ambient-greenhouse (ambient) and shade. (Mean ± SEM, $n = 5$). The chlorophyll *a/b* ratio is shown in **A** above each bar.

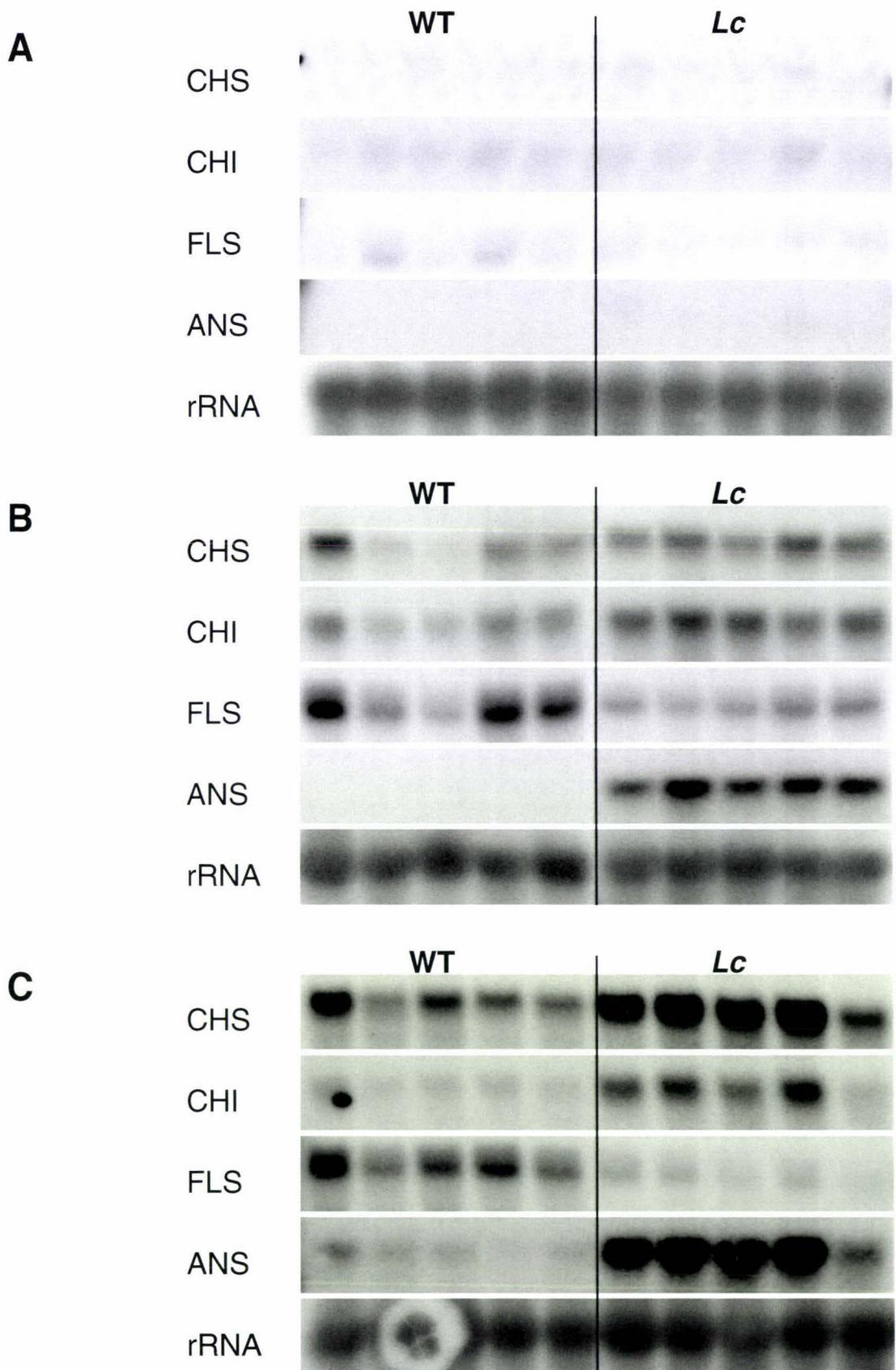


Figure 4.6: Northern blot analysis of flavonoid structural gene expression in wild type Mitchell and *Lc* plants under **A** shade, **B** ambient-greenhouse or **C** high-light treatments. Each lane represents a different individual plant within each treatment.

co-expressed in Mitchell petunia, but no transcript was detected for *ANS*. In *Lc* petunia, *CHS* and *CHI* expression was enhanced slightly compared to Mitchell, while there was a clear reduction in the level of *FLS* expression, and strong activation of *ANS* expression. The *rRNA* control shows RNA loading to be approximately equal.

Structural gene expression of Mitchell and *Lc* plants grown under high-light conditions is shown in Figure 4.6C. In Mitchell plants, *CHS* and *FLS* were co-expressed, and some *ANS* transcripts were detected. The levels of *CHI* were reduced compared to those of Mitchell under ambient-greenhouse conditions. The *Lc* plants show enhanced activation of *CHS* and *CHI* levels compared to Mitchell. *FLS* expression in *Lc* plants was reduced compared to Mitchell, while *ANS* levels were very strong. The *rRNA* control shows RNA loading to be approximately equal.

In lanes 2 and 3 of the wild type Mitchell (WT) samples in Figure 4.6C, an air-bubble, which occurred during RNA-membrane transfer, can be observed in the *rRNA* blot. In lane 1 of the WT samples on the *CHI* blot, there is a mark caused by a speck of luminescent crayon, used to mark the orientation of the blot on the autoradiograph, which resulted in exposure of the autoradiograph. Despite detracting slightly from the clarity of the picture, these factors do not alter the interpretation.

4.4 Discussion

Lc petunia displayed a strong light-induced anthocyanin pigmentation phenotype, which appears to be dose dependent: plants grown under shade were non-pigmented while those grown under ambient-greenhouse or high-light conditions had weak or strong pigmentation phenotypes respectively (Figure 4.1). The light-induced anthocyanin pigmentation observed in *Lc* petunia has also been observed in some other *Lc* transgenic plants. Transgenic CaMV35S::*Lc* alfalfa (*Medicago sativa*) displayed a light-induced anthocyanin pigmentation phenotype (Ray et al., 2003), and CaMV35S::*Lc* tomato (*Lycopersicon esculentum*) plants did not have enhanced anthocyanin pigmentation, unless grown under natural intense sunlight (Goldsbrough et al., 1996). Similar findings have also been observed with other anthocyanin regulating bHLH transcription

factors. Light enhanced the activation of flavonoid genes required for condensed tannin production in lotus (*Lotus corniculatus*) over-expressing the bHLH transcription factor *Sn* (Paolocci et al., 2005).

The light quality responsible for the light-induced anthocyanin pigmentation has not been determined for *Lc* petunia, or indeed *Lc* tomato or alfalfa. In *Lc* petunia, it is unlikely to be a UV light response, as the greenhouse used in this study is constructed of double skin plastic, which would largely exclude UV wavelengths (Ballaré et al., 1996), and plants grown under these ambient conditions became pigmented. Similarly, in the growth cabinet used for the high-light condition, UV wavelengths are probably excluded by the thick perspex plastic between the bulbs and the growth chamber. There are contrasting examples of light qualities which induce anthocyanin pigmentation in different species. In apple, red and UVB synergistically induce pigmentation in exposed apple skins (Arakawa, 1988). Red, blue and green wavelengths were all effective at inducing anthocyanin pigmentation in petunia flowers. This response was found to be a high light, phytochrome-mediated response (Moscovici et al., 1996). Red, and to a lesser extent blue, wavelengths have been found to be involved in anthocyanin regulation in tomato (Kerckhoffs and Kendrick, 1997). Red light was found to be the most effective wavelength to stimulate anthocyanin pigmentation in cranberry fruits (Zhou and Singh, 2002). The early biosynthetic genes in many species, including *Arabidopsis* and parsley, (and those required for flavonol production) have been shown to be induced by UVA/blue and UVB wavelengths (Lozoya et al., 1991; Hemm et al., 2004). Light quality and quantity is an environmental trigger that induces a range of responses in plants. Flavonols and anthocyanins have roles as photo-protectants, so it is perhaps not surprising that a light-induced phenotype was observed in *Lc* petunia.

The level of other flavonoids (identified as flavonols) present in Mitchell and *Lc* petunia leaves was similar under each of the light treatments (Figure 4.2A). The flavonol levels increased with increasing light level (Figure 4.2A), which is consistent with reports in many species that flavonols are induced by light, particularly short wavelengths (UVA/blue and UVB) (Lozoya et al., 1991; Reuber et al., 1996; Ryan et al., 1998; Merzlyak et al., 2002; Hemm et al., 2004; Jaakola et al., 2004) and provide a photoprotective role to the plant by absorbing UV wavelengths (Li et al., 1993; Middleton and Teramura, 1993). The plastic greenhouse or perspex in the growth

cabinet may exclude UV wavelengths but are unlikely to exclude blue light, which may explain the induction of flavonols in Mitchell and *Lc* petunia (this study). The flavonol profile and concentrations found in Mitchell and *Lc* petunia were consistent with previous analyses. The flavonols had been previously identified as various acylated kaempferol and quercetin-glycosides (Bloor et al., 1998). The similar levels of leaf flavonols in *Lc* and Mitchell petunia reported in this study (Figure 4.2A), contrast with the results of Bradley *et al.* (1998). They reported a slight reduction in the level of flavonols present in leaves of *Lc* petunia, possibly as a result of a re-direction of metabolites away from flavonol production towards anthocyanin production. The reasons for the differences observed between the present study and the results of Bradley *et al.* (1998) are not known. They may result due to the imposition of different growing conditions (e.g. glasshouse compared to a plastic-greenhouse), or may just be part of the variation for plants grown in different years.

Previous analysis of the anthocyanins present in the leaves of *Lc* petunia revealed there were three major anthocyanins present: petunidin-3-(*p*-coumaroylrutinoside) 5-glucoside, petunidin-3-rutinoside-5-glucoside acylated with caffeic acid and petunidin-3-rutinoside-5-glucoside acylated with *p*-coumaric acid. In addition to these three major peaks, various petunidin and delphinidin glycosides were also found (Bloor et al., 1998). The results from this study are consistent with these previous findings. It was also interesting that the anthocyanins present in Mitchell leaves were the same as *Lc* petunia, albeit at significantly lower levels. This suggests that *Lc* has enhanced the existing pigmentation patterns, by enhancing the existing expression pattern of the structural genes required to make these specific anthocyanins (e.g. F3'5'H required to make trihydroxylated anthocyanins). This is consistent with the idea that *Lc* is enhancing the activation of structural genes targeted by endogenous regulatory factors.

The concentration of anthocyanins in the leaves of high-light grown *Lc* petunia ($\sim 3 \text{ mg g}^{-1}\text{DW}$) determined in this study, is similar to previous analyses of *Lc* petunia (Bradley et al., 1998), although growing conditions and zygosity of the transgene probably contribute to the slight variation. It is hard to compare pigment levels in *Lc* petunia with other species, as features such as leaf thickness will have a large effect on the final concentration (on a per g basis), and pigmentation is usually limited to epidermal, or sub-epidermal layers (petunia has relatively thick leaves). *Lc* petunia (3

mg g⁻¹DW, which approximates to 0.3 mg g⁻¹FW) does, however, appear to be similarly pigmented to intensely pigmented leaves in *Perilla frutescens* (7.5 mg g⁻¹FW) (Saito and Yamazaki, 2002) or *Quintinia Serrata* (1.8 mg g⁻¹FW) (Gould et al., 2000), despite appearing to have a lower pigment concentration when expressed on a per gram basis.

It was anticipated that chlorophyll and carotenoid levels may vary between light treatments, but the differences in the chlorophyll and in the carotenoid levels between Mitchell and *Lc* petunia plants grown under high-light, were not expected (Figure 4.5). The observed differences are most likely to be associated with the increased levels of anthocyanins, rather than simply the presence of the transgene, otherwise similar responses should have occurred under lower light conditions (when *Lc* plants were not pigmented). The carotenoid and chlorophyll biosynthetic pathways are distinct from the flavonoid pathways, and do not share any common metabolites or enzymes. An examination of the carotenoid and chlorophyll biosynthetic genes may be warranted in future experiments.

The levels of total chlorophylls reduced with increasing lighting intensity (Figure 4.5A). Chlorophyll biosynthesis is responsive to its light environment, so that light harvesting can be optimised for photosynthesis. Under high-light conditions, light levels exceed the requirement for photosynthesis, and the size of the light harvesting complexes containing chlorophyll are reduced to reduce the amount of energy absorbed, which if not used can cause photoinhibition (Huner et al., 1998). This explanation is consistent with the levels of total chlorophylls observed in Mitchell and *Lc* petunia plants grown under the various light treatments. The differences observed in chlorophyll levels between Mitchell and *Lc* petunia are not great and may not be biologically significant. The largest differences in chlorophyll levels between Mitchell and *Lc* petunia was when they were grown under ambient-greenhouse conditions. *Lc* plants under this condition were only slightly pigmented, and it would be unlikely that such a modest amount of anthocyanin would alter the light environment of underlying cells sufficiently to result in changes in chlorophyll concentration.

The chlorophyll *a/b* ratios observed in Mitchell and *Lc* petunia grown under shade and ambient-greenhouse conditions are consistent with reports of increasing chlorophyll *a/b* ratio with increased lighting intensity (Willows, 2004). However, when the petunia

plants were grown under high-light conditions within the growth cabinet, the chlorophyll *a/b* ratios dropped to levels reported for shade adapted plants. In cotton, anthocyanin pigmentation was negatively correlated to chlorophyll *a/b* ratio (Wells, 2001), suggesting that the accumulation of anthocyanins reduced the chlorophyll *a/b* ratio (possibly by reducing light levels to underlying tissues). However, there is no clear explanation for the difference in chlorophyll *a/b* ratio between Mitchell and *Lc* petunia grown under the high-light treatment (Figure 4.5A). It is interesting that the differences in the chlorophyll *a/b* ratio between Mitchell and *Lc* petunia arise from changes in concentration of both chlorophyll *a* and chlorophyll *b* (data not shown). It is possible this is an aberrant effect due to the experimental system (e.g. lighting in growth cabinet) and it would be useful to repeat the experiment.

The greatest difference in carotenoid levels occurred under high-light conditions (Figure 4.5B), when *Lc* petunia had less than half the level of Mitchell. It is possible that this is also an aberrant effect and again it would be useful to confirm these results with further experiments. However, it is also possible that as the leaves of *Lc* petunia accumulate high levels of anthocyanins, they act as a filter, screening photosynthetic tissues from excess light, as proposed by Neill and Gould (2003). This photoprotection role by the anthocyanins may partially overlap with that of the carotenoids (Havaux and Kloppstech, 2001), and may result in slightly reduced levels of carotenoids. Carotenoid levels were reduced in young anthocyanic leaves of *Rosa* sp. and *Ricinus communis* L. compared to mature green leaves (Manetas et al., 2002). It was proposed that anthocyanins provided a screening role reducing photoinhibition, compensating for the low levels of carotenoids (Manetas et al., 2002), which appears consistent with the findings in this study. A reduction of carotenoid and chlorophyll biosynthesis was not observed, however, in purple leaves of *Quintinia serrata* (Gould et al., 2000). This may be due to the optical properties of the type of anthocyanins produced in *Lc* petunia (acylated petunidin-diglucosides) compared to those produced in *Q. serrata* (simple cyanidin-monoglucosides), as acylated anthocyanins have stronger absorption in UV/blue region compared to simple anthocyanins (Giusti et al., 1999). Blue light perception has been linked to carotenoid induction (Rau and Schrott, 1987; Giliberto et al., 2005), so perhaps the accumulation of acylated anthocyanins in *Lc* petunia grown under high-light conditions reduced the induction of carotenoids.

Northern analyses revealed that in Mitchell petunia, the expression of the early biosynthetic genes, *CHS* and *CHI*, and the flavonol specific gene *FLS*, were coordinated and responsive to light (Figure 4.6), suggesting that they are activated by a common, light-induced regulatory complex. *Lc* petunia displayed enhanced expression of *CHS* and *CHI* when light levels increased, as well as strong activation of the late biosynthetic gene *ANS* (Figure 4.6B/C). The very weak signal for *ANS* observed in shade grown *Lc* petunia (Figure 4.6A), is consistent with the observation that while these plants were overall green in appearance, they did contain a small amount of anthocyanin pigmentation on the margins of the leaves (below the level of detection in Figure 4.2A). The enhanced expression of the early biosynthetic genes and activation of the late biosynthetic gene *ANS* in *Lc* petunia when light levels increased, suggest that the presence of LC is only effective under such conditions, and that there is some other factor involved leading to anthocyanin production. This response may not be limited to high light, but other stresses reported to induce anthocyanins such as nutritional deficiency, cold, dehydration and salt-stress may also stimulate a similar response in *Lc* petunia, although this has not been examined. The hypothesis that LC is interacting with endogenous light-induced regulatory factors, is supported by the light-dependent enhanced activation of early biosynthetic genes and *de novo* activation of the late biosynthetic genes, which results in anthocyanin production.

The anthocyanin concentrations determined in the leaves (Figure 4.2B) were consistent with the visible accumulation of anthocyanins and activation of the late biosynthetic gene, *ANS* (Figure 4.1), in Mitchell and *Lc* petunia. The weak activation of *ANS* in Mitchell petunia grown under high-light (Figure 4.6C), may be a result of the strong activation of the flavonoid biosynthetic pathway such that, under high-light conditions, light-regulated endogenous transcription factors may reach a threshold concentration, at which time the transcription factor complexes start to bind to low affinity promoter targets (e.g. *ANS*), which results in modest anthocyanin production. Alternatively, an unidentified anthocyanin-specific transcription factor may be induced, to produce anthocyanins in leaves and stems (as observed in wild type Mitchell), to screen photosynthetic tissues from excess light energy.

Interestingly, wild type Mitchell plants under high-light conditions had weaker *ANS* expression than *Lc* petunia grown under ambient-greenhouse conditions (Figure 4.6),

yet had higher levels of anthocyanins (Figure 4.2B). This may reflect a greater flux of metabolites through the flavonoid biosynthetic pathway under high-light conditions, providing more substrates for ANS, and resulting in greater anthocyanin accumulation. The flavonol (other flavonoids) concentrations determined for both Mitchell and *Lc* petunia were similar for each growth condition, and they increased in a dose-dependent manner with increased lighting intensity (Figure 4.2A).

The enhanced expression of *CHI* observed in *Lc* petunia (Figure 4.6) was not observed in *Lc/CI* transgenic tomato (Bovy et al., 2002). One possible explanation is that in *Lc* petunia, LC may form a complex with an endogenous petunia Myb transcription factor, and that the promoter targets recognised by the endogenous Myb include *CHI*, which the LC/CI complex in tomato did not recognise. *CHI* expression was reduced in Mitchell petunia grown under high-light conditions, compared to plants grown under ambient-greenhouse conditions (Figure 4.6C). The reason for this reduction in *CHI* is not known, but this result has also been observed in both wild type and transgenic *Lc/CI* tomato plants where all other genes required for flavonol production were strongly activated, while *CHI* was not, despite accumulation of flavonols (Bovy et al., 2002). Mitchell petunia grown under high-light contained high levels of flavonols (Figure 4.2A). Therefore, the reduced gene expression of *CHI* did not prevent flavonol production. The reaction catalysed by CHI can occur spontaneously, without enzymatic intervention (Martin and Gerats, 1993; Holton and Cornish, 1995), and this may account for the high levels of flavonols produced even with reduced transcript levels of *CHI*. It is also possible that the changes observed in *CHI* transcript levels are not reflected at the protein level, due to changes in CHI stability. Alternatively, the existence of flavonoid multienzyme complexes, which allow the channelling of metabolites from one enzyme to the next, have increased the overall efficiency of the pathway (Winkel-Shirley, 1999), and therefore lower concentrations of CHI protein are not limiting flavonol production.

Multienzyme complexes result in very strong flux towards particular flavonoid branches, such as flavonols (Winkel-Shirley, 1999). Flavonol production in petunia flowers for example, is in strong competition with anthocyanin synthesis through the activities of FLS and DFR (Davies et al., 2003). Similarly, the strong flux towards flavonol production has posed an obstacle to engineering tobacco plants with enhanced isoflavonoid levels. A CHI-IFS (chalcone isomerise-isoflavone synthase) fusion protein

was made to overcome this obstacle, creating an engineered multienzyme complex in favour of isoflavonoid production (Tian and Dixon, 2006). It also appears that there is strong competition between flavonol and anthocyanin production in *Lc* petunia leaves. *FLS* expression was reduced in *Lc* petunia compared to wild type Mitchell plants in the high-light treatment (Figure 4.6B/C). Despite reduced expression of *FLS*, flavonol levels did not vary between *Lc* and Mitchell petunia. As proposed earlier for CHI, increased protein stability or a multienzyme complex, allowing efficient metabolite channelling towards flavonol production, are two possible explanations for the discrepancy between *FLS* expression and flavonol levels.

The changes in gene expression and anthocyanin accumulation in *Lc* petunia raise questions about the regulation of the flavonoid structural genes. *Lc* alone failed to activate *FLS* expression in Mitchell petunia, which suggests that either *Lc* specifically, or bHLH factors generally are not involved in flavonol regulation. The latter is consistent with studies in *Arabidopsis* where a complex of Myb and bZIP transcription factors is proposed for flavonol regulation, with no role for a bHLH partner (Hartmann et al., 1998; Hartmann et al., 2005). Promoter elements which are thought to bind bZIP, Myb and bHLH transcription factors have been identified in the early flavonoid biosynthetic genes in *Arabidopsis*. However, electromobility shift assays and promoter activation studies of the bZIP and Myb binding elements, suggest that a bZIP-Myb complex is involved in light-regulated activation of genes required for flavonol production (Figure 1.4B) (Hartmann et al., 2005). The adjacent bHLH binding sites present in the early structural genes may allow for developmentally regulated bHLH-Myb-WD40 activation of anthocyanin biosynthetic genes (Figure 1.4A) (Hartmann et al., 2005). In *Arabidopsis*, the flavonol specific gene, *FLS*, was not reported to contain the 'R' recognition element (recognised by bHLH transcription factors). If this is also the case in petunia, it may explain why LC failed to activate *FLS* expression in *Lc* petunia.

If we consider the model for the transcriptional regulation of anthocyanin biosynthetic genes (Figure 1.4A), a bHLH-Myb-WD40 ternary complex is required. In *Lc* petunia, the bHLH factor LC is constitutively expressed, and the WD40 protein AN11 (in petunia) has been shown to be expressed in leaves (de Vetten et al., 1997), which leaves the Myb factor missing from the complex. LC may, therefore, be interacting with an

unidentified light-induced endogenous Myb transcription factor in petunia, which is responsible for the light-induced anthocyanin pigmentation observed in *Lc* petunia.

The nature of this Myb is unknown, but a number of possibilities exist. It is possible that an unidentified anthocyanin-regulating Myb transcription factor is induced under high-light conditions. This Myb may already be involved in regulating the weak anthocyanin pigmentation observed in the stems and leaves of wild type Mitchell petunia plants (Figure 4.1E). It is also possible that a Myb not normally linked to anthocyanin biosynthesis is interacting with LC.

Flavonols are the predominant flavonoid produced in Mitchell and *Lc* petunia, and these appear to be regulated separately from anthocyanins, as discussed earlier. The northern results show reduced expression of *FLS* in *Lc* petunia under high-light, which may suggest that there was competition for a common regulatory factor (e.g. a Myb transcription factor) between anthocyanin and flavonol production in plants expressing LC. In *Arabidopsis*, AtMYB12 has been identified as the flavonol-regulating R2R3 Myb transcription factor which is responsible for co-regulating the early biosynthetic genes and *FLS* in response to light (Mehrtens et al., 2005), and acts independently of anthocyanin biosynthesis. AtMYB12 lacks the amino acid motif required for bHLH interaction (Zimmermann et al., 2004), so a petunia orthologue would not be expected to be able to interact with LC. However, it is possible that petunia differs from *Arabidopsis*, and a flavonol-regulating Myb transcription factor in petunia may have the ability to interact with a bHLH partner (such as LC), altering the target genes regulated and activating anthocyanin biosynthesis. Clearly, the normal function of any Myb transcription factor found to interact with LC would need further investigation.

The imposed light treatments confirmed that anthocyanin accumulation in *Lc* petunia is influenced by light. Plants grown under high-light showed a dramatic increase in the concentration of anthocyanins present in leaf tissue. The increased anthocyanin content also correlated with an increased expression of flavonoid structural genes, directing the pathway to anthocyanin biosynthesis, though not at the expense of flavonol production. Expression of the transgene *Lc* is not sufficient on its own to induce pigmentation, therefore, it would appear that LC must be interacting with a light-induced endogenous petunia regulatory factor. The missing component, in terms of the model anthocyanin

regulatory complex shown in Figure 1.4A, is a Myb transcription factor. This possibility is examined in Chapter 5.

Chapter 5

Isolation of endogenous petunia Myb transcription factors

5.1 Introduction

A proposed model for the regulation of anthocyanin biosynthesis (Figure 1.4A) has three components present in the regulatory complex that activates the structural genes required for anthocyanin formation. The complex consists of a bHLH transcription factor, a Myb transcription factor and a WD40 regulator. In *Lc* petunia, a bHLH transcription factor is supplied as the transgene and is constitutively expressed via the CaMV35S promoter, while the WD40 regulator is regarded as being ubiquitously expressed. This leaves the Myb transcription factor as a missing component which would be required for activation of anthocyanin biosynthesis. Under high-light conditions, *Lc* petunia is highly coloured, indicating activation of anthocyanin biosynthesis. If we consider this in relation to the proposed model, then it is possible that an endogenous, light-induced Myb transcription factor is forming a functional complex with LC and an endogenous WD40 regulator (AN11) to promote the observed anthocyanin production.

The main flavonoids produced in Mitchell leaf tissue are flavonols (Chapter 4). It is possible that an endogenous Myb transcription factor that regulates the early biosynthetic genes required to make flavonols, is light-responsive, and is interacting with LC. However, Mitchell petunia does produce a very modest amount of anthocyanin in the stems and leaves when grown under high-light conditions (Chapter 4), so it is also possible that there is an endogenous anthocyanin-regulating Myb transcription factor, that is expressed under these conditions, and that is interacting with LC.

The possibility that an endogenous Myb could be interacting with the transgene LC, was investigated by complementation experiments, introducing anthocyanin-regulating Myb transcription factors into shade grown *Lc* petunia tissue. Biolistic- and *Agrobacterium*-mediated transformations were carried out. Partial sequences of two putative endogenous Myb transcription factors were cloned using degenerate RT-PCR followed by 3'RACE.

5.2 Materials and methods

5.2.1 Plant material

The *Lc* (118C) and wild type Mitchell petunia plants have been described in Section 2.9. Plant material was grown under shade (Section 3.2.1) and green, non-induced leaves from *Lc* and Mitchell plants were collected for transformation.

5.2.2 Biolistic transformation of petunia leaves

5.2.2.1 DNA preparation

High concentration, high purity plasmid preparations (Section 2.5.2) of the Myb anthocyanin regulatory genes *An2* (*Petunia hybrida*), *Cl* (*Zea mays*) and *Rosea1* (*Antirrhinum majus*) (pPN81, pLN44 and pPN73 respectively, Appendix 3.2) were used for transient transformation assays. The Myb genes were present in pART7 cloning vectors, placing them under the control of the CaMV35S promoter, allowing strong gene expression inside the plant cells. A CaMV35S::GFP construct in a pUC based vector was used as a positive control for transformation (Appendix 3.3).

5.2.2.2 Growth and culture conditions

Petunia leaves (Section 5.2.1) were transferred onto agar media (Appendix 1.5.2) during biolistic transformation, as the media provides cushioning to the plant material. Leaves were transferred back onto moist filter paper following transformation, and maintained in a culture room at 25°C with a photoperiod of 16 h supplied by cool fluorescent tubes (25 $\mu\text{mol m}^{-2} \text{sec}^{-1}$).

5.2.2.3 Gold preparation

Gold particles of 1 μm diameter were prepared by transferring 50 mg gold to a 1.5 mL tube with 500 μL isopropanol. The gold/isopropanol mix was vortexed for 10 min. The gold particles were pelleted by brief centrifugation and isopropanol removed from the gold with a pipette. The gold was washed with 500 μL sterile water, vortexed and briefly centrifuged. This wash procedure was performed twice more to remove any traces of isopropanol. After the final wash step, 500 μL of sterile water was added to the gold and vortexed. Working quickly to keep the gold particles in suspension, 50 μL aliquots were transferred to 1.5 mL tubes. The 50 μL aliquots (approximately 5 mg gold) were stored at -20°C until required.

5.2.2.4 Particle bombardment

The DNA construct containing the Myb genes (10 μg) and 2 μg of the 35S::GFP construct (control) was added to each 50 μL gold preparation. DNA was precipitated onto the gold particles by simultaneously adding 20 μL of 0.1 M spermidine and 50 μL of 2.5 M CaCl_2 to the gold/DNA preparation. This mixture was vortexed, left on ice for 5 min and then centrifuged briefly. Ninety microlitres of the supernatant were removed, and the remaining gold mixture was re-suspended by flicking the tube. An aliquot of this mixture (5 μL) was used for each particle bombardment of the leaf tissue.

Biolistic-mediated transformation was performed using a modified helium particle inflow gun (Finer et al., 1992) with a high-speed direct current solenoid valve. The gold particles were accelerated in a helium stream under appropriate pressure in a partial vacuum of -90 kPa. A standard solenoid opening time of 30 ms was used for all experiments. Shooting pressure of 200 kPa was found to be the most effective, causing the least amount of bruising and cellular damage, yet still able to transform tissue.

5.2.2.5 Assessment

Plant tissue was assessed for anthocyanin production using an Olympus SZX12 light microscope and Leica Microsystems DC500 digital camera. GFP detection was performed with a blue light source and GFP filter set (Olympus) in addition to the microscope/camera. Anthocyanin production and GFP fluorescence were assessed as being present or absent.

5.2.3 *Agrobacterium*-mediated transformation

5.2.3.1 Plant material

Petunia leaves (Section 5.2.1) were sterilised in 15% (v/v) bleach + Tween 20 for 15 min. Sterilised leaves were washed twice in sterile water and cut in half for transformation. Explants (cut leaves) were maintained in a culture room (Section 5.2.2.2) during the culturing periods of transformation.

5.2.3.2 *Agrobacterium tumefaciens*

A. tumefaciens carrying binary-vector plasmids with anthocyanin regulatory Myb genes *Cl* or *Roseal* (pLN52, pLN83 respectively; Appendix 3.4), and the positive control GFP (pPN92 Appendix 3.5), were grown up from glycerol stocks onto YM-agar plates with antibiotic selection (Strep 200 mg L⁻¹, Spec 200 mg L⁻¹) (Section 2.4.1). The regulatory genes *Cl* and *Roseal* were present in the binary vector pART27, and the GFP control in the pBIN binary vector (pBIN-m-gfp5-ER) (Haselhoff et al., 1997), each

under the control of the CaMV35S promoter. The gene constructs were within the left- and right-border flanking sequences of the binary vectors, allowing transfer from the plasmid into the genome of the host plant.

Liquid cultures of *Agrobacterium* were started by picking single colonies and growing them in YM-broth with antibiotic selection (Strep 200 mg L⁻¹, Spec 200 mg L⁻¹) (Section 2.4.1); plasmid DNA was isolated (Section 2.5.4) and diagnostic restriction digests (Section 2.6.1) were performed to ensure the culture contained the correct plasmid. From the starter cultures, 48 h liquid cultures under antibiotic selection (Strep 200 mg L⁻¹, Spec 200 mg L⁻¹) were grown. After 48 h, cells were pelleted by centrifugation at 5000 g and the supernatant removed. Cells were gently re-suspended in liquid media (virulence-induction media, Appendix 1.5.3) to an optical density of 0.5 ($A_{600} = 0.5$) and left to recover at 28°C for 4 h. After recovery/induction of virulence, the *A.tumefaciens* culture was used for transformation.

To ensure that the transformation system was working, petunia were transformed with the binary vector pPN92, which contains CaMV35S::GFP, as a positive control. The plants transformed with pPN92 were then assessed for their expression of GFP. Anthocyanin pigments are readily detectable by eye, and therefore serve as their own marker.

5.2.3.3 Transformation

Sterilised petunia leaves were cut into small explants with a scalpel blade. The wound sites provide entry points for *Agrobacterium*, and in addition to this, endogenous phenolic compounds produced by the plant at wound sites, serve to further activate virulence (Gelvin, 2003). The leaf explants were placed into the liquid *Agrobacterium* culture and submerged for 5 min. Explants were blotted of excess bacterial culture and placed onto media #2 (Appendix 1.5.2), allowing co-culture with *Agrobacterium*, and left in the culture rooms under conditions described in Section 5.2.2.2.

After 2 d the explants were transferred onto fresh media #2 supplemented with cefotaxime (500 mg L⁻¹), which kills *Agrobacterium*. This was performed as the

formation of an *Agrobacterium* bio-film can damage plant material, initiating necrosis and preventing assessment of pigmentation. Explants were then left in the culture rooms and were assessed for pigment production after 3 d. The Leica microscope/camera (Section 5.2.2.5) was used to assess and photograph pigmented plant cells.

5.2.4 Cloning endogenous petunia Myb transcription factors

5.2.4.1 Degenerate primer sequences to the conserved Myb domain

The nucleotide sequence of the R2R3 Myb DNA-binding domains of known anthocyanin-regulating Myb transcription factors from dicotyledonous plants were aligned using the CLUSTAL W algorithm (DNASTAR software). Degenerate primers were designed based upon this alignment. The alignment and primer position is indicated in Figure 5.3. The oligonucleotide primers were sourced from SigmaGenosys: dicot fwd 5' TATGGWGAAGGVAARTGGCA3', dicot rvs 5'GTGTTCCARTARTTYTTSACRTC 3'.

5.2.4.2 cDNA synthesis

First strand cDNA was synthesised from 5 µg total RNA (Section 4.2.4) from shade-grown and high-light grown *Lc* and Mitchell petunia, using superscript III reverse transcriptase (Invitrogen) following the manufacturer's instructions. The primer used for cDNA synthesis was oligo(dT)₁₂₋₁₈ (Invitrogen). Incubations were performed in an Eppendorf Mastercycler gradient PCR machine.

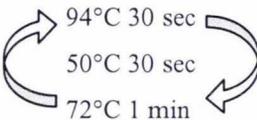
5.2.4.3 Reverse transcription-PCR (RT-PCR)

RT-PCR was performed to amplify the R2R3 Myb domain using 2 µL of cDNA from shade- and high-light-grown *Lc* and Mitchell plants with the 'dicot fwd' and 'dicot rvs' primers. As a negative control, a reaction without template was performed, and as positive controls, 1 µL (5 ng µL⁻¹) of the Myb genes *An2* and *Rosea1* were included. Thermo-cycling was performed in an Eppendorf Mastercycler gradient PCR machine.

PCR reaction:

2 μL cDNA
1 μL fwd primer (10 μM)
1 μL rvs primer (10 μM)
1 μL dNTPs (10 μM) (Invitrogen)
5 μL 10 \times PCR buffer (QIAGEN)
39.5 μL H_2O
0.5 μL *Taq* polymerase (5 U μL^{-1} , QIAGEN)

Thermo-cycling:

94°C 2 min (denaturation)
 94°C 30 sec (denaturation)
50°C 30 sec (primer annealing)
72°C 1 min (extension)
72°C 5 min (extension)

50 μL Total

5.2.4.4 Cloning PCR products

PCR products were purified (Section 2.7.1) and quantified (Section 2.6.3.1) before being cloned into the pGEMTeasy (Promega) cloning system following the manufacturer's instructions. An aliquot of the ligation mixture (2 μL) was transformed into NovaBlue *E. coli* by heat-shock transformation (Section 2.4.3), and cells were plated onto LB-agar plates containing Amp (100 mg L^{-1}). The plates also contained 12 μL IPTG (20 mg mL^{-1}) and 40 μL X-gal (20 mg mL^{-1}), allowing blue-white selection for insert-positive clones. Several white colonies were picked into 3 mL cultures, and grown overnight, before extracting plasmids by alkaline lysis (Section 2.5.1). The plasmid DNA was digested with *EcoRI* (Section 2.6.1) to drop out the plasmid insert, and the digest was analysed by gel electrophoresis (Section 2.6.2). Sigma GenElute plasmid minipreps (Section 2.5.3) were performed on bacterial cultures corresponding to clones that were positive for the 240 bp *EcoRI* fragment. The purified plasmid DNA was quantified by both gel-quantification (Section 2.6.3.1) and spectrophotometry (Section 2.6.3.2) before being sent for DNA sequencing (Alan Wilson Centre Genome Service, Massey University).

5.2.4.5 3'Rapid amplification of cDNA ends (3'RACE)

To clone the sequence beyond the R2R3 Myb DNA binding domain, 3'RACE PCR was performed, using nested gene-specific primers based upon the 240 bp clones.

The DNA sequences obtained for the shade and high-light clones were aligned, using the CLUSTAL W algorithm (DNASTAR software), and the consensus sequences established. Gene-specific primers were designed to nest downstream of the 'dicot fwd' primer, and are indicated in Figure 5.5. Primers were sourced from SigmaGenosys; 'shade fwd' 5' CTTTTGTCCGGAGGAAGTG 3', 'high light fwd' 5' CTCTGAGGATGAAGTAGATCTC 3'.

First strand cDNA was synthesised as described in Section 5.2.4.2 except that the primer used was 'adapter long' 5' GACTCGAGTCGACATCGA[T]₁₇ 3', resulting in 'tagged' cDNAs. The first round of 3'RACE was performed using 2 µL of the tagged cDNA, with the 'dicot fwd' primer, and 'adapter small' primer 5' GACTCGAGTCGACATCG 3' (which binds to the 3' tag). The PCR reactions and thermo-cycling conditions are the same as described in Section 5.2.4.3. The second (nested) round of 3'RACE was performed using 1 µL of the first round product mixture as the template, and the 'shade fwd' or 'high light fwd' and 'adapter small' primers. The PCR reactions and conditions remained unchanged. Aliquots from both rounds were taken and checked on an agarose gel (Section 2.6.2).

5.2.4.6 Cloning 3'RACE products

The remaining reaction mixtures from the second round of 3'RACE were run on a 1% (w/v) TAE agarose gel (Section 2.6.2) and the major PCR products were excised from the gel (as indicated Figure 5.6). The excised PCR products were gel-purified (Section 2.7.1) and cloned into pGEMTeasy (Section 5.2.4.4). Clones were screened and sent for DNA sequencing (Alan Wilson Centre Genome Service, Massey University).

5.2.4.7 Semi-quantitative RT-PCR of Myb gene expression

To determine the expression of the two partial Myb clones, *MybI* and *MybII*, semi-quantitative RT-PCR was used. Gene-specific primers designed to the 3' region of the genes.

RT-PCR was performed as described in Section 5.2.4.3, except that 3 μ L of cDNA was used, the annealing temperature was 48°C, and gene-specific primers were used:

'*MybI*' primers:

fwd 5' CACAAGGATGAATATAGCAAAC 3', rvs 5' TTACATGGGAATCAAAATGG 3'

'*MybII*' primers:

fwd 5' GATAAAGATGGAAGTAACAATG 3', rvs 5' TTTAATGAATGGATTCTCTTATT 3'

Actin was also amplified, as a cDNA loading control for semi-quantitative analysis, using the 'actin fwd' 5' TTCAGCCACTTGTCTGTGAC 3' and 'actin rvs' 5' CGACATCACATTTTCATGATGG 3' primers. PCR reactions and thermo-cycling were the same for both the Myb and actin amplifications, except that 2 μ L of cDNA was used for the actin reactions.

5.3 Results

5.3.1 Biolistic transformation

Myb transcription factors were introduced, using biolistic transformation, into green, non-induced *Lc* petunia leaves to investigate whether an endogenous Myb transcription factor could induce anthocyanin pigmentation in *Lc* petunia. Three different Myb anthocyanin transcription factors were tested; *Roseal* (*Antirrhinum*), *An2* (petunia) and *Cl* (maize), and each co-transformed with GFP. Both *Roseal* and *An2* successfully induced anthocyanin production. Anthocyanin producing cells were identified under a dissecting microscope, and these were then viewed under blue light to check if they were also positive for GFP (Figure 5.1). The cells indicated by the arrows in Figure 5.1 are positive for both anthocyanin pigments and GFP expression. The intense

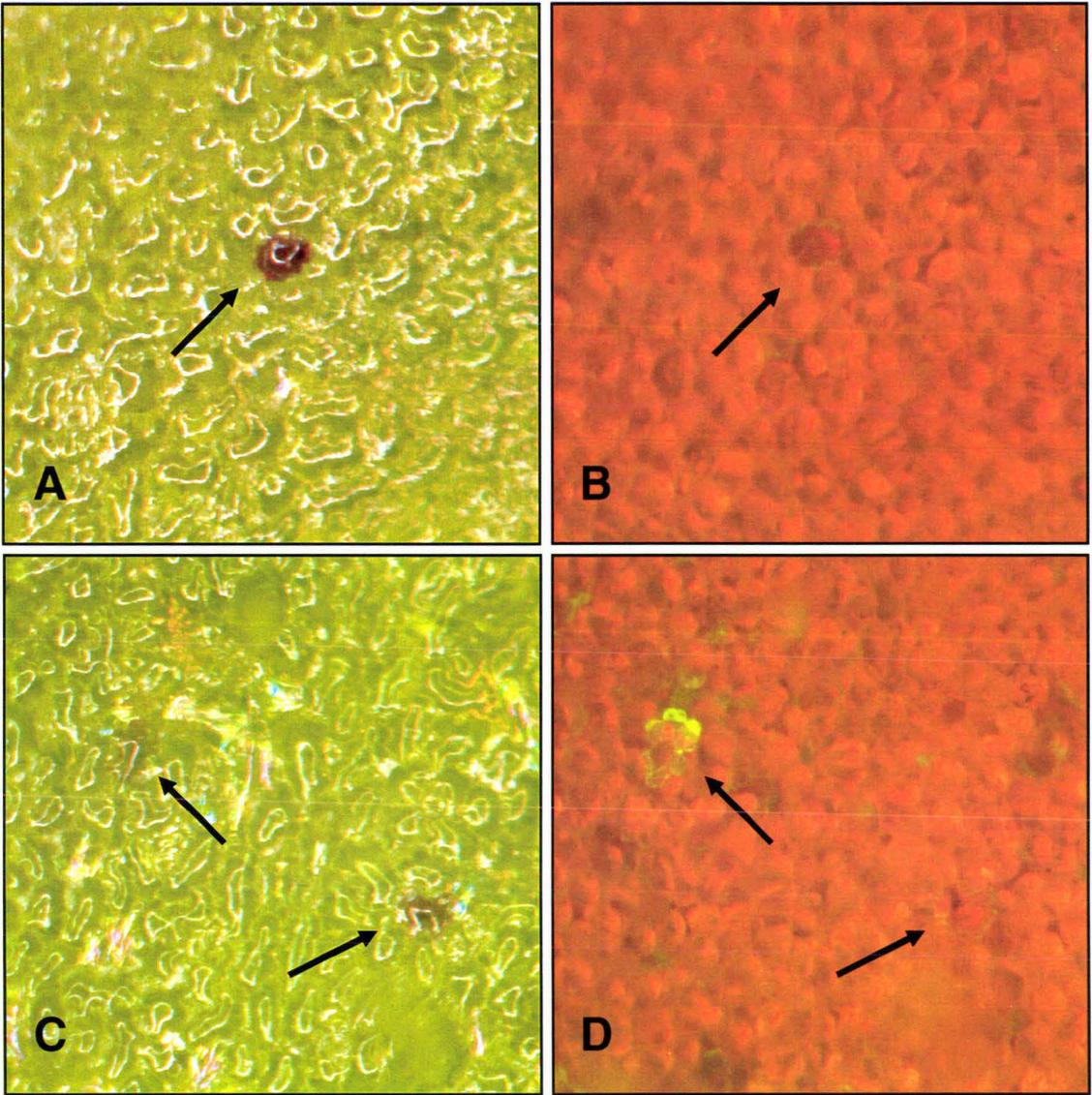


Figure 5.1: Biolistic transformation of non-induced *Lc* petunia leaves with the Myb gene *An2* and GFP. **A** and **C** are viewed under white light, **B** and **D** under blue light to reveal GFP fluorescence. Arrows indicate cells which are both anthocyanin-producing and positive for GFP expression.

anthocyanin pigmentation in the cell indicated in Figure 5.1A quenches some of the GFP fluorescence so that only a halo of GFP can be seen in B. In C there is one pigmented cell which has a faint halo of GFP, and there is one which is expressing GFP very strongly and has modest anthocyanin pigmentation; it is hard to see since the cell is located within the sub-epidermis. Transformation with *CI* did not produce any pigmented cells.

Biolistic-transformation of petunia leaves was difficult as the leaves are soft and bruise easily. A range of shooting parameters were altered, including shooting pressure and distance, but no significant improvement in the transformation efficiency was achieved. Autofluorescence of leaf material also proved problematic for the detection of GFP. Chlorophyll fluoresces red when viewed under blue wavelengths, which can obscure GFP detection. Chlorophyll fluorescence can be problematic, while the green-yellow fluorescence of wounded leaf tissue proved to make detection of GFP fluorescence even more difficult. Gold only (no DNA) negative controls, as well as GFP positive controls, were performed when establishing the shooting conditions. Transformed cells were few in number and tended to be located on the outskirts of the blast impact zone. It would be expected for a successful biolistic transformation to have more than 100 foci (individually transformed cells) expressing GFP per leaf. However, less than 5 cells per leaf were typically found.

5.3.2 *Agrobacterium*-mediated transformation

A second set of complementation experiments was performed using the Myb transcription factors *Roseal* (*Antirrhinum*) and *CI* (maize) under the control of the CaMV35S promoter and an *Agrobacterium*-mediated transformation protocol. Shade-grown Mitchell and *Lc* petunia leaves were used for transformation. Transformation with CaMV35S::GFP was performed as a positive control for transformation, and negative control for inducing pigmentation. Non-induced *Lc* petunia material was completely free of anthocyanin pigmentation prior to transformation.

Mitchell and *Lc* explants transformed with CaMV35S::GFP did not produce any anthocyanin producing cells as shown in Figures 5.2A and C, but GFP was detected along many cut surfaces, as shown in B and D. It was expected that the cut surfaces would be most efficiently transformed, as they provide entry sites for the bacteria to get access to the intercellular spaces and infect cells, and effect transformation. Cut surfaces were made with a sharp sterile scalpel, limiting the area of damaged tissue. The autofluorescence encountered with wounded cells in biolistic transformation was not a problem here, and clear GFP fluorescence was observed (Figure 5.2).

Mitchell plants transformed with CaMV35S::*Rosea1* did not produce any anthocyanins along the cut surfaces (Figure 5.2E) but it had previously been demonstrated that the tissue was competent to be transformed with GFP. When *Lc* petunia explants were transformed with *Rosea1*, anthocyanin pigments were detected along the cut surfaces of the leaves as seen in Figure 5.2F. Transformation of Mitchell and *Lc* petunia with CaMV35S::*Cl* did not result in anthocyanin pigmentation along the cut surfaces of the explants.

5.3.3 Isolation of endogenous Myb transcription factors

An exogenous Myb was able to complement the requirement for high-light to induce pigmentation in *Lc* petunia (Sections 5.3.1, 5.3.2). This prompted an investigation of whether endogenous Myb transcription factors were induced under high-light, resulting in the light-induced pigmentation in *Lc* petunia.

The nucleotide sequence of Myb transcription factors is not well conserved, except in the R2R3 Myb DNA binding domain. Nucleotide alignment of the R2R3 DNA binding domain of reported anthocyanin-regulating Myb transcription factors from selected dicotyledonous species was performed using the CLUSTAL W algorithm. This Myb alignment and the consensus sequence is shown in Figure 5.3. The consensus sequence was used to design degenerate forward and reverse primers, as indicated in Figure 5.3. Alignment of anthocyanin-regulating Mybs enabled more specificity for Mybs which

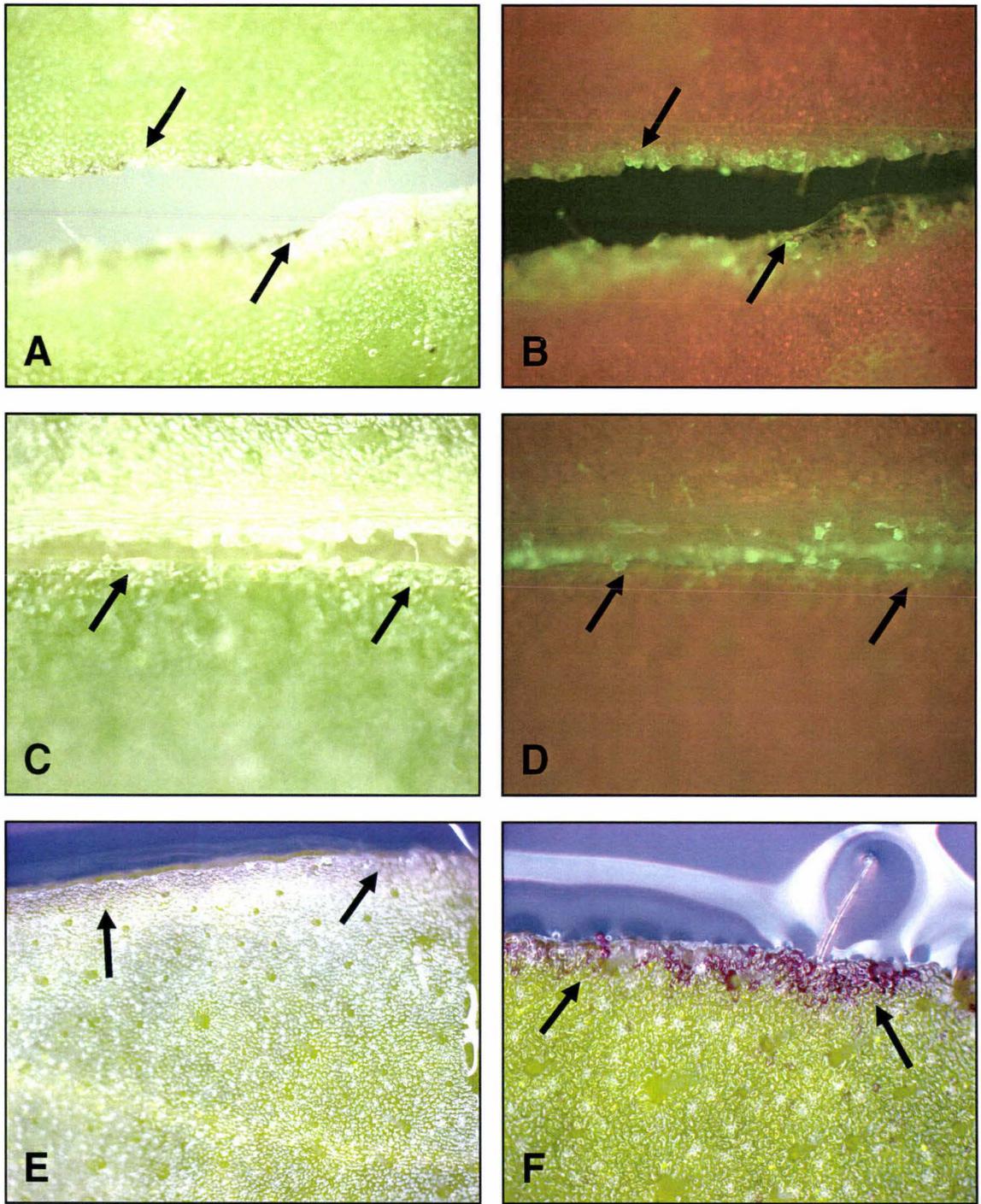


Figure 5.2: Light complementation experiments, utilising *Agrobacterium*-mediated transformation of *Lc* and wild type Mitchell petunia leaves. **A-D** leaf explants transformed with CaMV35S::GFP. **A** and **B** are Mitchell tissue, **C** and **D** are *Lc* tissue. **B** and **D** are viewed under blue light to reveal GFP fluorescence. **E** and **F** are Mitchell and *Lc* leaf explants respectively, transformed with CaMV 35S::Rosea1. Arrows mark the cut surfaces of the explants.

are likely to regulate anthocyanin to be amplified through PCR, particularly as the R2R3 DNA binding domain determines target promoter specificity.

Reverse transcription-PCR (RT-PCR) using the degenerate R2R3 Myb primers with cDNA from shade and high-light grown Mitchell and *Lc* plants was performed, as shown in Figure 5.4. The results from the RT-PCR show that a product of the expected 240 bp size was produced in the positive controls and from the cDNA from Mitchell and *Lc* petunia, from both light treatments.

The *Roseal* sequence and the 'dicot rvs' primer actually has one base mismatch at the 5' end of the primer (Figure 5.3) but, under the PCR conditions, this did not prevent amplification.

The PCR products were purified and cloned into pGEMTeasy, and multiple clones were sent for DNA sequencing. The sequences were run through BLASTn (NCBI, www.ncbi.nih.gov/) (Altschul et al., 1997), which aligned with known anthocyanin Myb regulators (*An2*, *Roseal*), revealing that the cloned PCR products from both shade and high-light grown plants coded for R2R3 Myb transcription factors. The consensus sequences for the shade and high-light clones are shown in Figure 5.5. The shade and high-light sequences are very similar, but they had several differences which were consistent among all the clones sequenced. These differences resulted in several amino acid changes, when the nucleotide sequence was translated, which suggests that these are likely to be two distinct Myb transcription factors.

3'RACE was performed to clone the 3' sequence beyond the R2R3 Myb DNA binding domain. The known sequence gained from the degenerate RT-PCR, enabled gene-specific primers to the Myb clones from shade and high-light to be designed as shown in Figure 5.5.

The first round of 3'RACE was performed with the degenerate forward primer to the R2R3 domain, and the gene specific forward primers ('shade fwd', 'high light fwd') were used for the second nested round of PCR. The results for the 3'RACE are given in Figure 5.6. The first round of 3'RACE resulted in a smear of products, although a faint band can be seen at approximately 800 bp, as indicated by the arrows. The second

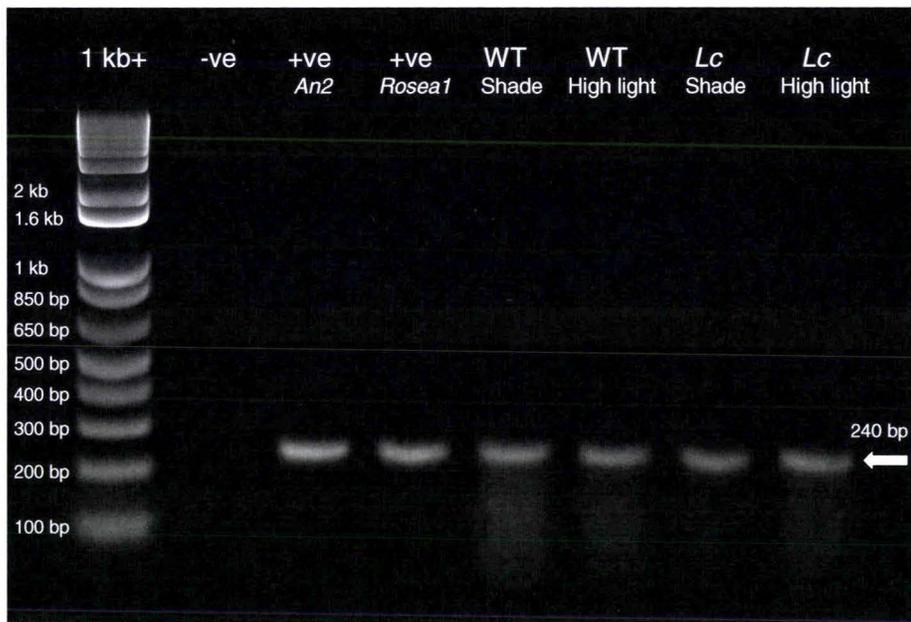


Figure 5.4: Amplification of Myb transcription factors using degenerate primers to the conserved R2R3 DNA binding domain. A sample (5 μ L) of each PCR was run on a 2% (w/v) TAE agarose gel. The size standard used was the 1kb+ ladder. A no-template negative control, and positive controls using the Myb genes *An2* and *Rosea1*, were performed. The sample templates for PCR were cDNA from shade and high-light grown wild type Mitchell and *Lc* plants respectively



Figure 5.5: Consensus sequences for the degenerate PCR clones of the R2R3 DNA domain of endogenous Myb transcription factors in petunia plants, grown under shade or high-light conditions. Nucleotide differences are highlighted in grey.

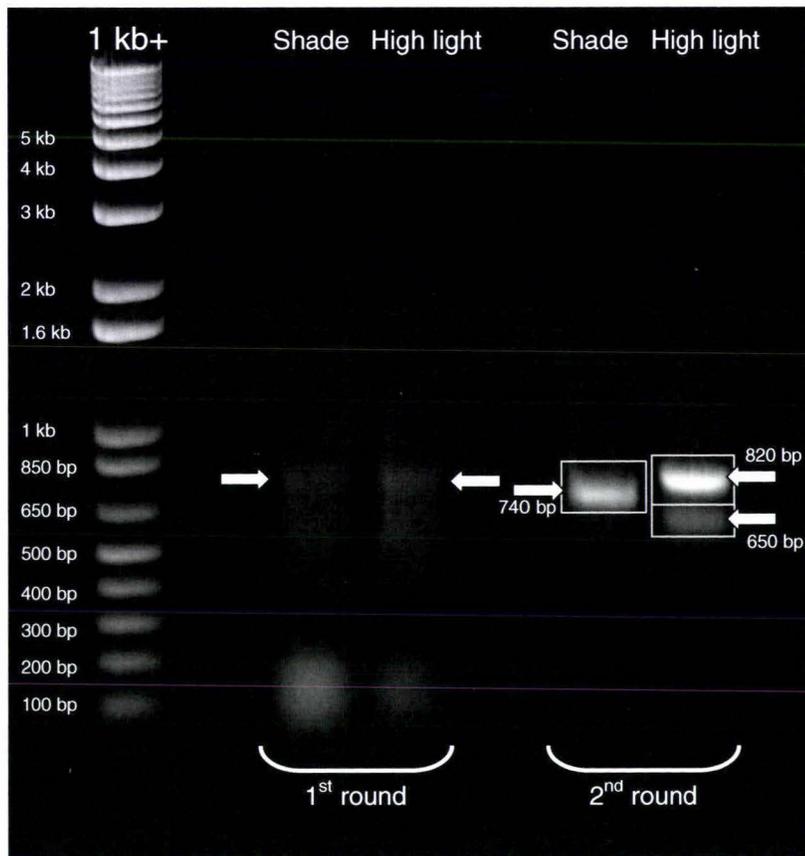


Figure 5.6: 3'RACE of Myb transcription factors from cDNA of shade and high-light grown petunia leaves. 5 μ L of each PCR reaction is loaded per lane on a 1% (w/v) TAE gel. The first round of PCR was performed using the degenerate 'dicot myb' forward primer, and the second, nested round of PCR was performed using an aliquot of the 1st round as the template, using the gene specific forward primers: 'high light' forward primer, 'shade' forward primer. The major bands present in the PCR products are indicated by arrows. The boxes around the major bands of the second round indicate where these bands were excised out of the gel for purification, cloning and DNA sequencing.

nested round of PCR resulted in very clear bands, which are indicated. The shade product was 740 bp, and in the high-light PCR there were two products of 650 bp and 820 bp. These products were purified, cloned into the pGEMTeasy vector system and sent for DNA sequencing.

Analysis of the DNA sequences revealed that the 740 bp product from the shade cDNA was indeed the same gene which had been amplified previously with the degenerate 240 bp PCR, and was named *MybI* for the purposes of this study. The high light 840 bp product, which was most intense on the gel (Figure 5.6) corresponded to the 240 bp high light clone obtained with the degenerate primers designed to the R2R3 Myb domain, and was named *MybII*. The high light 650 bp product contained a mixture of sequences. One clone was completely unrelated to Myb transcription factors, whilst a sequence which shares some similarities to Myb transcription factors was also found. This Myb-like sequence shared some conservation with the R2 helix of the DNA binding domain of Myb transcription factors, but nowhere else. Translation of the sequence found that it contained several stop codons within the open reading frame (data not shown), and it was not investigated further.

RT-PCR was performed using gene specific primers for both *MybI* and *MybII*. The results are given in Figure 5.7, and show that *MybI* was expressed constitutively under both shade and high-light conditions, while *MybII* was only expressed strongly under high-light conditions.

The sequences from the R2R3 DNA binding domain clones (240 bp high light and shade clones) and the 3'RACE clones were formed into contiguous sequences (Appendix 4) and translated, as shown in Figure 5.8.

5.4 Discussion

The results from the complementation experiments (both biolistic Figure 5.1 and *Agrobacterium* Figure 5.2) revealed that introduction of the anthocyanin-regulating

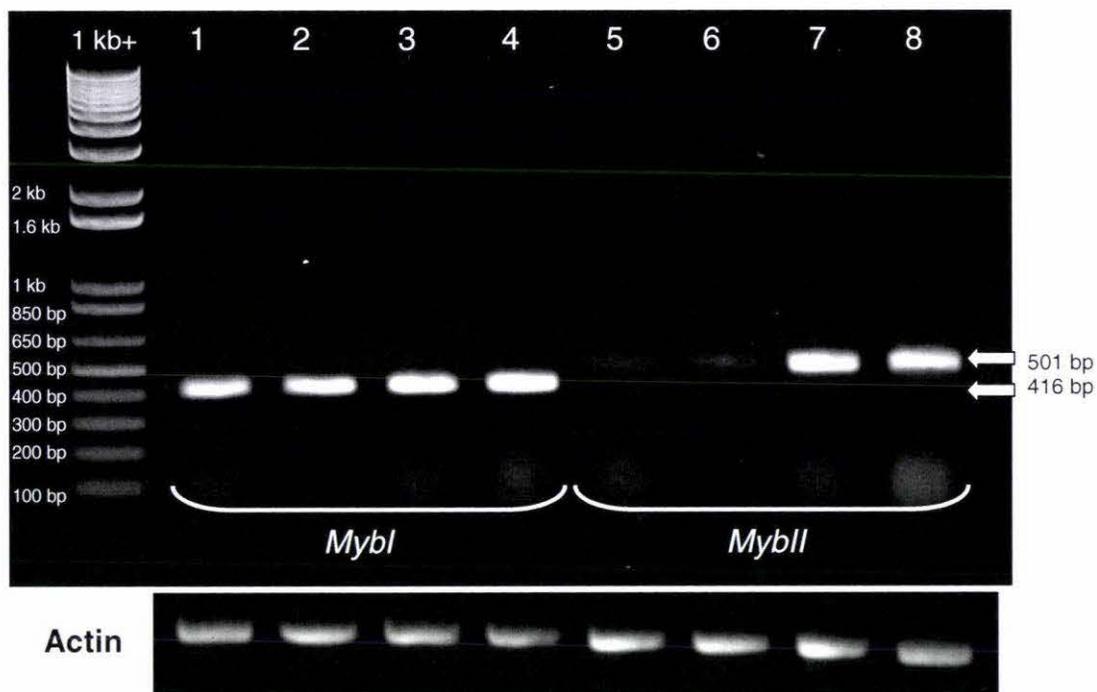
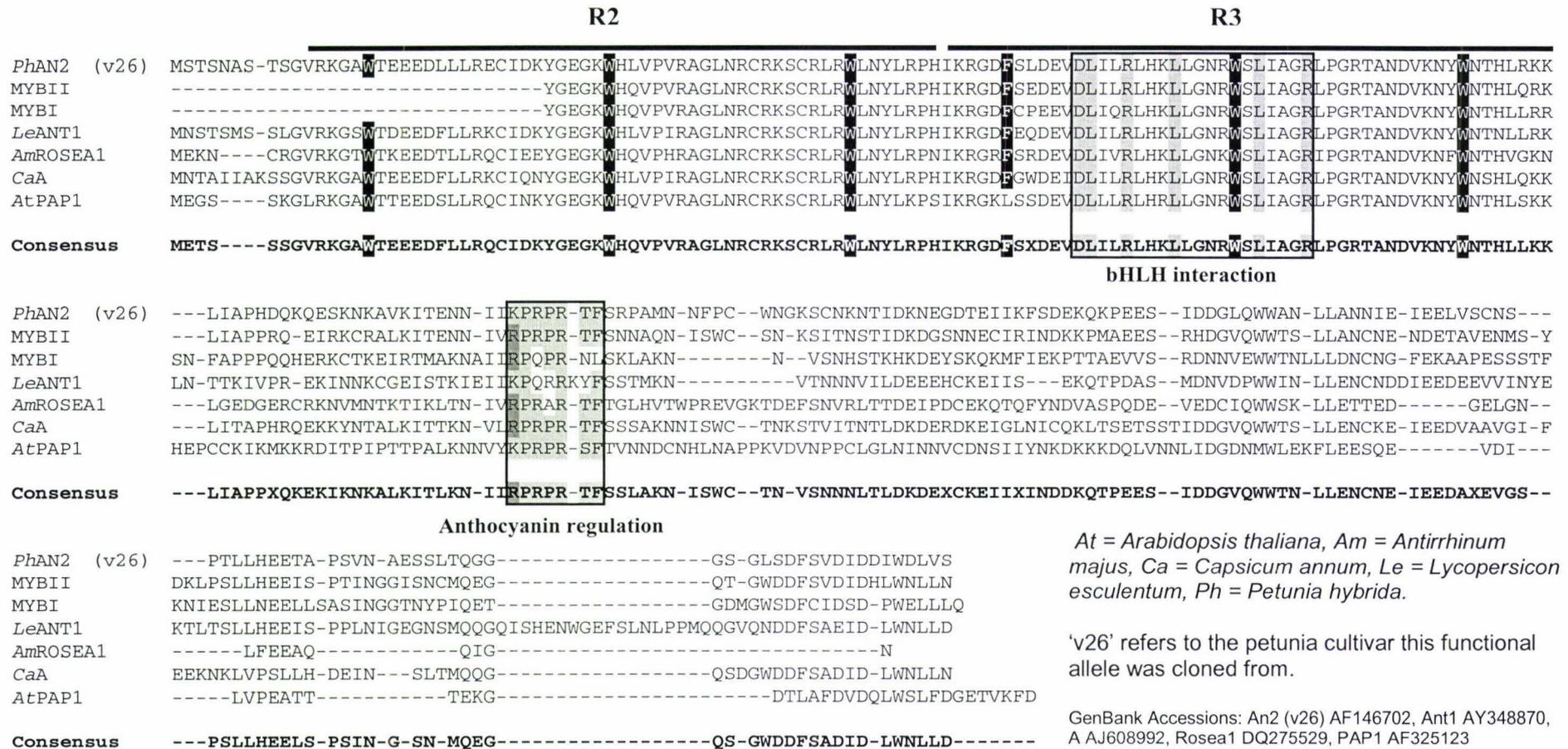


Figure 5.7: Semi-quantitative RT-PCR of the 3' region of *MybI* and *MybII*. Lanes 1-4 are the *MybI* PCR products (416 bp), 5-8 are the *MybII* PCR products (501 bp). Templates for PCR were: lanes 1 and 5 cDNA from shade-grown wild type Mitchell petunia; 2 and 6 cDNA from shade-grown *Lc* petunia; 3 and 7 cDNA from high-light-grown wild type Mitchell petunia; 4 and 8 cDNA from shade-grown *Lc* petunia. A 5 μ L aliquot of each PCR was run on a 1.5% (w/v) TAE agarose gel. PCR amplification of actin is shown as a loading control for cDNA concentration.



At = *Arabidopsis thaliana*, *Am* = *Antirrhinum majus*, *Ca* = *Capsicum annum*, *Le* = *Lycopersicon esculentum*, *Ph* = *Petunia hybrida*.

'v26' refers to the petunia cultivar this functional allele was cloned from.

GenBank Accessions: An2 (v26) AF146702, Ant1 AY348870, A AJ608992, Rosea1 DQ275529, PAP1 AF325123

Figure 5.8: Alignment of the deduced amino acid sequence of 'MybI' and 'MybII' with anthocyanin-regulating Myb transcription factors from dicotyledonous species. The R2 and R3 regions of the Myb DNA binding domain are indicated, with the residues that make up the imperfect repeats highlighted in black. The amino acid motif present in Myb TFs which interact with bHLH transcription factors is indicated, as is the amino acid motif present in Myb TFs which regulate anthocyanin biosynthesis. Residues highlighted in light grey fit the consensus motif. Residues highlighted in dark grey are highly similar residues to the consensus motif.

Myb transcription factors, *An2* or *Rosea1*, could complement the requirement for high-light for anthocyanin production in *Lc* petunia. This suggests that endogenous Myb transcription factors may be induced under high-light conditions, and form an active complex with LC enabling regulation of the biosynthetic genes required for anthocyanin production. Interestingly *C1*, an anthocyanin-regulating Myb gene from maize, was unable to complement the light requirement in *Lc* petunia (this study). A possible reason for this is that *C1* was unable to recognise at least some, if not all, of the promoter targets of biosynthetic genes required for anthocyanin biosynthesis in petunia. This contrasts to work previously performed in petunia which suggested that LC/*C1*, when co-expressed via biolistic transformation (driven by the CaMV35S promoter), could activate a *DFR::GUS* reporter construct in leaves and petals and induce anthocyanin pigmentation in petunia leaves (Quattrocchio et al., 1993; Quattrocchio et al., 1998). However, *C1* alone or LC/*C1* co-expressed (biolistic transformation) were unable to complement the *an2* mutation in petals, which results in acyanic/white flowers (although activation of a *DFR::GUS* construct was observed) (Quattrocchio et al., 1993; Quattrocchio et al., 1998), even though the endogenous bHLH factor AN1 should be present in petal tissue. This suggests that *C1* is not able to function effectively in petunia, as reported in this study.

Biolistic transformation can result in unnaturally high levels of transgene transcripts, due to high plasmid copy number of the CaMV35S driven constructs per plant cell (1000s per cell for transient expression, depending on the number of plasmids that precipitate onto each gold particle). Such high expression of a transcription factor may have allowed binding to, and activation of, low affinity promoter sites resulting in anthocyanin production (Schwechheimer et al., 1998), thereby producing results which may not be physiologically relevant. *Agrobacterium*-mediated transformation results in low transgene copy number (Deroles and Gardner, 1988), which accounts for the results reported here, and may also allow more physiologically relevant interactions to take place. The suggestion that *C1* may not recognise (with high affinity) petunia promoter targets is supported by ongoing work within the 'Plant Pigments Group' at Crop & Food Research, Palmerston North. There appears to be a divergence of the promoter targets recognised by Myb transcription factors from dicotyledonous and monocotyledonous species. Experimentally, transcription factors from dicotyledonous species were ineffective at inducing anthocyanin production in a monocotyledonous host, while

transcription factors from monocotyledonous species were effective in the monocotyledonous host (data unpublished).

Biolistic transformation of petunia leaves was difficult, as the tissue is soft and bruises easily. Once the tissue was bruised, browning, and localised necrosis occurred. Despite poor efficiency of transformation and autofluorescence problems, anthocyanin-producing, GFP-positive cells from *Rosea1* and *An2* transformations were observed. The biolistic results were supported by the *Agrobacterium* transformation results.

The outcome of the Myb complementation experiments (Figures 5.1, 5.2), reinforced the idea that endogenous R2R3 Myb transcription factors were induced by high-light and responsible for the light-induced anthocyanin production in *Lc* petunia. A combination of degenerate RT-PCR (Figure 5.4) and 3'RACE (Figure 5.6) was used to clone partial sequences of two putative Myb transcription factors. The partial cDNA clones were translated and compared to other Myb transcription factors. Both *MybI* and *MybII* contain the amino acid motif (Figure 5.8) required for bHLH transcription factor interaction (Zimmermann et al., 2004), as well as particular amino acid residues which are thought to be important for anthocyanin regulation (Stracke et al., 2001; pers. comm. Dr Kathy Schwinn, Crop & Food Research), as indicated in Figure 5.8. Both *MybI* and *MybII* clones share a high level of sequence identity to *An2* in the R2R3 DNA binding domain. However, MYBII shares significant amino acid similarity throughout the entire coding sequence with AN2, suggesting they may be very similar proteins. This level of similarity has also been seen for anthocyanin-regulating Mybs in *Antirrhinum*, where ROSEA1, ROSEA2 and VENOSA share a very high degree of similarity, encoding functionally similar proteins (Schwinn et al., 2006), and in maize where C1 and PL encode functionally equivalent proteins (Cone et al., 1993a). The sequence information would suggest that the *MybII* is likely to encode a novel anthocyanin regulator, similar to AN2.

Light-induced Myb transcription factors which regulate anthocyanin biosynthesis have been identified in several species. In maize, the R2R3 Myb transcription factor C1, responsible for regulating anthocyanin production in aleurone, is induced by a range of light qualities and environmental signals (Kao et al., 1996; Procissi et al., 1997; Piazza et al., 2002). In *Perilla frutescens*, a light inducible Myb transcription factor (MYB-P1)

has been cloned and characterised which is likely to be the determining factor for anthocyanin pigmentation in the red form of *Perilla* (Gong et al., 1999a). However, MYB-P1 lacks most of the R2 region of the Myb DNA binding domain (Gong et al., 1999a), although it has the required amino acid motif required for interaction with bHLH transcription factors (Zimmermann et al., 2004) and its bHLH counterpart, MYC-RP, has also been cloned (Gong et al., 1999b).

The expression pattern of the two Mybs (*MybI* and *MybII*) under shade and high-light conditions was investigated by RT-PCR, using gene-specific primers to each partial Myb gene. *MybI* was expressed constitutively in both shade and high-light grown *Lc* and Mitchell petunia (Figure 5.7). This suggests that this Myb transcription factor is probably not involved with the light-induced anthocyanin pigmentation observed in *Lc* petunia, despite its similarities to the *MybII*, and presence of a bHLH interaction domain. However, a very clear differential expression pattern was observed for *MybII*, which was expressed strongly in plants grown under high-light, but was barely detectable when amplified from cDNA of shade grown plants (even after 35 cycles of PCR, and with a high cDNA template concentration). This clear differential expression of *MybII*, combined with the *in silico* analysis of the translated sequence (bHLH interaction domain, anthocyanin-regulation residues) makes this a very good candidate for the Myb transcription factor responsible for the light-induced anthocyanin pigmentation observed in *Lc* petunia.

The *in silico* data of *MybII* suggests it is a novel anthocyanin regulator. Such an ascribed function appears to conflict with the northern results from Chapter 4, where *FLS* expression was significantly reduced in *Lc* petunia exposed to high-light (Figure 4.5). The possibility was raised that a flavonol-regulating Myb in petunia may be able to activate anthocyanin biosynthesis with LC, resulting in the observed competition between flavonol and anthocyanin biosynthetic genes (Figure 4.5). This is despite the current knowledge of flavonol regulation, which indicates that the known flavonol regulator AtMYB12 from *Arabidopsis* is unable to interact with a bHLH partner (Zimmermann et al., 2004; Mehrtens et al., 2005). The range of functions controlled by Myb transcription factors (Ramsay and Glover, 2005) highlights the importance of functional characterisation of the clone, before assigning a function to MYBII.

The identification of a differentially expressed *An2*-like Myb transcription factor, *MybII*, in petunia leaves under high-light conditions conforms with the model for the regulation of anthocyanins by a bHLH-Myb-WD40 complex (Figure 1.4A). It appears that the determining factor for anthocyanin production in *Lc* petunia is the expression of the MYBII transcription factor, although the function of this Myb transcription factor in wild type Mitchell petunia has yet to be determined.

Chapter 6

General discussion and future directions

This project continued the characterisation of *Lc* petunia begun by Bradley *et al.* (1998). The particular focus was on the light-induced anthocyanin pigmentation observed in these plants with an aim of extending our current understanding of gene regulation for flavonoid and anthocyanin biosynthesis in petunia. The specific hypothesis considered as part of this thesis was that the bHLH transcription factor LC is not able to activate anthocyanin biosynthesis alone, but requires a light-induced Myb transcription factor to regulate the anthocyanin structural genes as part of a Myb-bHLH-WD40 complex.

The results presented in this thesis give very clear evidence for a cooperative interaction between a bHLH and a Myb transcription factor regulating anthocyanin production. Complementation experiments showed that a Myb transcription factor could substitute for the requirement for high-light in *Lc* petunia leaves, and induce anthocyanin pigmentation. Subsequent to the complementation experiments, partial sequences of two Myb transcription factors were cloned. One clone in particular, *MybII*, was differentially expressed under high-light, and shares structural features with known anthocyanin-regulating Myb transcription factors. MYBII contains the amino acid motif required to interact with bHLH transcription factors (such as LC) as well as a motif which appears to be important for anthocyanin regulation, and is closely related to the Myb AN2 which regulates anthocyanin production in the corolla of petunia flowers. This data suggests that MYBII may be responsible for the light-induced anthocyanin production observed in *Lc* petunia. The results also support the proposed model for anthocyanin regulation (Figure 1.4A), and it is likely that MYBII forms a functional regulatory complex with LC and a WD40 protein (possibly AN11, the identified petunia WD40), which then activate the structural genes for anthocyanin biosynthesis.

Light-induced anthocyanin pigmentation was observed in *Lc* petunia when grown under high-light conditions, yet the plants remained green when grown under shade

conditions. This confirmed previous field and greenhouse observations, substantiating the idea that this was a light-induced response. The involvement of light for pigmentation in *Lc* petunia was noted by Bradley *et al.* (1998) but not explored. In this study, the light-induced anthocyanin pigmentation was characterised in terms of the levels and types of anthocyanins and other flavonoids present in *Lc* and wild type Mitchell petunia, and flavonoid structural gene expression. The types of anthocyanins were unchanged between Mitchell and *Lc* petunia, but the levels were increased eight-fold in *Lc* petunia, giving rise to the purple phenotype. The expression of the flavonoid structural genes increased in a dose-dependent manner relative to the light intensity. In *Lc* petunia, the late biosynthetic genes required for anthocyanin production were only activated under high-light conditions. The observations that *Lc* petunia had enhanced anthocyanin pigmentation in tissues which normally produced very modest levels of anthocyanins, but that the types of anthocyanins remained the same, suggests that LC was interacting with existing endogenous anthocyanin regulatory factors.

As well as a consideration of the regulatory factors involved in the light-induced pigmentation phenotype, this project had some more practical aims. One of these aims was to re-establish a standard hemizygous seed line of *Lc* petunia which would be suitable for investigating the light-induced pigmentation phenomenon. A hemizygous line was identified and characterised phenotypically and genetically. This was used for characterising the light-induced response, and a fresh hemizygous line was generated for future investigations. The expression of the *Lc* transgene, driven by the CaMV35S promoter, was found to be insensitive to light intensity and, therefore, not responsible for the light-induced anthocyanin pigmentation. This result was important for directing this study towards investigating the interactions of the transgenic transcription factor, LC, with existing endogenous petunia transcription factors.

Lc has been introduced into a range of species in attempts to enhance anthocyanin pigmentation. Pigmentation was enhanced in *Arabidopsis*, tobacco, alfalfa, tomato and petunia (Lloyd *et al.*, 1992; Goldsbrough *et al.*, 1996; Bradley *et al.*, 1998; Ray *et al.*, 2003), but not in lisianthus, pelargonium or chrysanthemum (Boase *et al.*, 1998; Bradley *et al.*, 1999). The responses of *Lc* petunia are very similar to those of CaMV35S::*Lc* tomato (Goldsbrough *et al.*, 1996) and alfalfa (Ray *et al.*, 2003), which also displayed light-induced anthocyanin pigmentation, perhaps indicating a common mechanism for

anthocyanin regulation in these plants. The failure of *Lc* to modify pigmentation in lisianthus and pelargonium (not chrysanthemum, as the transgene was silenced) may have been due to the lack of an endogenous Myb transcription factor which LC could functionally interact with. It is possible that these plants would have displayed enhanced pigmentation if placed under conditions where an endogenous Myb, capable of interacting with LC, was induced.

Myb-bHLH-WD40 complexes regulate a range of processes including flavonoid metabolism, epidermal cell fate and vacuolar pH (Ramsay and Glover, 2005). Interestingly, the Myb transcription factor appears to be the key determinant in specifying the target promoters regulated by these complexes. The bHLH transcription factors appear to be flexible, often able to interact with several different Myb transcription factors for different processes. The two bHLH transcription factors GLABRA3 (GL3) and ENHANCER OF GLABRA3 (EGL3) in *Arabidopsis*, regulate epidermal cell fates with the Mybs WEREWOLF (WER) and GLABROUS1 (GL1) (Payne et al., 2000; Bernhardt et al., 2003). However, these bHLH factors can also regulate anthocyanin production with the Myb PAP1 (Payne et al., 2000; Zhang et al., 2003). Similar observations have been made in petunia and maize. AN1 (bHLH) in petunia regulates anthocyanin production in the corolla with AN2 (Myb) and vacuolar acidification with PH4 (Myb) (Quattrocchio et al., 2006). In addition to regulating anthocyanin production, LC has been shown to regulate trichome formation when introduced into *Arabidopsis* and canola (*Brassica napus*) (Lloyd et al., 1992; Lloyd et al., 1994; Gruber et al., 2006). Such plasticity for transcription factor partners allows for combinatorial control of multiple processes with a limited set of transcription factors.

Light or stress regulated anthocyanin pigmentation is well documented, and is an important strategy for plants, as sessile organisms, to survive. Anthocyanins and other flavonoids have important roles in attracting pollinators and seed distributors, pollen fertility, insect resistance and photoprotection. Such a light-induced response poses challenges in situations where stable pigmentation is desired for commercial uses. Flowers with novel colours and interesting pigmentation patterns are in high demand. However, flowers or ornamental plants which have variable colour intensity or an unstable phenotype, depending on the light environment, are not desirable. If *Lc*

petunia were to be sold commercially as a highly pigmented ornamental, the light-induced pigmentation would need to be overcome, particularly if being promoted for indoor use. A similar situation occurs in horticultural crops such as pear, which produce a 'blush' of anthocyanins on fruit in response to cold-stress and high-light. 'Blushed' fruit are highly desirable while green pears have a significantly reduced value (Steyn et al., 2006). However, the horticulturist is unable to easily control the blushing on the pears, as it requires the correct timing of climatic factors just before harvest.

Anthocyanin pigments are important for the nutraceutical and food industries, to be used as antioxidant supplements and natural food colourants. Cell-culture systems are a convenient way to produce anthocyanin pigments at industrial scales. However, many cell cultures require light to produce anthocyanins (Deroles et al., 2006), which is both expensive and technically difficult to provide in commercial scale bio-reactors. The light regulation of anthocyanin pigmentation in *Lc* petunia may serve as a model providing insight into how the requirement for light may be overcome by molecular breeding, transgenic approaches, or treatments which can induce the same desired response.

If the light responsive factors could be identified and manipulated, plants which produce consistent responses at a range of light levels may be able to be developed. A factor to consider here may be altered plant vigour. *Lc* petunia showed no signs of reduced vigour while highly pigmented. This may not, however, always be the case. If pigmented foliage did limit photosynthetic activity, then under low light conditions plant vigour may also be limited. *Arabidopsis* plants overexpressing the Myb gene *PAP1* were purple with anthocyanins, and had increased insect resistance, yet inflorescence and silique production was reduced (Johnson and Dowd, 2004).

Future directions for this project include further characterisation of the light-induced pigmentation in *Lc* petunia and functional characterisation of *MybII*. This study has focussed on the role of light intensity for inducing anthocyanin production in *Lc* petunia but has not addressed the action spectrum for this response. Light quality is an important issue, which needs to be investigated, as it may provide insight into the function and regulation of *MybII* in wild type plants. Functional characterisation of the two Myb transcription factors identified in this study, especially *MybII* which appears to

be responsible for the light-induced pigmentation in *Lc* petunia, will extend the current understanding of the regulation of anthocyanin biosynthesis in petunia. It will also be interesting to investigate the promoter region of *MybII*, and identify *cis*-elements which confer light-responsiveness.

This project developed and expanded upon the initial questions raised regarding light-induced pigmentation in *Lc* petunia, successfully completing the original aims and identifying two new Myb transcription factors in petunia. The identification of *MybII* is particularly exciting, as it appears to be responsible for the light-induced pigmentation in *Lc* petunia, and possibly represents a novel anthocyanin-regulator in petunia.

Transgenic *Lc* petunia plants have proved a useful tool for broadening our understanding of light-regulated flavonoid gene regulation in petunia.

Appendix 1

Buffers, media and solutions

1.1 Bacterial media

Luria-Bertani (LB) broth and media

LB media contains 10 g L⁻¹ bactotryptone, 5 g L⁻¹ yeast extract, and 10 g L⁻¹ sodium chloride was dissolved in 900 mL water, made up to 1 L with water, then autoclaved. For LB-agar, 1.5% (w/v) agar (Difco) was added.

Yeast-mannitol (YM) broth and media

YM media contains 0.04% (w/v) yeast extract, 0.01% (w/v) sodium chloride, 0.02% (w/v) magnesium sulphate (MgSO₄.7H₂O), 0.05% (w/v) di-potassium hydrogen orthophosphate (K₂HPO₄) and 1% (w/v) mannitol made with water. The broth was sterilised by autoclaving, then stored at room temperature. For YM-agar, 1.5% (w/v) agar was added.

1.2 Alkaline lysis miniprep solutions

Solution I – Re-suspension solution

Solution I contains 50 mM glucose, 25 mM Tris-HCl (pH 8.0), 10 mM EDTA made up to 100 mL with sterile water then autoclaved. Stored at 4°C.

Solution II – Lysis solution

Solution II contains 0.2 M NaOH, 1% (w/v) SDS made up to required volume with water, and stored at room temperature.

Solution III – Neutralisation solution

Solution II contains 60 mL of 5 M potassium acetate, 11.5 mL glacial acetic acid and sterile water up to 100 mL and autoclaved. Stored at 4°C.

1.3 Electrophoresis reagents

1.3.1 Electrophoresis Buffers

10 × TBE buffer

10 × TBE buffer contains 0.89 M tris-base, 0.89 M boric acid and 20 mM EDTA and was made up with water.

10 × TAE buffer

10 × TAE buffer contains 0.4 M tris-base and 20 mM EDTA and was made up with water and adjusted to pH 7.8 with glacial acetic acid.

10 × NaB buffer – (Brody and Kern, 2004)

10 × NaB buffer contains 50 mM $\text{Na}_2\text{B}_4\cdot 10\text{H}_2\text{O}$ dissolved in water. The pH of the buffer was adjusted to 8.0 with acetic acid before it was autoclaved.

10 × MOPS buffer

10 × MOPS electrophoresis buffer contains 0.2 M MOPS, 50 mM sodium acetate, 10 mM EDTA, is made up with water and adjusted to pH 7.0 with NaOH. Autoclave, then store at room temperature.

1.3.2 Sample preparation

10 × loading dye

10 × loading dye contains 20% (w/v) Ficoll 400, 0.1 M EDTA (pH 8.0), 1% (w/v) SDS, 0.25% (w/v) bromophenol blue, 0.25% (w/v) xylene cyanol, made up with sterile water.

2 × RNA denaturing solution

2 × RNA denaturing solution contained: 500 µL 10 × MOPS, 500 µL formamide, 150 µL formaldehyde and 5 µL ethidium bromide (10 mg mL⁻¹). It was stored in a foil covered tube, and made fresh.

1.4 Antibiotics

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

Streptomycin solution (100 mg mL⁻¹) was made by dissolving 2 g of streptomycin sulphate into 20 mL of milliQ water. The streptomycin solution was filter sterilised and stored at -20°C.

Spectinomycin solution (100 mg mL⁻¹) was made by dissolving 2 g of spectinomycin dihydrochloride into 20 mL of milliQ water. The spectinomycin solution was filter sterilised (0.2 µm) and stored at 4°C.

Kanamycin solution (100 mg mL⁻¹) was made by dissolving 2 g of kanamycin sulphate into 20 mL of milliQ water. The kanamycin solution was filter sterilised (0.2 µm) and stored at 4°C.

1.5 Tissue culture/Transformation media

1.5.1 Shoot regeneration media

A solid modified MS media, optimised for petunia by scientists at Crop & Food Research, was used to regenerate shoots from petunia. Plant hormones BAP (cytokinin) and IAA (auxin) were added at a rate of 0.2 mg L⁻¹ and 3 mg L⁻¹ respectively. The media also contained cefotaxime at a final concentration of 500 mg L⁻¹. Kanamycin antibiotic was added at a rate of 100 mg L⁻¹ to the media when required.

1.5.2 Media #2

Solid media #2 used during biolistic-mediated transformation, and for co-culturing *A.tumefaciens* is a modified MS media (Murashige and Skoog, 1962).

1.5.3 Virulence induction media

Liquid virulence induction media contained 1 ×AB salts (1 g L⁻¹ NH₄Cl, 0.3 g L⁻¹ MgSO₄, 0.15 g L⁻¹ KCl, 0.01 g L⁻¹ CaCl₂), 240 mg L⁻¹ NaH₂PO₄, 10 g L⁻¹ glucose, 14.693 g L⁻¹ morpholinoethane sulfonic acid. Media was supplemented with 200 μM acetosyringone after sterilisation.

1.6 Nucleic acid extraction, blotting and hybridisation reagents

1.6.1 Genomic DNA extraction buffer – (Zhang, 1998)

The extraction buffer contained 2% (w/v) CTAB salt, 1% (w/v) PVP (soluble), 2% (w/v) SDS, 100 mM Tris-HCl (pH 8.0), 25 mM EDTA, 2 M NaCl made up with sterile water and autoclaved. The buffer was heated to 50°C, and mixed thoroughly before aliquots were taken.

1.6.2 20 × SSC

20 × SSC contains 3 M NaCl and 0.3 M Tri-sodium citrate made up with water. pH was adjusted with NaOH to 7.0, before sterilisation by autoclaving. This stock solution was diluted with sterile water as required.

1.6.3 Modified Church and Gilbert pre-hybridisation buffer – (Church and Gilbert, 1984)

7% (w/v) SDS, 0.5 M phosphate buffer pH 7.2, 10 mM EDTA. The solution was filter sterilised using a 0.45 μm filter.

1.7 Flavonoid extraction solvents

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

Appendix 2

The Wellburn equation

Chlorophyll and carotenoids concentrations were calculated by applying the Wellburn Equation for chloroform as the solvent (Wellburn, 1994):

Chlorophyll *a* $C_a = 10.91A_{666} - 1.2A_{648}$

Chlorophyll *b* $C_b = 16.38A_{648} - 4.57A_{666}$

Total Carotenoids $C_{x+c} = (1000A_{480} - 1.42C_a - 46.09C_b)/202$

Total Chlorophylls $C_a + C_b$

Chlorophyll *a/b* C_a / C_b

= $\mu\text{g mL}^{-1} \times \text{Dilution factor}$

= $\mu\text{g mL}^{-1} \times (\text{volume of extract}) \text{ mL}$

= $\mu\text{g} \times 1000$

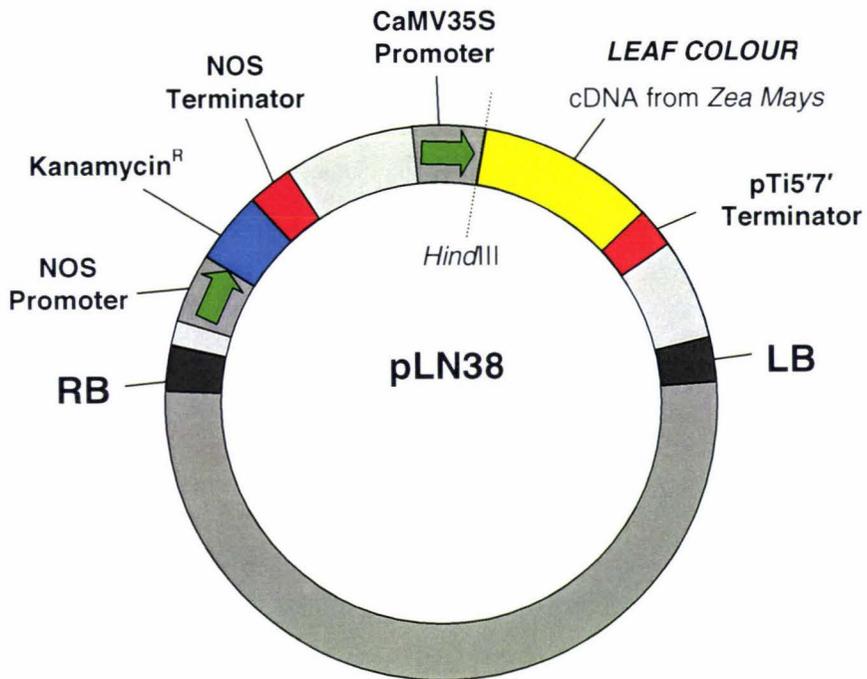
= $\text{mg} \div \text{weight (tissue) g DW}$

= $\text{mg g}^{-1} \text{ DW}$

Appendix 3

Plasmid Maps

3.1 Plasmid map for pLN38

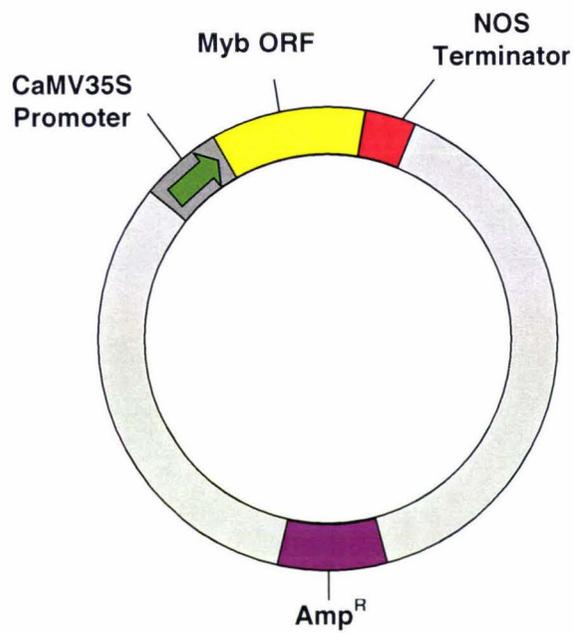


Leaf Colour cDNA from *Zea mays* from pLC349 in the binary vector pGA643.

Selection in plants: Kan 100 mg L⁻¹

Selection in *Agrobacterium*: Kan 50 mg L⁻¹

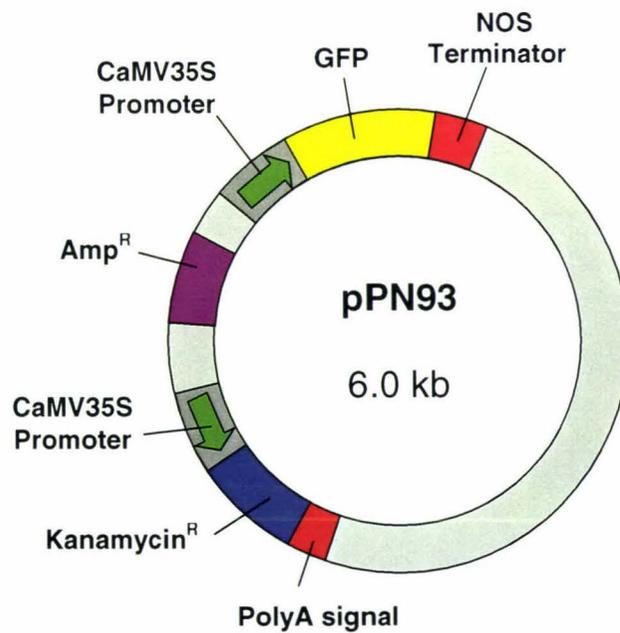
3.2 Plasmid Maps for pPN73, pPN81 and pLN44



pART7 vector with *Roseal* (pPN81), *An2* (pPN73) or *Cl* (pLN44) Myb ORFs.

E. coli selection: Amp 100 mg L⁻¹

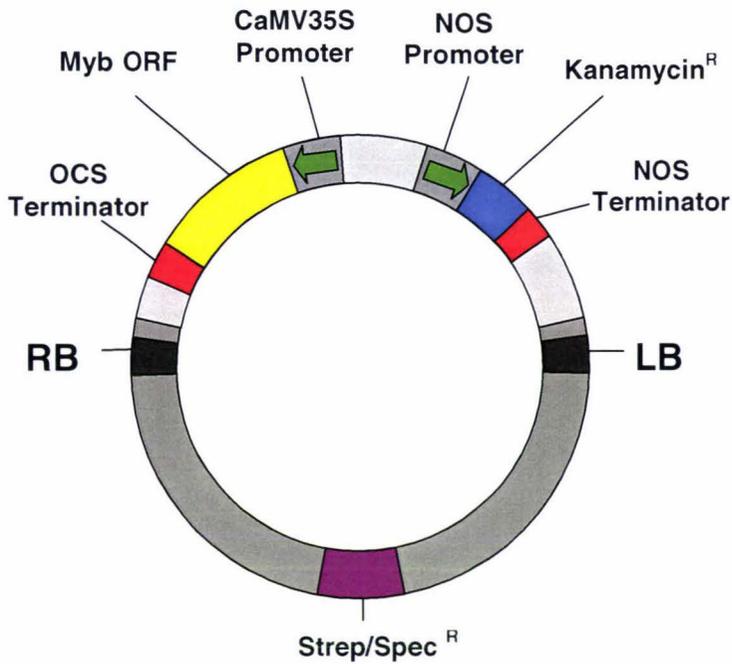
3.3 Plasmid map of pPN93



GFP in a pUC based vector.

Selection in *E. coli*: Amp 100 mg L⁻¹

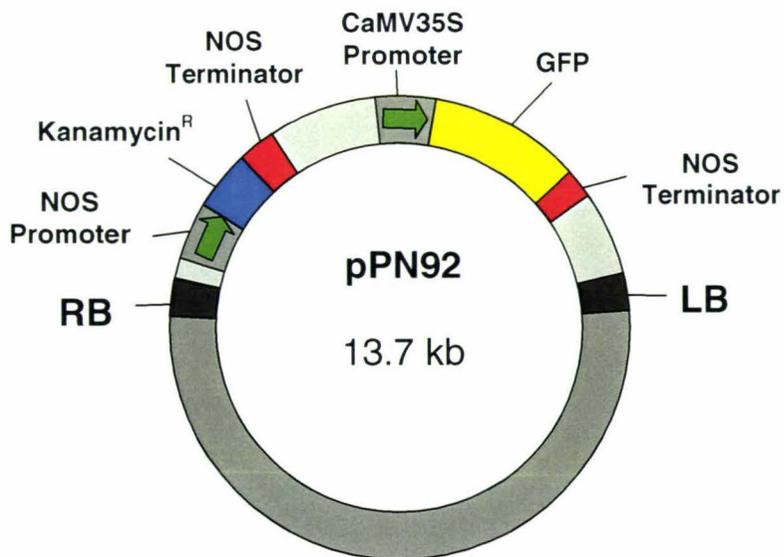
3.4 Plasmid map of pLN52, pLN83



pART27 binary vector with *Roseal* or *Cl* Myb ORFs, pLN83 and pLN52 respectively.

Selection in *Agrobacterium*: Strep 200 mg L⁻¹ Spec 200 mg L⁻¹
 Selection in plants: Kan 100 mg L⁻¹

3.5 Plasmid map of pPN92



GFP in the binary vector pBIN.

Selection in *Agrobacterium*: Strep 25 mg L⁻¹, Kan 50 mg L⁻¹
 Selection in plants: Kan 100 mg L⁻¹

Appendix 4

Partial nucleotide sequences of *MybI* and *MybII*

The sequence from 240 bp PCR products (Figure 5.4) and the 3'RACE products (Figure 5.6) were formed into contiguous sequences, which are given below.

MybI

```
TTTATGGAGAAGGVAAGTGGCATCAAGTTCCTGTTAGAGCTGGTTTAAATAGATGCAGGAAGAG
CTGCAGGCTAAGGTGGTTGAATTATCTGAGACCACATATAAAGAGAGGTGACTTTTGTCCGGAG
GAAGTGGACCTTATTCAGAGGCTTCATAAGCTTCTCGGCAACAGGTGGTCACTTATTGCCGGGA
GACTTCCGGGAAGAACGGCAAACGAYGTSAAAACTACTGGAAYACTCACCTTCTAAGGAGGTC
AACTTTGCTCCTCCTCCCCAGCAACACGAAAGGAAATGTACTAAAGAAATTAGGACCATGGCC
AAGAATGCCATAATAAGACCTCAACCTCGGAATCTCTCAAAATTAGCAAAGAATAACGTCTCAA
ACCACAGTACTAAACACAAGGATGAATATAGCAAACAGAAAATGTTTCATCGAGAAGCCAACAAC
GGCCGAAGTCGTGTGAGAGATAACAACGTTGAATGGTGGACGAAATTTACTGGATAACTGC
AACGGATTTGAAAAGGCAGCACCTGAAAGCTCTTCAACATTTAAGAACATAGAAAAGTTTGTAA
ACGAAGAACTATTATCAGCATCGATAAATGGTGGAACTACTATCCCATTCGAAGAACTGGAGA
CATGGGTTGGAGTGACTTTTGTATTGATTCTGACCCCTGGGAATTGCTACTCCAATGAATTACT
CAGATATATGATTGGTACTGTTATCTTGTACGTGTGATGTAATCTCAATAAATTGTCTGTGCTT
TATGTCCCAAGAACAACCTATTTAGGCCCATTTTGGATTCCCATGTAAAAAAAAAAAAAAAAAAAA
```

MybII

```
TTTATGGTGAAGGCAAGTGGCATCAAGTTCCTGTAAGAGCTGGTCTGAATAGATGCAGAAAGAG
CTGCAGACTTAGGTGGTTGAATTATCTAAGGCCACACATAAAGAGAGGGGACTTCTCTGAGGAT
GAAGTAGATCTCATTTTGAGGCTTCATAAGCTTCTAGGCAACAGATGGTCACTTATCGCGGGTA
GACTTCCGGGAAGAACAGCAAACGAYGTSAAAAAYTACTGGAACACACACCTGCAGAGGAAGTT
AATTGCTCCTCCGCGACAAGAGATAAGAAAGTGCAGAGCCCTTAAGATCACTGAGAACAACATA
GTAAGACCTCGACCTCGGACCTTCTCAAATAATGCACAGAATATTTCTTGGTGCAGCAACAAAA
GTATCACAAACAGCACTATAGATAAAGATGGAAGTAACAATGAATGTATAAGGATTAATGATAA
GAAGCCAATGGCCGAGGAGTCAAGACACGATGGAGTTCAATGGTGGACTAGTTTACTAGCTAAC
TGCAACGAAAATGATGAAACAGCAGTTGAGAACATGTCATATGATAAGTTACCGAGTTTGTTC
ACGAGGAAATATCACCAACGATAAATGGTGGAAATTAGCAACTGCATGCAAGAAGGACAACTGG
TTGGGATGACTTTTCTGTTGATATTGATCACCTATGGAATCTACTTAACTAGGTTTCATAACCA
TTAACTATATATTGAGTTATAATGATGGTATGGTAAACTTGTAATAGGGATACAACATTTTAAA
TAGTTTGCATGACTTTTGTATCCCAAGTACAAGTCGAGCCTATTCTATCCCTATTTGTAAAAAG
GGTTTATTTGTTAGTACGAATTTTGGCTCTATATCTCTTGGATTTTGAATGAATAAGAGAATCCA
TTCATTAATAAAAAAAAAAAAAAAAAAAAA
```

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