Appendix D

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Degree: PhD Dept/Institute/School: IMBS

Thesis title: Two novel MYB transcriptional activators regulate floral and vegetative anthocyanin pigmentation patterns in Petunia

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As author of the above named thesis, I request that my thesis be embargoed from public access until (date) 12.3.2012 for the following reasons:

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Please explain here why you think this request is justified:

The gene sequences identified are commercially sensitive with respect to controlling pigmentation in ornamental species. Release of this thesis would allow competing labs to publish similar findings or report on the function of the novel genes I identified and characterised before I have been able to publish.

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Two novel MYB transcriptional activators regulate floral and vegetative anthocyanin pigmentation patterns in *Petunia*

A thesis presented in partial fulfilment of the requirements for the degree of

Doctor of Philosophy in Plant Molecular Biology

At Massey University, Palmerston North, New Zealand

Nick William Albert 2009

Abstract

This thesis investigates the roles of two putative anthocyanin regulators for determining pigmentation patterns in *Petunia*. Partial cDNAs for two novel MYB transcription factors had been previously identified and full cDNAs for these two new MYB genes, *DEEP PURPLE* (*DPL*) and *PURPLE HAZE* (*PHZ*), were obtained and shown to encode MYB transcription factors homologous to *Anthocyanin2* (*An2*) and *Anthocyanin4* (*An4*).

The activity of *PHZ* is linked to light-induced vegetative and floral pigmentation phenotypes. *PHZ* transcripts were highly induced with high light treatment in leaves, and promoter activity co-localised to light regulated anthocyanin pigmentation patterns in leaves, stems and developing flower buds. *DPL* is expressed constitutively throughout vegetative and floral tissues and the *DPL* promoter activity is tightly associated with the vasculature in stems and leaves. The identification of a petunia line that lacks flower tube venation, and contains a transposon insertion allele of *DPL* (*DPL::LTR*) provided further genetic evidence that *DPL* determines flower tube venation pigmentation patterning.

DPL and *PHZ* were confirmed as anthocyanin regulators using forwards genetic approaches. Biolistic transformation of *Antirrhinum rosea*^{dorsea} petals with *35S*$_{pro}$*:DPL* or *35S*$_{pro}$*:PHZ* restored anthocyanin production, and stable *DPL* or *PHZ* over-expressing petunias were highly pigmented with anthocyanin throughout vegetative and floral organs. Interestingly, hierarchical interactions were shown to occur in the MYB over-expressing petunias, resulting in ectopic expression of the bHLH partner *Anthocyanin1* (*An1*) and a recently identified competitive inhibitor of anthocyanin synthesis, *MYBx*.

The related MYB anthocyanin regulators *An2*, *DPL* and *PHZ* were shown to differentially regulate flavonoid biosynthetic genes, and appear to have bHLH partner specificity for regulating target genes. In particular, DPL was shown to activate the promoters of *Hydroxylation at five1* (*Hf1*) and *CHALCONE SYNTHASE* (*CHS-A*) more

than AN2 and PHZ. The WD repeat protein ANTHOCYANIN11 (AN11) and bHLH AN1 were found to be essential for anthocyanin regulation. It was surprising that the bHLH factor *JOHNANDFRANCESCA13* (*JAF13*) could not substitute for *An1*, given that JAF13 was able to activate the promoters of anthocyanin biosynthetic genes with a MYB partner. This suggests one or more genes, essential to anthocyanin biosynthesis or transport, escapes regulation by *JAF13*, which *An1* normally regulates.

The roles for two repressors of anthocyanin synthesis were also examined, in addition to the MYB activators that have been the main focus of this study. The small R3 MYB, *MYBx*, appears to provide feedback repression upon synthesis and accumulation of anthocyanin in vegetative and floral tissues. The putative active repressor of anthocyanin synthesis, *MYB27*, may actively prevent leaves from accumulating anthocyanin until times of stress. The expression profiles of *PHZ*, which activates anthocyanin synthesis, and *MYB27* were opposite, suggesting the expression of the anthocyanin biosynthetic genes in leaves is tightly regulated by a balance of MYB activators and repressors.

Thus, *DPL* and *PHZ* contribute to pigmentation patterning in *Petunia*, operating as part of a complex network of MYB transcriptional activators, MYB repressors and co-regulators.

Acknowledgments

I thank my supervisors, Dr David Lewis, Dr Huaibi Zhang, Dr Kathy Schwinn, Professor Paula Jameson and Professor Michael McManus. You have been my support team, providing encouragement when things were difficult, challenging me to excel and celebrating my successes. I've learned from you all, and recognise how generous you've all been as teachers, colleagues and peers.

I thank Dr Kevin Davies, Professor Cathie Martin and Dr Don Hunter for mentoring, sound advice, encouragement and support.

I am grateful for the support and advice from Dr Simon Deroles and Murray Boase while generating stable transgenic petunias and to Andrew Mullan, Margaret Young and Sriya Pathirana for tissue culture media. I thank Steve Arathoon and Jan Manson for running the MB lab to *perfection*, and Ian King and Julie Ryan – your green fingers have been invaluable! I thank Dr Carlos Lehnebach for his assistance with phylogenies and tree building, and Dr Andrew Clarke for genotyping advice. I thank Dr Erin O'Donoghue, Dr Jocelyn Eason, Dr Marian McKenzie and Dr Dave Brummell for help and advice at various stages of my PhD. I thank Duncan Hedderley for statistics advice, and Tony Corbett for his beautiful photography and help with poster preparations. A huge 'thank you' to the support staff at Plant & Food Research – Carol, Maryanne, Anne, Trevor, Colin, Darren, Ian B and Tom – you were always so willing to help with disobedient fax machines, invoices, internet glitches, finding missing journals and fixing endnote.

To my lab-mates, past and present, thank you for making each day so much fun: Phillip, John, Nady, Lei, Ronan, Yongjin, Sheryl, Tatyana, Hahn, Margaret, Lyn, Jun, Rubina, Juan, Jing, Alice, the MTM lab group in IMBS. And to Jieun Jung (JJ), your smile and laughter is remembered by us all.

To Sarah, Kathryn, Andy and Bevan – thank you for all the laughs, sanity breaks and Friday night takeaways. You are truly wonderful friends.

I thank my parents, Denis and Jill, my brothers, aunt, uncles, cousin and Grandma Nola for your love and support.

I thank the Tertiary Education Commission for funding me through my PhD with the Top Achiever Doctoral Scholarship and Massey University for initially awarding me a Massey Doctoral Scholarship. I thank the Royal Society of New Zealand, the New Zealand Society of Plant Biologists and the Institute of Molecular BioSciences (Massey University) for financial support for travel and conference attendance.

I thank Professor Ronald Koes for gift of the petunia lines W134, W225, V30 and W59 used in this study, from the Amsterdam petunia collection.

Finally, I wish to thank Crop & Food Research/Plant & Food Research for hosting me. To all the people behind the scenes that helped make this happen, thank you.

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Abbreviations

6-FAM	6 - Carboxyfluorescein
35S	*35S* promoter, from the cauliflower mosaic virus
A_{xxx}	absorbance, at the wavelength indicated by the numerical value $_{(xxx)}$
Amp	ampicillin
BAC	bacterial artificial chromosome
BAP	6-benzyl amino purine
bHLH	basic helix-loop-helix; class of transcription factor
bp	base pairs
bZIP	basic region leucine zipper; class of transcription factor
CaMV	cauliflower mosaic virus
cDNA	complementary deoxyribonucleic acid
cm	centimetre
cpm	counts per minute
CTAB	cetyltrimethyl ammonium bromide

°C	degrees Celsius
d	days
DHK	dihydrokaempferol
DHM	dihydromyricetin
DHQ	dihydroquercetin
DNA	deoxyribonucleic acid
DMF	di-methyl-formamide
DMSO	di-methyl-sulfoxide
dNTP	deoxy-nucleotide-triphosphate
DTT	dithiothreitol
DW	dry-weight
EAR	ERF-associated amphiphilic repression motif
EBG	early biosynthetic gene
EDTA	ethylenediaminetetraacetate
EST	expressed sequence tag
FW	fresh-weight
g	gram
g	gravity, or g-force
GFP	green fluorescent protein, originally from *Aequorea victoria*
GMO	genetically modified organism
GUS	β-glucuronidase
iGUS	intron-GUS; β-glucuronidase containing an intron
h	hours
HPLC	high performance liquid chromatography
IAA	indol acetic acid
Indel	insertion/deletion
IPTG	isopropyl-β-D-thiogalactopyranoside
Kan	kanamycin
kb	kilobases
kPa	kiloPascal
LARDs	large retrotransposon derivatives
LB	Luria-Bertani (agar or broth)
LBG	late biosynthetic gene
LUC	Luciferase; firefly luciferase

LRU	light regulatory unit *syn.* light regulatory element (LRE)
M	molar; moles per litre
MBW	MYB-bHLH-WDR transcription factor complex
WDR	WD repeat protein *Syn.* WD40
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
MP	Mitchell petunia
ms	millisecond
MS	Murashige and Skoog: tissue culture media
MYB	class of transcription factor
N	normal
NAxxx	primer number; see appendix 2
NaOAc	sodium acetate
ng	nanograms
nm	nanometre
OCS	terminator from the *Agrobacterium* octopine synthase gene
OE	over-expression
ORF	open reading frame
PA	proanthocyanidin *syn.* condensed tannin
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
pXXX	plasmid; see appendix 3
PVP	polyvinyl pyrrolidone
qRT-PCR	quantitative, reverse transcription PCR. *Syn.* qPCR, QPCR
RACE	rapid amplification of cDNA ends

REN	Renilla; Renilla luciferase
RNA	ribonucleic acid
RNase	ribonuclease
rpm	revolutions per minute
RT	reverse-transcription
RRE	'R' response element
rRNA	ribosomal ribonucleic acid
SDS	sodium dodecyl sulphate
s	second
SNP	single nucleotide polymorphism
SSC	sodium chloride-sodium citrate buffer
Spec	spectinomycin
Strep	streptomycin
TAE	tris acetate EDTA buffer
TBE	tris borate EDTA buffer
T-DNA	transfer DNA; DNA transferred from *Agrobacterium tumefaciens* into the host genome
TE	tris-EDTA buffer
TF	transcription factor
TLC	thin layer chromatography
Tm	melting temperature
Tris	tris(hydroxymethyl) aminomethane
U	enzyme units
UC	untransformed control
UV	ultra violet
V	volt
V26	petunia cultivar Violet26
V30	petunia cultivar Violet30
W59	petunia cultivar White59
W115	petunia cultivar White115; *syn.* Mitchell petunia
W134	petunia cultivar White134
W225	petunia cultivar White225
v/v	volume/volume
w/v	weight/volume

WT	wild type
WD40	WD-repeat protein; *syn.* WDR
WDR	WD-repeat protein
X-gal	5-bromo-4-chloro-3-indolyl galactopyranoside
X-gluc	5-bromo-4-chloro-3-indolyl glucuronide
YM	yeast-mannitol

Abbreviations of genes, loci and proteins

Loci/genes

An1	*Anthocyanin1*; encoding a bHLH transcription factor (*Petunia*)
An2	*Anthocyanin2*; encoding a MYB transcription factor (*Petunia*)
An3	*Anthocyanin3*; encoding flavanone 3-hydroxylase (*Petunia*)
An4	*Anthocyanin4*; encoding a MYB transcription factor (*Petunia*)
An6	*Anthocyanin6*; encoding dihydroflavonol 4-reductase, DFRa (*Petunia*)
An9	*Anthocyanin9*: encoding glutathione-*S*-transferase (*Petunia*)
An10	*Anthocyanin10*; putatively encoding cinnamate 4-hydroxylase (*Petunia*)
An11	*Anthocyanin11*; encoding a WD40 protein (*Petunia*)
DPL	*DEEP PURPLE*; a MYB transcription factor (*Petunia*)
Fa	*Fading*; locus conferring fading of anthocyanins in petals: dominant trait. The gene(s) associated with this locus have yet to be cloned. (*Petunia*)
Fl	*Flavonol*; encoding flavonol synthase (*Petunia*)
JAF13	*JOHNANDFRANSCESCA13*; a bHLH transcription factor (*Petunia*)
Hf1	*Hydroxylation at five1*; encoding flavonoid 3′5′-hydroxylase (*Petunia*)
Hf2	*Hydroxylation at five2*; encoding flavonoid 3′5′-hydroxylase (*Petunia*)
Ht1	*Hydroxylation at three1*; encoding flavonoid 3′-hydroxylase (*Petunia*)
Ht2	*Hydroxylation at three2*; encoding flavonoid 3′-hydroxylase (*Petunia*)
MYB27	MYB27; encoding an R2R3 MYB transcription factor of unknown function (*Petunia*)
PHZ	*PURPLE HAZE*; a MYB transcription factor (*Petunia*)
Po	*Pollen*; encoding chalcone isomerase, CHIb (*Petunia*)

Rt	*Rhamosylation at three*; encoding 3-rhamnosyl transferase (*Petunia*)
Ve1	*Venation1*; putatively encoding a MYB transcription factor (*Petunia*)
Ve2	*Venation2*; locus involved in flower limb venation. Dominant alleles prevent venation on the limb. The gene(s) associated with this locus have yet to be cloned. (*Petunia*)
Ve3	*Venation3*; locus involved in flower limb venation. The gene(s) associated with this locus have yet to be cloned. (*Petunia*)
Wha	*White anther*; encodes chalcone synthase, CHSa (*Petunia*)
C1	*Colourless1*; encoding a MYB transcription factor (*Zea mays*)
Lc	*Leaf colour*; encoding a bHLH transcription factor (*Zea mays*)
Del	*Delila*; encoding a bHLH transcription factor (*Antirrhinum majus*)
Mut	*Mutabilis*; encoding a bHLH transcription factor (*Antirrhinum majus*)
Ros1	*Rosea1*; encoding a MYB transcription factor (*Antirrhinum majus*)
Ros2	*Rosea2*; encoding a MYB transcription factor (*Antirrhinum majus*)
Ven	*Venosa*; encoding a MYB transcription factor (*Antirrhinum majus*)

Enzymes

PAL	phenylalanine ammonia lyase
4CL	4-coumarate:CoA ligase
C4H	cinnamate 4-hydroxylase
CHS	chalcone synthase
CHI	chalcone isomerase *syn*. Chalcone flavanone isomerase (CFI)
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
ANR	anthocyanidin reductase
LAR	leucoanthocyanidin reductase

MT	methyl transferase
GT	glucosyl-transferase
3RT	3-rhamnosyl transferase
UF3GT	UDP-glucose flavonoid 3-glucosyl transferase; *syn.* 3GT
UF5GT	UDP-glucose flavonoid 5-glucosyl transferase; *syn.* 5GT
GST	glutathione-*S*-transferase

Promoters, plasmids, gene fusions, insertions and deletions

Abbreviations for promoters, plasmids, gene fusions, insertions and deletions are based on the guidelines for authors set out in 'The Plant Cell' journal.

Promoters	e.g. *35S$_{pro}$*
Promoter fusion	e.g. *35S$_{pro}$:LUCIFERASE*
Plasmid	e.g. pNWA1
Insertion	e.g. *An1*::*dTph1*

Primers e.g. 'NA xxx' are listed in Appendix 2
Plasmids are listed in Appendix 3

Chapter 1

Introduction

1.0 Background

Anthocyanins have been used as a model system for over a century to help understand some of the most fundamental processes in biology. Gregor Mendel used flower and seed colours (anthocyanins and proanthocyanidins, respectively) as visual markers to investigate inheritance and describe the laws of segregation and independent assortment (Mendel, 1986). More than 80 years later, Barbara McClintock discovered transposable elements, present within anthocyanin biosynthetic genes of maize, and recognised them as a powerful tool to generate mutants for studying gene function (McClintock, 1950). The first plant transcription factor discovered, *Colourless1* (*C1*), controls anthocyanin production in maize (Paz-Ares et al., 1986; Paz-Ares et al., 1987). The investigations outlined in this thesis use many of these landmark discoveries to further our understanding of the regulation of genes involved in anthocyanin pigment biosynthesis.

The roles of two new putative anthocyanin regulators, and their contribution to regulating floral and vegetative pigmentation patterns in petunia, were examined in this thesis.

1.1 Petunia as a model system

Petunia hybrida is a species derived from inter-specific crosses between the naturally occurring species *Petunia axillaris* and *Petunia integrifolia*, although there are no known wild hybrids (Ando et al., 2001). Petunias are facultative long day, (typically) 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 is one of the classic floral model systems used to investigate anthocyanin biochemistry and regulation, due to the existence of a wide range of colours and collections of mutants. Petunia rivals other systems (such as *Arabidopsis*) because it has complex pigmentation patterns, is relatively easy to transform, and there is an increasing set of resources available, including 3D-transposon databases and EST libraries (Gerats and Vandenbussche, 2005; Vandenbussche et al., 2008). As a member of the Solanaceae, it also provides a suitable model for important crops such as potato, capsicum and tomato.

Previous investigations into anthocyanin regulation in petunia have been limited to floral pigmentation. The corolla is fused in petunia, forming a tube and limb (Figure 1.1A). The tube and limb often display different pigmentation patterns, including vein-associated anthocyanin pigmentation (venation). In addition to petal colour, the anthers and pollen are often coloured purple or blue with anthocyanins. In this study, *P. hybrida* and Mitchell petunia (*P. axillaris* × [*P.axillaris* × *P. hybrida* cv. 'Rose of heaven']) were used extensively, as these have different morphologies and attributes (Figure 1.1B). Some of these differences include the lack of colour in the petal limb and a long narrow flower tube in Mitchell petunia, which is derived from the moth-pollinated *P. axillaris* progenitor. *P. hybrida* cultivars, such as V26, have many features of *P. integrifolia* including a coloured corolla and large flowers with a wider, more open tube, reflecting the pollination syndrome of the wild *P. integrifolia* progenitor, which is predominantly bee pollinated (Stuurman et al., 2004; Hoballah et al., 2007).

Mitchell petunia is used primarily due to its transformation efficiency, high fertility and vigorous growth. It is also a double-haploid, which can be particularly useful for distinguishing unique genes from alleles of the same gene.

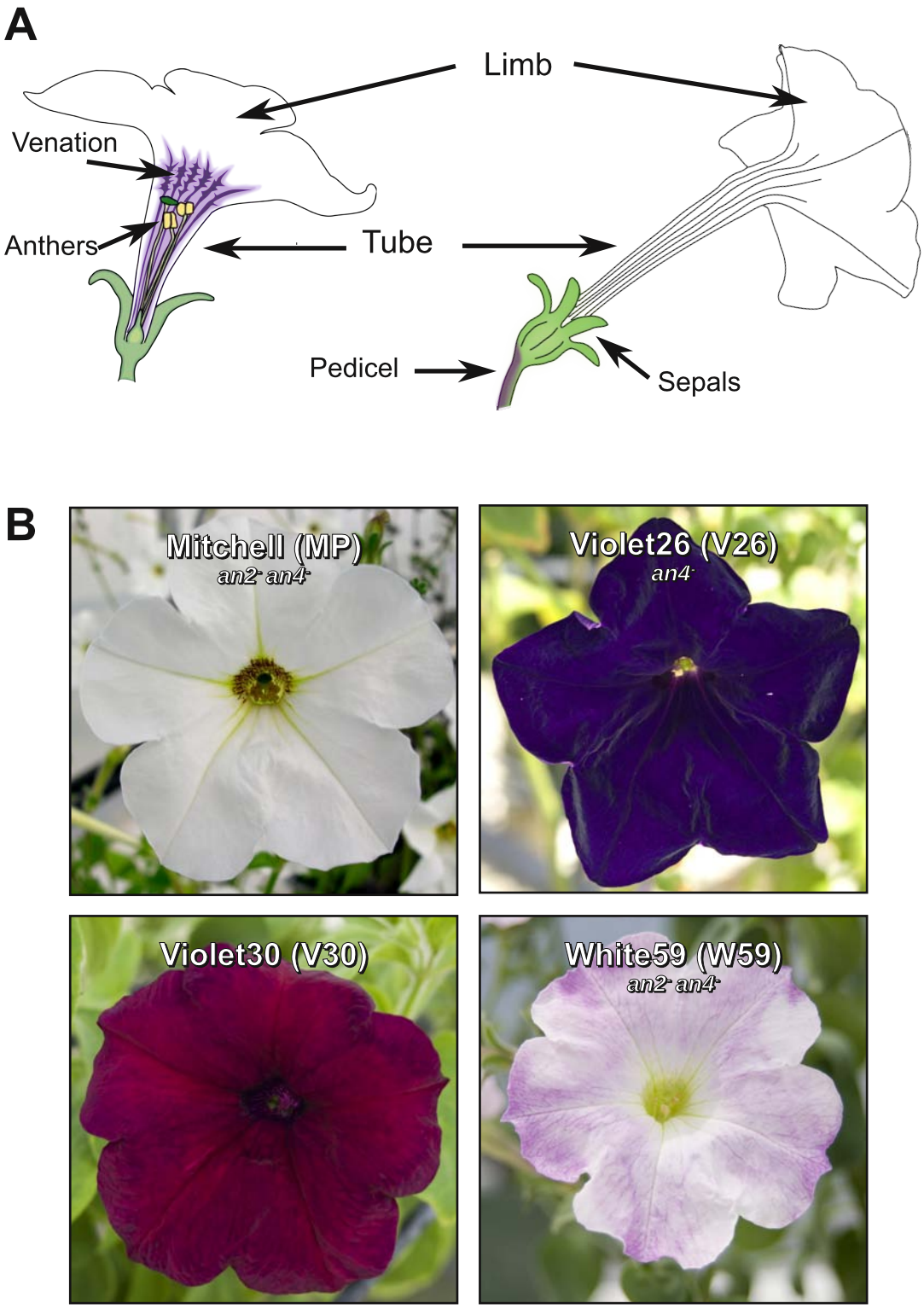


Figure 1.1 A. Flower form of petunia. The petals are fused, and can be separated into tube and limb. **B.** Flowers of the petunia cultivars used in this investigation are shown, with the genotype, in terms of anthocyanin regulatory loci, indicated.

1.2 Plant pigments

Plant pigments comprise a range of secondary metabolic compounds that absorb visible light. These pigments impart perceived colour to the cells, tissues, or organs which accumulate them. The most common plant pigments are the chlorophylls (green), carotenoids (yellow-red) and anthocyanins (red-blue). Additionally, betalains (yellow-purple), chalcones (yellow) and aurones (yellow) accumulate in some species (Yoshida et al., 2004; Davies et al., 2006; Tanaka et al., 2008).

Chlorophylls and carotenoids are required for light capture and energy dissipation during photosynthesis, and are located within the thylakoid membranes of plastids. Carotenoids can also be produced in non-photosynthetic tissues, such as flowers and fruit, where they act as visual cues to pollinators and seed distributors, respectively (Grotewold, 2006).

Anthocyanins are the major coloured compounds produced from the highly branched flavonoid biosynthetic pathway. They range in colour from orange-red through to purple and blue, depending on the side chain substitutions (hydroxylation, glycosylation, acylation and methylation), vacuolar pH, interactions with metal ions and co-pigmentation with other flavonoids (Mol et al., 1998; Ellestad, 2006; Grotewold, 2006). Anthocyanins are synthesised in the cytoplasm, and are subsequently transported to the central vacuole, by a mechanism which is still poorly understood (Grotewold and Davies, 2008). Within the vacuole, they can exist as both soluble and insoluble forms (anthocyanic vacuolar inclusions, AVIs) (Markham et al., 2000; Zhang et al., 2006).

Anthocyanins are produced in flowers, fruits and vegetative tissues. In flowers and fruits, they act as visual cues to pollinators and seed distributors, respectively (Gould and Lister, 2006; Hoballah et al., 2007). In vegetative tissues, they appear to provide protection from various stresses. Sub-epidermal anthocyanins in leaves can screen light from photosynthetic tissues by absorbing blue-green wavelengths (500 nm - 600 nm), which can reduce photoinhibition (Feild et al., 2001; Neill and Gould, 2003; Hughes et al., 2005; Merzlyak et al., 2008; Albert et al., 2009). *De novo* synthesis of anthocyanins in the leaves of deciduous species during autumn may

serve a photoprotective role, allowing successful retrieval of nutrients from senescing leaves (Feild et al., 2001; Hoch et al., 2003). Anthocyanins are also potent antioxidants, which raises the possibility that these pigments may also act to scavenge reactive oxygen species generated through respiration and photosynthesis (Neill and Gould, 2003; Hatier and Gould, 2009).

Plants produce colourless flavonoids in addition to coloured anthocyanins. For example, flavones and flavonols are UV absorbing compounds that often accumulate to high levels in leaves and flowers, and contribute to UV protection (Li et al., 1993; Middleton and Teramura, 1993; Mol et al., 1998; Casati and Walbot, 2005). They can also act as co-pigments, intensifying or modifying the perceived colour of anthocyanins (Aida et al., 2000; Shiono et al., 2005). The UV absorptive properties of these compounds are also recognised by many insects, and many plants accumulate flavonols in a spatial manner in their petals to act as nectar guides to attract pollinators (Thompson et al., 1972; Gronquist et al., 2001).

Proanthocyanidins (PAs) are flavonoid compounds derived from the polymerisation of anthocyanidin precursors (Dixon et al., 2005). PAs are produced in the testa of seeds in many species, including the model species *Arabidopsis thaliana*. While these compounds do not accumulate to provide colour, their biochemistry and regulation is partially shared with anthocyanins. Petunia seeds are presumed to contain PAs, as hydrolysis of the seed coat releases anthocyanidins (Koes et al., 1990; Verweij et al., 2008).

1.3 The flavonoid biosynthetic pathway

The flavonoid biosynthetic pathway (Figure 1.2) is the best characterised secondary metabolic pathways in plants, and has been extensively reviewed (Grotewold, 2006; Tornielli et al., 2008). The first committed step to flavonoid metabolism, from the greater phenylpropanoid pathway, is catalysed by chalcone synthase (CHS). Subsequently, the pathway branches off to produce a range of flavonoid compounds, including aurones, chalcones, flavones, flavonols, isoflavonoids, proanthocyanidins

Figure 1.2 The phenylpropanoid and flavonoid biosynthetic pathways. The first committed step to flavonoid metabolism is catalysed by CHS. Bold lines indicate biosynthetic steps required to make flavonols or anthocyanins. Dotted lines indicate side-branches to produce various phenylpropanoids or flavonoids. 'Anthocyanins' are glycosylated anthocyanidins. Flavonols are glycosylated, and may also be acylated (not shown). The 'early' flavonoid biosynthetic genes (EBGs) are *CHS*, *CHI* and *F3H*. The 'late' biosynthetic genes (LBGs) are *F3'H*, *F3'5'H*, *DFR*, *ANS*, *3RT*, *5GT*, *MT* and *GST* (*ANR* and *LAR* are LBGs for proanthocyanidin synthesis). *FLS* is specific to flavonol production, but appears to be co-regulated with the EBGs, as well *3GT* and *AT*.

PAL phenylalanine ammonia lyase, C4H cinnamate 4-hydroxylase, 4CL 4-coumarate:CoA ligase, CHS chalcone synthase, CHI chalcone isomerase (*syn.* chalcone flavanone isomerase), F3H flavonoid 3-hydroxylase, F3′H flavonoid 3′-hydroxylase, F3′5′H flavonoid 3′5′-hydroxylase, DFR dihydroflavonol 4-reductase, LAR leucoanthocyanidin reductase, ANS anthocyanidin synthase (*syn.* LDOX leucoanthocyanidin dioxygenase), ANR anthocyanidin reductase (*syn.* BANYULS) 3GT 3-glucosyl transferase, 3RT 3-rhamnosyl transferase, 5GT 5-glucosyl transferase, MT methyl transferase, AT acyl transferase, GST glutathione-S-transferase, FLS flavonol synthase, DHK dihydrokaempferol, DHQ dihydroquercetin, DHM dihydromyricetin.

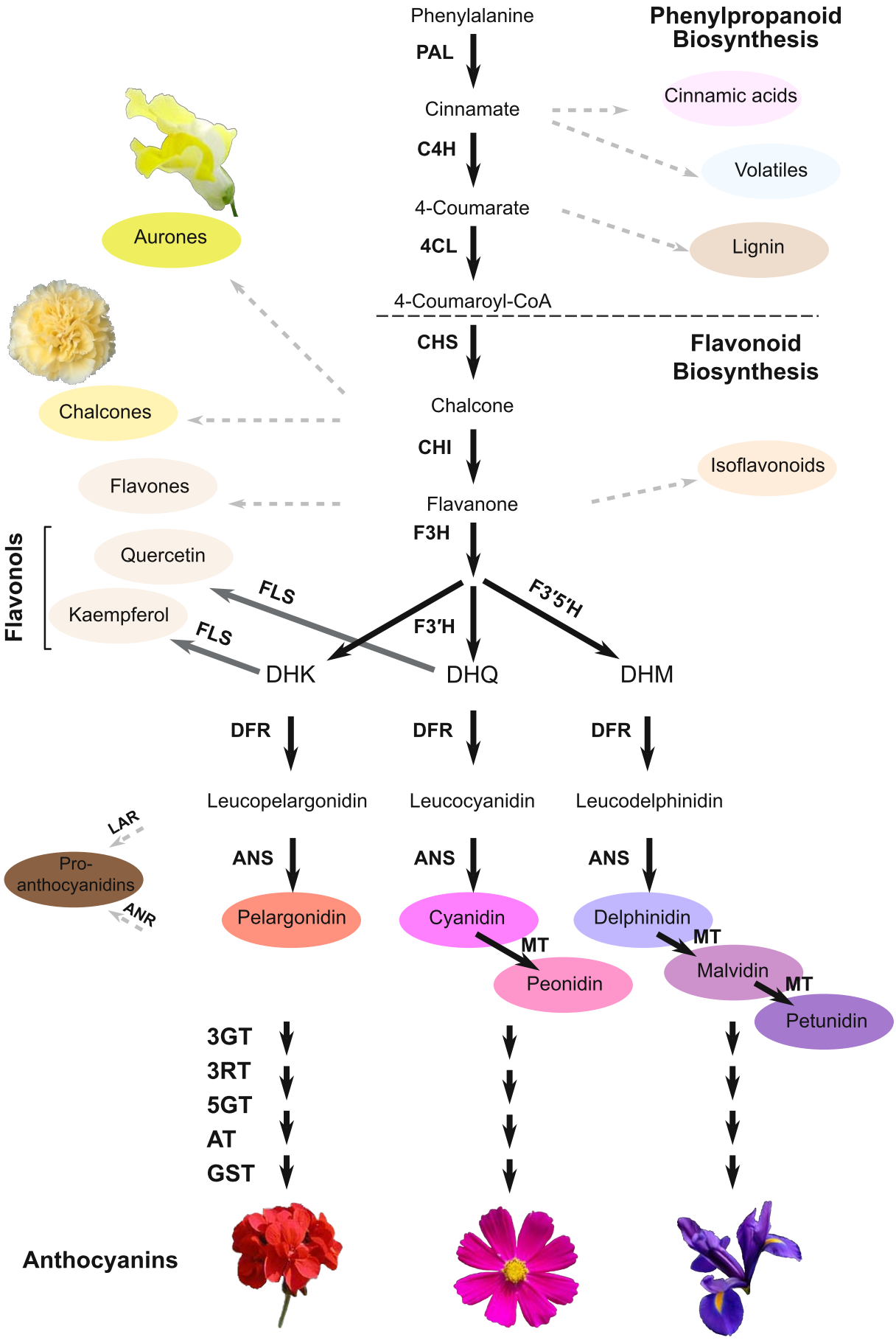


Figure 1.2 The phenylpropanoid and flavonoid biosynthetic pathways. Full legend is on the preceding page.

(condensed tannins) and anthocyanins. The first enzymatic steps in the pathway catalysed by CHS, CHI and F3H are shared by multiple branches. The genes coding for these enzymes are often referred to as 'early biosynthetic genes' (EBGs), while those which are specific to anthocyanin (or proanthocyanidin) production, and show reduced expression in mutants of anthocyanin regulatory loci, are called 'late biosynthetic genes' (LBGs) (e.g. *DFR*, *ANS*). In petunia, flavonols and anthocyanins are the two predominant flavonoids produced in flowers, leaves and other vegetative tissues, although anthocyanidin-derivatives (proanthocyanins) are produced in the seedcoat.

Flavonoid enzymes are often encoded by multi-gene families. Isoforms of enzymes are common, and genes may be under distinct regulatory mechanisms. For example, *CHS-A* accounts for 90% of *CHS* transcripts in petunia flowers, but its expression is only slightly reduced in anthocyanin regulation mutants, while *CHS-J* appears to be under the direct control of the anthocyanin regulators (Koes et al., 1989b; Koes et al., 1989a; Koes et al., 1990). Gene family members have also been shown to have distinct expression domains in petunia flowers. *Hydroxylation at five1* (*Hf1*) and *Hf2* encode F3′5′H (Holton et al., 1993b), and *Hydroxylation at three1* (*Ht1*) and *Ht2* encode F3′H (Brugliera et al., 1999): *Hf1* and *Ht1* are both expressed in both the flower tube and limb, while *Hf2* is only active in the limb, and *Ht2* is only active in the tube (Wiering, 1974; Martin and Gerats, 1993). These distinct expression domains suggest these gene family members are regulated differentially.

Due to the branched nature of the flavonoid biosynthetic pathway, competition for substrates can occur. In many plants, flavonols are often produced in addition to anthocyanins, and these share dihydroflavonol substrates. The key branch-point enzyme for anthocyanin synthesis, DFR, has substrate specificity in many solanaceous species. Petunia DFR catalyses dihydromyricetin (DHM) preferentially, can utilise dihydroquercetin (DHQ), but not dihydrokaempferol (DHK) (Johnson et al., 2001). Flavonol synthase (FLS) competes for dihydroflavonols, and is particularly active with DHK and DHQ as substrates (Forkmann et al., 1986). These preferences result in competition for DHQ by both DFR and FLS, with the subsequent separation of metabolite pools for flavonols and anthocyanins. The competition for DHQ was demonstrated in Mitchell petunia (white), where either

enhancing *DFR* expression or reducing *FLS* gave pink (DHQ-based) anthocyanin pigmentation (Davies et al., 2003). *FLS* antisense also enhanced the accumulation of anthocyanins in coloured varieties of *Nicotiana* and petunia (Holton et al., 1993a). This competition highlights the importance of F3′5′H activity (required to generate DHM) for anthocyanin production in solanaceous species.

1.4 Regulation of the flavonoid biosynthetic pathway

Anthocyanins and other flavonoid compounds are produced in response to a wide range of developmental signals and environmental cues (Grotewold, 2006). Developmental signals may include layers of higher order regulation established by the floral organ identity genes (e.g. MADS box transcription factors)(Bey et al., 2004). Environmental cues are well known to control the accumulation of flavonoid compounds (Gould, 2004). Autumnal senescence, and associated anthocyanin accumulation in many deciduous plants is a classic example. Conditions of intense light combined with cold temperature, typical of autumn mornings, produce the most vibrant displays of autumn colour. These conditions are particularly effective at generating reactive oxygen species, and this stress is likely to be a major contributing factor inducing anthocyanin production, which may be exacerbated by the developmental changes e.g. chloroplast disassembly (which increase sensitivity to excess light) during senescence. Flavonols are also induced with increasing light intensity, reflecting their role as UV photoprotectants (Albert et al., 2009; Matus et al., 2009).

The model plant species traditionally used to study anthocyanin regulation have been *Zea mays* (maize), *Petunia* sp (petunia), *Ipomoea* (morning glory) 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 anthocyanin metabolism is achieved primarily through transcriptional regulation of the

biosynthetic genes mediated by bHLH and MYB transcription factors, and a WD repeat protein (WDR) (summarised in Table 1.1).

Table 1.1: Summary of the transcription factors involved in anthocyanin regulation from a range of plant species.

Species	**R2R3 MYB** (activator)	**R2R3 MYB** (repressor)	**R3 MYB** (repressor)	**bHLH1**	**bHLH2**	**WDR**
Petunia	AN2 AN4 MYBB	PhMYB27	MYBx	JAF13	AN1	AN11
Ipomoea sp. (Morning glory)	C-1 InMYB2 InMYB3			InDEL InbHLH3	IVS	CA
Antirrhinum	ROSEA1 ROSEA2 VENOSA			DELILA	MUTABILIS	AmWDR
Zea mays	C1 PL**			R B SN LC	IN (repressor)	PAC1
Arabidopsis	PAP1 PAP2 AtMYB113 AtMYB114	AtMYBL2*	TRY CPC ETC1, 2,3 TCL	GL3 EGL3	TT8	TTG1
Perilla Fructescens	MYB-P1			MYC-GP/RP	MYC-F3G1	PFWD
Fragaria* × *ananassa		FaMYB1				

* MYBL2 is described as an R3 MYB (Dubos et al., 2008; Matsui et al., 2008), but is larger than the other R3 repressors and shows significant similarity to the R2R3 repressors. **PL should not be confused with P which is a distinct R2R3 MYB transcription factor that regulates 3-deoxyflavonoid synthesis.

There is considerable interest into 'what regulates the regulators' of flavonoid biosynthesis, as 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).

However, the focus on flavonoid regulation for the purposes of this study was on the transcription factors that act directly on the biosynthetic genes.

1.4.1 The bHLH transcription factors

bHLH transcription factors are characterised by a basic helix-loop-helix (bHLH) domain and share structural features shared with MYC transcription factors from animals. The *R* gene family in maize comprises a family of bHLH factors, that are expressed both spatially and temporally to regulate anthocyanin pigmentation patterns throughout the plant. The *R* gene family includes *Red* (*R*) (Chandler et al., 1989), *Leaf colour* (*Lc*) (Ludwig et al., 1989), *Scutellar node* (*Sn*) (Tonelli et al., 1991) and *Booster* (*B*) (Chandler et al., 1989). bHLH anthocyanin regulators have been identified in a range of genera. These include *Petunia*, *Ipomoea* and *Antirrhinum*, the classic systems for investigating floral pigmentation, as well as *Arabidopsis*.

Petunia has two known bHLH factors, *Anthocyanin1* (*An1*) and *JOHNANDFRANCESCA13* (*JAF13*). *An1* regulates anthocyanin synthesis in the petal tube and limb, anthers and proanthocyanidin accumulation in the seedcoat (Spelt et al., 2000; Spelt et al., 2002). *JAF13* was identified based on its similarity to *R* from maize (Quattrocchio et al., 1998). It is expressed in both pigmented (petals) and non-pigmented organs (leaves), and its expression profile in flowers does not match that of the other anthocyanin regulators. Despite this, it has been shown to activate a *DFR* promoter (the branch-point enzyme for anthocyanins) in transient assays, indicating that it may have a role in regulating anthocyanin production. The lack of a null *jaf13* mutant with a clear phenotype may indicate that JAF13 activity is redundant with AN1. The situation is not reciprocal, however, as *An1* is absolutely required for pigmentation (Spelt et al., 2000). It was recently reported that a *jaf13* mutant had been identified, which had a small but reproducible effect on floral pigmentation, but a full report has yet to be published (Tornielli et al., 2008).

Antirrhinum has two bHLH factors, *Delila* (*Del*) and *Mutabilis* (*Mut*) (Goodrich et al., 1992; Schwinn et al., 2006). *Del* controls anthocyanin production in the flower

tube and lobes (limb). In the absence of *Del*, anthocyanin accumulation is restricted to the lobes due to the activity of *Mut*. A null mutant has recently been identified for *Mut*, which can only be observed in a *del* mutant background (Prof. Cathie Martin, *pers. comm.).* It appears that in *Antirrhinum*, DEL and MUT are at least partially functionally redundant. The bHLH factors which regulate anthocyanins in *Arabidopsis* are also shared with trichome development and seed mucilage production. This overlap in function is proposed to occur within the asterids (which includes *Arabidopsis*), while trichome initiation occurs by a distinct mechanism in the rosids (*Antirrhinum*, petunia, *Ipomoea*) (Serna and Martin, 2006). *GLABRA3* (*GL3*) and *ENHANCER OF GLABRA3* (*EGL3*) are expressed throughout vegetative tissues (Bernhardt et al., 2003). In addition to these bHLH factors, *TRANSPARENT TESTA8* (*TT8*) controls proanthocyanidin production in seeds, and *tt8* mutants also show a small reduction in vegetative pigmentation, suggesting TT8 also contributes to anthocyanin production.

The bHLH factors can be further separated into two clades, bHLH1 and bHLH2. bHLH1 factors are homologous to *R* from maize, and include *Delila* (*Antirrhinum*) and *JAF13* (petunia), while bHLH2 factors are more like *An1* from petunia, and include *Mutabilis* (*Mut*; *Antirrhinum*), and *Intensifier* (*In*; maize) (Burr et al., 1996; Spelt et al., 2000; Koes et al., 2005; Schwinn et al., 2006). These two clades are found in very diverse species, from the grasses to flowering monocots and dicots, suggesting they diverged early during plant evolution. The bHLH2 factors, *An1* and *IVS*, from *Petunia* and *Ipomoea*, respectively, control anthocyanin biosynthesis in petals and proanthocyanidins in seeds. Mutations in these genes cause a severe phenotype. In *Antirrhinum*, *Del* is the predominant bHLH1 factor, and in maize, the bHLH1 gene family has replicated several times generating the *R* gene family. The bHLH2 from maize, IN, acts as repressor, and is proposed to inhibit anthocyanin production by binding to *R*-like bHLH activators, and sequestering them into inactive complexes (Burr et al., 1996), rather than through direct repression. IN contains part of the activation domain present in R/B (Burr et al., 1996), which may suggest IN was once an activator that has since acquired mutations rendering it a repressor, as is the case for the *c1-I* allele (MYB) (Paz-Ares et al., 1990). Despite this, the *R* gene family members (bHLH1) are sufficient to activate anthocyanin

production. It appears functional specialisation of the bHLH factors has occurred independently in a range of species.

1.4.2 MYB transcription factors

The second major class of flavonoid regulators are MYB transcription factors. These proteins 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 with 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). The diverse functions of MYB transcription factors are still being investigated, but 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).

Colourless1 (*C1*) was the first transcription factor identified in plants, and encodes an R2R3 MYB transcription factor that regulates anthocyanin synthesis (Cone et al., 1986; Paz-Ares et al., 1986). A *C1* paralogue, *Purple leaf* (*Pl*), that is also present in maize, 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) and provides a mechanism for differential tissue-specific regulation of anthocyanins.

The *Anthocyanin2* (*An2*) locus in petunia encodes an R2R3 MYB transcription factor homologous to *C1/Pl*, which regulates anthocyanin pigmentation in the corolla of the flower (Quattrocchio et al., 1998; Quattrocchio et al., 1999). The *Anthocyanin4* (*An4*) regulatory locus also encodes an R2R3 MYB protein linked to anthocyanin

synthesis in the flower tube and anthers (Wiering, 1974; Quattrocchio et al., 1998; Quattrocchio et al., 1999; Kroon, 2004; Koes et al., 2005). An additional *MYB* clone, *MYBb*, has also been identified that is homologous to *An4* and is expressed in flowers (Kroon, 2004).

Similarly, *R2R3 MYB* genes that regulate anthocyanin biosynthesis have also been identified in *Antirrhinum*, namely *Rosea1* (*Ros1*), *Rosea2* (*Ros2*), and *Venosa* (*Ven*) (Schwinn et al., 2006). These are required for pigmentation, and their spatial expression provides pigmentation patterning. ROS1 and ROS2 regulate anthocyanin production in both lobes of the flower, while VEN is responsible for a striking pigmentation pattern that is limited to the epidermal cells overlaying the veins (Schwinn et al., 2006; Shang, 2006). *Arabidopsis* has four anthocyanin regulatory *R2R3 MYB* genes, *PRODUCER OF ANTHOCYANIN PIGMENT1* (*PAP1*, *AtMYB75*), *PAP2* (*AtMYB90*) (Borevitz et al., 2000), *AtMYB113* and *AtMYB114*, as well as others such as *TRANSPARENT TESTA2* (*TT2*) which regulate proanthocyanidin biosynthesis (Nesi et al., 2001; Debeaujon et al., 2003).

1.4.3 WDR regulator

The third class of regulatory factor involved in anthocyanin regulation are the WD repeat proteins (WDR), also known as WD40 proteins. These are not transcription factors *per se*, as they have no intrinsic enzymatic activity and do not bind directly to the promoters of flavonoid structural genes (Ramsay and Glover, 2005). The proteins do contain structural features which facilitate multiple protein-protein interactions (i.e. docking sites), and are involved in a wide variety of processes, from signal transduction to regulating developmental processes (Smith et al., 1999; Ramsay and Glover, 2005). The WDR 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; Morita et al., 2006; Albert et al., 2009).

WDR proteins involved in anthocyanin (and PA) regulation have been identified in several of the model species, including *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), *Ca* from *Ipomoea* (Morita et al., 2006) and *Perilla frutescens WD* (*PFWD*) from *Perilla frutescens* (Sompornpailin et al., 2002).

The role of the WDR regulator is not conclusively established. Fractionation experiments suggest AN11 is primarily localised within the cytoplasm (de Vetten et al., 1997). However, transient expression of a PFWD-GFP fusion protein localised to the cytoplasm, but localised to the nucleus when a bHLH factor (MYC-RP) was co-transformed (Sompornpailin et al., 2002). In a recent review, it was reported that a JAF13-GFP (bHLH) fusion protein was localised to the cytoplasm in an *an11*$^{-}$ mutant, but if *An11* was co-transformed, JAF13-GFP was transported to the nucleus (Tornielli et al., 2008). Both WDR and bHLH factors contain putative nuclear localisation signals (Sompornpailin et al., 2002), but perhaps these domains are inaccessible until bHLH-WDR factors bind each other, at which stage correct translocation occurs. These observations suggest that the WDR factor may be involved in TF complex assembly, and is subsequently transported to the nucleus with bHLH partners.

1.4.4 MYB-bHLH-WDR (MBW) ternary 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 are 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 maize tissues (Goff et al., 1990; Lloyd et al., 1992), and subsequent promoter activation assays showed that the co-introduction 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). The maize transcription factors C1 (MYB) and B (bHLH) have been shown to physically interact in yeast, suggesting that the synergistic effects of co-transformation of MYB and bHLH regulators is due to the formation of a regulatory complex that activated the flavonoid biosynthetic genes (Goff et al., 1992). In a series of domain-swapping experiments, Grotewold et al. (2000) were able to confer bHLH responsiveness to PERICARP COLOUR (P), a MYB which normally acts independently of a bHLH partner to regulate 3-deoxyflavonoid synthesis. The amino acid motif required for MYB factors to bind their bHLH partners was subsequently identified (Grotewold et al., 2000; Zimmermann et al., 2004), suggesting these two proteins function as a complex.

The *An1* (bHLH), *An2* (MYB) and *An11* (WDR) loci in petunia, are required for anthocyanin pigmentation in the corolla (Quattrocchio et al., 1993), and in *Arabidopsis* the *TT8* (bHLH), *TT2* (MYB) and *TTG1* (WDR) 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. At the protein level, the formation of a MYB-bHLH-WDR (MBW) complex has been demonstrated in *Arabidopsis* to be required to activate the biosynthetic genes for proanthocyanidin synthesis in the seed coat. A combination of yeast two- and three-hybrid experiments showed that TT8, TT2 and TTG1 interacted together, in a complex. TTG1 (WDR) 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 (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).

The MBW transcription factor complex may regulate anthocyanin structural genes in the manner shown in the model in Figure 1.3. Similar models have been published in Baudry et al. (2004), Broun (2005) and Ramsay and Glover (2005). The model suggests that MYB, bHLH and WDR proteins form a complex (MBW) and the transcription factors bind to elements within the target promoters and activate

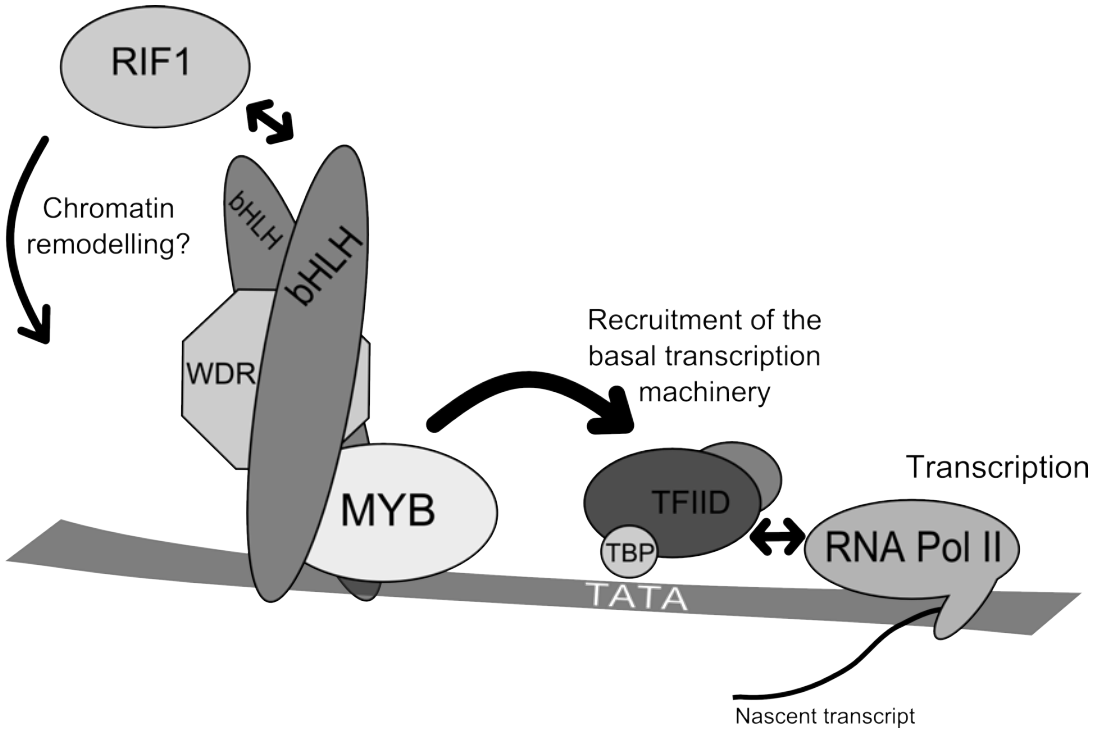


Figure 1.3 Model for the regulation of the anthocyanin biosynthetic genes. A transcriptional activation complex consisting of MYB, bHLH and WDR transcription factors binds *cis*-elements within the promoter region of target anthocyanin biosynthetic genes. The activation domains within the MYB and bHLH transcription factors recruit the basal transcription machinery to initiate transcription. The bHLH transcription factors may also recuit proteins involved in chromatin remodelling (e.g. RIF1), to promote accessibility to the DNA. TBP = TATA-binding protein.

transcription. Hartmann et al. (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 MBW 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 MBW complex to regulate these genes. Supporting this, MYB and bHLH binding elements have been identified in the *Bronze2* gene (a late biosynthetic gene) required for anthocyanin synthesis in maize, and are both found to be required for activation by R (bHLH) and C1 (MYB) (Bodeau and Walbot, 1996).

bHLH factors in the MBW complex may recruit proteins involved in histone modification. R-interacting-factor-1 (RIF1) was recently identified from maize, during a screen for proteins that interact with the bHLH factor R (Hernandez et al., 2007). RIF1 contains domains present in proteins known to have roles in chromatin modification, and was shown to be necessary for the activation of flavonoid biosynthetic genes by R and C1 (MYB) in the native chromatin environment, but not in transient assays. These findings highlight the importance of chromatin modifications for the accessibility of transcription factors to bind the promoters of target genes, activating transcription. Chromatin modification represents an additional level of regulation upon flavonoid biosynthetic genes that is still relatively poorly understood.

MBW complexes also control processes distinct from anthocyanin synthesis. In *Arabidopsis*, epidermal cell fates (trichomes, root hairs) and seed mucilage are also controlled by MBW complexes, and share bHLH and WDR factors in conjunction with process-specific R2R3 MYB factors e.g. WEREWOLF for root hairs (Lee and Schiefelbein, 1999); GLABROUS1 for trichomes (Oppenheimer et al., 1991; Larkin et al., 1994) and *At*MYB5 for seed mucilage (Li et al., 2009). In addition to anthocyanin regulation, AN1 (bHLH) in petunia controls vacuole acidification in petals with AN11 (WDR) and a pH specific R2R3 MYB transcription factor *PH4* (Quattrocchio et al., 2006).

1.4.5 MYB repressors

Repressive MYB transcription factors also regulate anthocyanin production. In addition to the R2R3 MYB activators, a distinct class of R2R3 MYB factors have been shown to repress target pathways. 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). *AmMYB308* and *AmMYB330* in snapdragon are believed to encode repressive MYB transcription factors which repress lignin and hydroxycinnamic acid production, respectively (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 sinnapate esters, which are effective UVB protectants (Jin et al., 2000). Repression by AtMYB4 was active, required DNA binding and is mediated through the C terminal domain containing the 'subgroup 4' motif, which fits the consensus ERF-associated amphiphilic repression (EAR) motif (Jin et al., 2000; Kazan, 2006).

FaMYB1 in strawberry was identified as a repressor of anthocyanin biosynthesis and contains an EAR repression motif in the C terminal (Aharoni et al., 2001). FaMYB1 was shown to be able to interact with the petunia bHLH factors AN1 and JAF13 in yeast-two-hybrid assays, suggesting it may form a complex with bHLH factors to bind target promoters to repress transcription. In the same study, PhMYB27 from petunia was shown to share many structural features to the previously mentioned repressive R2R3 MYBs, including FaMYB1. However, it has yet to be shown directly that PhMYB27 is a repressor of anthocyanin or flavonoid biosynthesis, although it appears to be regulated by the anthocyanin regulator *An1* (Spelt et al., 2000).

A third class of MYB transcription factor has recently been shown to play a role in regulating anthocyanin production. The R3 MYB factors lack the first two MYB repeats, but contain a DNA binding domain and residues to interact with bHLH transcription factors. These small R3 MYBs were first identified as being involved

in trichome and root-hair development, processes that are regulated by MBW complexes and share bHLH and WDR factors with anthocyanin synthesis. These factors act as inhibitors by competing with R2R3 MYB activators for bHLH partners and include *AtCAPRICE* (*CPC*), *AtTRIPTYCHON* (*TRY*), *AtENHANCER OF TRIPTYCHON AND CAPRICE1* (*ETC1*), *ETC2*, *ETC3* (Simon et al., 2007; Tominaga et al., 2007) and *AtTRICHOMELESS1* (*TCL1*) (Wang et al., 2007). *CPC* was recently shown to negatively regulate anthocyanin synthesis in *Arabidopsis* (Zhu et al., 2009). *MYBx* was recently identified in petunia, and encodes an R3 MYB that is homologous to TRY and CPC (Kroon, 2004; Koes et al., 2005). It interacted with AN1 and JAF13 (bHLHs), suggesting it may also act as a competitive inhibitor. Over-expression of *MYBx* inhibited anthocyanin synthesis in petunia flowers, and also affected vacuolar pH (activities regulated by AN1) thus supporting a role as a repressor (Kroon, 2004).

Recently a new R3 MYB, AtMYBL2, that actively represses anthocyanin and proanthocyanidin synthesis was characterised in *Arabidopsis* (Dubos et al., 2008; Matsui et al., 2008) . Unlike the R3 MYBs discussed so far, MYBL2 is larger, containing part of the R2 region, and has an active repression domain (Matsui et al., 2008). The protein can bind the bHLH partners GL3, EGL3 and TT8 (Sawa, 2002; Matsui et al., 2008) and either sequesters bHLH partners (Dubos et al., 2008) (passive repression) or it may be incorporated into MBW complexes that bind the promoters of target genes (active repression), and switch the complex to repress, rather than activate, transcription (Matsui et al., 2008). It appears MYBL2 is more closely related in both sequence and function to the R2R3 MYB (active) repressors than the R3 MYBs.

1.4.6 Regulation of flavonols and flavones

The flavonoid pathway also produces colourless flavonols and flavones in response to environmental cues and developmental signals. These flavonoids provide UV protection, and their production is often induced by light and stress (e.g. pathogen attack, nutrient deficiency, cold) (Martens and Mithofer, 2005; Albert et al., 2009).

In addition to photoprotective roles, there is an increasing awareness that certain flavonoids can affect plant form and development. This occurs through poorly understood mechanisms, but involves the polar transport of auxin (Peer and Murphy, 2007; Santelia et al., 2008).

The early biosynthetic genes are regulated by a distinct class of MYB transcription factor. Light-regulation of the early biosynthetic genes and flavonol synthase (or flavone synthase) is co-ordinately regulated in response to light in many species including *Arabidopsis* and petunia (Hartmann et al., 2005; Albert et al., 2009). AtMYB12 and two related transcription factors (AtMYB11, AtMYB111) were recently identified in *Arabidopsis* as regulators of flavonol production (Mehrtens et al., 2005; Stracke et al., 2007). These MYB transcription factors are distinct from those involved with anthocyanin regulation, as they lack the amino acid motif required for interacting with bHLH transcription factors (Zimmermann et al., 2004). Further, they show similarity to P from maize, which acts independently of a bHLH partner. AtMYB12 was recently shown to activate flavonol production in tomato, suggesting flavonols are regulated by a similar mechanism in diverse species (Luo et al., 2008). In some species, such as *Antirrhinum* and *Gentiana*, flavones accumulate instead of flavonols but perform similar roles (UV protection, co-pigmentation) (Martin et al., 1991; Dixon and Paiva, 1995; Nakatsuka et al., 2005). Recently a P-like MYB was cloned from *Antirrhinum*, which appears to regulate flavone synthesis (Dr Kathy Schwinn, *pers. comm.*).

The EBGs are shared with multiple pathways, and are controlled by overlapping regulatory systems. The flavonoid biosynthetic genes are separated into 'early' and 'late' based on the effects on transcript abundance by mutations in anthocyanin regulators (MYB, bHLH, WDR). It was initially proposed that the MBW regulators did not act upon these early steps in the pathway, because transcripts for the EBGs accumulated in MBW mutants. However, it was recently shown that the EBGs in *Arabidopsis* contain multiple *cis*-elements in their promoters to confer light-mediated/anthocyanin-independent regulation, and MBW-dependent regulation (Hartmann et al., 2005). These elements include a MYB Recognition Element (MRE), a bHLH recognition element named the R-Response Element (RRE), and an ACGT Containing Element (ACE) shown to bind bZIP transcription factors. Light

regulation required the MRE and ACE, suggesting MYB12 and a bZIP may control these genes for flavonol production. The MRE and RRE were both required to confer regulation by C1 (MYB) and SN (bHLH), suggesting that the anthocyanin regulators can act redundantly upon the EBGs. In support of this, the triple mutant of *Arabidopsis*, *myb11*$^{-}$ *myb12*$^{-}$ *myb111*$^{-}$, does not make flavonols, but anthocyanins accumulate (Stracke et al., 2007), indicating that in the absence of the flavonol regulators, the anthocyanin regulators can act upon the early biosynthetic genes. The idea that the anthocyanin regulators act redundantly upon the EBGs is still debated, as much of the early data suggesting the anthocyanin regulators act upon the EBGs came from ectopic expression of anthocyanin regulators (e.g. *35S*$_{pro}$*:PAP1*) (Gonzalez et al., 2008).

1.5 *Petunia* as a model for pigmentation patterning

Transgenic petunias ectopically expressing the bHLH anthocyanin regulator *Leaf colour* (*Lc*), under the control of the *CaMV35S* promoter, display enhanced anthocyanin pigmentation throughout vegetative and floral tissues (Bradley et al., 1998). This pigmentation is dependent upon the growth conditions of the plants, particularly light intensity (Albert, 2006; Albert et al., 2009). When grown under low light conditions (50-350 µmol m^{-2} s^{-1}), *Lc* petunias remain green, producing very little anthocyanin, yet under high light conditions (750 µmol m^{-2} s^{-1}) become deeply pigmented with anthocyanins. While the pigmentation levels were enhanced in *Lc* petunia, the pigmentation patterns were not altered, suggesting other endogenous factors were required to regulate anthocyanin production. The proposed model for the regulation of the anthocyanin biosynthetic genes suggests that both bHLH and MYB transcription factors are required. This led to the hypothesis that an anthocyanin-regulating MYB transcription factor may be induced under high light conditions, which could interact with LC to activate anthocyanin production. Expression of *Rosea1* (MYB) in *Lc* petunia leaves complemented the requirement for high light to induce anthocyanin production, suggesting endogenous MYB factors may determine the light-induced phenotype (Albert et al., 2009).

Reverse-Transcription-Polymerase-Chain-Reaction (RT-PCR) experiments using degenerate primers designed to amplify anthocyanin-regulating *MYB* transcription factors, followed by 3′RACE, resulted in the identification of two novel *MYB* genes in petunia, *MYBI* and *MYBII*.

MYBI was cloned from cDNA of shade-grown petunia leaves, while *MYBII* was cloned from cDNA from high light grown leaves. Subsequent RT-PCR experiments suggested that *MYBI* was expressed constitutively in both shade- and high light-grown leaves, while *MYBII* expression was differentially expressed under high light and *MYBII* may be responsible for the light-induced pigmentation observed in *Lc* petunia (Albert, 2006).

1.6 Two novel R2R3 MYB factors from petunia

The major objective for the project presented in this thesis was to determine the function of the two novel MYB transcription factors, MYBI and MYBII, and their involvement in regulating anthocyanin pigmentation in petunia. *MYBII* was found to be differentially expressed under high light conditions, and is, therefore, the candidate for regulating light-induced anthocyanin synthesis. *MYBI* was not differentially expressed, and yet striking sequence similarity suggested that this gene encoded a transcription factor homologous to *MYBII* and other known anthocyanin-regulating MYB transcription factors. It was surprising that co-expression of both MYBI and LC under shade conditions did not activate anthocyanin synthesis. This raised questions about the specificity of these transcription factors for their partners, the target genes they regulate, and hierarchy between the regulators.

The following hypotheses were proposed:

1. MYBI and MYBII are anthocyanin regulators which contribute to vegetative and floral pigmentation patterns.

2. *MYBII* is induced by light stress in vegetative tissues and MYBII regulates light-induced anthocyanin production.

3. MYBI and MYBII have separate specificities for transcription factor partners and the target genes which they regulate.

A series of experimental objectives were proposed to test these hypotheses, forming the basis of this study. They include:

- Isolation of full length cDNA clones for *MYBI* and *MYBII*. Once obtained, the cDNA sequences would be used for phylogenetic studies, and to perform both transient and stable transformation experiments in petunia and heterologous host species.

- Characterisation of expression patterns for *MYBI*, *MYBII*, and other anthocyanin regulators in leaves and in flowers at different developmental stages.

- Development of petunia plants over-expressing *MYBI* and *MYBII* and characterisation of these transgenic plants phenotypically, genetically and biochemically for effects on anthocyanin and flavonoid regulation and accumulation.

- The identification of stable mutants in *MYBI* or *MYBII*.

- Determination of the spatial expression patterns of *MYBI* and *MYBII* by generating petunia plants containing promoter:iGUS reporter constructs.

- Examination of transcription factor partner specificity by crossing stable MYB over-expression lines into defined mutants for anthocyanin regulators (bHLH, WDR), and assessing the effects on anthocyanin production.

- Determining the transcription factor targets by promoter activation assays.

The overall aim of this research project was, therefore, to confirm the function of MYBI and MYBII as anthocyanin regulators, and determine their roles in the wider context of anthocyanin pigmentation in *Petunia*.

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 100 kPa, 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 are included in Appendix 1, and antibiotic stocks in Appendix 1.4.

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. Antibiotic and hormone stocks were filter sterilised (0.2 μm).

2.4 Bacterial manipulation

2.4.1 General bacterial growth conditions

The *Escherichia coli* strains used for general lab manipulations were NovaBlue (Novagen) or XL1-blue (Stratagene). 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* strains used were LBA4404 (Invitrogen) and GV3101. *A. tumefaciens* LBA4404 was grown on YM-agar plates and YM-broth (Appendix 1.1);

GV3101 was grown on LB-agar plates and LB-broth (Appendix 1.1), with appropriate antibiotic selection at 28°C. Liquid cultures were grown at 28°C in a shaking incubator at 250 rpm. The *Agrobacterium* strains used contain chromosomal resistance to rifampicin and the disarmed T_i plasmids pAL 4404 and pMP90 present in strains LBA4404 and GV3101, respectively, contain resistance to streptomycin/spectinomycin and gentamicin, respectively.

2.4.2 Chemically competent *E. coli* cells

Chemically competent *E. coli* cells were prepared by the method of Inoue et al. (1990). The antibiotic selection for XL1-blue and Novablue cells was tetracycline 10 mg L^{-1}.

2.4.3 Heat-shock transformation of *E. coli*

Heat-shock transformation using chemically competent *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 - 5 µL of a ligation reaction) on ice for 20 min. After 20 min, cells were heat-shocked in a 42°C water bath for 1 min, 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.

2.4.4 Electro-competent GV3101 *A. tumefaciens* cells

Electro-competent *Agrobacterium* GV3101 cells were prepared according to the method of McCormac et al., (1998). Cells were grown at 28°C in LB media with selection: 20 mg L^{-1} rifampicin for *Agrobacterium* and 20 mg L^{-1} gentamicin for the disarmed T_i plasmid MP90 (Appendices 1.1 and 1.4). Cells were grown to mid log-phase (A_{600} = 1.0), harvested by centrifugation at 700 × *g*, washed three times in ice cold 10% (v/v) glycerol, and finally resuspended in 0.01 volumes of 10% (v/v) glycerol. Cells were dispensed into 100 µL aliquots, snap frozen in liquid nitrogen and stored at -80°C.

2.4.5 Electro-competent LBA4404 *A. tumefaciens* cells

Electro-competent LBA4404 *Agrobacterium* cells were sourced from Invitrogen. LBA4404 is inherently less competent to receive plasmid DNA and therefore commercially prepared cells were used.

2.4.6 Electroporation of *A. tumefaciens*

Agrobacterium cells (50 µL) were transformed with approximately 0.5 µg of plasmid DNA by electroporation. Freshly thawed *Agrobacterium* competent cells and plasmid DNA were placed between the electrodes of a pre-chilled electroporation cuvette (Biorad). Cells were electroporated at 300 V (12.5 $KVcm^{-1}$) in a Biorad Gene pulser, then transferred to a tube containing 1 mL of Terrific broth (Appendix 1.1), recovered at 28°C with shaking (250 rpm) for 6 h, before being plated onto agar plates with appropriate selection. GV3101 cells with the pART27 based binary vector were selected on media containing 100 mg L^{-1} spectinomycin, 20 mg L^{-1} rifampicin and 20 mg L^{-1} gentamicin (Appendix 1.4). LBA4404 cells with the pART27 based binary vector were selected on media containing 200 mg L^{-1} streptomycin and 200 mg L^{-1} spectinomycin.

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 of solution I, lysed with 250 µL of solution II, and the solution was neutralised with 350 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, desalted with 70% (v/v) ethanol, and air-dried. 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 Axygen (California, USA) midiprep plasmid kit was used to prepare large volumes of high concentration, high purity plasmid DNA. 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 30 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 in 300 µL sterile water.

2.5.3 *E. coli* plasmid preparation – small volume

The Axygen (California, USA) 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 µL 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 culture was used. The plasmid DNA extracted from *Agrobacterium* was routinely back-transformed into *E. coli* for diagnostic digests, due to poor yield and contaminating T_i-plasmid.

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, 500 ng of plasmid DNA was digested with 2.5 U restriction-endonuclease enzyme(s) (Roche), in a total volume of 15 µL in 1 × 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 (or as otherwise required), 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. Loading dye (10 ×) (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 and TBE (Appendix 1.3.1), and gels were run at 4.5 V cm^{-1} or 6 V cm^{-1}, 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 the 1 kb Plus ladder (Invitrogen).

2.6.3 Standard PCR procedure

Standard PCR was performed routinely for a number of applications, including cloning, probe templates, screening *E. coli* colonies and *in vitro* transgenic plant material. The specific primers are listed in Appendix 2, each being numbered "NA ×××", or as named by the person who designed them. Oligonucleotides were sourced from SigmaGenosys. The precise cycling conditions varied depending on the primer pairs, template and product size, but the general reaction and cycling conditions are given below.

1 µL	template (e.g. 100 ng genomic DNA; 1st strand cDNA)
0.5 µL	10 mM dNTPs (200 µM each)
0.5 µL	10 µM sense primer (0.2 µM)
0.5 µL	10 µM antisense primer (0.2 µM)
2.5 µL	10 × PCR buffer containing 15 mM $MgCl_2$ (1.5 mM final conc.)
18 µL	sterile H_2O
0.2 µL	***Taq*** polymerase (1 U)
25 µL	

Initial denaturation	94 ℃	2 min	
Denaturation	94 ℃	30 s	× 35
Annealing	55 ℃	30 s	
Extension	72 ℃	1 min	
Final extension	72 ℃	5 min	
Hold	12 ℃	Hold	

2.6.4 General cloning of PCR products

PCR products were generated for a number of applications, such as cloning cDNAs for use as radiolabelled probes and sequencing PCR products, and were routinely cloned into the pGEMteasy vector system. This system takes advantage of the terminal transferase activity of *Taq* polymerase, which preferentially adds an adenine nucleotide to the 3′ ends of PCR products, allowing them to be efficiently ligated into a vector with thymine overhangs. PCR products were purified by gel extraction (Section 2.6.7) or column purification (Section 2.6.8) and ligated into the pGEMteasy vector (Promega)

following the manufacturer's instructions. This vector allows blue-white selection, and as such transformed *E. coli* cells were plated onto LB-agar plates containing ampicillin (vector selection), 5-bromo-4-chloro-3-indolyl galactopyranoside (X-gal) and isopropyl-β-D-thiogalactopyranoside (IPTG).

2.6.5 Ligation

Ligation reactions to generate constructs were performed using the Rapid ligation kit (Roche) the following manufacturer's instructions. Typically, the vector was prepared by digesting it with two different restriction endonucleases to allow directional cloning. PCR products generated with primers containing restriction sites for cloning were prepared by digesting the PCR product with appropriate restriction enzymes, heat-inactivating the enzymes (where possible) or column purifying (Section 2.6.8) the reaction prior to ligation. Vector preparations were routinely dephosphorylated with Shrimp Alkaline Phosphatase (Roche), following the manufacturer's instructions, to prevent intra-molecular ligation.

The insert and vector were quantified (Section 2.6.6) and the amount of vector: insert was determined by the following formula:

$$\frac{50 \text{ ng vector} \times x \text{ kb insert}}{y \text{ kb vector}} \times 3 = z \text{ ng insert required}$$

__	µL	insert (*z* ng)
__	µL	vector (50 ng)
4	µL	5 × DNA dilution buffer
10	µL	2 × Rapid ligation buffer
__	µL	H_2O
1	µL	T4 ligase
20	µL	

The reaction was left to incubate at room temperature for a minimum of 15 min prior to transformation. Typically 5 µL of the ligation reaction was used for heat-shock transformation of *E. coli* (Section 2.4.3).

2.6.6 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.

2.6.6.1 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) similarly prepared. The amount of DNA in the unknown samples was estimated by the fluorescent intensity of ethidium bromide staining compared to the mass ladders.

2.6.6.2 Spectrophotometric method

1 - 2 µL of nucleic acid solution was measured on the Nanodrop spectrophotometer (Thermo Scientific). The absorbances at 260, 280 and 230 nm were recorded, and the concentration and purity were determined:

A_{260} × 50* × dilution factor = ng µL^{-1}

A_{260}/A_{280} >1.8 indicates a sample free of protein

A_{260}/A_{230} > 2 indicates a sample free of carbohydrate or solvents

* assumes that 50 µg mL^{-1} of double stranded DNA has an A_{260} = 1.0.

Spectrophotometry was also performed for RNA samples, except that the multiplier was 40 (40 µg mL^{-1} RNA A_{260} = 1.0).

2.6.7 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 Zymoclean™ Gel DNA Recovery Kit (Zymo Research) 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-based wash, and DNA was eluted from the membrane in 10 µL of elution buffer.

2.6.8 PCR reaction clean-up

PCR reactions were purified using the AxyPrep™ PCR Cleanup Kit (Axygen Biosciences), 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.

2.6.9 DNA sequencing

Sequencing was performed routinely upon plasmids containing DNA sequences of interest. Plasmid DNA was prepared (Section 2.5.3) and 300 ng was used per sequencing reaction with 3.2 pmol of the sequencing primer. The diluted template and primer were supplied to either the Allan Wilson Centre Genome Service (Massey University) or Waikato University DNA sequencing facility, where the sequencing reactions and reads were performed using the BigDye Terminator V3.1 chemistry. The sequence data obtained was trimmed of vector sequence and sequencing errors edited. Sequence editing, alignments and contigs were performed with the DNASTAR Lasergene 7 suite of bioinformatics tools.

2.7 Hybridisation and probing of northern and Southern blots

2.7.1 Pre-hybridisation

Before northern or Southern blot membranes were probed, they were pre-hybridised in Church and Gilbert buffer (Appendix 1.6.6) 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.

2.7.2 Probing

To make probes for gene expression analysis for northern blots, or to detect transgene presence and copy number for Southern blots, ^{32}P radioactively labelled probes were made. Probes were generated by primer extension of random hexamers with 100 ng of cloned cDNA inserts, excised from plasmids, using Klenow enzyme lacking 3′ and 5′ exonuclease activity (New England BioLab) (Feinberg and Vogelstein, 1983). Unincorporated radioactive-nucleotides were removed with ProbeQuant G50 micro-columns (GE Healthcare). 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.

2.7.3 Stringency washes

Membranes for both northern and Southern blots were washed following incubation with a radioactive probe. This was done to remove excess probe that was bound non-specifically to the membranes or nucleic acids. The standard washing protocol was to incubate membranes in a series of saline sodium citrate (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 × SSC to 0.1 × SSC, 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 if the radioactivity dropped below 5 cpm; typically membranes were washed to 0.5 × SSC.

2.7.4 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 the manufacturer's instructions (Kodak).

2.7.5 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.

2.8 Plant material and growing conditions

Mitchell petunia (*Petunia axillaris* × [*Petunia axillaris* × *Petunia hybrida* cv. 'Rose of Heaven']) (Ausubel et al., 1980) was originally obtained from Professor Richard Gardner at the School of Biological Sciences, University of Auckland, New Zealand. Violet 26 (V26) petunia seed was obtained from Dr Tim Robbins (Nottingham University, UK). Violet 30 (V30), White 134 (W134), White 225 (W225) and White 59 (W59) petunia lines were gifted by Professor Ronald Koes from the petunia mutant collection held at Vrije Universiteit (Amsterdam, The Netherlands).

The genotypes of the petunia lines used in this study, in terms of anthocyanin regulation, are shown in Table 2.1.

Table 2.1: Petunia genotypes used in this study, in terms of anthocyanin regulatory loci.

Petunia cultivar	Genotype	Description of mutation(s)
Mitchell petunia (MP) (W115: Vrije mutant collection)	***an2⁻, an4⁻***	MYB mutant (corolla and anthers)
V26	***an4⁻***	MYB mutant (anthers)
V30	-	-
W59	***an2⁻, an4⁻***	MYB mutant (corolla and anthers)
W134	***an11⁻***	WD40 mutant
W225	***an1-***	bHLH mutant

Plants were grown in a potting mix of bark:pumice (60:40 v:v) 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}).

Transgenic plants were grown within the PC2 containment greenhouse facility at Crop & Food Research, Palmerston North. The mutant petunia lines were grown within a standard glasshouse.

2.8.1 Description of plant organs/tissues sampled

Leaf – unless otherwise stated, mature expanded leaves were collected.
Petals – the corolla of the flower is made up of fused petals, and can be further separated into *tube* and *limb*. These two tissues have been shown to display differential expression of flavonoid regulatory and biosynthetic genes in petunia flowers. In

addition, the floral tube contains additional pigmentation patterning in many lines. A distinctive venation pattern is observed in the tube of MP, V26 and V30 lines.

2.9 Stable transformation and regeneration of *Petunia*

The transformation protocol is based upon that of Conner et al (2008) and was adapted to meet the requirements of the plants in culture.

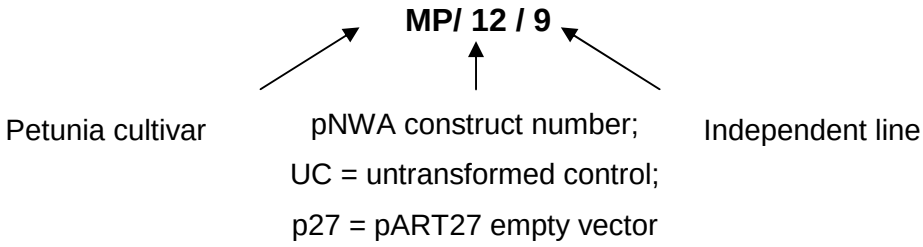
Young, newly emerged petunia leaves (Mitchell and/or V26) were collected and sterilised for 10 min in 15% (v/v) Janola™ bleach (final conc. 0.63 % (w/v) sodium hypochlorite, 15 mM NaOH) and a few drops of Tween20, followed by three washes with sterile water. Leaf discs (6 mm) were made from the leaves with a sterile hole-punch and were immersed for 5 min in an early log-phase culture of *Agrobacterium* harbouring the binary vector to be integrated (100 leaf discs were used per construct). The *Agrobacterium* culture was grown to an OD_{600} = 0.6, at which stage cells were harvested, gently re-suspended in virulence induction media (Appendix 1.5) containing 100 µM acetosyringone, and cultured at 28°C with shaking for 4 h. After immersion in the bacterial culture, the leaf discs were blotted dry on sterile filter paper and transferred to co-cultivation media (Appendix 1.5). The leaf discs were then transferred to a dark culture room (25°C) and allowed to co-culture with *Agrobacterium* for two days. After co-cultivation, leaf discs were transferred to selective shoot regeneration media (Appendix 1.5) containing 500 mg L^{-1} cefotaxime to inhibit the growth of *Agrobacterium,* and kanamycin to inhibit regeneration of non-transformed cells.

The leaf discs were grown in a 25°C culture room with white light (30 µmol m^{-2} s^{-1}, 16 h photoperiod). Leaf discs were transferred to fresh selective media every two weeks, while callus and shoots developed. Once shoots emerged, they were excised from the disc and placed onto selective shoot regeneration media to propagate clonal material. To ensure each transgenic line was generated from an independent transformation event, one shoot per disc was collected. Shoots were screened by PCR to confirm they contained the transgene. The propagated clonal shoots (4 - 5) were excised from the callus/shoot mass, dipped in IAA solution (100 mg L^{-1}) and transferred to root

regeneration media (Appendix 1.5). For V26 petunia, which appears particularly sensitive to kanamycin, rooting was inhibited in many transgenic lines with kanamycin selection. The shoots were sub-cultured several times with selection and failed to develop roots. Selection during rooting was reduced to 50 mg L^{-1} kanamycin, with successful rooting of about half the lines. For the remaining lines selection was completely removed, allowing development of roots.

2.9.1 Plant lines

Plant lines are referred to in the following format:



2.9.2 PCR screening regenerated shoots

Leaf/shoot material was collected from each putative transgenic line (approx 0.1 g), which was ground in liquid nitrogen to a fine powder and added to extraction buffer (200 mM Tris pH 7.5, 250 mM NaCl, 25 mM EDTA, 0.5% (w/v) SDS). Genomic DNA was extracted once with phenol:chloroform, precipitated with one volume of isopropanol and 0.1 volumes of 3 M NaOAc (pH 5.0), pelleted by centrifugation at 14 000 × *g*, desalted with 70% ethanol, dried, and dissolved in 50 µL sterile water containing RNase A (0.2 mg mL^{-1}). Genomic DNA was used as a template for PCR, using primers corresponding to the gene of interest. The binary vector used for transformation was used as a positive control, and no template and non-transformed petunia samples were used as negative controls. The transgenic product was differentiated from the endogenous genomic copy of each gene by size, based on the cDNA used in the constructs compared to the genomic copy containing introns. For the promoter:iGUS lines, a specific primer to the 5′ UTR/promoter combined with a primer specific to the GUS reporter gene was used, preventing amplification from the

endogenous promoter. Primers used are described in Sections 5.1.1 and 6.1.3, and listed in Appendix 2.

2.10 Nucleic acid extraction from *Petunia*

2.10.1 RNA extraction

Total RNA was extracted from approximately 0.5 - 1.0 g FW of frozen, ground, plant tissue by guanidine/acidic phenol extraction using a modified method of Chomczynski and Sacchi (1987). Frozen tissue was added to guanidine extraction buffer (Appendix 1.6.1), to which 0.1 volumes of 2M NaOAc (pH 4.0), 1 volume of unbuffered phenol (Appendix 1.6.2) and 0.3 volumes of chloroform:isoamyl alcohol (49:1) were sequentially added, vortexing well after each addition. The phases were separated by centrifugation at 10 000 × *g*; the aqueous phase was collected and RNA was precipitated with 1 volume isopropanol. The RNA was collected by centrifugation, and the RNA pellet was desalted with 70% (v/v) ethanol, centrifuged, ethanol removed and air-dried. RNA was dissolved in an appropriate volume of sterile water (typically 200 µL), and stored at -20°C.

2.10.2 Genomic DNA extraction

Genomic DNA was extracted using alkaline guanidine/phenol for applications where very clean, high molecular weight DNA was required. The protocol was adapted from the RNA extraction protocol in Section 2.10.1. Genomic DNA was extracted from 0.5 - 1.0 g FW of frozen, ground, leaf tissue in extraction buffer (Appendix 1.6.1.), extracted with phenol:[chloroform:isoamyl alcohol] (Appendix 1.6.3) followed by centrifugation at 10 000 × *g*. The aqueous phase was collected, 0.1 volumes of 3M NaOAc (pH 5.5) was added, and DNA was precipitated with 1 volume of isopropanol. The genomic DNA silks were spooled and collected, desalted with 70% (v/v) ethanol, air-dried, and dissolved in 500 µL of TE buffer (Appendix 1.6.7). Contaminating RNA was removed by digestion with RNase A (0.8 mg mL^{-1}) incubated at 37°C for 45 min, followed by extraction with 1 volume of chloroform/isoamyl alcohol (24:1). The aqueous phase was collected and DNA was precipitated with 0.1 volumes of 3M NaOAc (pH 5.5) and 0.8

volumes of isopropanol. The genomic DNA was desalted with 70% (v/v) ethanol and pelleted by centrifugation. The alcohol was removed and the DNA was air-dried and dissolved in an appropriate volume of TE. Genomic DNA was stored at 4°C.

2.11 Amplifying petunia cDNA clones for probes

The cDNA clones corresponding to UDP:rhamnose anthocyanidin-3-glucoside rhamnosyl transferase (3RT; *Rt* locus), UDP:glucose anthocyanin 5-*O*-glucosyltransferase (UF5GT), a putative anthocyanin methyl transferase (MT), a putative anthocyanin acyl transferase (AT) and dihydroflavonol reductase (DFRa) from petunia were amplified by RT-PCR from mixed stage flower bud cDNA from V26 petunia. Primers were designed based on publically available sequence data: *3RT* (Genbank X71059) NA54/NA55; *5GT* (Genbank AB027455) NA50/NA51; *AT* (Genbank AR944737; US patent 7105719) NA52/NA53; *MT* (Genbank DD178352; JP patent 2005514950-A/1) NA56/NA57; *DFR-A* (Genbank X79723) NA58/NA59 (Appendix 2). The primers included restriction sites to allow directional cloning into the pART7 expression vector, generating the plasmids pNWA23 (3RT), pNWA24 (AT), pNWA25 (UF5GT), pNWA26 (MT) and pNWA27 (*DFRa*) (Appendix 3).

cDNA clones corresponding to petunia *PAL* (pPN296), *C4H* (pPN303), *CHS-A* (pPN297), *CHI-A* (pPN300), *F3H/An3* (pNWA17), *F3'H* (pPN301), *F3'5'H/Hf1* (pNWA18), *FLS/Fl* (pPN302), *ANS* (pPN299), *UF3GT* (pNWA20) and *GST/An9* (pNWA19) were held within the 'Plant Pigments' plasmid collection at Crop & Food research (Appendix 3). Those clones which were only present as plasmid stocks were transformed into *E. coli* (Section 2.4.3) and assigned pNWA numbers. Full enzyme names, and petunia loci associated with these are described in the list of abbreviations pp xvii-xviii and Figure 1.2.

2.12 Gene walking – cloning promoters

The Universal GenomeWalker™ kit (Clonetech) was used to clone the upstream sequence, including the promoter region, of known gene sequences. The promoters of *DEEP PURPLE*, *PURPLE HAZE*, *An1*, *MYBx*, *Hf1* (F3′5′H) and *Hf2* (F3′5′H) were cloned. The primers and template source is shown in Table 2.2. Amplification was performed with i-StarMAX™ II DNA Polymerase (iNTRON Biotechnology), a hot-start *Taq*-based enzyme mix containing proof-reading polymerases to allow long-range PCR. Primer design and PCR conditions were performed following the manufacturer's instructions. The major bands generated from the PCR reactions (secondary round) were separated by agarose gel electrophoresis, excised from the gel, extracted (Section 2.6.7), cloned into pGEM®-T Easy (Promega), transformed into *E. coli*, and positive clones were sent for DNA sequencing. The sequences from each library were formed into contiguous sequences with each other, and the known sequence for each gene.

Table 2.2: Primers and template used for gene-walking. Primer sequences are listed in Appendix 2.

Gene	Gene-specific primers 1°; 2° PCRs	Template DNA
DEEP PURPLE (MYB)	NA60; NA61	Mitchell petunia
PURPLE HAZE (MYB)	NA99; NA98	Mitchell petunia
An1 (bHLH)	NA76; NA77	Mitchell petunia
MYBx (R3 MYB)	NA129; NA130	Mitchell petunia
Hf1 (F3′5′H)	NA66; NA67	V26 petunia*
hf1-1 (F3′5′H)		Mitchell petunia
Hf2 (F3′5′H)	NA100; NA101	Mitchell petunia

* V26 was chosen as the template, as Mitchell petunia contains the ***hf1-1*** allele which is expressed at reduced levels, while V26 has functional copies for ***Hf1***.

Chapter 3

Cloning *DEEP PURPLE* and *PURPLE HAZE*

3.0 Introduction

Two partial cDNA clones encoding MYB transcription factors were originally identified during a screen for MYB factors that may mediate a light-induced anthocyanin pigmentation phenotype in petunia (Albert, 2006). These clones were amplified previously by degenerate PCR, using primers designed to the MYB DNA binding domain of known MYB anthocyanin regulators, and subsequently the 3′ portion of the cDNAs were obtained by 3′RACE. The two clones were named '*MYBI*' and '*MYBII*' and preliminary expression studies showed that *MYBII* is induced by high-light treatment in leaves, correlating with the pigmentation phenotype under investigation, while *MYBI* was shown to be expressed constitutively (Albert, 2006). The missing 5′ region of the cDNAs, containing coding sequence and the 5′ untranslated region (5′UTR), was required to enable functional studies on the two putative anthocyanin regulators *MYBI* and *MYBII* to be performed. *MYBI* and *MYBII* were renamed *DEEP PURPLE* (*DPL*) and *PURPLE HAZE* (*PHZ*), respectively.

3.1 Materials and methods

3.1.1 Cloning *DEEP PURPLE* and *PURPLE HAZE*

Partial cDNA clones for *DEEP PURPLE* (*DPL*) and *PURPLE HAZE* (*PHZ*) were previously cloned using degenerate PCR to the conserved MYB DNA binding domain, followed by 3′RACE (Albert, 2006). 5′RACE was performed to obtain full length cDNA clones for both these genes, using the 5′RACE System for Rapid Amplification of cDNA Ends, version 2.0 (Invitrogen) following the manufacturer's instructions, with nested gene-specific primers NA18 and NA19 for *DPL* and NA16 and NA17 for *PHZ*. 5′RACE amplification is based upon the 5′ tailing of cDNAs with poly-cytosine byterminal transferase, allowing priming with a complementary abridged primer and

specific amplification with nested gene-specific primers. First strand cDNA was synthesised from total RNA (1-5 µg) using Superscript III reverse transcriptase (Invitrogen) primed with NA16 or NA18 for *DPL* and *PHZ*, respectively. Total RNA from shade-grown (50-350 µmol m^{-2} s^{-1}) or high-light-grown (750 µmol m^{-2} s^{-1}) Mitchell petunia plants, that was previously extracted (Albert, 2006; Albert et al., 2009), was used as template for cDNA synthesis for 5′RACE for *DPL* and *PHZ*, respectively. Amplification was performed with *Taq* polymerase (QIAGEN), and cycling conditions for both rounds were as follows: 94°C, 2 min; 94°C, 30 s, 52°C, 30 s, 72°C, 1 min × 30 or 35 cycles (for 1° and 2° rounds, respectively); 72°C, 5 min; 12°C hold. PCR products were run on agarose gels, the bands excised, gel purified (Sections 2.6.2 and 2.6.7), cloned into pGEMteasy (Section 2.6.4) and sent for DNA sequencing (Section 2.6.9). The sequences obtained from 5′RACE were formed into contiguous sequences with the sequences previously obtained by 3′RACE and degenerate PCR, giving full length cDNAs. Primers specific to the 5′ and 3′ ends were designed and the full length cDNAs were amplified by PCR with the primers NA27/NA38 for *DPL* and NA26/NA30 for *PHZ*. Proofreading polymerases were not used to obtain the final sequence, as they proved unable to amplify either of these cDNAs. Full cDNAs for *DPL* and *PHZ* were amplified at least three times, from the same cDNA template, cloned and sequenced, enabling the detection of any errors introduced by *taq* polymerase.

Genomic sequences for *DPL* and *PHZ* were amplified from Mitchell petunia genomic DNA by PCR with the primers NA27/NA38 and NA26/NA30, respectively with FastStart High Fidelity PCR system polymerase mix (Roche). The amplified products were cloned into pGEM®-T Easy (Promega) (Section 2.6.4) and sequenced. The genomic sequence was then compared to the full cDNA sequence, and the transcription start site, size and position of introns determined. Subsequently, genomic clones of *DPL* and *PHZ* were cloned from V26, V30 and W59 petunia, in the same manner as the Mitchell petunia clones. *An4*, a related gene to *DPL*, was also amplified from MP and V30 petunia. Sequences for the *DPL*, *PHZ* and *An4* alleles are provided in Appendix 4.

3.1.2 Southern blotting

Southern blotting was performed on 50 µg genomic DNA isolated from Mitchell and V26 petunia (Section 2.10.2) digested with *Eco*RI, *Eco*RV or *Hind*III with λ *Hind*III fragments loaded as a molecular marker. DNA was separated on 0.8% (w/v) agarose gels containing ethidium bromide, and transferred to Hybond N^+ membrane (GE Healthcare) by capillary action with 0.4 M NaOH. The membrane was visualised under UV light, ensuring complete transfer to the membrane, and the location of the molecular size standards marked. The membranes were hybridised with [α^{32} P] dATP labelled probes for *DPL* and *PHZ* generated by PCR labelling. To do this, template DNA was prepared by PCR using the primers NA10/NA12 and NA13/NA15 upon the plasmids pNWA3 and pNWA4, respectively. PCR labelling was performed by setting up a 20 µL PCR reaction containing 100 ng of template DNA, 3.1 µM each of dTTP, dCTP, dGTP and [α^{32}P] dATP, 0.2 µM primers, *Taq* polymerase (Roche) in 1 × PCR buffer containing 1.5 mM $MgCl_2$. Amplification was performed 94°C 2 min; 94°C 30 s, 50°C 30 s, 72°C 1 min × 10 cycles. Unincorporated nucleotides were removed using a ProbeQuant G50 column (GE healthcare). Pre-hybridisation, hybridisation, washes and exposure to film were performed as described in Sections 2.7.1, 2.7.2, 2.7.3 and 2.7.4, respectively.

3.1.3 Phylogenetic analysis of *DEEP PURPLE* and *PURPLE HAZE*

The translated coding sequence for DEEP PURPLE and PURPLE HAZE were aligned to other MYB sequences (below) using ClustalX (Thompson et al., 1997). Maximum Parsimony analysis was performed using PAUP* 4.0b8 software (Swofford, 1993), assuming unordered character states and equal character state weighting. The relative support for each node was examined with non-parametric bootstrapping, 1000 replicates (Felsenstein, 1985).

The GenBank accession numbers for additional sequences used in the phylogenetic tree were: PhAN2 (AF146702); PhMYB1 (Z13996); PhMYB2 (Z13997); PhMYB3 (Z13998); PhODORANT1 (AY705977); AmROSEA1 (DQ275529); AmROSEA2 (DQ275530); AmVENOSA (DQ275531); AmMIXTA (X79108); CaA (AJ608992);

SlANT1 formerly LeANT1 (AY348870); AtPAP1 (AF325123); AtPAP2 (AF325124). *PhAN4* and *PhMYBb* sequences were from Kroon (2004). Assistance preparing the phylogenetic tree was kindly given by Dr Carlos Lehnebach, (Allan Wilson Centre for Molecular Ecology and Evolution, Palmerston North).

3.2 Results

The 5′ ends of the cDNAs for *MYBI* and *MYBII* were obtained by 5′RACE. The second (nested) round 5′RACE products are shown in Figure 3.1. The major products for both *MYBI* and *MYBII* were approximately 380 bp, which was a reasonable fragment length based on the predicted number of missing amino acids and 5′UTR compared to related MYB factors such as AN2. The major bands were cloned and sequenced, and found to form a contiguous sequence with the cDNA clones obtained previously. Primers were subsequently designed to the beginning of the new 5′ sequences to amplify the full cDNAs for *MYBI* and *MYBII* using gene specific primers. The amplified product was the same size as the predicted *in silico* sequence, and subsequent sequencing confirmed the full cDNA sequences for *MYBI* and *MYBII*. The genes were re-named *DEEP PURPLE* (*MYBI* clone) and *PURPLE HAZE* (*MYBII* clone) based on phenotypes in functional studies (Chapter 6).

Amplification of *DEEP PURPLE* (*DPL*) and *PURPLE HAZE* (*PHZ*) from genomic DNA, corresponding to the full cDNA regions, was performed. The PCR products were cloned and sequenced (Appendix 4). The gene structure, position and size of introns are shown in Figure 3.2 A. Two introns are present in the coding sequence, corresponding to the DNA binding domain in *DPL* and *PHZ*. In addition to these two introns, *PHZ* also contains a small (~100 bp) intron in the 5′UTR region.

Southern blots were prepared from Mitchell (*P. axillaris* × [*P. axillaris* × *P. hybrida*]) and V26 (*P. hybrida*) petunia genomic DNA. Probes to *DPL* or *PHZ* were designed to include the 3′UTR and part of the C-terminal region, excluding the highly conserved MYB DNA binding domain (Figure 3.2A). The probe for *DPL* hybridised to 5-7 bands in Mitchell petunia, and 3-4 bands in V26 petunia (Figure 3.2B). The genomic clones of *DPL* from Mitchell and V26 lack *Eco*RI and *Hind*III sites in the region the probe was

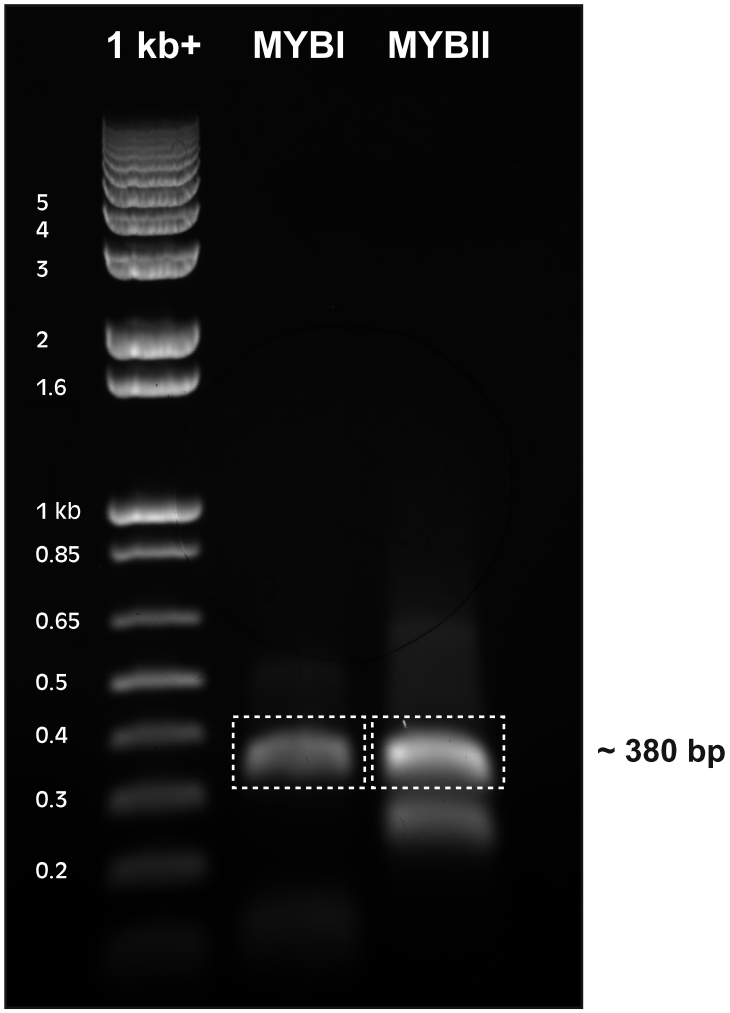


Figure 3.1 Second round 5′RACE products, corresponding to the missing 5′ portion of cDNAs for *MYBI* and *MYBII*. *MYBI* and *MYBII* were subsequently renamed *DEEP PURPLE* and *PURPLE HAZE*, respectively. The major bands were excised from the gel (as indicated), cloned and sequenced.

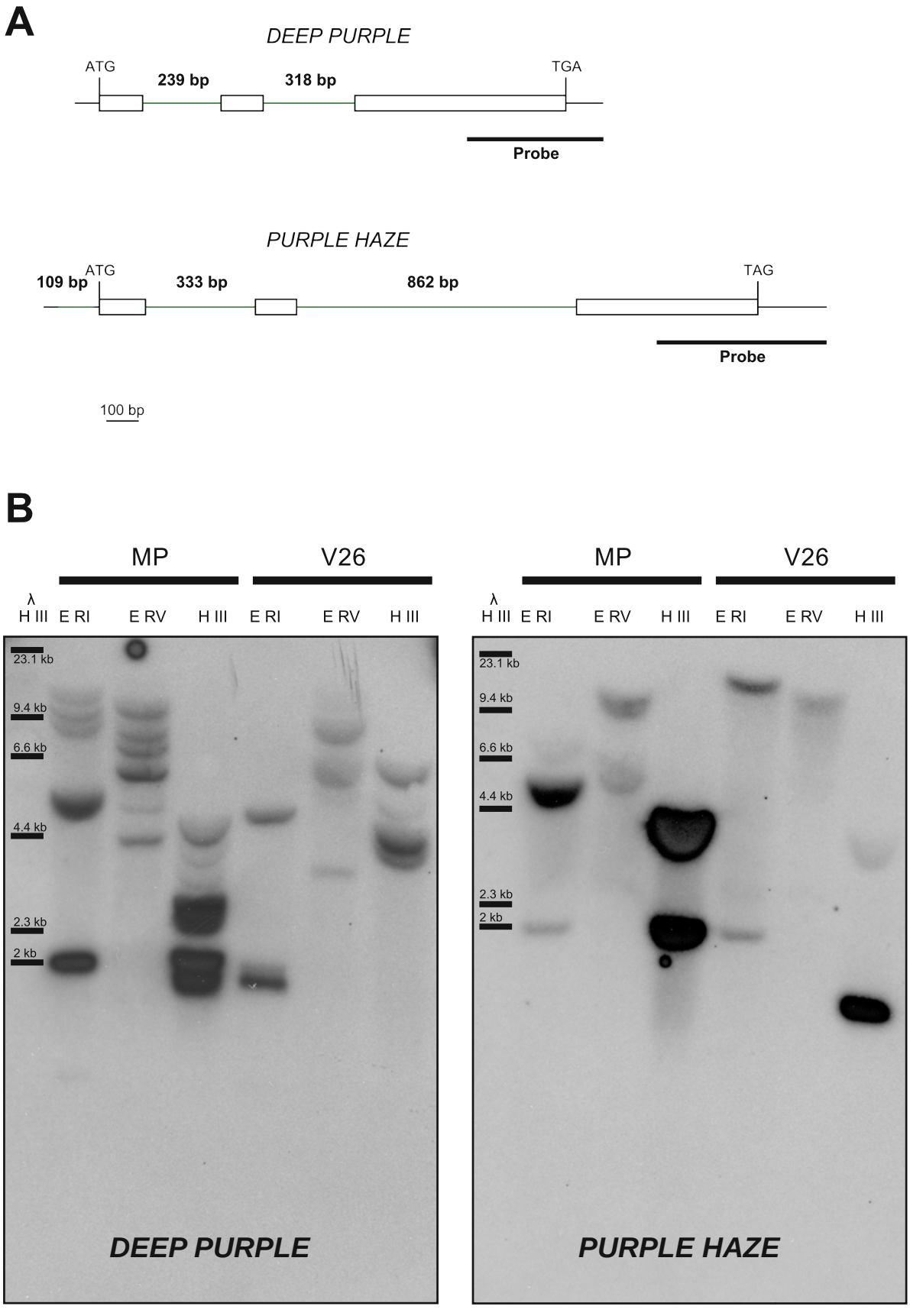


Figure 3.2 A. Gene structures for *DEEP PURPLE* and *PURPLE HAZE*. Coding sequences are shown as boxes, start and stop codons are indicated. The region used to probe Southern blots in **B** is indicated. **B.** Southern blots of Mitchell (MP) and V26 petunia DNA, probed with the C terminal and 3′UTR region of *DEEP PURPLE* or *PURPLE HAZE*. DNA was digested with *Eco*RI (ERI), *Eco*RV (ERV) or *Hind*III (HIII) and compared to λ *Hind*III fragments (approximate positions indicated). Blots were washed to high stringency (0.1× SSC, 60°C).

made to, and *Eco*RV is completely absent. The probe for *PHZ* hybridised to 2-3 bands in Mitchell petunia and 2 bands in V26 petunia (Figure 3.2B). The genomic clones of *DPL* from Mitchell and V26 lack *Hind*III sites in the region the probe was made from, and *Eco*RI and *Eco*RV are completely absent.

The translated amino acid sequence of the coding sequence for *DPL* and *PHZ* were aligned by CLUSTALX to other MYB transcription factors, generating the phylogeny shown in Figure 3.3. Both DPL and PHZ cluster tightly to MYB transcription factors involved in anthocyanin regulation, particularly to other anthocyanin-MYB transcription factors from solanaceous species (e.g. tomato, capsicum, petunia). DPL and PHZ cluster with the known petunia anthocyanin regulators AN2, AN4 and a petunia clone called MYBb. *DPL* is very similar to *An4* and *MYBb*, sharing 93% and 97% nucleotide identity, respectively, while *PHZ* shares 79% nucleotide identity with *An2*.

Amplification of *An4* and *MYBb* was also performed from Mitchell (MP) and V30 petunia. *An4* was amplified from both lines. The amplification products for *MYBb* matched those sequences previously amplified using primers designed for *DPL* (Appendix 4).

3.3 Discussion

Two novel MYB transcription factors, DPL and PHZ, have been cloned from petunia. These two transcription factors were identified previously in a screen for anthocyanin-regulating MYB transcription factors involved in mediating light-induced vegetative anthocyanin production (Albert, 2006). The full-length cDNA sequences corresponding to these genes have now been cloned and sequence alignments show *DPL* and *PHZ* to be very closely related to other known anthocyanin regulators. In addition, they also share the amino acid motif ([DE]Lx_2[RK]$x_3Lx_6Lx_3R$), which has been identified as being important for interaction with bHLH type co-regulators (Zimmermann et al., 2004). The intron positions within MYB DNA binding domain of *DPL* and *PHZ* are conserved for anthocyanin-regulating MYB transcription factors (Paz-Ares et al., 1987;

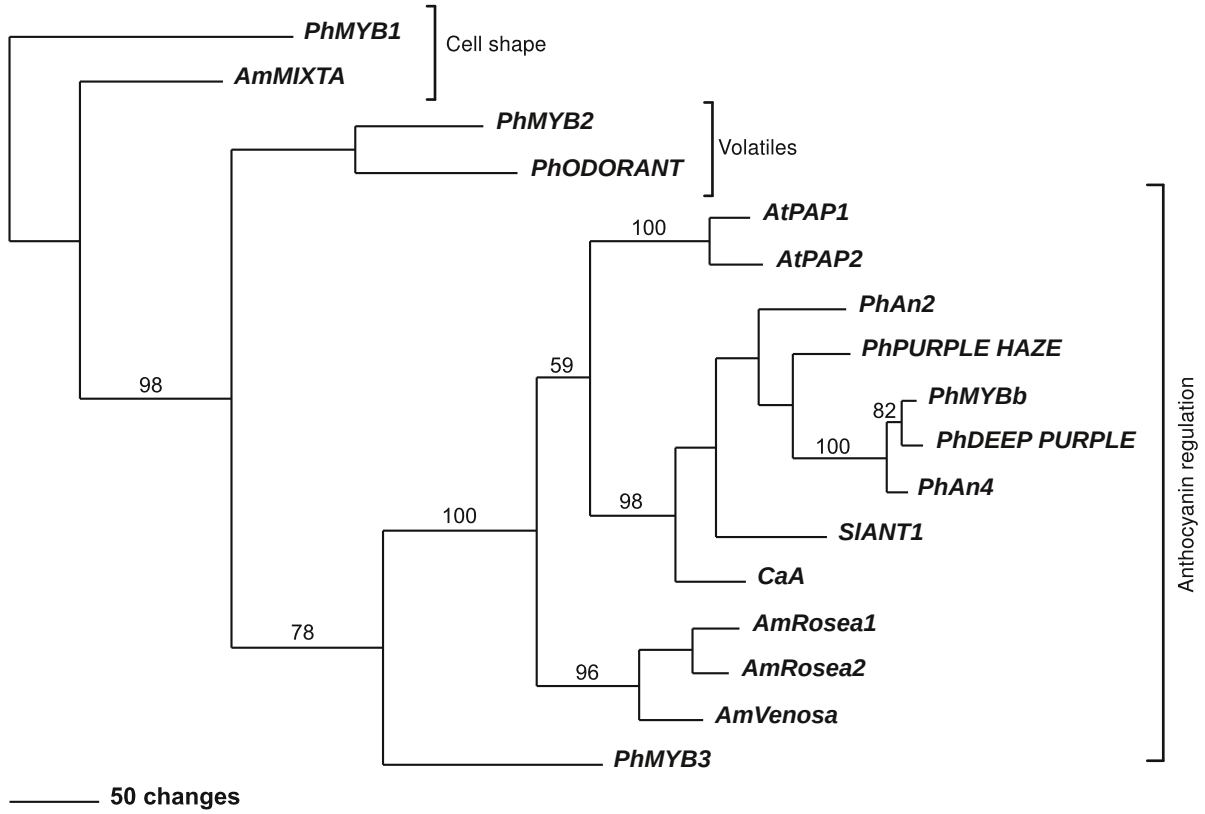


Figure 3.3 Phylogenetic tree of selected R2R3 MYB transcription factors, involved in regulating a range of products and developmental processes, including anthocyanins, floral volatiles (scent) and floral epidermal cell morphology. Bootstrap values (percentage) are shown, from 1000 replicates. *Ph Petunia hybrida, Am Antirrhinum majus, Ca Capsicum annuum, Sl Solanum lycopersicum, At Arabidopsis thaliana*

Quattrocchio et al., 1999; Schwinn et al., 2006), which together with other structural features, supports a putative role in anthocyanin regulation.

Southern analysis of *DPL* and *PHZ* suggests that each exists as a small gene family of highly related genes in petunia. Southern blots were replicated, with independent DNA extractions and digestions, indicating the banding patterns are not the result of incomplete digestion. The possibility of generating multiple bands per copy of either *DPL* or *PHZ* due to the restriction endonucleases used has also been excluded, as none of the enzymes used to digest the genomic DNA have sites within the sequence used as probes. In Mitchell petunia, the number of bands hybridising under high stringency to the *DPL* probe is twice that of V26 petunia. Mitchell petunia (*P. axillaris* × [*P. axillaris* × *P. hybrida*]) is a double haploid generated from inter-specific crosses to the wild *Petunia axillaris* species (Ausubel et al., 1980), which while useful as a true homozygous line, has a complex genome. The level of nucleotide identity between *An4* and *DPL* is such that cross-hybridisation, even at high stringency, is possible. However, Mitchell petunia is *an4*$^{-}$ (Cornu and Farcy, 1981; Quattrocchio et al., 1998), suggesting that this is distinct from *DPL*.

Kroon (2004) recently isolated two *MYB* genes, and showed one of them segregated with the *An4/an4*$^{-}$ locus. In the same study another clone, *MYBb*, was identified which shares 94% nucleotide identity to *An4*, but was shown to be genetically distinct from AN4. Transcript expression patterns for *An4* and *MYBb* showed *An4* was expressed in anthers, while *MYBb* was expressed predominantly in the flower tube, with no transcripts for *An4* or *MYBb* detected in leaves. The sequence identity between *DPL* and *MYBb* is very high, raising the possibility that these may be allelic. *DPL* was cloned from leaves, and previous expression studies using semi-quantitative RT-PCR showed *DPL* transcripts were constitutively expressed in leaves (Albert, 2006). *An4* and *DPL* were both amplified from MP and V30 petunia, suggesting these two related genes are distinct. However, amplification of *MYBb* and *DPL* in MP and V30 resulted in a single product indicating *MYBb* and *DPL* may be allelic.

PURPLE HAZE appears to be present in two copies in the petunia genome. While it has a high degree of sequence similarity at the amino acid level to other anthocyanin

regulators, it is clearly distinct from any previously identified MYB from petunia, or any other EST on publically available databases.

Chapter 4

Transcription factor expression and pigmentation patterning in *Petunia*

4.0 Introduction

Two new putative anthocyanin regulators, *DEEP PURPLE* (*DPL*) and *PURPLE HAZE* (*PHZ*), have been isolated from petunia. The sequence similarity to other MYB factors known to regulate anthocyanins suggests both *DPL* and *PHZ* have a role in controlling pigmentation patterns. *DPL* and *PHZ* appear to belong to a small gene family of anthocyanin regulators that includes *An2* and *An4*, which have previously been shown to regulate anthocyanin synthesis in the corolla and anthers. The expression of *DPL* and *PHZ* was examined to link the activity of these two genes to floral and vegetative pigmentation patterns.

The anthocyanin biosynthetic genes are activated by MYB-bHLH-WDR (MBW) transcription factor complexes and, therefore, the expression of *DPL* and *PHZ* was compared to that of known MYB (*An2*), bHLH (*An1*, *JAF13*) and WDR (*An11*) factors. In addition to transcription factors that activate anthocyanin synthesis, the expression of the putative repressors, *MYBx* (R3 MYB) and *MYB27* (R2R3 MYB), was also examined. Transcript abundance for the transcription factors involved in anthocyanin regulation was determined by quantitative, real-time PCR. The sensitivity and specificity of qRT-PCR has allowed the determination of transcript abundance for transcription factors, even though they are often expressed at very low levels.

Petunia species and cultivated varieties exhibit several floral pigmentation patterns, including flower tube venation, flower limb venation, bud-blushing, coloured anthers and fully coloured petals. The MYB factor *An2* regulates the fully coloured petal phenotype, and *An4* controls flower tube and anther colour (Wiering, 1974; Quattrocchio et al., 1999). The more subtle floral pigmentation patterns (e.g. venation,

bud blushing) are best examined in *an2*$^{-}$ *an4*$^{-}$ backgrounds, such as Mitchell and W59 petunia, where strong pigmentation is absent. The possible involvement of *DPL* and *PHZ* for determining floral pigmentation patterns was investigated in Mitchell and W59 petunia, and compared to V26, a fully coloured cultivar.

The expression patterns for *DPL* and *PHZ* were examined in different stages of flower bud development in three petunia cultivars, and in the leaves of shade- and high light-grown petunias.

4.1 Materials & Methods

4.1.1 Sequence alignments

The MYB domain region of the R2R3 MYB activators, R2R3 MYB repressors and R3 MYB repressors were aligned by CLUSTALW. PhMYB27 (Murr, 1995), AtMYBL2 (Genbank NM_105772) , AtTRY (Genbank AY161286), AtCPC (Genbank NM_130205), PhMYBx (Kroon, 2004), PhAN4 (Kroon, 2004), PhAN2 (Genbank AF146702), AtPAP1 (Genbank AF325123), AtPAP2 (Genbank AF325124) were included in the alignment.

4.1.2 Cloning *DPL* and *PHZ* from W59

The W59 petunia alleles of *DPL* and *PHZ* were amplified from W59 genomic DNA, as described in Section 3.1.1.

4.1.3 Quantitative reverse transcription PCR (qRT-PCR)

For quantitative, real-time, reverse-transcription PCR (qRT-PCR) RNA was extracted (Section 2.10.1) from corolla tissue (tube and limb combined) from stages 1-5 flower buds of V26, W59 and Mitchell petunia with three biological replicates. Each sample consisted of three to six pooled buds. RNA was also used which had been extracted previously. Pooled leaves of Mitchell petunia grown under shade (50 - 350 µmol m^{-2} s^{-1}) or high-light conditions (750 µmol m^{-2} s^{-1}) were collected, with four biological replicates per treatment, as described in Albert et al. (2009). Total RNA (1 µg) was treated with RNase-free DNase 1 (Invitrogen) and this was used as template

for first strand cDNA synthesis, prepared with Transcriptor reverse transcriptase (Roche) and oligo dT_{12-18} (10 µM). cDNA was diluted 20-fold for qRT-PCR. Amplification upon DNase treated RNA without reverse-transcription (-RT control) was performed to ensure the removal of contaminating genomic DNA by DNAse 1 was effective.

Primers were designed to have a T_m = 60°C (± 1°C), a minimal secondary structure, to be unable to form stable dimers, and to amplify a product ranging in size from 80 – 250 bp for efficient detection with SYBR Green I. The primer pairs used *An11*, NA82/NA83; *An1*, NA84/NA85; *JAF13*, NA86/NA87; *DPL*, NA90/NA91; *PHZ*, NA88/NA89; *MYBx*, NA103/NA104 and *An2*, NA105/NA106. The primers for the housekeeping genes *Actin* (ACT2) PhACT2F/PhACT2R, *Histone H4* (HisH4) PhHisH4F/PhHisH4R and *Elongation Factor 1-α* (EF1α) PhEF1αF/ PhEF1αR were previously designed by Snowden et al., (2005). Primer pair specificity was determined by performing amplification upon cloned cDNAs for target genes and upon genomic DNA. Melt-curve analysis for the amplified qRT-PCR products was always performed.

qRT-PCR was performed using LightCycler® 480 SYBR Green I Master (Roche) reagents and the Rotor Gene 3000 real-time PCR machine and Rotor Gene 6000 series software 1.7 (Corbett Research), with three replicates of each cDNA sample. Fluorescence measurements were performed at 72°C each cycle and continuously during final melting. Relative transcript abundance was determined by comparative quantification (Pfaffl, 2001) to the geometric mean of the most stable housekeeping genes (ACT2 and EF1α), which was determined using '*BestKeeper*' (Pfaffl et al., 2004). The qRT-PCR reactions were set up as a master mix, for all reactions (excluding template). The reaction master mix and cDNA templates were dispensed into a 72 well Gene Disc (Corbett Research) with the CAS-1200 liquid handling system (Corbett Robotics).

qRT-PCR reaction:

0.5 µL 10 µM S primer (0.5 µM)	Master mix
0.5 µL 10 µM AS primer (0.5 µM)	
1.5 µL H_2O	
5.0 µL 2× SYBR Green I Master (Roche)	
2.5 µL diluted cDNA	
10 µL Total	

qRT-PCR was performed under the following cycling parameters:

Initial denaturation / enzyme activation	95℃	10 min	
Denaturation	95℃	10 s	× 40
Annealing	60℃	15 s	
Extension	72℃	20 s	
Melting curve	72℃ – 95℃		

4.2 Results

4.2.1 Vegetative pigmentation and transcript abundance

Mitchell petunia plants (MP) grown under shade conditions were green, and completely lacked anthocyanin pigments (Figure 4.1A). However, once exposed to high light the leaves of MP accumulated anthocyanins, particularly on the stems and the cells overlaying the veins (Figure 4.1A; Albert et al., 2009).

Transcript abundance for transcription factors implicated in anthocyanin regulation was determined in the leaves of MP, by qRT-PCR (Figure 4.1B). The high light-induced anthocyanin production positively correlated with an increase in transcripts for the two MYB factors *PHZ* (>60-fold induction), and *DPL* (5-fold induction). The putative active repressor of anthocyanin biosynthesis, *MYB27*, had an inverse expression profile

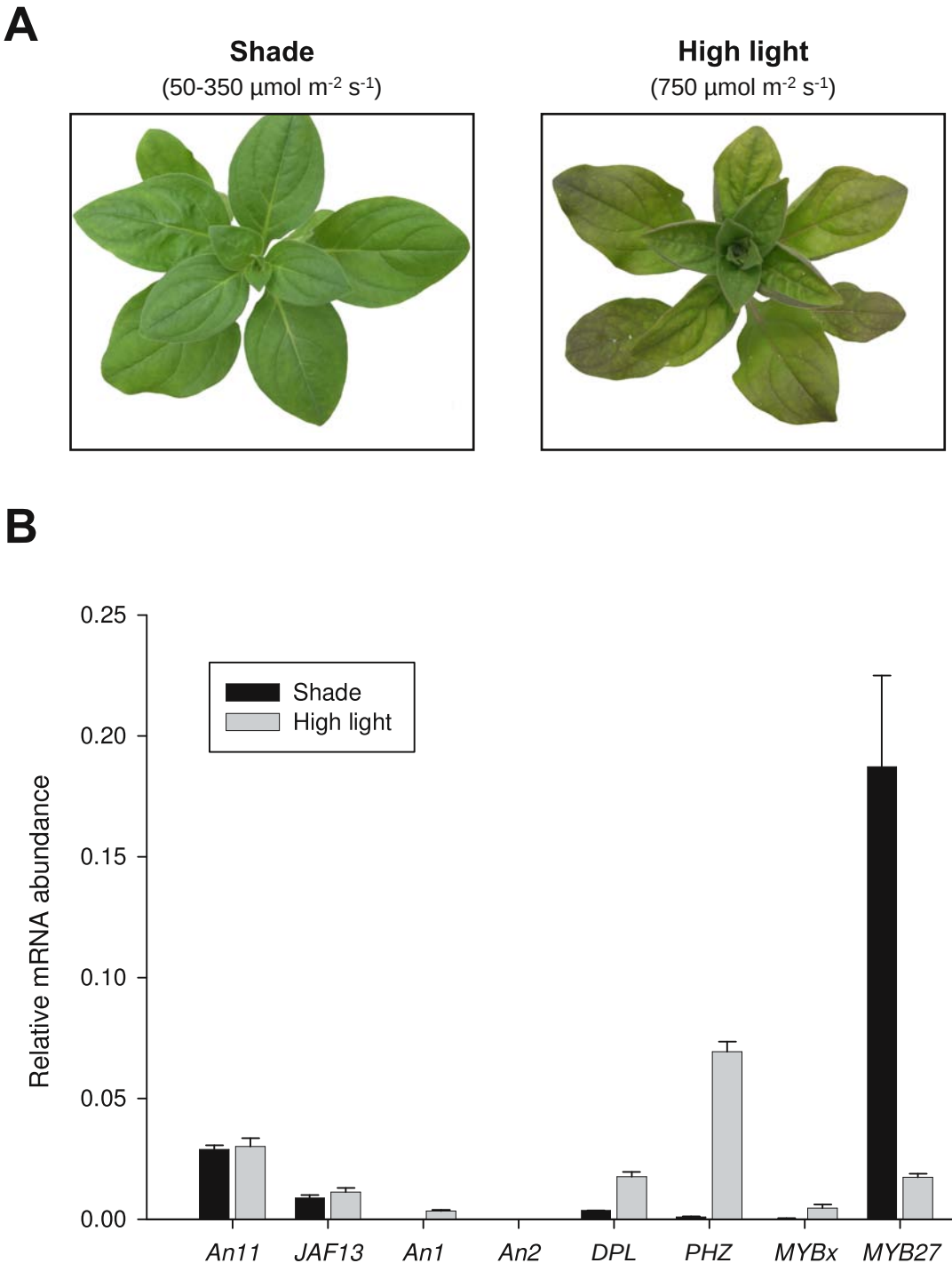


Figure 4.1 A. Vegetative phenotypes of Mitchell petunia plants grown under shade or high light. **B.** Relative transcript abundance of transcription factors involved in anthocyanin regulation, in shade- or high light-grown Mitchell petunia plants. Transcript abundance was determined by qRT-PCR, normalised to the geometric mean of *ACT2* and *EF1α*. Means ± SE, $n = 4$ are reported.

to that of the MYB activators. *MYB27* transcripts were detected at high levels in shade-grown leaves and reduced 16 fold with high-light treatment.

Transcripts for *An2*, the principal MYB factor that determines floral pigmentation, were not detected in leaves. Transcripts for the bHLH factor *An1* were not detectable in shade-grown leaves, but were induced with high light. Similarly, the R3 MYB repressor, *MYBx*, was also slightly induced with high light, while *An11* and *JAF13* were expressed irrespective of lighting treatment.

4.2.2 Floral pigmentation and transcript abundance

Transcript abundance of anthocyanin regulators was determined in the petals of developing MP flower buds (Figure 4.2A). The flower developmental series is shown in Figure 4.2B. MP is a white flowered petunia variety due to mutations in *an2* (corolla) and *an4* (petal tube, anthers). Petals do, however, show vein-associated anthocyanin production on the external petal surfaces, which occurs from stage 1. The inner flower tube also has highly reticulated vein-associated anthocyanin pigmentation which becomes coloured by stage 3 (See Chapter 1, Figure 1.1). MP buds can occasionally accumulate a faint blush of anthocyanin on the petal limb in response to light, which fades to white upon flower opening.

DPL and *PHZ* were expressed early during bud development and declined as the flowers developed. This is in contrast to *An2*, whose expression peaked at stage 4. *An1* was expressed throughout the stages, with a sharp increase at stage 5. *JAF13*, *An11* and *MYBx* were expressed consistently throughout bud development, with a small increase at stage 5. *MYB27* was expressed early, in a similar manner to *DPL* and *PHZ*, except that it increased slightly at stage 5.

Transcript abundance of anthocyanin regulators was determined in the petals of developing V26 petunia flower buds (Figure 4.3A). The developmental series is shown in Figure 4.3B. V26 petunia is a dark violet coloured cultivar. The flower tube has vein associated pigmentation, but in addition to this the corolla also becomes deeply

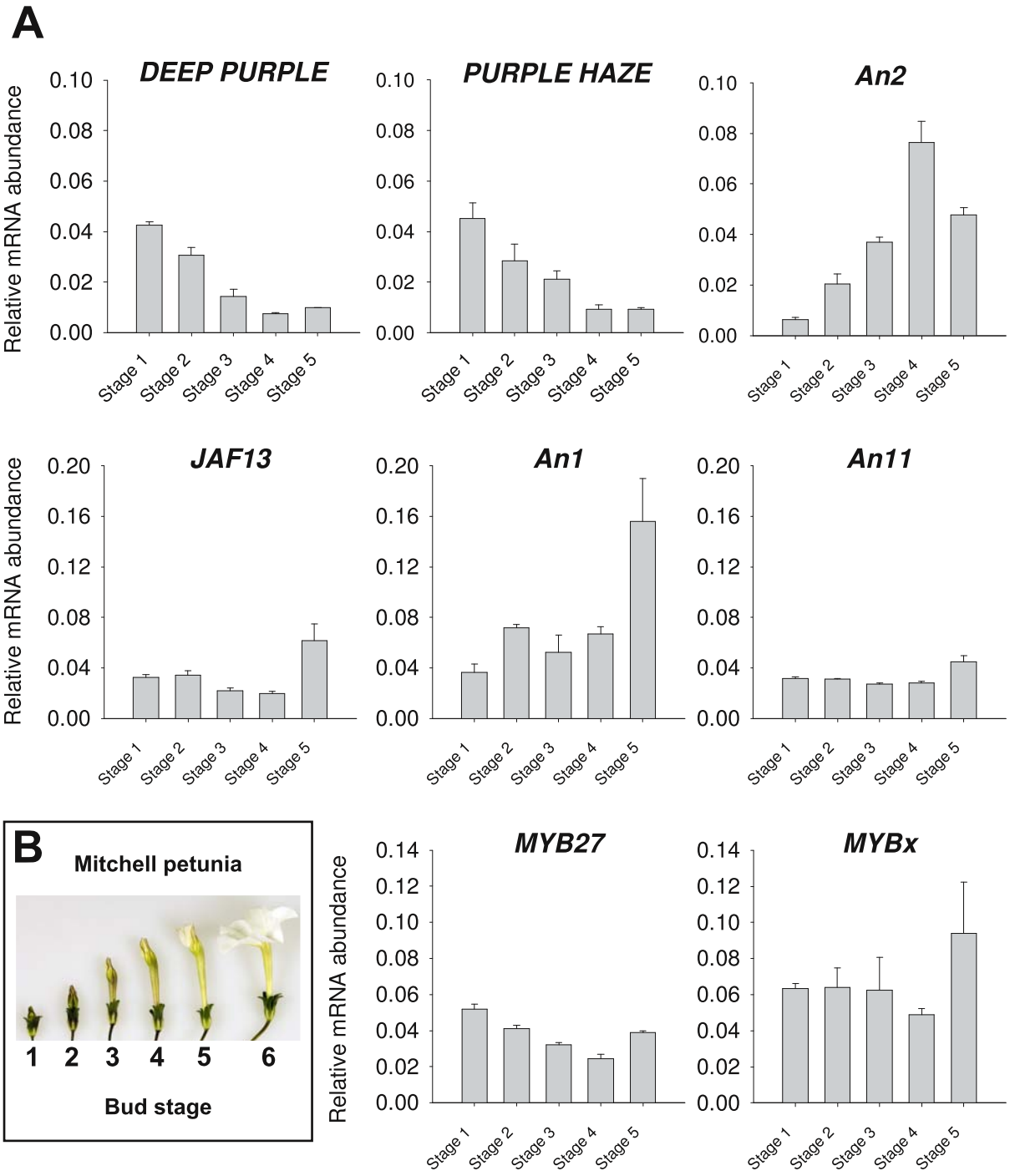


Figure 4.2 A. Transcript abundance of transcription factors involved in anthocyanin regulation in petals of developing Mitchell petunia flower buds. Relative transcript abundance was determined by qRT-PCR, normalised to the geometric mean of *ACT2* and *EF1α*. Means ± SE, *n*=3 are reported. **B.** Developmental flower bud series used for Mitchell petunia samples.

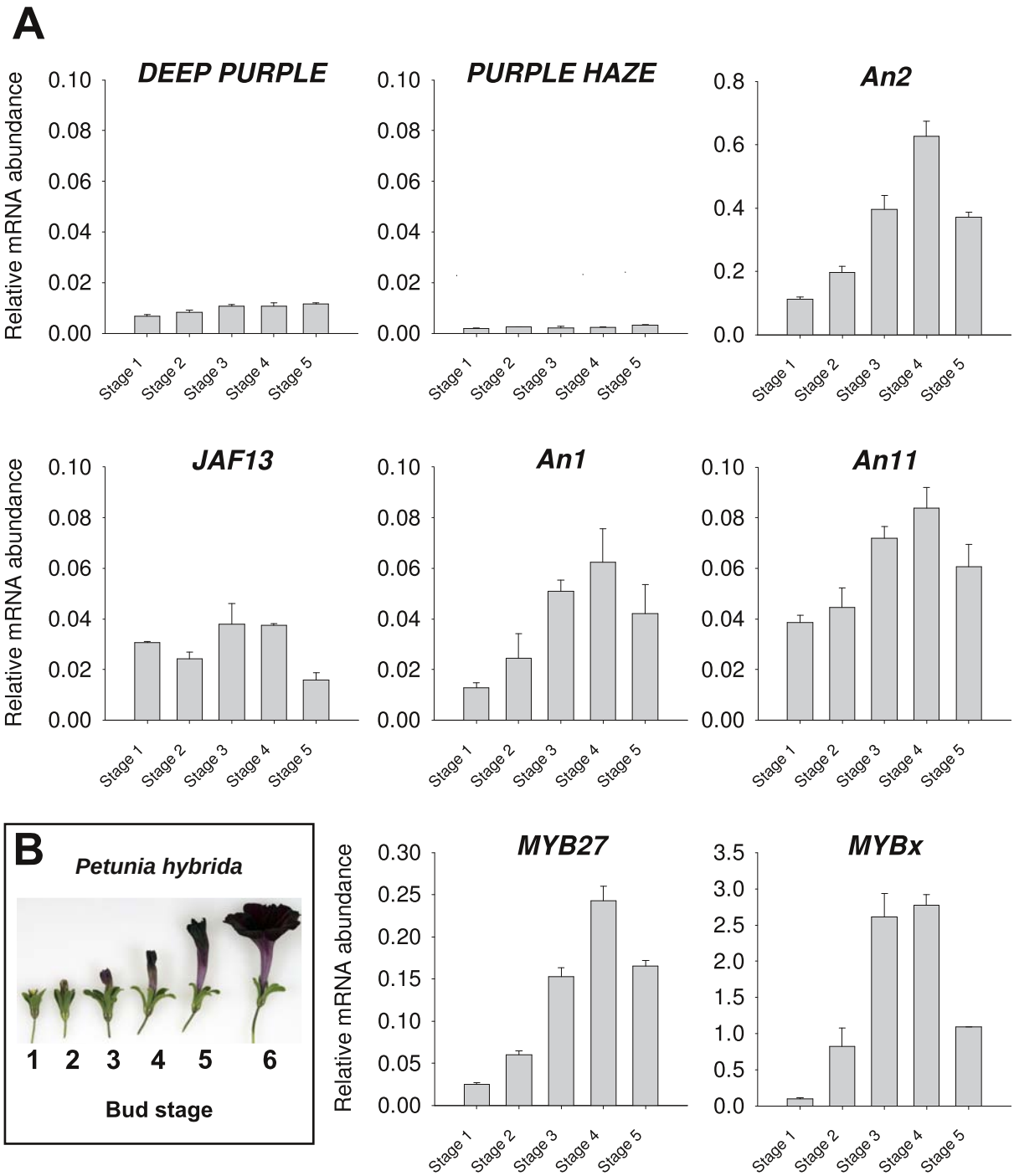


Figure 4.3 A. Transcript abundance of transcription factors involved in anthocyanin regulation in petals of developing V26 petunia flower buds. Relative transcript abundance was determined by qRT-PCR, normalised to the geometric mean of *ACT2* and *EF1α*. Means ± SE, *n*=3 are reported. **B.** Developmental flower bud series used for V26 petunia samples.

pigmented, masking these vein-associated pigmentation phenotypes later in bud development. V26 petunia buds are visibly coloured with anthocyanin by stage 3.

DPL and *PHZ* were expressed at a low level throughout all stages of flower bud development. *An2* was expressed at very high levels, peaking at stage four. *An1* and *MYB27* also shared the same expression profile as *An2*, with expression peaking at stage 4. *An11* was expressed throughout bud development, but also peaked at stage 4. *MYBx* was expressed to extremely high levels, reaching a maximum at stages 3 and 4, before declining. *JAF13* was expressed throughout flower bud development.

W59 is a petunia line which harbours mutant alleles of *an2* (corolla) and *an4* (flower tube, anthers). The floral phenotypes of W59 petunia is shown in Figure 4.4A. Flowers have a blotchy 'tie-dyed' appearance, with anthocyanin accumulation on the abaxial petal surface. This pigmentation occurs early during flower development (stages 2 to 3, not shown). It appears to be induced by light because in opening flower buds, petal tissue that was folded within the bud, and protected from light, remain white, while exposed petal tissue is purple with anthocyanin. W59 flowers lack reticulate vein-associated pigmentation (venation) in the inner flower tube, which is seen in many petunia cultivars (e.g. V30, V26, MP). Despite lacking venation on the inner petal surface, weak parallel venation on the outer surface of the flower tube is still visible.

The W59 alleles of *DPL* and *PHZ* were amplified from genomic DNA, cloned and sequenced. The W59 allele of *PHZ* coding sequence appeared free of mutations, insertions or deletion, suggesting that this allele is functional. The *DPL* allele from W59 contains a 1.4 kb insertion within intron two (Figure 4.4B), and closer analysis showed that this insertion contains a putative long terminal repeat (LTR) retrotransposon with 226 bp inverted terminal repeats. Further sequence analysis of the LTR insertion showed no similarity to any known retrotransposons, with no identifiable open reading frames.

Transcript abundance of *DPL*, *PHZ* and *An1* was determined in W59 petunia, to assess the relationship between pigmentation phenotypes and the activities of *DPL* and *PHZ* (Figure 4.4C). Transcripts for *DPL* were detected at very low levels, which reduced further during flower bud development from stage 3. *PHZ* transcripts were abundant,

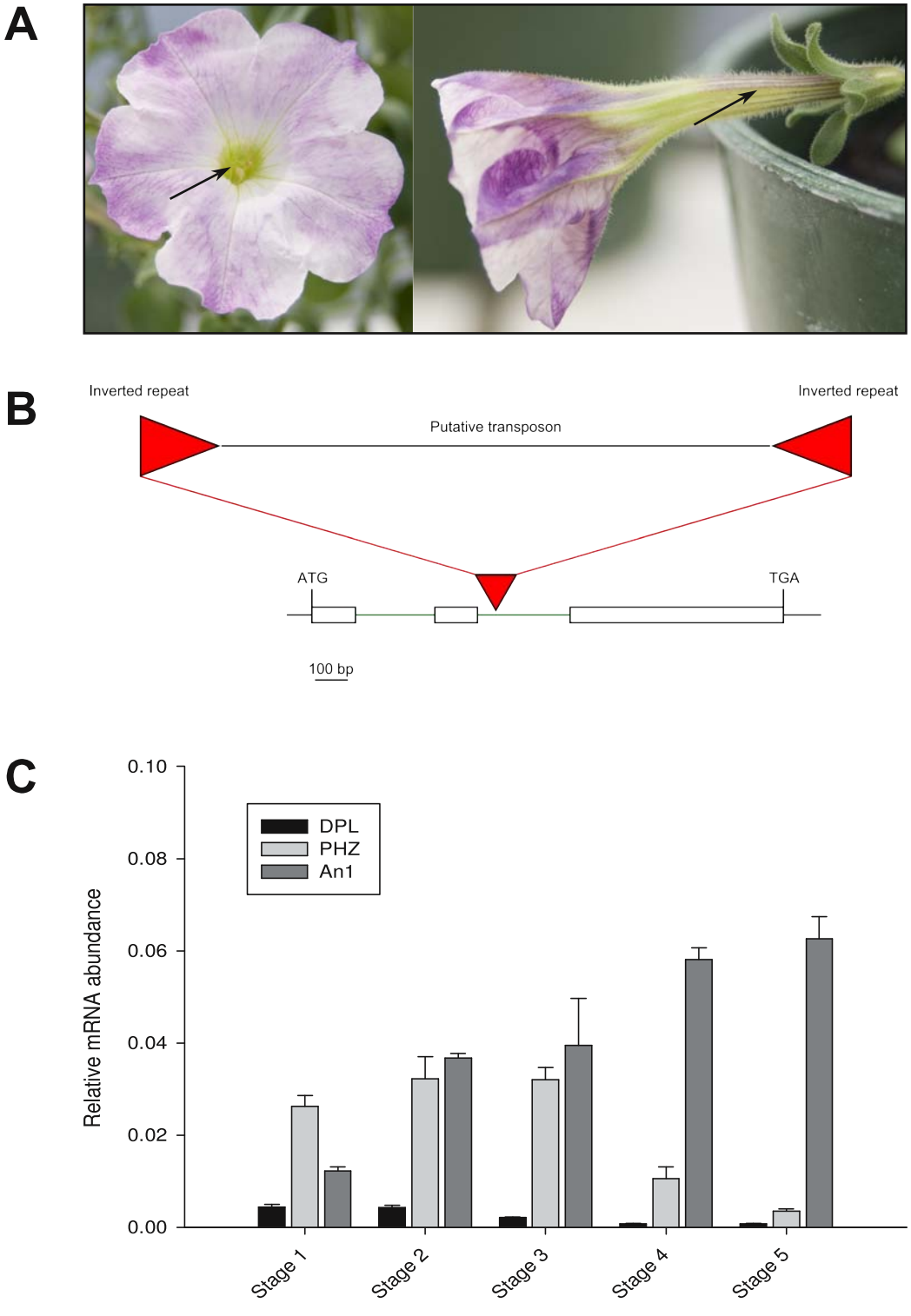


Figure 4.4 A. Floral pigmentation phenotypes of W59 petunia, a fully open flower and an opening bud. Arrows indicate the lack of adaxial flower tube venation, and parallel venation on the abaxial surface. **B.** The gene structure of the W59 *DEEP PURPLE* allele, containing a putative Long Terminal Repeat (LTR) retrotransposon within intron 2. **C.** Transcript abundance for *DEEP PURPLE*, *PURPLE HAZE* and *An1* in developing flower buds of W59 petunia (refer to Figure 4.3 B for *P. hybrida* bud series). Relative transcript abundance was determined by qRT-PCR, normalised to the geometric mean of *ACT2* and *EF1α*. Means ± SE, *n*=3 are reported.

and expressed early, especially from stages 1 to 3. *An1* transcripts increased during bud development. Overlap in the expression of *PHZ* and *An1* occurred during stages 1 to 3, reaching similar expression levels during stages 2 and 3.

4.2.3 MYB repressors

The MYB DNA binding domain of MYB activators and repressors involved in anthocyanin regulation in petunia and *Arabidopsis* were aligned (Figure 4.5). The DNA binding domains of DPL and PHZ are similar to the known anthocyanin regulators PhAN2 and PhAN4 from petunia, and to the *Arabidopsis* anthocyanin regulators AtPAP1 and AtPAP2.

The putative R2R3 repressor PhMYB27 from petunia shares a high level of sequence similarity to the MYB activators, particularly in the R3 region. The R3 repressor AtMYBL2 is similar to MYB27 and the anthocyanin activators in the R3 region, but is truncated, lacking the R2 domain. The amino acids required for interaction with a bHLH partner were conserved in all the aligned MYBs.

AtCAPRICE (CPC) and AtTRIPTYCHON (TRY) from *Arabidopsis*, and PhMYBx from petunia, are small R3 MYB factors. These factors show similarity to one another, but share less sequence similarity to the other MYBs discussed.

4.3 Discussion

4.3.1 Light-induced vegetative pigmentation

The expression of the MYB factor *PHZ* strongly correlated to light-induced vegetative pigmentation in Mitchell petunia. This gene was initially identified as a putative anthocyanin regulator that was differentially expressed under high light, but not shade conditions (Albert, 2006). Quantitative transcript analysis confirmed that *PHZ* was induced strongly by high light (Figure 4.1), which confirmed previous semi-quantitative results (Albert, 2006). *Lc* petunia constitutively express a bHLH anthocyanin regulator

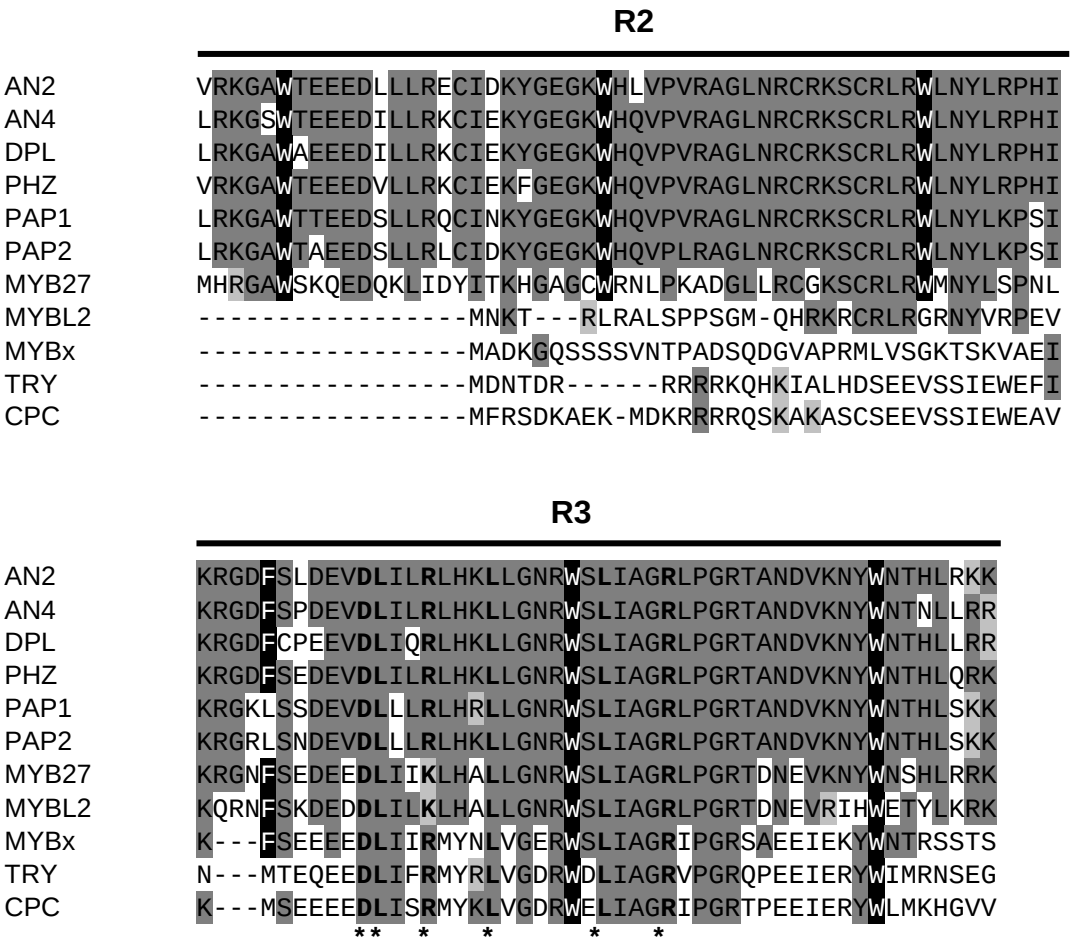


Figure 4.5 CLUSTALW alignment of the MYB domain from petunia and *Arabidopsis* MYB activators and repressors. Conserved residues are highlighted in grey. Residues that make up the MYB repeats are highlighted in black. Residues involved with bHLH interaction are in bold-type and indicated *

(*Leaf colour*), and have enhanced light-induced pigmentation. Under shade conditions, *Lc* petunia were almost completely green (Albert et al., 2009). However, with high light treatment, *PHZ* was induced (Albert, 2006) and the *Lc* petunias became deeply pigmented with anthocyanin. This strongly suggests that *PHZ* mediates light-induced vegetative pigmentation, in both MP and *Lc* petunias. The MYB factor *DPL* was expressed under shade conditions, but was also induced 5-fold with high light (Figure 4.1B). The light induction of *DPL* was not detected previously by semi-quantitative methods (Albert, 2006). This suggests *DPL* may also contribute to light-regulated anthocyanin pigmentation, although its function in shade conditions is unclear.

The bHLH factor *An1* is regulated by high light in petunia leaves. *An1* has previously been shown to be an essential regulator of anthocyanin production in flowers (Spelt et al., 2000), although the involvement of this factor in vegetative pigmentation patterns was previously unknown. Transcripts for *An1* were not detected in leaves of shade-grown petunias, but high light induced the expression of *An1*. While the total level of *An1* transcripts may appear low, this correlates well with the weak pigmentation phenotype, which was limited to sub-epidermal cells (Bradley et al., 1998; Albert et al., 2009). The other known bHLH factor *JAF13* was expressed constitutively along with the WDR factor *An11*. The involvement of *JAF13* in anthocyanin regulation is unclear. It is homologous to known bHLH factors, *Leaf colour* (maize) and *Delila* (*Antirrhinum*) (bHLH1 class), but is not functionally redundant with *An1* (bHLH2 class), as *an1*$^{-}$ mutants completely lack anthocyanin in flowers, despite expression of *JAF13* (Spelt et al., 2000). It is also interesting that in shade-grown leaves, *DPL* (MYB), *JAF13* (bHLH) and *An11* (WDR) are all expressed, yet anthocyanins fail to accumulate. It is not known if these three factors are expressed in the same tissues or cell types. The induction of *An1* by high light suggests this bHLH factor is likely to play a central role in regulating light-induced anthocyanin pigmentation, together with the MYB factors PHZ and DPL and the WDR, AN11.

The putative active repressor *PhMYB27* was expressed highly in shade-grown leaves, and was down-regulated by high light. The expression pattern for *PhMYB27* in shade- and high light-grown leaves supports a role for preventing anthocyanin production in shade-grown leaves. Under high light conditions, down-regulation of *PhMYB27* and up-regulation of the MYB activators *PHZ* and *DPL* provides a mechanism that accounts

for the activation of the anthocyanin biosynthetic genes. The down-regulation of a MYB repressor and up-regulation of MYB activators for anthocyanin synthesis has recently been shown in *Arabidopsis*. Here, *AtMYBL2* transcripts were reduced with high light treatment, while the MYB activators *AtPAP1* and *AtPAP2* were up-regulated along with the bHLH factor *AtTT8* (homologous to *An1*) (Dubos et al., 2008). The results shown here in petunia are consistent with the findings in *Arabidopsis*.

PhMYB27 contains an EAR motif in its C terminal region, which supports the putative function as a repressor. PhMYB27 encodes a highly similar protein to that of *FaMYB1* from strawberry, which was shown to bind the petunia bHLH factors JAF13 and AN1 and repress anthocyanin synthesis (Aharoni et al., 2001). *PhMYB27* also shares similarity to *AtMYBL2,* which also contains the residues in the R3 MYB domain required to bind bHLH partners (Figure 4.5). In addition to the bHLH interaction residues, the DNA binding domains of AtMYBL2 and PhMYB27 are very similar to their MYB activator counterparts, which might suggest they bind the same or similar DNA sequences.

The proposed models for the repressive function of AtMYBL2 suggest AtMYBL2 sequesters bHLH partners into inactive complexes (Dubos et al., 2008; Matsui et al., 2008) or binds through a bHLH to a pre-existing activation complex ($MYB_{activator}$ - bHLH-WDR) where it exerts its repressive activity (Matsui et al., 2008). Repression may be achieved through these means. However, if sequestration of the bHLH factor alone was required, then an active repression motif would be redundant. Truncated *At*MYBL2ΔC which lacked the C terminal repression domain was still able to bind bHLH partners, but failed to repress target genes and failed to complement a *mybl2* mutant (Matsui et al., 2008). The proposed models do not allow for direct DNA binding by MYBL2 with a bHLH partner to allow active repression to occur. It was suggested that MYBL2 may not bind DNA, as removal of the C terminal domain (containing repressive motifs), and the addition of a viral activation domain failed to activate a DFR_{pro}*:LUC* reporter construct (Matsui et al., 2008). However, MYBL2 may require a bHLH partner to help facilitate DNA binding, and then require the motifs in the C terminal to actively repress transcription.

Based on these collective findings, the model proposed in this thesis is that a $MYB_{repressor}$ - bHLH-WDR complex forms (WDR through bHLH), which binds target genes and actively represses transcription through repressive C-terminal motifs, possibly by inhibiting the basal transcription machinery and/or recruiting histone modification enzymes (Figure 4.6A), as has been proposed for repression through EAR motifs (Kazan, 2006).

The small R3 MYB, ***MYBx***, was induced by high light treatment in Mitchell petunia leaves. The modest transcript levels under high light correlated to the weak pigmentation in leaves. MYBx is proposed to act as a competitive inhibitor (Figure 4.B), as it was shown to bind the bHLH factors JAF13 and AN1 and because over-expression suppressed floral anthocyanin production (Kroon, 2004; Koes et al., 2005). Similar findings have recently been shown in *Arabidopsis*, where *CAPRICE* (*CPC*) over-expression was shown to repress the expression of the anthocyanin biosynthetic genes, particularly the late biosynthetic genes that are regulated by MYB-bHLH-WDR complexes (Zhu et al., 2009). Furthermore, *cpc* mutants produce higher levels of anthocyanins than wild type plants during stress conditions, suggesting CPC normally functions as a repressor of anthocyanin synthesis, in addition to its roles in regulating epidermal cell fates (Zhu et al., 2009). Induction of ***MYBx*** when tissues begin to accumulate anthocyanin pigments could provide feedback inhibition, to prevent more anthocyanin than is needed to be made. The competitive nature of this type of repression could lead to dampening of the anthocyanin induction response, providing a mechanism for fine control.

The synthesis of anthocyanins in leaves is a balance between photoprotection, and inhibiting plant growth and development. The accumulation of high levels of anthocyanins in leaves results in internal shading as blue-green light is screened from under lying photosynthetic cells (Hughes et al., 2005; Albert et al., 2009). Under high light conditions, this can provide photoprotection from excess quanta, but if light is limiting, the presence of anthocyanins could severely affect energy capture, limiting plant growth. The repression of anthocyanin synthesis by MYB27 under low light conditions, and feedback repression by MYBx, upon anthocyanin synthesis, would allow for strict control of anthocyanin accumulation in photosynthetic tissues.

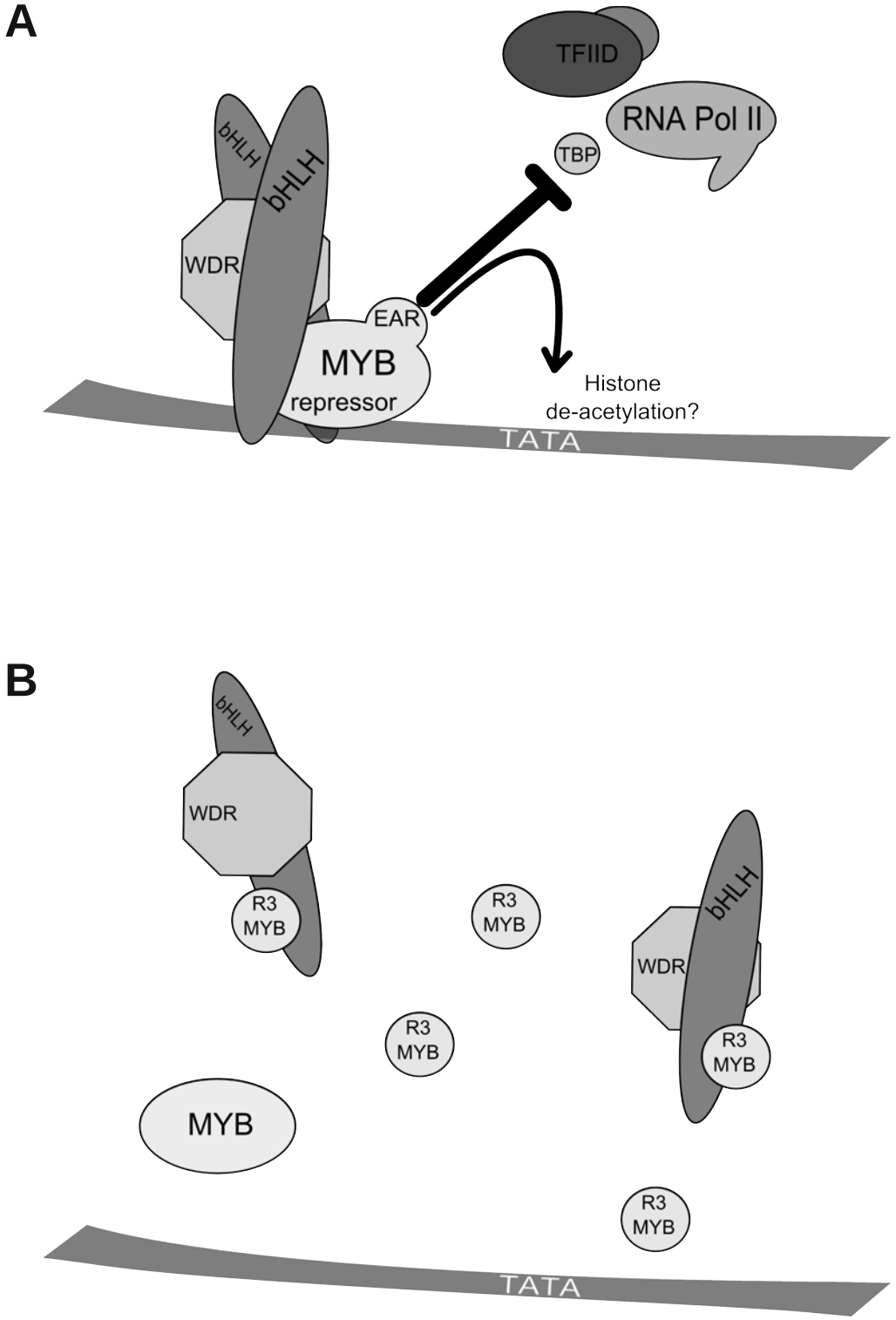


Figure 4.6 Models for repression of anthocyanin biosynthetic genes. **A.** A transcriptional repression complex, consisting of bHLH, WDR and R2R3 MYB repressors. The MYB repressor actively represses transcription through the EAR motif in the C terminal. Repression may occur by inhibiting the basal transcription machinery, and/or by recruiting histone deacetylases to make the DNA less accessible. **B.** Competitive inhibition by small R3 MYBs. The R3 MYB factors compete with R2R3 MYB activators for bHLH binding partners.

4.3.2 Floral pigmentation phenotypes

Mitchell petunia (*an2*$^{-}$ *an4*$^{-}$) has white flowers, with vein-associated anthocyanin pigmentation. Petals are white due to a null allele of *an2* (frame-shift), the dominant MYB factor controlling anthocyanins in petals, although the transcripts for this gene are expressed normally (Figure 4.2). The flower tube of MP is coloured with a highly reticulated patterning of anthocyanin associated with the veins (venation). Venation is a pigmentation phenotype distinct from tube colour controlled by the *An4* locus (Wiering, 1974). Venation develops early during flower bud development, and the expression profile of either *DPL* or *PHZ* could account for this patterning (Figure 4.2). Occasionally, MP buds show weak bud blushing that is induced by light, which fades upon flower opening. This bud blushing is most noticeable in *Lc* petunia (MP background) (Bradley et al., 1998), which enhances the existing pigmentation patterns. A complicating feature of MP is that it appears to contain the dominant *Fading* locus, which destabilises, or degrades anthocyanin from the petal limb, although flower tube venation appears to be somewhat resilient (de Vlaming and van Eekeres, 1982). The venation and bud blushing phenotypes may be due to activities of *DPL* and *PHZ*.

W59 is an *an2*$^{-}$ *an4*$^{-}$ petunia line that lacks flower tube venation, but displays light-induced bud-blushing. *PHZ* transcripts were shown to be expressed early during flower bud development, and significantly overlapped with the bHLH partner *An1* at stages 1 to 3, which coincides with the development of the bud-blush pigmentation pattern (Figure 4.4C). The light-induced nature of this pigmentation, the expression profile in flowers and the prior demonstration that *PHZ* is highly induced by light (Figure 4.1B), supports the hypothesis that *PHZ* controls the bud blushing pigmentation pattern in petunia flowers.

Bud blushing is a common floral pigmentation pattern in many ornamental species. *Antirrhinum* displays bud blushing on the dorsal petal that encloses the developing bud, in response to lighting intensity, but is only observable in lines which lack *Rosea1* (MYB) activity, such as *rosea*^{dorsea} genotypes (Schwinn et al., 2006). Vegetative pigmentation and bud blushing is linked to the complex *Rosea* locus, which contains two identified MYB transcription factors, *Ros1* and *Ros2* (Schwinn et al., 2006). The

molecular basis for the *rosea*^{dorsea} phenotype is not known, although it is hypothesised that a third MYB factor may be located at the *Rosea* locus (Pathirana, 2007). The pigmentation phenotype of W59 petunia phenocopies *Antirrhinum rosea*^{dorsea}, suggesting similar regulatory systems exist to regulate complex pigmentation patterns.

W59 petunia lacks flower tube venation, and contains a transposon-insertion allele of *DPL* (*DPL::LTR*). Flower tube venation phenotypes occur independently of *An2* and *An4* in petunia (Wiering, 1974), raising the possibility that a distinct MYB regulator, possibly *DPL*, determines this pigmentation pattern. The insertion of the LTR element lies within intron two, and does not affect the coding sequence for *DPL*. However, while the splice-donor and -acceptor sites are intact, the insertion of the LTR element close to the intron boundary has displaced additional splicing signals (5′ UA rich element) which may have prevented correct splicing (Lorković et al., 2000). This potentially resulted in a reduced level of correctly processed, functional transcripts, which may account for the loss of venation phenotype. qRT-PCR showed the level of *DPL* was low (Figure 4.4C). However, the primers used would not have discriminated between splice variants, so the abundance of fully spliced functional transcripts may indeed be much lower. It appears the transposon insertion allele has resulted in a loss of venation phenotype.

The venation phenotype has been described previously, with at least three loci involved in venation patterns in different parts of the flower; *Venation1* (*Ve1*), *Venation2* (*Ve2*) and *Venation3* (*Ve3*) (Wiering, 1974; Wiering et al., 1979; Wiering and de Vlaming, 1984). However, the detailed descriptions of the phenotypes suggest only *Ve1* is active in the flower tube, and dominant alleles confer venation. The description of a close reticulate venation in the flower tube in *Ve1*$^{+}$ individuals, and weak longitudinal pigmentation along veins in *ve1*$^{-}$ individuals (Wiering, 1974; Wiering et al., 1979) accurately matches the phenotype observed in W59 petunia. I speculate *Venation1* encodes *DPL*. It is difficult to confirm unequivocally that *DPL* resides at the *Ve1* locus, without extensive genetic resources to enable genotyping and segregation analyses.

Venation phenotypes are a common pigmentation pattern in flowers. Vein-associated anthocyanin pigmentation in the petals of *Antirrhinum* is controlled by a MYB transcription factor *Venosa* (*Ven*) (Schwinn et al., 2006). The spatial expression of *Ven*

radiates from veins (Shang, 2006), indicating the localisation of this MYB transcription factor determines the pigmentation patterning. *Ven* can function with either bHLH factor in *Antirrhinum*, *Delila* (*JAF13* orthologue) or *Mutabilis* (*An1* orthologue) (Schwinn et al., 2006). *Del* is expressed in the tube and lobes (limb) while *Mut* appears only to be expressed in the lobes (Goodrich et al., 1992; Schwinn et al., 2006). The venation patterning in *Antirrhinum*, therefore, appears to be determined by *Ven*. In petunia, *Venation1* (*Ve1*) controls vein-associated pigmentation in the flower tube, and so it is possible this is also expressed in a vein-associated manner.

V26 petunia contains functional copies for all known anthocyanin regulators, with the exception of the MYB factor that controls flower tube and anther pigmentation, *an4*. The high activity of *An2* results in very dark petal pigmentation (Figure 4.3), which masks more subtle pigmentation phenotypes such as bud blushing and venation. The expression of both *DPL* and *PHZ* is reduced in V26 compared to MP. If the flower bud is already highly pigmented with anthocyanin, then internal shading by the anthocyanins may prevent full induction and expression of *PHZ*, which was shown to be induced by light in leaves. The V26 alleles of *DPL* and *PHZ* have no obvious defects, and V26 plants show light-induced vegetative pigmentation (Appendix 4), which suggest that DPL and PHZ are functional.

The transcription factors *An1*, *JAF13* and *An11* were expressed during flower development. Expression of *An1* and *An11* matched that of *An2*, in fully coloured V26 petunia (Figure 4.3A), consistent with previous findings (de Vetten et al., 1997; Quattrocchio et al., 1998; Spelt et al., 2000). The expression of *JAF13* did not match that of the other regulators (Figure 4.2A and Figure 4.3A) (Quattrocchio et al., 1998; Spelt et al., 2000). The role of JAF13 in anthocyanin regulation is unclear, as its function is not redundant with AN1 (Spelt et al., 2000).

The putative repressor *MYB27* is expressed in a similar pattern to the *MYB* activators, which may provide feedback repression. *MYB27* was expressed consistently throughout bud development in MP, which accumulates very little anthocyanin (Figure 4.2). However, in V26 petunia, *MYB27* was highly expressed and in a similar pattern to *An2*. Previously, *MYB27* expression was shown to be highly reduced in *an1*$^{-}$ mutants, which fail to produce anthocyanins (Spelt et al., 2000). Ectopic expression of an AN1-

glucocorticoid receptor fusion protein, with cyclohexamide treatment and dexamethasone induction restored expression of *MYB27* in *an1*$^{-}$ petals. This suggests that AN1 is a direct regulator of *MYB27*. The similarity of *MYB27* expression to *An2* in V26, and to *DPL* and *PHZ* in MP may indicate that AN1 regulates *MYB27* expression with a MYB partner. The requirement for an active repressor of anthocyanin production in petals is unclear, and further functional characterisation of MYB27 is required. It is interesting to note that the anthocyanin repressor *FaMYB1* from strawberry is also expressed during colour development in fruit, during ripening, and has been suggested to provide feedback regulation (Aharoni et al., 2001).

MYBx is expressed in response to anthocyanin accumulation. The level and timing of *MYBx* transcripts correlates with the accumulation of anthocyanins in leaves and flowers (Figures 4.1B, 4.2A and 4.3A), suggesting MYBx could provide feedback repression. V26 flowers are deeply pigmented and have high levels of *MYBx* transcripts from stage three, when buds become coloured (Figure 4.3A). Previously *MYBx* transcripts were shown to be severely reduced in *an1*$^{-}$ or *an11*$^{-}$ mutants, in which anthocyanins are completely absent (Kroon, 2004). This raised the hypothesis that *MYBx* may be regulated by a MBW complex. However, mutants in *an2* still accumulated *MYBx* transcripts, which is consistent with the findings in MP flowers (*an2*$^{-}$, *an4*$^{-}$) which have reduced levels of anthocyanins, but are not completely absent (Figure 4.2A). *MYBx* may be regulated by *DPL* or *PHZ* in the absence of *An2*, or alternatively, *MYBx* may be expressed in response to anthocyanin accumulation. The hierarchical interactions of these transcription factors will be examined further in Chapter 7.

4.3.3 Isolation of a novel retrotransposon

The W59 allele of *DPL* contains a putative LTR retrotransposon. This LTR element contains 226 bp inverted terminal repeats, a poly purine tract adjacent to one LTR, and a putative primer binding site which shows some similarity to tRNAs and viral sequences. Most characterised LTR retrotransposons are autonomous, and contain open reading frames (ORFs) for proteins involved in replication, movement and packaging (e.g.

reverse transcriptase). The LTR transposon within *DPL* appears to lack these ORFs and BLAST analysis of the *DPL::LTR* sequence shows multiple hits to BAC clones from other solanaceous species such as potato, but the region of similarity was limited to the inverted terminal repeats. Non-autonomous LTR transposons, which lack some or all of the coding sequences for transposition, have been described previously. These include *Zeon-1* and *Bs1* from maize, which lack a full complement of coding sequences for replication and movement (Jin and Bennetzen, 1989; Hu et al., 1995). More recently, a non-autonomous LTR transposon, *Dasheng*, was characterised in rice, which lacks all characteristic coding sequences of autonomous LTR retrotransposons (Jiang et al., 2002b; Jiang et al., 2002a). *Dasheng* appears to be activated by the autonomous LTR retrotransposon *RIRE2*, which may provide the machinery needed for replication and transposition. The LTR element found within *DPL* appears to fall into the same category as *Dasheng*, and related *Sukkula* elements from barley, which have, subsequently, been called large retrotransposon derivatives, or LARDs (Kalendar et al., 2004), which is the first element of this type to be identified in petunia.

The expression patterns of *DPL* and *PHZ* correlate to vegetative and floral pigmentation phenotypes. *DPL* expression is linked to vein-associated pigmentation in flowers, while *PHZ* has been linked to light-induced pigmentation in both leaves and developing flower buds. To further establish the link between the expression of *DPL* and *PHZ* to these pigmentation phenotypes, information about the spatial expression patterns for these genes is required. This will be examined in Chapter 5.

Chapter 5

Spatial localisation of *DEEP PURPLE* and *PURPLE HAZE* promoter activity

5.0 Introduction

A family of MYB transcription factors controls pigmentation patterning in petunia. *An2* controls the predominant, fully coloured petal phenotypes, while *An4* controls anthocyanin synthesis in anthers and the flower tube (Wiering, 1974; Quattrocchio et al., 1999; Kroon, 2004). The expression patterns for *DPL* and *PHZ*, shown in Chapter 4, suggest that these two MYB factors regulate pigmentation patterns in both vegetative and floral tissues. The expression of *DPL* was linked to venation pigmentation in the flower tube of petunias (Figures 4.2 and 4.4). In *Antirrhinum*, the MYB transcription factor *Venosa* controls vein-associated anthocyanin pigmentation, and is expressed in the cells adjacent to the vasculature (Schwinn et al., 2006; Shang, 2006). The venation patterns in petunia and *Antirrhinum* may be determined by similar mechanisms. The activity of *PHZ* was linked to light-induced vegetative pigmentation patterns. However, the floral bud-blushing pattern observed in W59 petunia also correlated with the expression pattern for *PHZ* (Figure 4.4). The spatial expression patterns for *DPL* and *PHZ* would further link these putative anthocyanin regulators to pigmentation patterns observed in petunia.

The *GUSa* gene (encoding β-glucuronidase) from *E.coli* was first established as a reporter gene in plants by Jefferson et al., (1987), and has since been used extensively to determine gene expression patterns and to establish transformation protocols. The sensitivity and robustness of this system, has resulted in GUS becoming the reporter gene of choice, particularly when weak promoters are being investigated. Fluorescent proteins (e.g. green fluorescent protein, GFP) are an alternative, commonly used reporter system. However, low sensitivity and autofluorescence of plant tissues can make detection of fluorescent proteins difficult (de Ruiter et al., 2003; Shang, 2006).

The most commonly used substrate for the histochemical detection of GUS activity is X-gluc (5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid). Upon cleavage of X-gluc by GUS, the soluble colourless product must dimerise and undergo oxidation to form a coloured insoluble indigo dye, which enables visual detection of GUS activity (Jefferson, 1987). The rate of oxidation is important, as diffusion of the primary cleavage product prior to oxidation can result in inaccurate localisation of GUS activity. Indeed, to overcome the problem of diffusion, the oxidation catalysts potassium ferricyanide ($K_3Fe(CN)_6$) and potassium ferrocyanide ($K_4Fe(CN)_6$) are commonly included (Lojda, 1970; Jefferson, 1987). GUS activity assays *in vitro*, using the fluorescent substrate 4MUG (4-methylumbelliferyl-β-D-glucuronide), is an alternative commonly used substrate (Jefferson, 1987; Jefferson et al., 1987). However, the MUG assay is not suitable for histochemical detection and, therefore, cannot provide information about spatial expression patterns.

The spatial expression patterns for *DPL* and *PHZ* were investigated by generating stable transgenic petunias expressing promoter:intronGUS (iGUS) reporter constructs. The spatial localisation of GUS activity was determined in petunia seedlings and flowers by histochemical detection with X-gluc.

5.1 Materials and methods

5.1.1Promoter:iGUS reporter lines

The promoters for *DPL* and *PHZ* were isolated by gene-walking (Section 2.12). The promoter and 5′UTR for *DPL* (1.7 kb) and *PHZ* (1 kb) were amplified from MP genomic DNA with the primers NA78/NA79 and NA81/NA102 respectively. The primers introduced restriction sites to the ends of the promoter, so that the promoter could be directionally cloned into pDAH3 which had been prepared with *Nhe*I and *Nco*I (removing the existing *35S* promoter), placing the promoter in front of an intron-GUS (iGUS) reporter gene (*uidA*) encoding β-glucuronidase. The presence of the intron within the GUS coding sequence prevents bacterial expression. The promoter was cloned in-frame with iGUS, through the *Nco*I restriction site, to ensure premature start

codons were not introduced that may compromise the activity of the construct. The *DPL*$_{pro}$:iGUS and *PHZ*$_{pro}$:iGUS constructs in pDAH3 were named pNWA33 and pNWA34 respectively. The *Not*I fragments of pNWA33 and pNWA34 were cloned into the pART27 binary vector (Gleave, 1992), generating pNWA35 and pNWA36 respectively. The control constructs were 2×*35S*$_{pro}$:iGUS/pART27 (pNWA37) and pART27 empty vector. The binary vector constructs were transformed into *Agrobacterium* GV3101 by electroporation (Section 2.4.4). Stable transgenic Mitchell petunia plants carrying the *DPL*$_{pro}$:iGUS, *PHZ*$_{pro}$:iGUS, 2×*35S*$_{pro}$:iGUS and pART27 (empty vector) constructs were generated by leaf-disc transformation (Section 2.9). Shoots were confirmed to contain the transgene cassette by PCR, using the primers NA27/GUSAS (*DPL*$_{pro}$*:iGUS*), NA26/ GUSAS (*PHZ*$_{pro}$*:iGUS*) and CMF/GUSAS (*2×35S*$_{pro}$*:iGUS*).

5.1.2 Plant material and growth conditions

Fifteen independent lines (four clones each) were exflasked and grown in the GMO greenhouse during Summer 2008/2009. Plants were sampled and stained for GUS activity.

The primary transgenic lines MP/37/1, MP/p27/d, MP/35/1, MP/35/2, MP/35/3, MP/36/1, MP/36/2 and MP/36/3 were self-crossed, and the seed was collected. Seed was sterilised for 10 min in 15% (v/v) Janola™ bleach (final conc. 0.63 % (w/v) sodium hypochlorite, 15 mM NaOH) and a few drops of Tween20, followed by three washes with sterile water. The seed was imbibed overnight in 100 mg L^{-1} GA_3. The GA_3 solution was removed and the seed was suspended in sterile 0.1% (w/v) agarose and plated onto solid half-strength MS media (Appendix 1.5). The seed was allowed to germinate within a tissue culture room 25 °C, 30 µmol m^{-2} s^{-1} light, and 16 h photoperiod. Seedlings were stained for GUS activity four d after germination.

5.1.3 Histochemical detection of GUS

β-glucuronidase activity was detected in fresh petunia tissues with the histochemical substrate 5-bromo-4-chloro-3-indolyl glucuronide (X-gluc).

Tissues were prepared for staining by dipping stem, leaf and flower tube pieces in diethyl ether for 20 s, rinsing for 30 s in 90% (v/v) ice cold acetone and then rinsing twice in 50 mM phosphate buffer (pH 7) (Appendix 1.8.1). The samples were then submerged in X-gluc staining solution (Appendix 1.8.2) and placed in a vacuum chamber for 10 min, to infiltrate the staining solution throughout the samples. Samples were incubated at 37°C overnight. Samples were decolourised with ethanol: acetic acid (95:5 v:v) at 55°C for 30 min, and stored in 70% (v/v) ethanol. A modified X-gluc staining solution was supplemented with 0.1% (v/v) 2-mercaptoethanol so that GUS activity could be detected in tissues with a high phenolic content.

A modified procedure was used in order for petal limb tissue to be successfully stained for GUS activity. Petal limb tissue was pre-treated with diethyl ether for 30 s, rinsed for 1 min in 90% (v/v) ice cold acetone and rinsed twice in 50 mM phosphate buffer (pH 7) prior to staining. Petal limb tissue was then covered in X-gluc staining solution (Appendix 1.8.2) that was supplemented with 1 mM $K_3Fe(CN)_6$ and 1 mM $K_4Fe(CN)_6$, and vacuum infiltrated for 10 min. Samples were then incubated overnight at 37°C. Samples were stored in 70% (v/v) ethanol.

Germinated seedlings were stained for GUS activity by washing in ice-cold 90% (v/v) acetone for 5 min, and rinsing twice in 50 mM phosphate buffer (pH 7) prior to staining. Seedlings were covered in X-gluc staining solution (Appendix 1.8.2), vacuum infiltrated for 10 min, and incubated overnight at 37°C. Seedlings were destained with ethanol: acetic acid (95:5 v:v) at 55°C for 30 min. Seedlings were also stained in X-gluc solution supplemented with 1 mM $K_3Fe(CN)_6$ and 1 mM $K_4Fe(CN)_6$ (Jefferson, 1987), which were prepared, stained and cleared in the same way.

5.2 Results

5.2.1 Establishing promoter:iGUS petunia lines

More than 20 independent transgenic lines were generated harbouring *DPL_{pro}:iGUS* or *PHZ_{pro}:iGUS*, and 5 independent lines for 2×*$35S_{pro}$:iGUS* or pART27 (empty vector) controls. Each line was confirmed by PCR.

Preliminary staining of regenerated, rooted-shoots from three independent lines, maintained in tissue-culture, was performed for both *DPL_{pro}:iGUS* and *PHZ_{pro}:iGUS*, along with a positive control (*2×$35S_{pro}$:iGUS*) (Figure 5.1). *DPL_{pro}*:iGUS directed GUS activity in stems and leaves, and appeared to be associated with the vasculature. The *PHZ* driven GUS staining was weaker, and appeared as a faint blush to the apex, and to the leaf margins. The *2×$35S_{pro}$:iGUS* positive control stained throughout the leaves and roots, but staining in the stem was variable and patchy.

Following the preliminary staining, 15 independent petunia lines for each of *DPL_{pro}:iGUS* and *PHZ_{pro}:iGUS*, and four 2×*$35S_{pro}$:iGUS* and four pART27 lines were exflasked and grown in the GMO greenhouse, each with four clones.

5.2.2 Optimising GUS histochemical staining in petunia

The lack of staining in greenhouse grown *2×$35S_{pro}$:iGUS* lines, which had previously stained well in tissue culture, indicated that many tissues in petunia plants, including leaves, stems and trichomes, produce inhibitors of GUS activity. The failure to stain tissues from the *2×$35S_{pro}$:iGUS* positive control lines could not be attributed to substrate penetration alone: pre-treatment with diethyl ether enabled good penetration with phosphate buffer/methanol/bromophenol blue, yet this treatment alone did not enhance the detection of GUS activity with X-gluc in leaves or stems. Freshly sectioned stem and leaf pieces failed to stain despite direct exposure of cut surfaces to X-gluc, whereas petal limb and ovary tissue stained well. It was noted that tissues that failed to stain became brown, and could not be cleared with acetic acid/ethanol (Figure 5.2). This indicated that oxidation of phenolic compounds was occurring (Lagrimini,

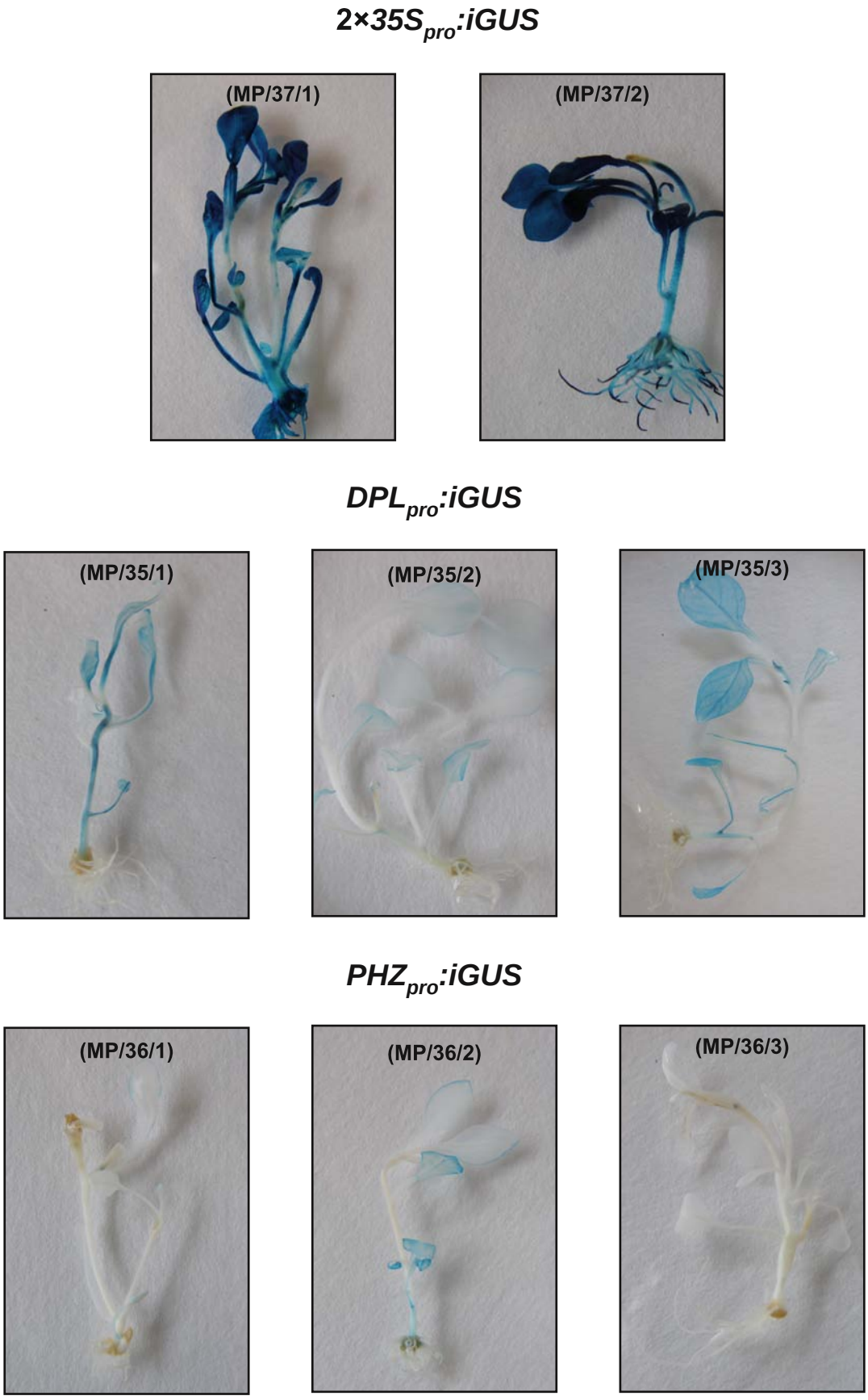


Figure 5.1 Regenerated rooted-shoots in tissue culture stained for GUS activity. *2×35S:iGUS* (MP/37/1, 2), *DPL*$_{pro}$*:iGUS* (MP/35/1, 2, 3) and *PHZ*$_{pro}$*:iGUS* (MP/36/1, 2, 3).

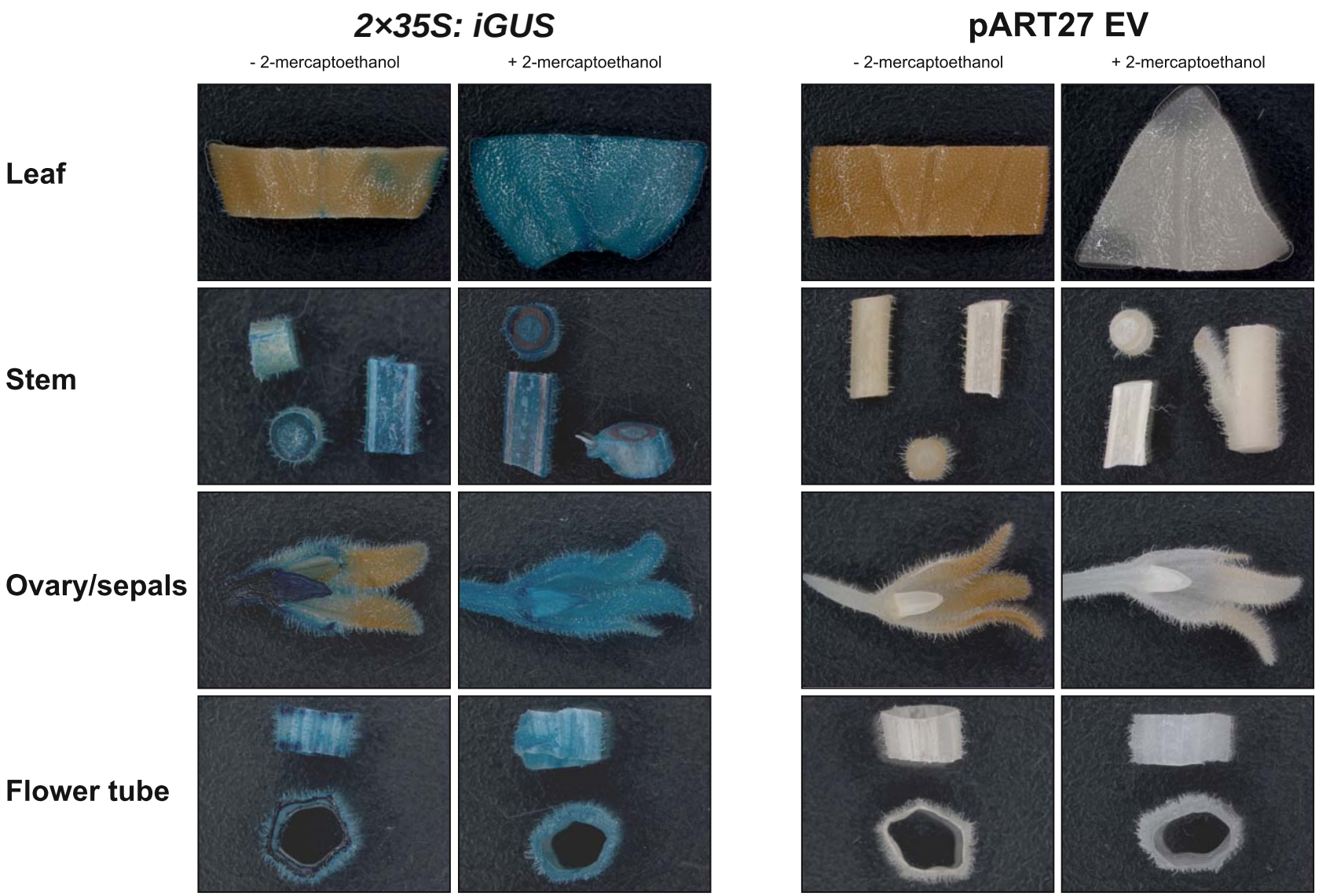


Figure 5.2 Oxidised phenolics inhibit histochemical detection of GUS activity in petunia. Fresh tissues of 2 × *35S$_{pro}$: iGUS* or pART27 empty vector petunia line stained for GUS activity with X-gluc ± 0.1% (v/v) 2-mercaptoethanol.

1991; Richard-Forget and Gauillard, 1997; Robards et al., 1999; Tomás-Barberan and Espín, 2001) and given that oxidised phenolics are known to bind proteins and nucleic acids (Pierpoint, 2003) were a likely cause of inhibition of GUS activity. Inclusion of either sodium ascorbate or 2-mercaptoethanol to the staining solution prevented oxidation of the phenolic compounds, thus preventing browning, and allowed GUS activity staining with X-gluc to occur. 2-mercaptoethanol was found to give more consistent prevention of browning, and therefore more consistent GUS staining with X-gluc (data not shown).

Subsequently, leaves, stems and flower tubes from *2×35S$_{pro}$:iGUS* and pART27 empty vector lines were stained for GUS activity, with and without 2-mercaptoethanol (Figure 5.2). In the absence of 2-mercaptoethanol, browning occurred, especially in the leaves, and indigo was not formed. The inclusion of 2-mercaptoethanol resulted in the detection of GUS activity with X-gluc, with dark, evenly stained tissues and no evident browning.

The flower tubes of the *2×35S$_{pro}$:iGUS* lines were unevenly stained. The cut surface of the tube, immediately accessible to the staining solution, was always more intensely stained. This initially appeared to be a substrate accessibility issue, but further modifications to sample preparation and staining/infiltration with dyes suggested penetration of the staining solution was occurring. The uneven tube staining was most noticeable when lower concentrations of X-gluc were used (data not shown).

The leaves, stems and flower tubes of *DPL$_{pro}$:iGUS* and *PHZ$_{pro}$:iGUS* petunia lines did not stain in standard X-gluc solution. However, inclusion of 2-mercaptoethanol prevented the oxidation of phenolic compounds, and allowed the detection of GUS activity. The staining was diffuse, and prevented reliable localisation of GUS activity (data not shown). It did appear that *PHZ$_{pro}$* was likely to be light responsive, as stem pieces that were coloured with anthocyanin on one side, once stained, showed more indigo on one side of the stem.

Because of the diffuse nature of the GUS staining in mature plants with modified X-gluc staining solution (containing 2-mercaptoethanol), shoots propagated in tissue culture were stained. The established cultures were sub-cultured on antibiotic-free

media and grew robustly. The initial staining results from the *DPL$_{pro}$:iGUS* and *PHZ$_{pro}$:iGUS* lines could not be reliably replicated. The 2×*35S$_{pro}$:iGUS* lines stained well in the roots and lamina, but failed to stain well in the stems and vasculature in leaves, and de-staining showed these tissues had browned.

5.2.3 Spatial expression of *DEEP PURPLE* and *PURPLE HAZE* promoters

Flower limb tissues were able to be stained for GUS activity, without the addition of antioxidants. However, tissue preparation was necessary to enable penetration of the staining solution into the petals. Brief treatment with diethyl ether was found to be the most effective treatment to ensure penetration of the cuticle by the X-gluc substrate.

Petals from eight independent lines for the *DPL$_{pro}$:iGUS* and *PHZ$_{pro}$:iGUS* were stained in standard X-gluc solution. The *DPL$_{pro}$:iGUS* lines stained throughout the petal limb. The staining appeared strongest in the fine veins, but was not exclusively associated with the vasculature of the limb. While the staining intensity varied between independent transgenic lines, the staining pattern was the same. The *PHZ$_{pro}$:iGUS* lines were weakly stained, but consistently showed a tie-dyed pattern of GUS staining on the abaxial petal surface on the petal limb. The 2×*35S$_{pro}$:iGUS* lines were always intensely, evenly coloured, while pART27 controls were never coloured.

The oxidation catalysts $K_3Fe(CN)_6$/ $K_4Fe(CN)_6$ were added to the staining solution to improve the spatial resolution of GUS localisation. The GUS localisation driven by *DPL$_{pro}$* and *PHZ$_{pro}$* activity in petal limb tissue is shown with 1 mM $K_3/K_4Fe(CN)_6$ added to the X-gluc staining solution (Figure 5.3). The same localisation patterns were observed for all independent lines, even when up to 3 mM $K_3/K_4Fe(CN)_6$ was added. Increasing concentrations of $K_3/K_4Fe(CN)_6$ reduced overall GUS staining intensity, especially for *PHZ$_{pro}$* lines, but still gave the same spatial localisation. Tissue browning and GUS inhibition was observed at the junction between the petal tube and limb, particularly over the major veins. This was exacerbated when high concentrations of oxidation catalysts (up to 3 mM) were included.

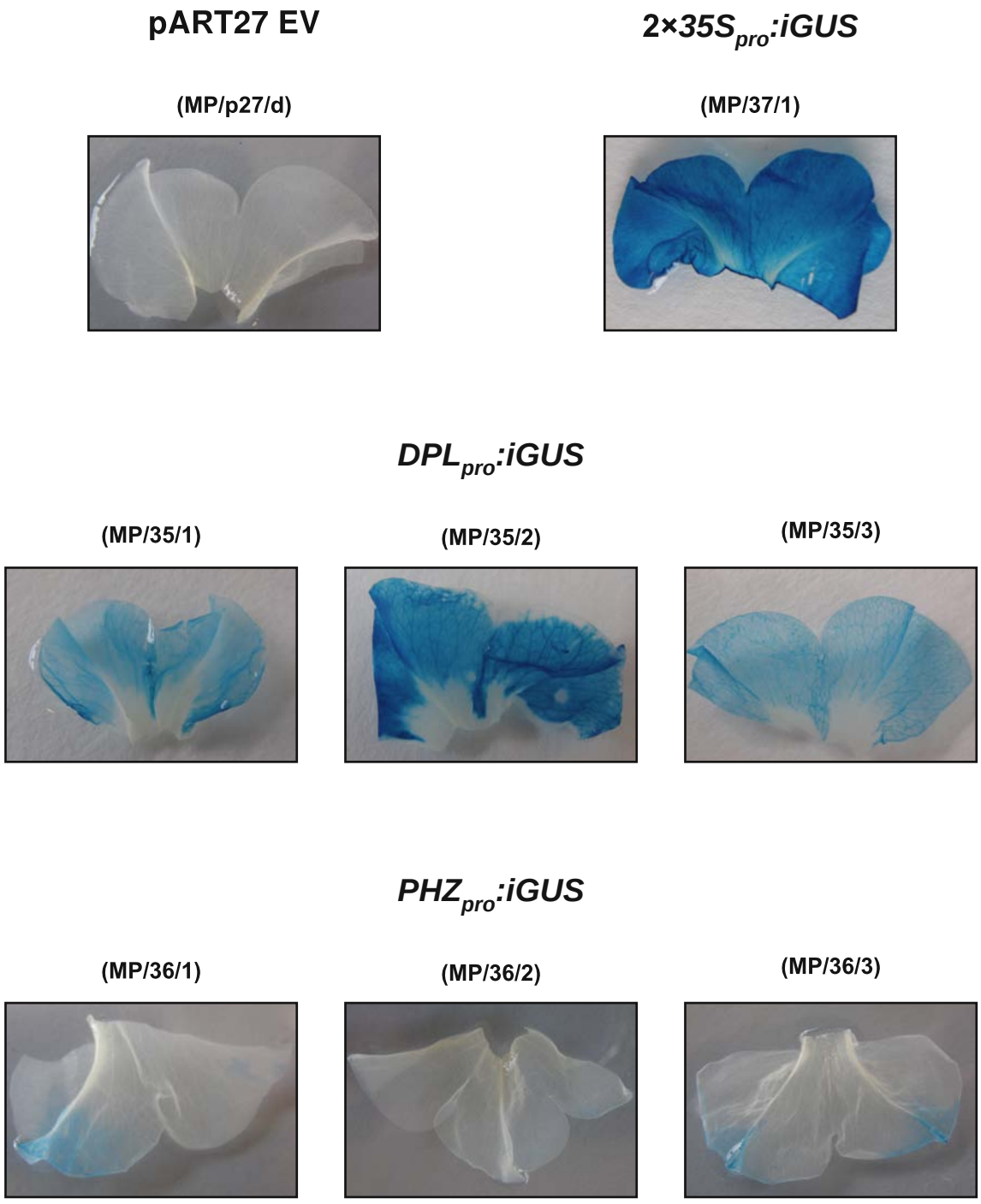


Figure 5.3 Petal limb tissue from independent transgenic petunia lines stained for GUS activity with X-gluc, in the presence of 1 mM K_3/K_4 $Fe(CN)_6$. The petunia lines stained were pART27 EV, *2×35S:iGUS* (MP/37/1), , DPL_{pro}*:iGUS* (MP/35/1, 2, 3), PHZ_{pro}*:iGUS* (MP/36/1, 2, 3).

It was proposed that germinated seedlings may contain lower levels of phenolics, which would allow the detection of GUS activity and localisation of the *DPL* and *PHZ* promoter activities in vegetative tissues. Three independent lines of DPL_{pro}*:iGUS* and PHZ_{pro}*:iGUS* were selected for further investigation. These lines had initially given the same spatial expression pattern in tissue culture, displayed a range of staining intensities, and had representative flower limb staining. One 2×$35S_{pro}$*:iGUS* and one pART27 line were also selected to include as controls. Plants were selfed and seed was collected.

Seeds were germinated and grown in tissue culture, and seedlings were stained for GUS activity (Figure 5.4). For the 2×$35S_{pro}$*:iGUS* seedlings, intense staining was observed throughout the cotyledons, apex, hypocotyl and roots (Figure 5.4). Staining was never observed in the pART27 (negative control) seedlings. Staining in the PHZ_{pro} lines was weak, but GUS activity was shown to be highest in the leaf margins and apex. The DPL_{pro} reporter lines showed that the expression in the cotyledons and hypocotyls and closer inspection showed that the GUS staining was localised around the vasculature, but had diffused slightly (Figure 5.4). The inclusion of 1 mM $K_3/K_4Fe(CN)_6$ in the staining solution reduced the overall staining intensity, but showed that the expression of GUS was restricted to the vasculature throughout the seedling (Figure 5.5). This vascular-specific staining was most easily seen in the hypocotyl.

5.3 Discussion

5.3.1 Spatial localisation of *DEEP PURPLE* and *PURPLE HAZE*

The *PHZ* promoter directed GUS expression to the apex and margins of the cotyledons, and appeared to be located sub-epidermally (Figure 5.4). This expression pattern was the same as was seen initially in the rooted-shoots in tissue culture (Figure 5.1), where leaves, particularly at the margins and in apical structures, had the highest GUS activity. It should be noted that in both these cases, the rooted-shoots and seedlings grown in tissue culture were exposed to low light. The distribution of the GUS signal was similar to that of weak anthocyanin accumulation which can occur in response to stress

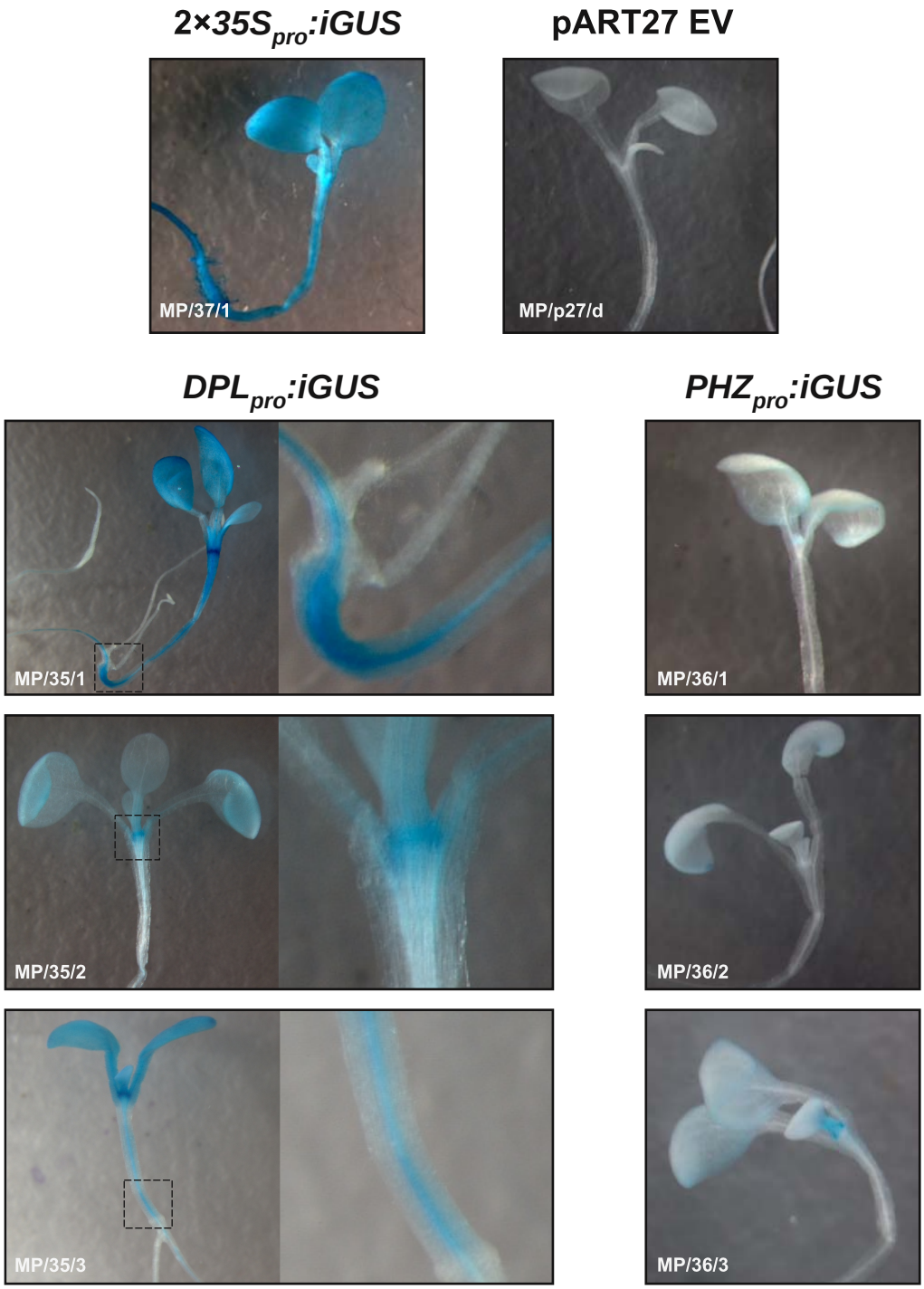


Figure 5.4 Histochemical localisation of GUS activity in pART27 (p27) empty vector, *2×35S*$_{pro}$*:iGUS* (MP/37/1), *DPL*$_{pro}$*:iGUS* (MP/35/...) and *PHZ*$_{pro}$ *:iGUS* seedlings. GUS activity was localised with X-gluc, in the absence of oxidation catalysts. Boxes indicate magnified regions.

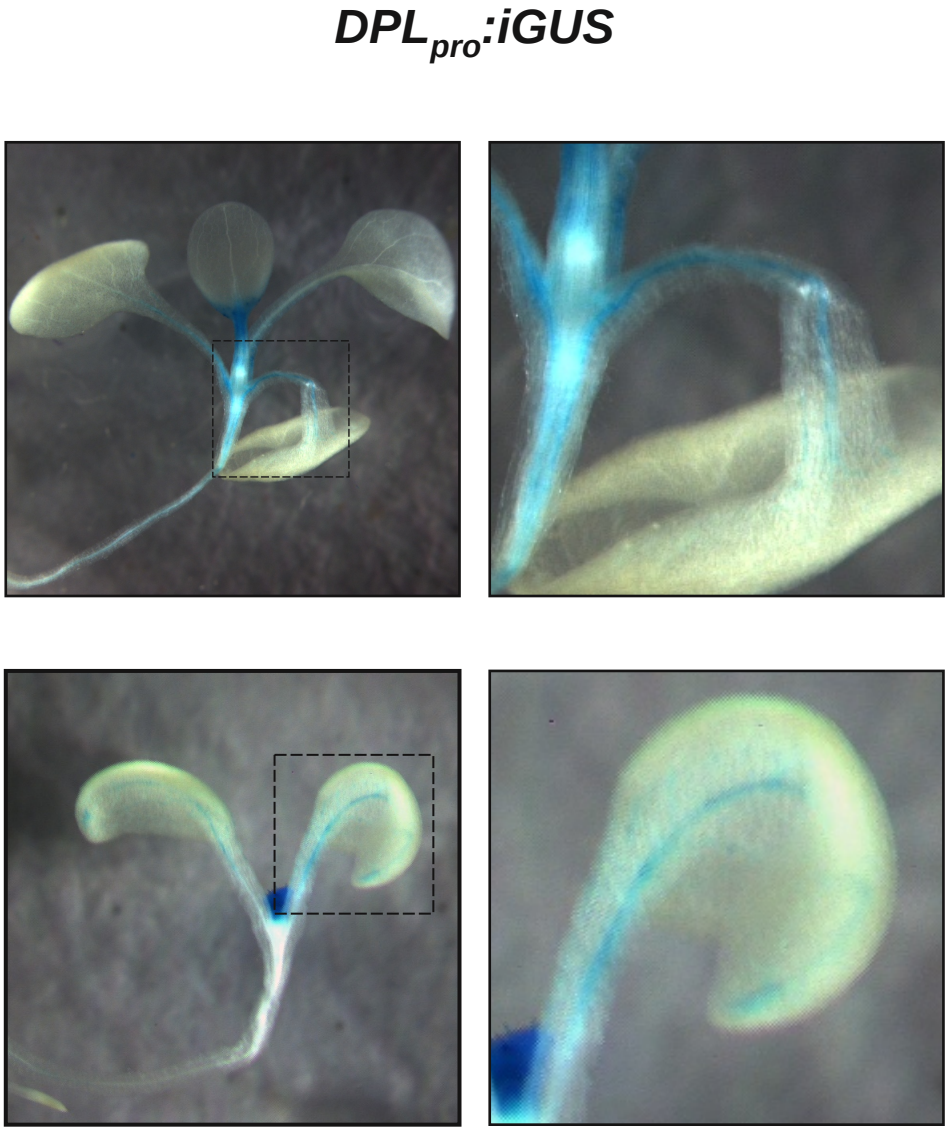


Figure 5.5 Histochemical localisation of GUS activity in *DPL$_{pro}$:iGUS* seedings,with X-gluc, in the presence of 1 mM $K_3/K_4Fe(CN)_6$. Refer to Figure 5.4 for *DPL$_{pro}$:iGUS* seedlings stained for GUS activity in the absence of $K_3/K_4Fe(CN)_6$. Boxes indicate the magnified region.

and light, even in tissue culture. This link to light-induced pigmentation in vegetative tissues was not able to be explored further by high light treatment of seedlings or in mature plants due to the presence of phenolic compounds that inhibited GUS activity. However, the spatial expression patterns observed in the *PHZ_{pro}:iGUS* lines fits with the previous transcript analysis and induction of *PHZ* (Chapter 4, Figure 4.1). In total, these results suggest PHZ regulates light-induced anthocyanin production in vegetative tissues.

In floral tissues, the *PHZ* promoter directed GUS activity to the petal limb, in a tie-dyed manner on the abaxial epidermal surface (Figure 5.3). This GUS localisation pattern matches the bud blush anthocyanin pigmentation pattern observed in W59 petunia, where *PHZ* transcript abundance was tightly correlated to the phenotype (Chapter 4, Figure 4.4). Thus, the spatial localisation of the *PHZ* promoter activity is strong evidence that *PHZ* is the MYB factor responsible for determining the floral bud blush phenotype in petunia flowers.

In vegetative tissues, the *DPL* promoter directed vascular-associated GUS activity in cotyledons, hypocotyls, stem and leaves (Figure 5.4). The inclusion of $K_3/K_4Fe(CN)_6$ in the GUS staining solution greatly enhanced the resolution, confirming a tight association with the vasculature (Figure 5.5). Transcript abundance showed *DPL* was expressed in non-pigmented leaves that were grown under shade conditions (Chapter 4, Figure 4.1). The activity of the *DPL* promoter correlated well with the transcript analysis (Chapter 4).

The physiological role for an anthocyanin regulator in the vasculature of non-stressed leaves is not known. The expression of *DPL* in the vasculature of vegetative tissues was unusual since anthocyanins or proanthocyanidins do not accumulate here normally. Anthocyanin synthesis may not occur under shade conditions due to the absence of the correct bHLH partner. High light treatment was shown to induce the expression of the bHLH *An1*, which correlated tightly to light-induced anthocyanin production (Chapter 4, Figure 4.1). Alternatively, the active repressor, *MYB27*, may prevent the activation of the anthocyanin biosynthetic genes, despite expression of *DPL*. Interestingly, an anthocyanidin glucosyl transferase and a glutathione-*S*-transferase (TT19), required for anthocyanin synthesis and transport, respectively, were shown to be expressed in

association with the vasculature in *Arabidopsis* leaves, even though anthocyanins were not produced (Wenzel et al., 2008). Both these genes are direct targets of the anthocyanin regulators (e.g. *PAP1*) (Tohge et al., 2005; Gonzalez et al., 2008; Rowan et al., 2009) which may suggest a subset of anthocyanin biosynthetic genes are activated by MYB anthocyanin regulators in the presence of the active repressor AtMYBL2. It is interesting to note that in petunia, the *DFRa* promoter activity was also localised to the vasculature of the pedicel (Van der Eycken et al., 2000), even though anthocyanins or proanthocyanidins are not produced. It is thus possible that *DPL* regulates this expression.

The petunia cultivar W59 lacks flower tube venation and contains a transposon insertion allele of *DPL* that is expressed at very low levels in flowers, linking the activity of *DPL* to venation pigmentation patterns (Chapter 4, Figure 4.4). In vegetative tissues, the *DPL* promoter was shown to direct vascular-associated expression. Staining for *DPL* promoter activity in the flower tube was unsuccessful, due to the presence of phenolic compounds that are proposed to inhibit GUS activity. However, the link to vascular expression indicates *DPL* may determine flower tube venation through spatial expression surrounding the vasculature in the flower tube. In *Antirrhinum*, *Venosa* (MYB) controls anthocyanin venation patterning in flowers. *Venosa* transcripts are expressed associated with the vasculature and (Schwinn et al., 2006; Shang, 2006), suggesting floral venation patterns occur through conserved mechanisms in *Antirrhinum* and petunia.

DPL_{pro}-directed GUS activity indicates that the *DPL* promoter is active throughout the petal limb. The petal limb contains a network of very fine veins, but *DPL*-directed GUS activity was not exclusively associated with the vasculature (Figure 5.5), even when high concentrations of oxidation catalysts were included. The expression of *DPL* in petal limbs may be an additional expression pattern to that of vegetative tissues (and presumably the petal tube), or it may be that the signal(s) from the vasculature, that control *DPL* expression, is more diffuse in the petal limb due to the fine network of veins. The signal(s) that may control the expression are unknown for *DPL* in petunia, or for *Venosa* in *Antirrhinum*. Candidate signals include sucrose and auxin, which are both transported in the phloem. Sucrose has been previously shown to be important for anthocyanin synthesis in petunia petals (Weiss, 2000), and the MYB anthocyanin

regulator *PAP1* from *Arabidopsis* is highly induced by sucrose (Teng et al., 2005), although its expression has not been associated with the vasculature. Auxin is a candidate due to the links between flavonoids and polar auxin transport (Lazar and Goodman, 2006; Santelia et al., 2008).

Transcript expression studies in various petunia lines showed *DPL* was expressed in developing petunia flower buds (Chapter 4), and *DPL* has been linked to flower tube venation pigmentation. The GUS localisation of *DPL* promoter activity to the petal limb suggests *DPL* may also contribute to flower limb pigmentation. Venation pigmentation patterns are known to occur in the petal limb of petunia. However, flower limb venation is complex, with three known loci involved: *Venation1* (*Ve1*), *Venation2* (*Ve2*) and *Venation3* (*Ve3*). *Ve1* is required for both flower tube and limb pigmentation (Wiering, 1974; Wiering et al., 1979; Cornu and Maizonnier, 1983), and may encode *DPL* (Chapter 4). In the flower limb, two additional loci affect the phenotypic expression of venation patterns. Dominant alleles of *Ve2* inhibit venation patterns in the limb (not tube), while dominant *Ve3* alleles determine the extent of venation in the flower limb (Wiering, 1974). The involvement of *DPL* for conferring venation phenotypes in the petal limbs of cultivated petunias requires further investigation.

5.3.2 Oxidised phenolics inhibit GUS

GUS reporter activity in greenhouse grown petunias was inhibited by endogenous phenolic compounds. Tissue browning occurs due to the oxidation of phenolic compounds (Lagrimini, 1991; Richard-Forget and Gauillard, 1997; Robards et al., 1999; Tomás-Barberan and Espín, 2001). Oxidised phenolic compounds are known to bind and interfere with proteins and nucleic acids, which is one reason why reducing agents are added to extraction buffers (Pierpoint, 2003). The presence of oxidised phenolics in many petunia tissues inhibited the *in situ* GUS assay. The inclusion of antioxidants prevented tissue browning and restored GUS activity to the *2×35S$_{pro}$:iGUS* petunia tissues, which strongly supports a causal link between oxidised phenolics and inhibition of GUS activity. Indeed, leaves and flower tubes, which are prone to browning, contain

high levels of the phenolic compound rosmarinic acid (Bloor et al., 1998; Albert et al., 2009) linking the presence of this phenolic compound to GUS inhibition.

The occurrence of oxidised phenolics in petunia tissues and the requirement to include a reducing agent meant that the GUS staining was more diffuse. This reduced the resolution at which GUS activity could be localised, because the formation of the coloured, insoluble, indigo requires oxidation – the inclusion of antioxidants, therefore, is incompatible with this fundamental requirement. Oxidation catalysts, which are often used to improve resolution of GUS localisation (Lojda, 1970; Jefferson, 1987), exacerbate the oxidation of phenolic compounds, and the inhibitory effects upon GUS activity.

Alternative substrates, that do not require oxidation, may prove suitable for the histochemical detection of GUS activity in plant tissues with high phenolic content, and in the presence of reducing agents. A range of Sudan-based glucuronide substrates have been synthesised that are suitable for GUS detection (Guivarc'h et al., 1996; Van der Eycken et al., 2000). The major benefit for the Sudan-based substrates is that upon cleavage of the glucuronide moiety, the large hydrophobic Sudan dye is immediately insoluble, unlike X-gluc which requires oxidative dimerisation (Van der Eycken et al., 2000). The SudanIV glucuronide was shown to provide superior localisation to an optimised X-gluc solution, which had a more diffuse localisation of indigo, even though oxidation catalysts were included (Van der Eycken et al., 2000). The Sudan-glucuronide substrate should be compatible with the inclusion of a reducing agent to prevent oxidation of phenolic compounds that would otherwise inhibit GUS activity. Unfortunately, the Sudan-based substrates are not commercially available and due to the expense of custom synthesis, were not examined as part of this thesis.

The inclusion of positive (e.g. *$35S_{pro}$:GUS*) and negative controls is rarely published for GUS localisation, which is highly questionable, given the many artefacts (e.g. substrate penetration and diffusion of GUS cleavage intermediates) which can occur with the histochemical detection of GUS. The problems encountered in petunia are not unique, yet this is not widely reported in the literature. Highly erratic GUS staining of various tissues in cranberry (*Vaccinium macrocarpon*) has also been attributed to the interference by phenolic compounds (Serres et al., 1997). Tissue-specific and

developmental effects have also been observed, which likely reflect varying phenolic concentration and content (Dixon and Paiva, 1995). GUS activity was confirmed in cranberry tissues that failed to stain (with X-gluc) using a modified MUG assay (Serres et al., 1997). The MUG assay was modified to include PVPP in the extraction, to absorb interfering phenolic compounds, and was originally developed for carnation, which also contained inhibitors of GUS activity (Vainstein et al., 1993).

The observed inhibition of GUS activity by phenolic compounds is of significance as it may give inaccurate localisation of promoter activity, due to false-negative staining. In petunia, the promoter of the floral homeobox gene *Apetala3* driving GUS resulted in petal and sepal staining (Verdonk et al., 2008). The sepal stained unevenly with browning observed in the apical portion, which failed to stain. This browning matches that observed in the 2×*35S$_{pro}$:iGUS* sepals (Figure 5.2, - 2ME) where the apical portion of the sepal failed to stain and was dark brown due to oxidised phenolics. *35S$_{pro}$:GUS* petunias were previously reported not to stain for GUS activity with X-gluc in the trichomes, epidermis or cortex of stems, and had erratic, uneven staining in other plant tissues (van der Meer et al., 1992). Anecdotal reports that the *CaMV35S* promoter (detected with a GUS reporter) is only expressed in ovules in alfalfa suggests that similar inhibitory effects to those observed in petunia may also occur in other species. Notably, the petunia ovules stained very well, indicating that they are free from inhibitors of GUS activity (Figure 5.2, - 2ME). The *CaMV35S* promoter is known to direct strong and constitutive expression in plants (Odell et al., 1985; Hraška et al., 2008), including alfalfa (Peel et al., 2009), suggesting that these observed patterns were artefacts, and may be due to the inhibitory effects of oxidised phenolic compounds upon GUS activity.

The level of inhibition of GUS activity is likely to vary with species, tissue type and environmental conditions. Plants produce a wide range of phenolic compounds in response to developmental and environmental signals (Dixon and Paiva, 1995), which may make some tissues more susceptible to inhibition of GUS activity. The petal limb tissues of greenhouse-grown petunias do not accumulate the phenolic compound rosmarinic acid and stained well, while the petal tube and leaves do accumulate this compound and were prone to GUS inhibition due to oxidised phenolics. However, it was thought that newly germinated seedlings grown *in vitro* would contain lower levels

of phenolic compounds, which may allow the detection of GUS reporter gene activity. This was the case and some information about the spatial expression patterns for *DPL* and *PHZ* was able to be determined.

In future studies, the use of alternative methods to detect the GUS reporter gene may allow spatial expression patterns for promoters to be determined. Reporter gene approaches are often favoured over other methods, such as *in situ* hybridisation, to avoid problems with cross-hybridisation between gene-family members, and to provide a macro scale view of expression patterns throughout an organ or the entire plant. While problems with interfering phenolic compounds may prevent enzyme activity being detected *in situ*, the GUS protein itself can still be detected. The GUS protein is foreign to plants, providing unique epitopes that could be localised by immunodetection using commercially available antibodies. Recently, immunolocalisation of a phloem-associated protein, SUT1, was performed on fresh and sectioned tissues of tomato, tobacco and potato. SUT1 was detected using conjugated fluorescent secondary antibodies and confocal microscopy (Schmitt et al., 2008). This powerful and sensitive system may provide fine, cellular resolution of expression patterns for the GUS reporter genes, in tissues that cannot be stained with X-gluc.

Despite the many difficulties encountered using GUS in petunia, the localisation patterns of *DPL* and *PHZ* were determined in both vegetative and floral tissues. The localisation of *PHZ* supports a role in determining light-induced, sub-epidermal vegetative anthocyanin pigmentation, and floral bud blushing.

The expression pattern for *DPL* was localised to the vasculature in vegetative tissues. This localisation and the genetic evidence from W59 petunia (*DPL::LTR* insertion allele) suggest that *DPL* determines venation pigmentation patterning in the flower tube. Further work to localise *DPL* transcripts, or localise DPL_{pro}-directed GUS protein accumulation in tissues that could not be stained for GUS activity (flower tube) may provide more conclusive evidence. The role for *DPL* in vegetative tissues is unclear, although the lack of the bHLH *An1* in vegetative tissues (except under stress conditions) may prevent anthocyanin synthesis from occurring. However, these transcription factors have not been confirmed unequivocally as anthocyanin regulators, despite the level of sequence similarity to known regulators, and expression patterns linked to

pigmentation patterns. Thus, the use of reverse genetics approaches to provide further evidence that *DPL* and *PHZ* are indeed anthocyanin regulators, are examined in Chapter 6.

Chapter 6

MYB over-expression petunia lines

6.0 Introduction

The genes *DPL* and *PHZ* encode MYB transcription factors, and are putative anthocyanin regulators. The transcript expression patterns for *PHZ* correlate well with light-induced vegetative anthocyanin production, as well as a bud-blush floral phenotype (Chapter 4, Figure 4.4). The transcript expression pattern for *DPL* did not correlate to pigmentation in vegetative tissues, as the gene is expressed in the leaves of non-pigmented shade-grown plants (Chapter 4, Figure 4.1). However, it was linked to floral pigmentation, as transcripts for *DPL* in W59 petunia, which contain a transposon insertion allele of *DPL* (*DPL::LTR*) and lack flower tube venation, were markedly reduced compared to V26 or MP (Chapter 4, Figure 4.4), linking the activity of *DPL* to flower tube venation pigmentation.

Gene function can be determined by both forward (stable mutants, RNAi) and reverse genetics approaches (over-expressing a gene). While reverse genetics approaches are often favoured, finding appropriate mutants, gene redundancy, and inefficient RNAi knockdown can prevent these approaches from being used successfully. Reverse genetics by over-expression is a complementary approach, and can help overcome some of these problems, but this requires careful interpretation and possible off-target effects should be considered. The ability for *DPL* and *PHZ* to function as anthocyanin regulators was tested by ectopically expressing these MYB factors in the petals of *Antirrhinum rosea*^{dorsea} flowers, which are white due to reduced MYB activity. In addition, stable transgenic petunias over-expressing these two genes were generated to test directly if these transcription factors are capable of regulating anthocyanin production in petunia. In this instance, these reverse genetics approaches were used to complement the gene expression and localisation data.

Two petunia cultivars were chosen for the transgenic over-expression experiments. Mitchell petunia is the most routinely used cultivar in transformation experiments, due to its robust growth and ease of transformation and regeneration. Mitchell petunia (*P. axillaris* × [*P. axillaris* × *P. hybrida*]), however, has a complex genetic background, being a double-haploid of an inter-specific cross, and it was possible this background contained mutations in loci important for the activity of *DPL* or *PHZ*. The defined *P. hybrida* cultivar Violet 26 (V26) was therefore included in the over-expression studies. Generation of the over-expression lines enabled pigmentation phenotypes, biosynthetic gene expression and flavonoid biochemistry to be assessed.

6.1 Materials and methods

6.1.1 Over-expression constructs

Over-expression constructs were made by amplifying the coding sequence for *DPL* or *PHZ* and cloning into *Kpn*I and *Bam*HI sites of pART7 (Gleave 1992), placing the coding sequences under the control of the *CaMV35S* promoter and OCS terminator. The constructs in the resulting plasmids pNWA1 (*35S$_{pro}$:DPL*) and pNWA2 (*35S$_{pro}$:PHZ*) were then subcloned by *Not*I digestion and ligation into the *Not*I site of pART27 binary vector (Gleave 1992), generating the plasmids pNWA12 (*35S$_{pro}$:DPL*) and pNWA14 (*35S$_{pro}$:PHZ*). These were transformed into *Agrobacterium* LBA4404 by electroporation (Section 2.4.5). The *Agrobacterium* cultures harbouring the binary vectors were then used for leaf-disc transformation of petunia.

6.1.2 Biolistic transformation

Antirrhinum rosea^{dorsea} petals were transformed with pNWA1 or pNWA2 by biolistic transformation. Dorsal petals were collected from *rosea^{dorsea} Antirrhinum* plants prior to dehiscence and surface sterilised (Section 2.9). Plasmid DNA (5 µg) of pNWA1 or pNWA2 and the internal control pPN93 (*35S$_{pro}$:GFP-ER*) (1 µg) was precipitated onto 1 µm gold particles with spermidine and $CaCl_2$ as described by Schwinn et al., (2006). Biolistic transformation was performed with a particle inflow gun, as described by Shang et al. (2007). Petals were maintained on solid MS media (Appendix 1.5) at 25°C under a16 h photoperiod (25 µmol m^{-2} s^{-1} photons). Images of the transformed petals were collected 3 d post-transformation with an Olympus SZX12 light microscope and Leica Microsystems DC500 digital camera. GFP detection was performed with an

Olympus SZX-RFL coaxial fluorescence attachment, consisting of a mercury lamp, blue wavelength excitation filter (BP460-490), a dichroic mirror (DM505), and a long-pass barrier filter that blocks wavelengths below 510 nm (BA510IF).

6.1.3 Stable transgenic petunia lines

Stable transgenic petunia lines over-expressing *DPL* or *PHZ* were generated by *Agrobacterium*-mediated transformation of V26 and Mitchell petunia leaf discs with pNWA12 or pNWA14, respectively (Section 2.9). Shoots were screened by PCR (Section 2.9.2) using the primers NA34/NA35 (pNWA12/*DPL*) or NA24/NA25 (pNWA14/*PHZ*). Three clones each, from ten independent lines, for each construct, in each petunia cultivar, were regenerated and grown in the GMO greenhouse facility at Plant & Food Research Palmerston North. Plants were assessed for their pigmentation phenotypes.

6.1.4 RNA extraction

Mature leaves and corolla tissue from stage four flower buds (refer Figure 4.2B), separated into tube and limb (Section 2.8.1, Figure 1.1A), were collected and immediately frozen in liquid nitrogen. Tissue was collected at the same time of day for sampling (late afternoon), to avoid any diurnal or circadian effects upon flavonoid gene expression. The pooled tissue was ground to a fine powder with a mortar and pestle in liquid nitrogen and stored at -80°C. Total RNA was extracted from 1 g FW (Section 2.10.1).

6.1.5 Northern blotting

Total RNA (15 µg) was diluted to a final volume of 15 µL. One volume of 2 × 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 × 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.

The gel tank and apparatus were soaked in RNAse decontamination solution (Appendix 1.3.1). 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 (Appendix 1.3.1). 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 enabled 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 1.6.5). The membrane used was Hybond N^+ Membrane (GE Healthcare). After transfer, the blot was disassembled, and the membrane was dried. The membrane was then subjected to UV-C cross-linking (Hoefer) for 1 min at 70 000 mJ cm^{-2}. The membrane was visualised under UV light, ensuring complete transfer to the membrane, and the location of the molecular size standards marked. The membranes were pre-hybridised, hybridised, washed and exposed to film as described in Sections 2.7.1, 2.7.2, 2.7.3 and 2.7.4, respectively.

Radiolabelled probes were generated by random primed labelling (Section 2.7.2) upon the cDNA inserts isolated from the following plasmids: pPN296 (*PAL*); pPN303 (*C4H*); pPN297 (*CHS-A*); pPN300 (*CHI-A*); pNWA17 (*F3H/An3*); pPN301 (*F3'H*); pNWA18 (*F3'5H/Hf1*); pPN302 (*FLS/Fl*); pNWA27 (*DFR-A*); pPN299 (*ANS*); pNWA20 (*UF3GT*); pNWA23 (*3RT/Rt*); pNWA25 (*UF5GT*); pNWA24 (*AT*); pNWA26 (*MT*); pNWA19 (*GST/An9*); pNWA9 (*Actin*); pTIP6 (*26S rRNA*); pNWA1 (*DPL*); pNWA2 (*PHZ*).

6.1.6 Pigment extraction and analysis

Mature leaves and corolla tissue from stage five flower buds (refer to Figure 4.2B), separated into tube and limb (Section 2.8.1), were collected and stored at -20°C. The tissue was ground to a fine powder with a mortar and pestle in liquid nitrogen and freeze-dried. Total flavonoids, including anthocyanins, were extracted from 50 mg DW tissue with in 2 mL of acidified 70% (v/v) methanol (Appendix 1.7) 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 the extracts used further for HPLC analysis.

6.1.6.1 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:CH_3CN:H_3PO_4:H_2O (20:24:1.5:54.5)] and 1.5% (v/v) H_3PO_4 (solvent B) and a linear gradient starting with 35% A, increasing to 67% A at 20 min, 90% A at 23 min and 100% A at 29.3 min, remaining at 100% A for a further 10 min (Bloor ***et al.*** 1998). 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.

6.1.6.2 Anthocyanin aglycones

Anthocyanin aglycones (anthocyanidins) were extracted from leaf and flower limb samples: MP/UC/4 (untransformed control), MP/12/9 (*$35S_{pro}$:DPL*) and MP/14/1

(*35S$_{pro}$:PHZ*). Anthocyanidins were extracted from 25 mg DW tissue in 3N HCl for 45 min at 100°C in an oil bath. Particulate matter was removed by centrifugation, and anthocyanidins were partitioned with 1 mL ethyl acetate. The aqueous fraction was collected, and the anthocyanidins were extracted with isoamyl alcohol. The isoamyl alcohol fraction was collected, and this was used directly for thin-layer chromatography. For HPLC analysis, an aliquot of the isoamyl alcohol/anthocyanidin extract was dried under nitrogen and dissolved in 90 % (v/v) acidified methanol (Appendix 1.7). HPLC was performed as in Section 6.1.6.1 except that anthocyanidin standards (pelargonidin, cyanidin, delphinidin, peonidin and malvidin) were run as a comparison for the retention times of the aglycones, for identification.

6.1.6.3 Thin-layer chromatography

The isoamyl alcohol/anthocyanidin fractions (Section 6.1.6.2) were spotted onto thin-layer chromatography plates directly along with authentic standards (pelargonidin, cyanidin, delphinidin, peonidin and malvidin) until a visible coloured spot was visible at the origin. The TLC plate was run in formic acid: H_2O: HCl (5:3:2; v:v:v). An authentic standard for petunidin was not available.

6.1.7 Fresh sections

Fresh sections of tissues from the MYB over-expression lines were made by hand using a sharp scalpel blade. Transverse (TS) and longitudinal (LS) sections of stems, and TS sections through leaves were made, mounted in water and photographed with a dissecting microscope and digital camera, as described in Section 6.1.2.

6.2 Results

6.2.1 Biolistic transformation of *Antirrhinum rosea*^{dorsea} petals

Antirrhinum rosea^{dorsea} petals are pale (white except a blush of anthocyanin on the outer epidermis of the dorsal petal) due to reduced expressed of endogenous MYB anthocyanin regulators. Transient transformation of the inner epidermis of the dorsal petal with *35S$_{pro}$:DPL* or *35S$_{pro}$:PHZ* restored anthocyanin synthesis to transformed cells (Figure 6.1). A *35S$_{pro}$:GFP* construct was co-transformed as an internal control for transformation efficiency. GFP fluorescence co-localised to anthocyanin producing cells.

6.2.2 Pigmentation phenotypes of MYB over-expression petunia lines

Petunia lines over-expressing *DPL* or *PHZ* were generated by *Agrobacterium*-mediated leaf disc transformation. Within 48 hours of incubating the leaf discs with *Agrobacterium* culture containing the *MYB* over-expression constructs, the edges of the leaf discs turned purple with anthocyanin. The leaf discs subsequently gave rise to highly pigmented shoots, which were then propagated into independent transgenic lines. Ten independent lines of each construct, each with three clones were exflasked and grown in the GMO greenhouse. The plants became even more highly pigmented within a day of exflasking, than when grown in tissue culture.

The Mitchell petunia untransformed controls had green leaves (Figure 6.2A). The flower buds of Mitchell petunia have weak blushing of anthocyanin on the pedicel and sepals, and faint anthocyanin pigmentation is visibly associated with the major veins on the abaxial epidermis (Figure 6.2B). The petal limb was white, and lacked anthocyanin, but the flower tube had a highly reticulated venation pigmentation pattern (Figure 6.2C).

Over-expression of *DPL* (MP/12/…) resulted in intense anthocyanin production throughout the stems and leaves (Figure 6.3A). There were two phenotypes which were equally represented in the chosen lines: dark bronze (MP/12/5, MP/12/6) and solid

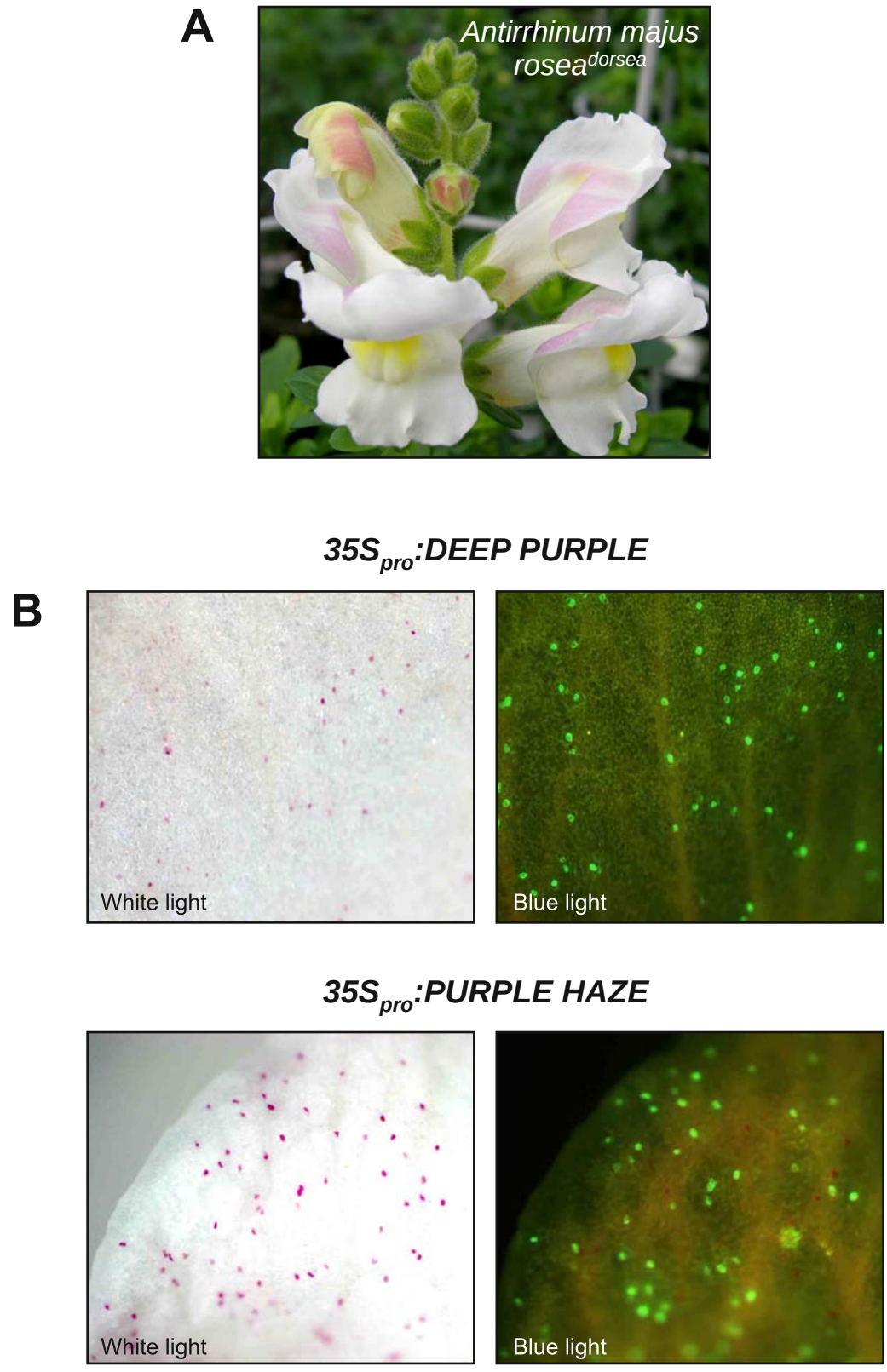


Figure 6.1 Complementation of the *rosea*^{dorsea} phenotype by biolistic transformation. **A.** *Antirrhinum majus rosea*^{dorsea} inflorescence. **B.** Biolistic transformation of *rosea*^{dorsea} petals with *35S*$_{pro}$*:DEEP PURPLE* or *PURPLE HAZE*. *35S*$_{pro}$*:GFP* was cotransformed. Pigmented cells were viewed under a white light source, GFP fluorescence was viewed under a blue light source.

Figure 6.2 Pigmentation phenotypes of Mitchell petunia. **A.** Vegetative tissues. **B.** Mature flower bud. **C.** Opening flower.

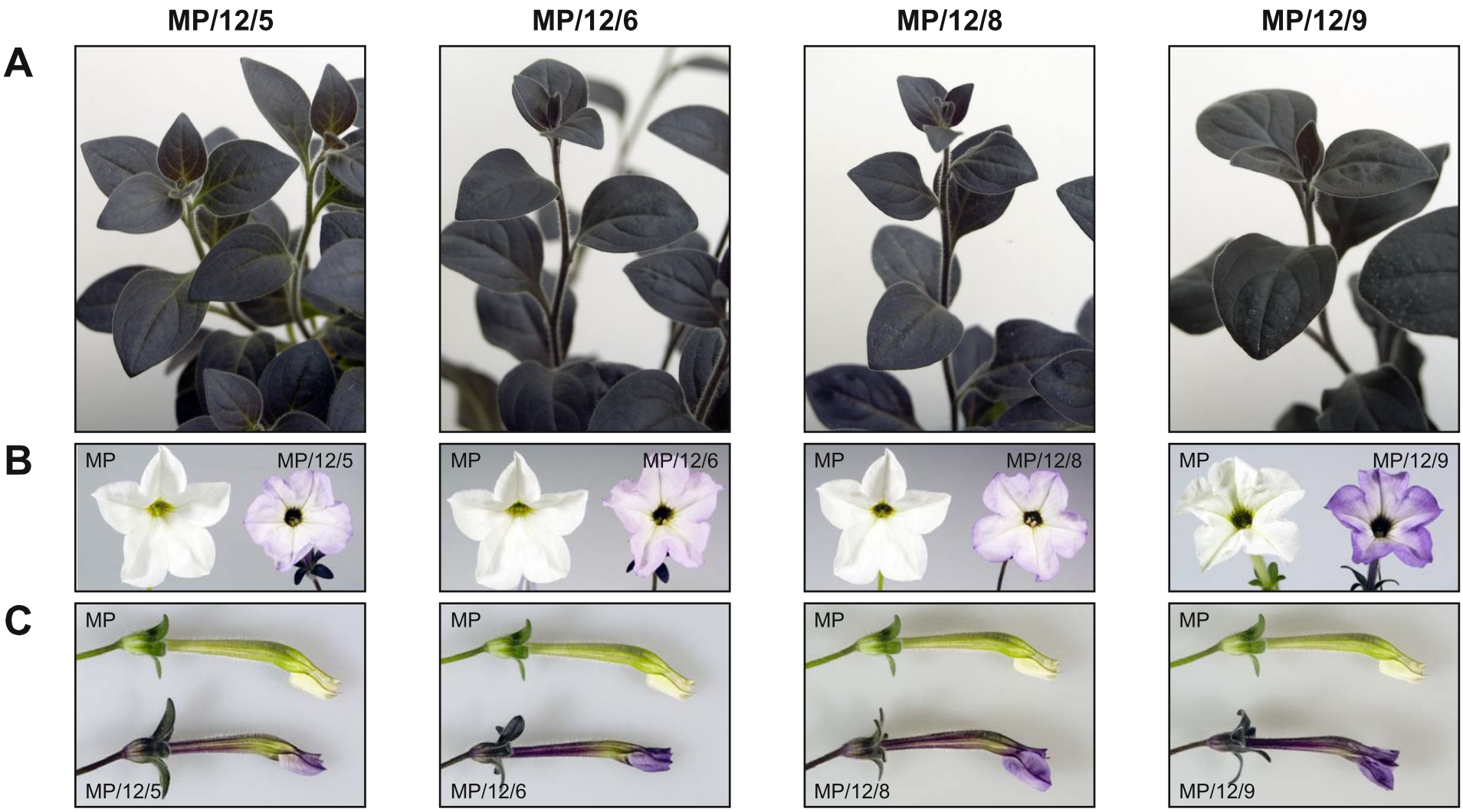


Figure 6.3 Pigmentation phenotypes of Mitchell petunia plants over-expressing *DEEP PURPLE* (MP/12/...). **A.** Vegetative phenotypes. **B.** Opening flower. **C.** Mature flower bud. For flower phenotypes, an untransformed Mitchell flower (MP) is shown for comparison.

intense purple (MP/12/8, MP/12/9). The floral phenotypes (Figure 6.3B and C) showed purple anthocyanins accumulated in the flower tube and limb. The flower buds faded very soon after opening. Mature flower buds clearly showed the tube and limb accumulate anthocyanins. Upon close inspection prior to dehiscence, anthocyanin was also visible on the anthers of all *DPL* over-expression lines, but pollen was yellow. Untransformed Mitchell controls have white flowers that produce anthocyanin in a reticulate venation pattern in the tube region, and also very faint pigmentation on the veins of the abaxial surface of the flower bud. The anthers are never coloured, and produce yellow pollen. The sepals and pedicel of untransformed Mitchell flowers can sometimes also accumulate a visibly detectable amount of anthocyanin in response to light-intensity.

Over-expression of *PHZ* (MP/14/…) resulted in a consistent dusky-bronze phenotype in stems and leaves (Figure 6.4A). This phenotype can intensify somewhat in response to increasing light intensity, but the dusky phenotype shown is typical of healthy robust plants grown under normal conditions. The floral phenotypes (Figure 6.4B and C) at first inspection may appear white, but they are in fact very pale purple. This is most evident in the mature buds (Figure 6.4C) where the flower tube is clearly more coloured, and the unfolding limb tissue is pale mauve. Upon close inspection prior to dehiscence, anthocyanin was also visible on the anthers of all *PHZ* over-expression lines, but pollen was yellow.

Line MP/14/5 was an exception and did not produce an anthocyanin pigmentation phenotype. This line was PCR positive for the presence of the *PHZ* cDNA and grew roots easily in the presence of 150 mgL^{-1} kanamycin. It was included for further analysis, as the only phenotype that differed from the dusky bronze phenotype. It performed in every way like the untransformed controls, and provided an internal (negative) control in later analyses.

The V26 lines showed the same vegetative pigmentation phenotypes as the Mitchell lines. Due to the difficulties maintaining the V26 transformants due to low vigour, the masking of possible floral phenotypes resulting from *DPL* or *PHZ* over-expression, and prior biochemical knowledge and expertise with the Mitchell line, any further biochemical and molecular analyses were not performed on the V26 transformants.

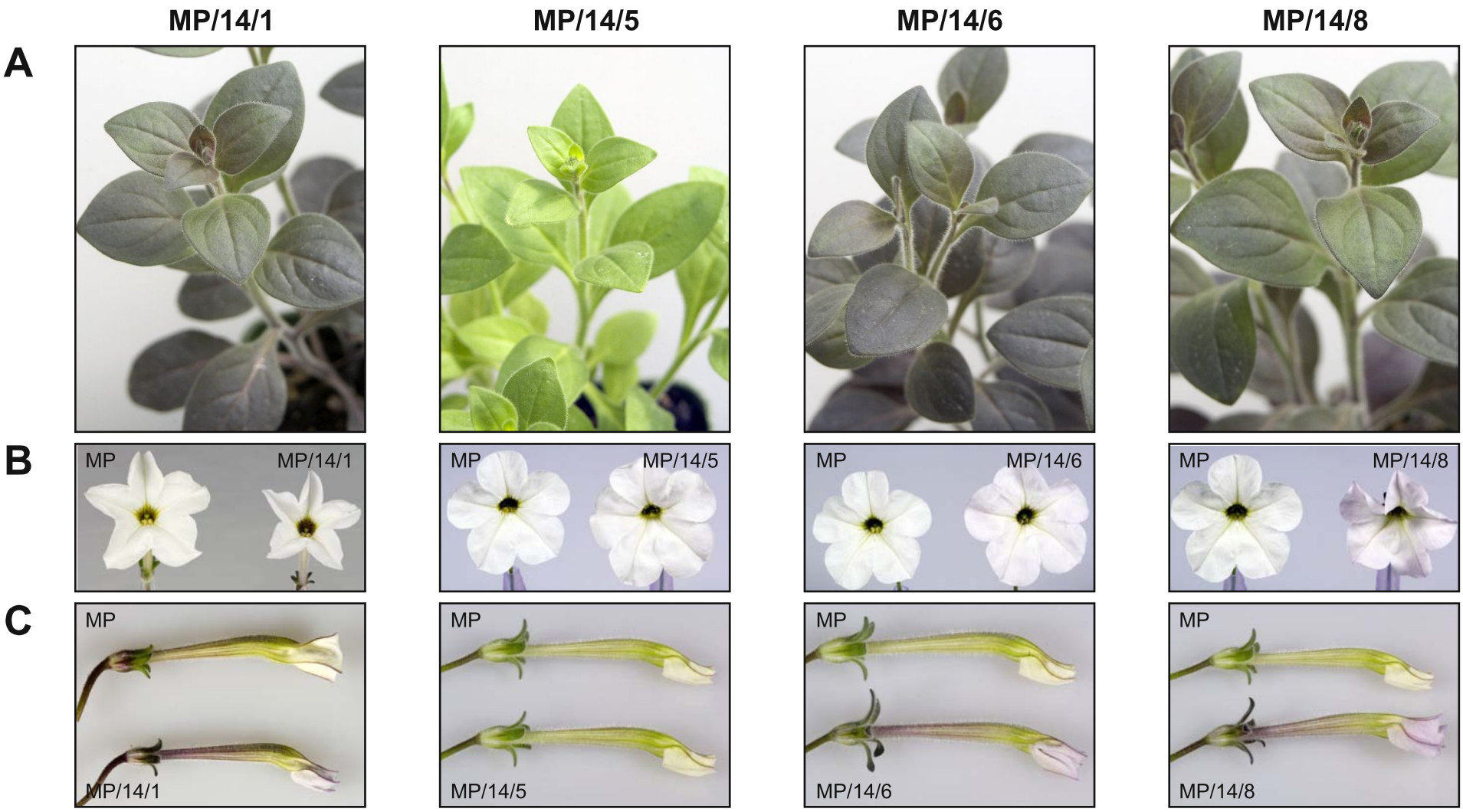


Figure 6.4 Pigmentation phenotypes of Mitchell petunia plants over-expressing *PURPLE HAZE* (MP/14/...). **A.** Vegetative phenotypes. **B.** Opening flower. **C.** Mature flower bud. For flower phenotypes, an untransformed Mitchell flower (MP) is shown for comparison.

Vegetative phenotypes are shown in Appendix 5. The flowers of wild type V26 are dark violet (Chapter 1, Figure 1.1B), masking any contribution from *DPL* or *PHZ* over-expression. The flowers from *DPL* lines did appear slightly darker, but this was very subtle and was not quantified.

Fresh hand sections of leaves and stems from the Mitchell *DPL* and *PHZ* over-expression (OE) lines were made, to observe the spatial distribution of anthocyanin production. Labelled line drawings of the tissues visible in the hand sections are shown in Figure 6.5. Anthocyanins were located in sub-epidermal mesophyll and pith cells of the *DPL* OE lines (MP/12/5, MP/12/9) (Figure 6.6). In the weaker MP/12/5 line, anthocyanin production occurred predominantly in the palisade mesophyll cells in both leaves and stems, including the abaxial palisade layer, while anthocyanins were present throughout palisade and spongy mesophyll layers in the stronger MP/12/9 line. The *DPL* OE lines also produced anthocyanins in cells adjacent to the xylem of both the stem and the midrib of the leaves, but anthocyanins were never observed in the vascular rays that connect the pith and cortex. Anthocyanins were located in the adaxial palisade mesophyll and pith cells of *PHZ* OE lines (MP/14/1, MP/14/8). In addition, anthocyanins were also produced in the cells adjacent to the xylem, and in the vascular rays connecting the pith and cortex: this is visible as files of scarlet cells in the vasculature of the TS leaf section and as pink streaks across the secondary xylem (white) of the TS and LS sections of stem (Figure 6.6). Anthocyanins were not observed in the untransformed wild type control. If wild type plants are exposed to intense light (especially combined with other stresses such as cold and nutrient deficiency) the stems and leaves can produce anthocyanins, which are usually located in the palisade mesophyll (not shown).

6.2.3 Pigment biochemistry

Anthocyanins and other flavonoids (flavonols) were extracted from the leaves and flowers of the *MYB* OE petunia lines (Figure 6.7). Anthocyanins accumulated to high levels in the leaves of the *DPL* and *PHZ* over-expression lines. The anthocyanin concentrations correlated to the pigmentation phenotypes: MP/12/9 was the most darkly

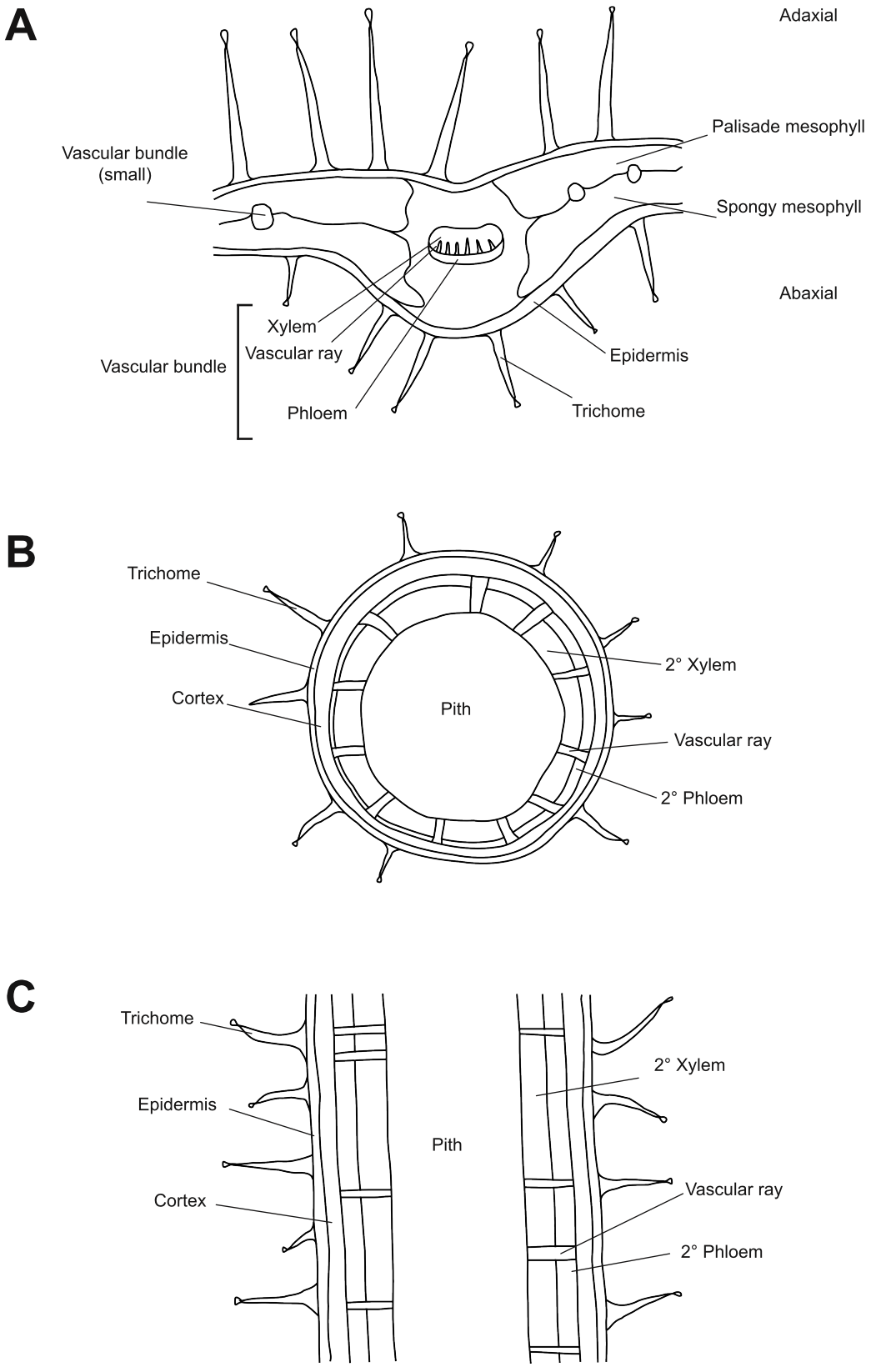


Figure 6.5 Labelled line drawings of tissues in leaf and stem sections. **A.** Transverse section through a leaf. **B.** Transverse section through stem. **C.** Longitudinal section through stem.

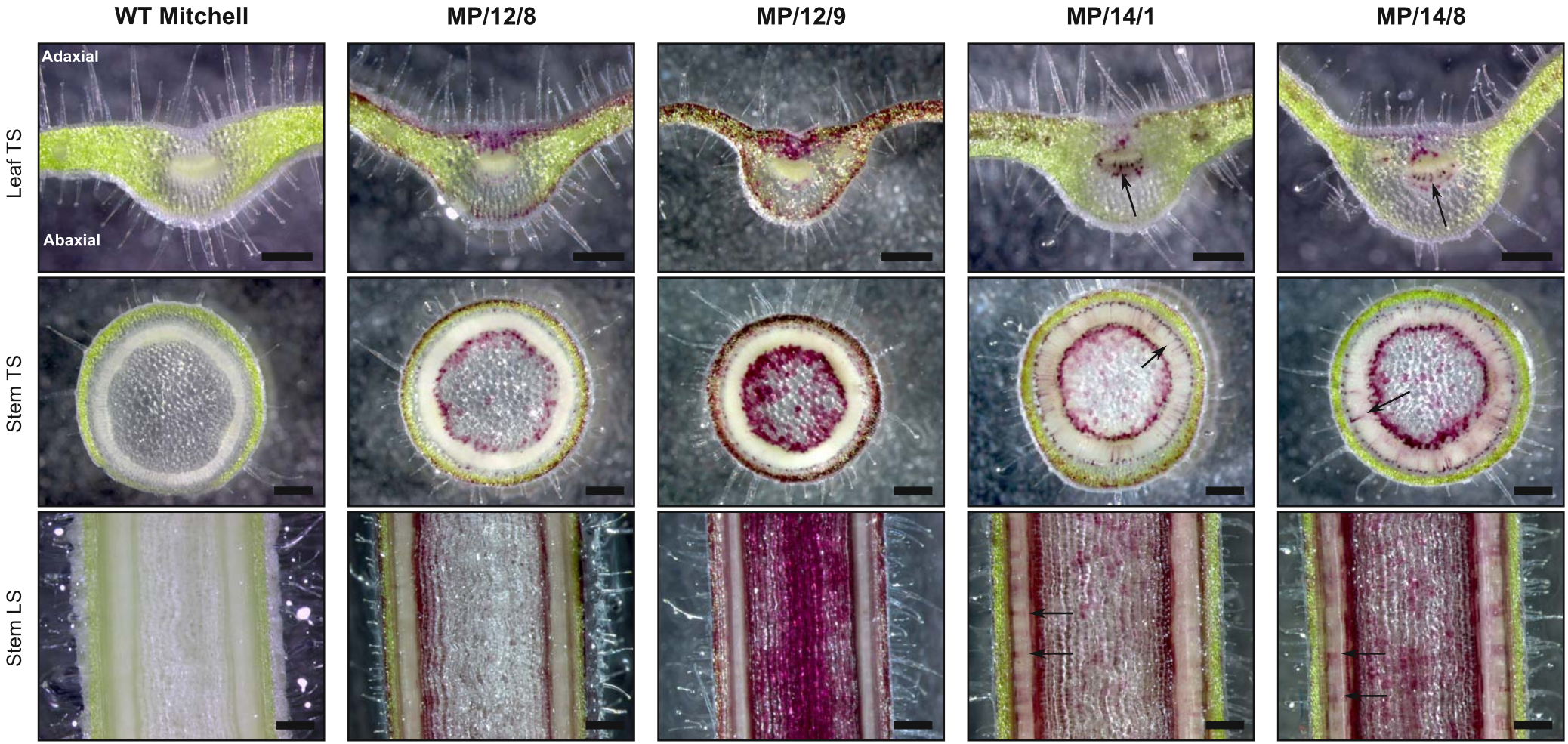


Figure 6.6 Anthocyanin distribution in fresh hand sections of stems and leaves in *35S$_{pro}$:DPL* (MP/12/...) and *35S$_{pro}$:PHZ* (MP/14/...) petunia lines. TS = transverse section, LS = longitudinal section. Arrows mark coloured vasular rays. Scale bar represents 0.5 mm.

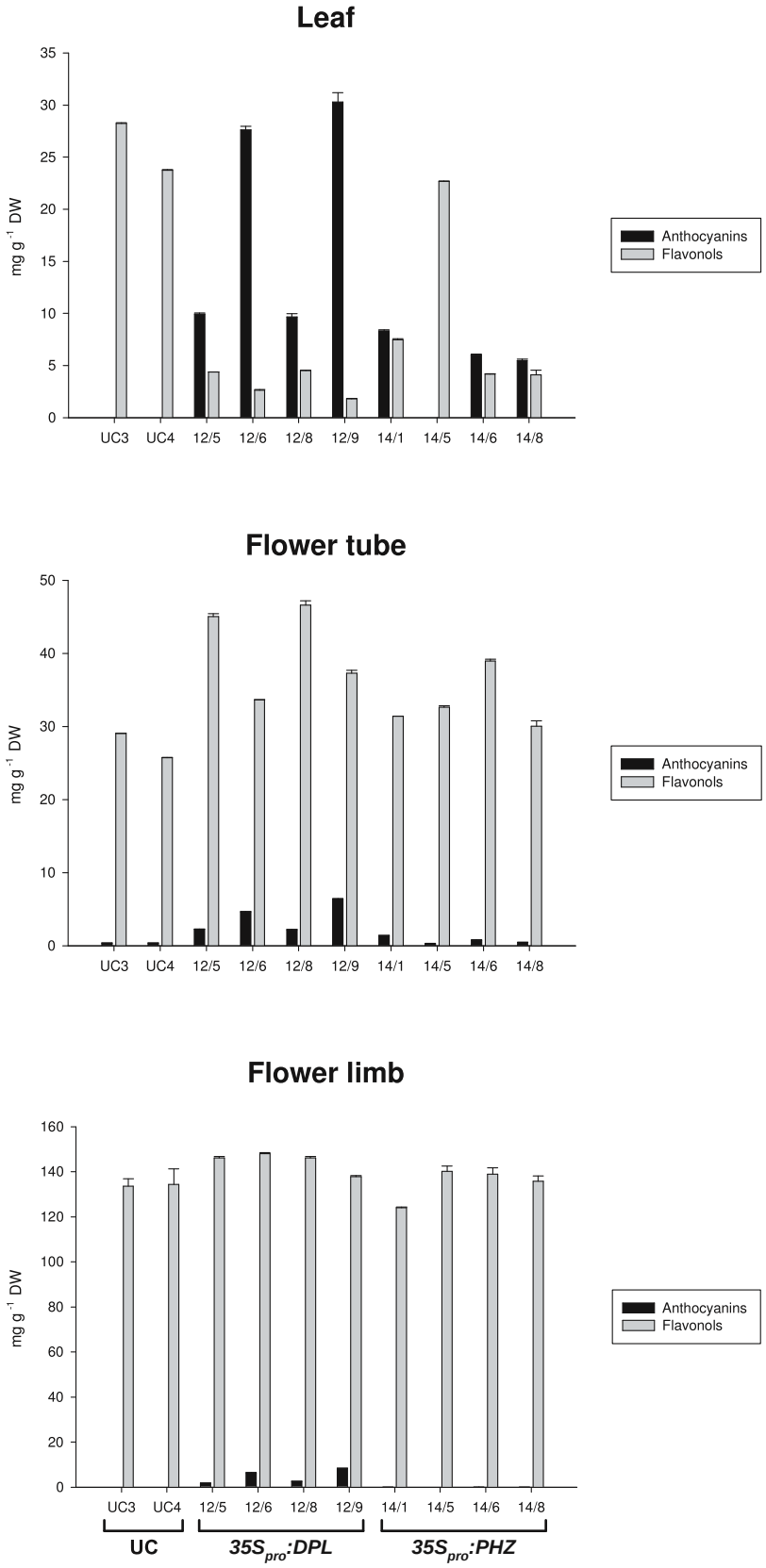


Figure 6.7 Concentration of anthocyanins and other flavonoids (flavonols) in different organs of untransformed Mitchell petunia (UC), *35S$_{pro}$:DPL* or *35S$_{pro}$:PHZ* petunias, as determined by high performance liquid chromatography of leaf extracts. Samples were extracted in duplicate. Mean concentration ± SE, *n*=2.

coloured petunia line and had the highest concentration of anthocyanins, while the untransformed controls and line MP/14/5 had green leaves and had no detectable anthocyanin pigments. Flavonol levels in leaves were severely reduced in all petunia lines that accumulated anthocyanins.

Flavonols were produced to high levels in the flowers of control and *MYB* OE lines (Figure 6.7). Anthocyanins accumulation was enhanced in the flower tube in the both *DPL* and *PHZ* OE lines. In the flower limb, flavonols accumulated to very high concentrations. *DPL* lines had increased anthocyanins in the flower limb, while *PHZ* lines had a very small, but detectable, increase in anthocyanin accumulation in the flower limb, which correlated to the off-white/pale mauve phenotype (Figure 6.7). Flavonols were the predominant flavonoids produced in flowers, even in the *MYB* OE lines.

Anthocyanin aglycones (anthocyanidins) were extracted from leaf and flower limb tissues of representative petunia lines over-expressing *DPL* or *PHZ*. Thin-layer chromatography separation of the aglycones showed two predominant anthocyanidins were present in all the samples (Figure 6.8A). The first band migrated with the delphinidin authentic standard, and had the characteristic purple hue. The second, predominant band did not migrate with any of the authentic standards. An authentic standard for petunidin was not available, but previous analysis of the anthocyanins present in Mitchell petunia suggested that the anthocyanins are a mixture of delphinidin- and petunidin-glycosides. HPLC separation of the aglycone extracts from leaves of representative *DPL* and *PHZ* OE lines is shown in Figure 6.8B. Anthocyanidins were not detected in the leaf extracts of the untransformed controls (not shown). The major anthocyanidins present in the leaves of *DPL* and *PHZ* OE lines had the same retention times and spectral properties as delphinidin and petunidin. Trace levels of malvidin were also detected.

Flavonoid aglycones were also extracted from flowers and leaves, and analysed by HPLC, and found to be various glycosides of kaempferol and quercetin (flavonols) (data not shown).

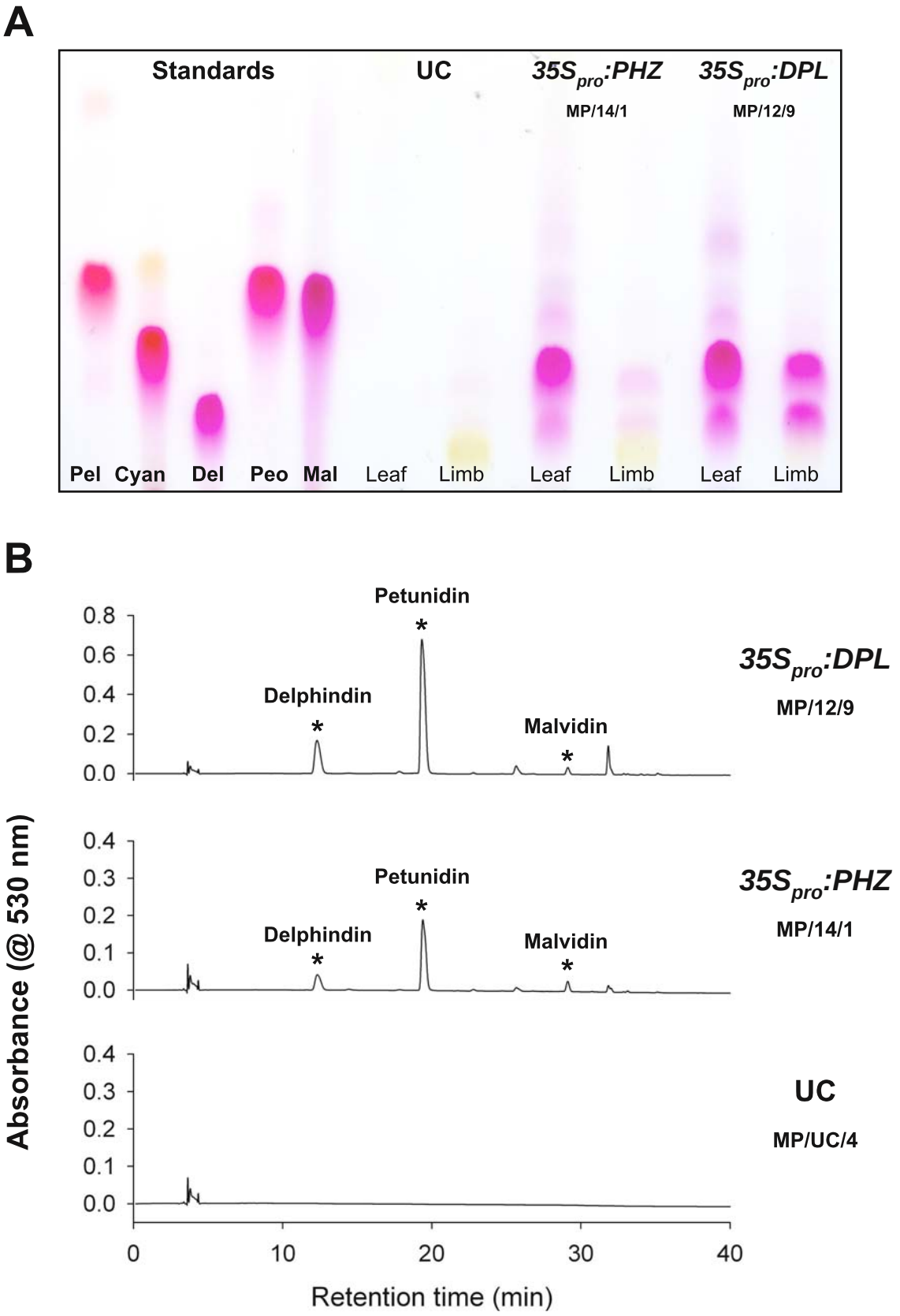


Figure 6.8 Identification of anthocyanin aglycones (anthocyanidins) present in untransformed Mitchell petunia (UC), *35S$_{pro}$:PHZ* or *35S$_{pro}$:DPL* petunias. **A.** Thin layer chromatography of leaf and flower limb extracts. **B.** High performance liquid chromatography of leaf extracts. **Pel** pelargonidin, **Cyan** cyanidin, **Peo** peonidin, **Mal** malvidin.

6.2.4 Gene expression

Transgene expression was confirmed in the *MYB* over-expression lines by northern blotting (Figure 6.9). Blots probed with *DPL* showed high levels of hybridising transcripts in the *35S$_{pro}$:DPL* lines in both flowers (Figure 6.9A) and leaves (B). In addition to detecting the *DPL* transgene, endogenous hybridising transcripts were detected with the *DPL* probe in petals of the untransformed controls, and the *PHZ* OE lines. Hybridising transcripts were also detected in the leaves of the *35S$_{pro}$:PHZ* lines, which were absent in the untransformed controls and in line MP/14/5. Blots probed with *PHZ* hybridised to a single band in the leaves of *35S$_{pro}$:PHZ* lines (Figure 6.9B), with the exception of MP/14/5, which doesn't appear to express the transgene. The *PHZ* probe also hybridised to transcripts of the same size in the leaves and flowers of the *35S$_{pro}$:DPL* lines. Hybridising bands were also detected by the *PHZ* probe in flowers of the untransformed controls (Figure 6.9A). The detection of endogenous hybridising transcripts distinct from the transgene was not unexpected, as *DPL*, *PHZ* and *An2* were shown previously to be expressed in Mitchell flowers (Chapter 4, Figure 4.2A), and *DPL* was shown to be expressed in leaves (Chapter 4, Figure 4.1B).

Transcript abundance for flavonoid biosynthetic genes was determined in the leaves of MYB over-expression lines by northern blotting (Figure 6.10). Over-expression of *DPL* resulted in enhanced transcript levels for *PAL*, *C4H*, *CHS*, *CHI*, *F3H*, *F3′H*, *F3′5′H*, *DFR*, *ANS*, *3RT*, *5GT*, *MT GST*, but not for *FLS*, *3GT* or *AT* (refer to Figure 1.2 for pathway). Similarly, these same genes appeared to be activated in *PHZ* over-expression lines, though at lower levels to that of the *DPL* lines. The MP/14/5 line, which failed to express the *PHZ* transgene, performed as the untransformed controls, and showed low levels of transcripts for *PAL*, *CHI*, *FLS* and *3GT*.

Flavonoid transcripts abundance was also determined in flower tube and limb tissue (Figure 6.11). *PAL*, *C4H*, *CHS*, *CHI*, *F3H*, *FLS*, *3GT* and AT were all expressed in flower tube and limb of untransformed Mitchell, *DPL* OE and *PHZ* OE lines. Transcripts for *PAL*, *CHS*, *CHI* and *F3H* show a small enhancement in expression levels in the flower tube of *DPL* OE lines. *F3′H*, *F3′5′H*, *DFR*, *ANS*, *3RT*, *5GT*, *MT* and *GST* show enhanced transcript levels in both *DPL* and *PHZ* over-expression lines

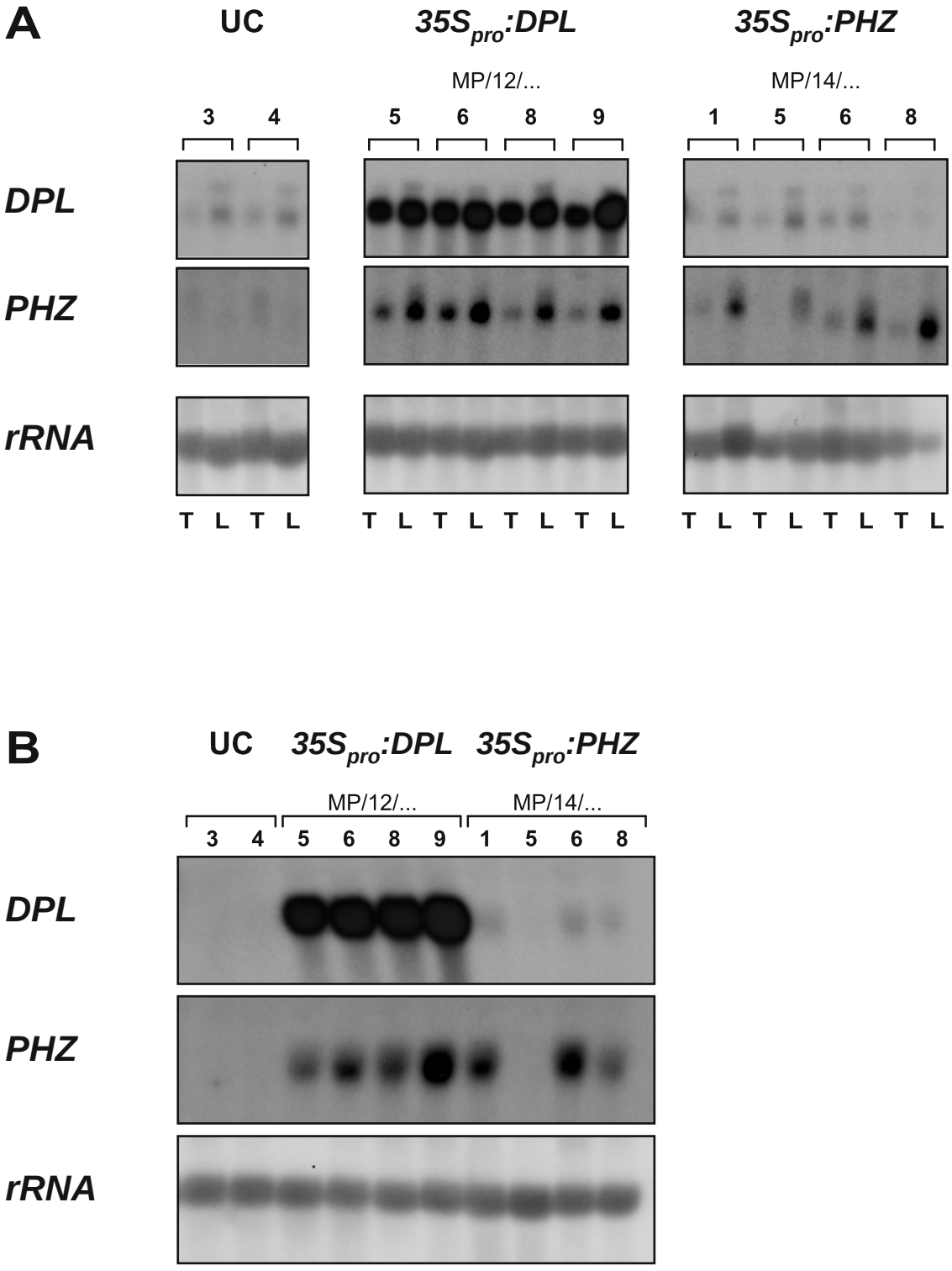


Figure 6.9 Northern analysis of transgene expression. **A.** Flower tube (T) and limb (L) tissue. **B.** Leaves. A total RNA loading control (26s rRNA) is shown.

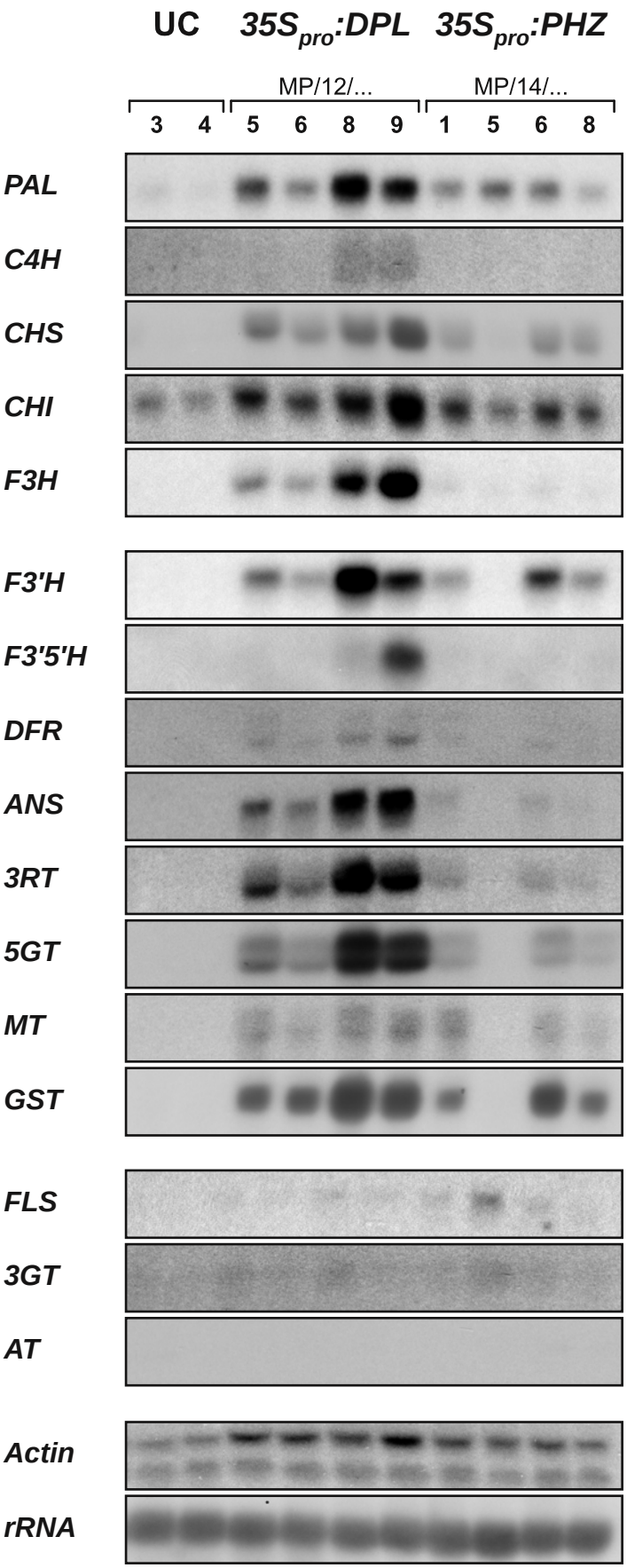


Figure 6.10 Northern blots of flavonoid biosynthetic genes in leaves of *35S$_{pro}$:DPL* and *35S$_{pro}$:PHZ* and untransformed Mitchell petunia (UC). A total RNA loading control (26S rRNA) and an mRNA loading control (Actin) are shown.

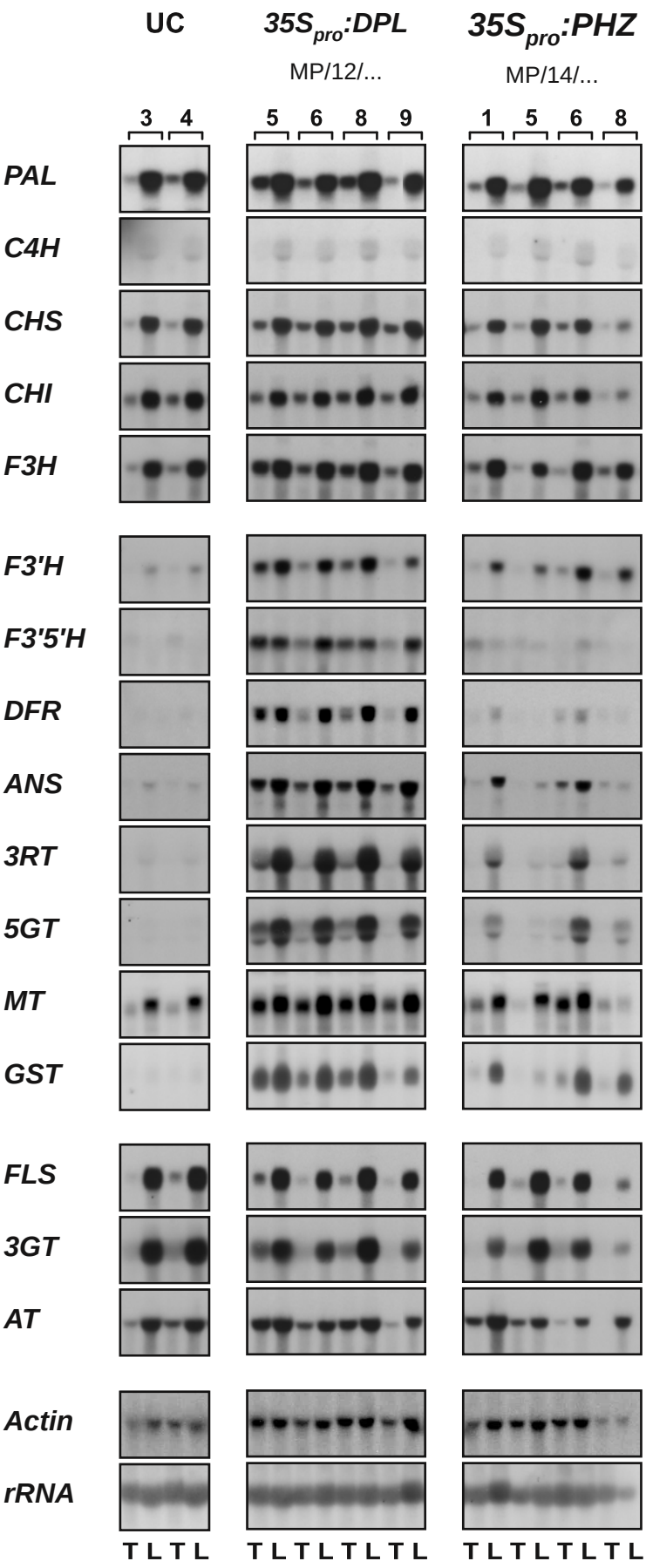


Figure 6.11 Northern blots of flower tube (T) and limb (L) tissue from *35S$_{pro}$:DPL*, *35S$_{pro}$PHZ* and untransformed Mitchell petunia (UC). Total RNA loading control (26S rRNA) and mRNA loading control (Actin) are shown.

(except MP/14/5). These 'late' anthocyanin biosynthetic genes were expressed at low levels in the flowers of untransformed Mitchell petunia.

6.3 Discussion

Over-expression of *DPL* or *PHZ* in petunia resulted in ectopic synthesis of anthocyanin pigments in vegetative tissues. In addition, anthocyanin pigmentation was enhanced in floral tissues, and the *an2* and *an4* mutations (MYB) in Mitchell petunia were complemented. This is consistent with findings in *Arabidopsis*, where the MYB anthocyanin regulator *PAP1/AtMYB75* was discovered through activation tagging (Borevitz *et al.* 2000). The *PAP1-D Arabidopsis* lines were intensely pigmented throughout the entire plant (Borevitz, et al. 2000), and subsequently null *pap1* and RNAi knockdown lines (targeting all related MYBs: *PAP2/AtMYB95*, *AtMYB113* and *AtMYB114*) were shown to have reduced anthocyanin content (Gonzalez *et al.* 2008, Teng *et al.* 2005).

DPL and PHZ both activated anthocyanin synthesis in heterologous host species. *DPL* and *PHZ* were both able to complement the *Antirrhinum rosea*^{dorsea} mutant phenotype (MYB mutant), restoring anthocyanin production to transformed petal cells. Transient transformation with either *DPL* or *PHZ* and a bHLH partner was able to activate anthocyanin synthesis in potato, sunflower (Dr Simon Deroles, *pers. comm.*), onion (Dr Colin Eady, *pers. comm.*) and *Cymbidium* (not shown). This corroborates the findings from stable petunia *DPL* and *PHZ* over-expression lines characterised in this study, that DPL and PHZ regulate anthocyanin synthesis.

However, the pigmentation phenotypes of the *DPL* and *PHZ* over-expression petunia lines differed. Over-expression of *PHZ* consistently resulted in a dusky-bronze vegetative phenotype in Mitchell and V26 petunia, while *DPL* consistently gave a darker phenotype. The level of activation of the flavonoid biosynthetic genes reflected these differences in pigmentation intensity, with *DPL* OE lines consistently showing greater increases in transcripts than the *PHZ* lines. The level of transgene expression, however, complicates the assessment of the pigmentation patterns derived as a result of

over-expressing these transcription factors. The *DPL* OE lines examined express the transgene to a much higher level than the *PHZ* OE plants. The first suggestion of this came when northern blots were probed with the coding region for either *DPL* or *PHZ*. *DPL* gave a very strong signal in the *DPL* OE lines, with only weak cross-hybridisation to the *PHZ* transgene (Figure 6.9). Blots probed with *PHZ* showed equal hybridisation to *DPL* and *PHZ* lines (except MP/14/5 which doesn't express the transgene). This reflects the relative abundance of the *DPL* transgene compared to that of the *PHZ* transgene. Subsequently, quantitative real-time PCR confirmed this, revealing a 10-fold difference in transgene expression (Chapter 7, Figure 7.4). The consistency in *PHZ* transgene expression (and phenotype) between independent lines suggests either *i)* features of the pNWA14 *PHZ* construct have affected its expression level or *ii)* expression of the *PHZ* transgene at higher levels may have inhibited the regeneration of transgenic shoots. The latter suggestion is favoured, since transient expression of the same *DPL* and *PHZ* binary constructs in petunia leaves by *Agrobacterium* infiltration results in pigmentation of similar intensity (data not shown). Further evidence the $35S_{pro}$:*DPL* and $35S_{pro}$:*PHZ* constructs (as well as $35S_{pro}$:*An2*) perform similarly is presented in Chapter 7, through promoter activation assays.

The floral pigmentation phenotypes of the *DPL* and *PHZ* over-expression petunia lines were also different. *DPL* OE lines have purple petals, which fade upon opening, probably due to the activity of the *Fading* locus in Mitchell petunia (de Vlaming and van Eekeres 1982). By contrast, *PHZ* OE petunias have pale mauve flowers, producing very little anthocyanin, which fade to white upon opening. Interestingly, both *PHZ* and *DPL* lines accumulate the same anthocyanins (delphinidin, petunidin based), in both flowers and leaves, indicating the same biosynthetic genes were targeted. Northern blots showed that *DPL* and *PHZ* OE lines have enhanced transcript levels for the same anthocyanin biosynthetic genes in flowers and leaves. The level of activation was consistently lower in the *PHZ* lines, matching the weaker pigmentation phenotypes, which may reflect the level of transgene expression.

In previous research by other researchers, $35S_{pro}$*:An2* petunias were generated (also in MP background), which had a very pale vegetative phenotype and pink flowers (Quattrocchio *et al.* 1998). The vegetative phenotype was enhanced if $35S_{pro}$*: JAF13* was co-transformed, but it was still weaker than the vegetative pigmentation generated

by expressing *DPL* or *PHZ* alone. The *An2* petunias had pink flowers suggesting cyanidin-based pigments were produced. This contrasts to the purple delphinidin and petunidin anthocyanins that accumulated in the *DPL* and *PHZ* petunias. The production of delphinidin and petunidin based anthocyanins requires the activity of Flavonoid 3′5′ hydroxylase (F3′5′H). Petunia has two loci encoding F3′5′H, *Hf1* and *Hf2*. Mitchell petunia contains the *hf1-1* allele, which is not null, but is expressed predominantly in the flower tube (*Hf1* is normally expressed in the tube and limb). It is not known if MP contains a functional *Hf2* allele, though it is thought only to act only in the flower limb (Tornielli *et al.* 2008). The differences in floral pigmentation between *DPL*, *PHZ* and *An2* OE petunia lines indicates that these closely related transcription factors have different activities, particularly to be able to activate *F3′5′H*. If AN2 was unable to activate *F3′5′H* in $35S_{pro}$*:An2* Mitchell petunia, then competition for dihydroquercetin between DFR and FLS may account for the weak vegetative pigmentation. The possibility that DPL, PHZ and AN2 act differently upon *F3′5′H* genes is examined further in Chapter 7.

Anthocyanin synthesis had different effects on flavonol production in flower and leaves. In flowers, flavonols accumulate to high levels (Figure 6.7), and the flavonol biosynthetic genes are highly expressed (*CHS*, *CHI*, *F3H*, *FLS*, *3GT*, *AT*) (Figure 6.11). *DPL* or *PHZ* over-expression had little or no effect on the levels of flavonols in the flowers, despite activating the late anthocyanin biosynthetic genes, and accumulating anthocyanins. In leaves, however, the biosynthetic genes required for flavonol synthesis were expressed at low levels (Figure 6.10), which was matched by the low total flavonol levels (Figure 6.7). Flavonol synthesis is induced by light in leaves, through the activation of the early biosynthetic genes, and those specific to flavonol production (Albert *et al.* 2009, Hartmann *et al.* 2005). The low level of flavonols in the leaves of the petunia lines reflects the lower light conditions the plants were grown under. Interestingly, the *DPL* or *PHZ* OE lines had even lower levels flavonols in their leaves than the controls (Figure 6.7). Flavonol and anthocyanin biosynthetic pathways compete for dihydroflavonol substrates (Davies *et al.* 2003) and so the activation of the late anthocyanin biosynthetic genes in the *DPL* and *PHZ* lines appears to have redirected flavonoid metabolites towards anthocyanin synthesis, and away from flavonol synthesis.

The *DPL* and *PHZ* OE petunia lines have a different spatial distribution of anthocyanins. In addition to differences in pigmentation intensity between the *DPL* and *PHZ* OE petunia lines, tissue-specific pigmentation patterns were observed. Fresh sections of stems and leaves showed anthocyanins to predominantly accumulate in the sub-epidermal cell layers of stems and leaves, and the pith and cortical cells adjacent to the vasculature. However, in addition to this, the vascular rays that connect the pith and cortex were pigmented in *PHZ* but not in *DPL* OE lines. Pigmentation patterns must arise due to the spatial expression patterns of the co-activators (bHLH, WDR) and repressors of anthocyanin synthesis, as the *MYB* transgenes were expressed from the constitutive *CaMV35S* promoter. The involvement of MYB27 (repressor) is unlikely to contribute to the vein-associated pigmentation, as this would require selective activity in *DPL* but not *PHZ* petunia lines, and *MYB27* expression was localised throughout all major tissues and cell types in seedlings and flowers of $MYB27_{pro}$*:GUS* petunias (Murr 1995). The WDR is considered to be expressed ubiquitously and constitutively (Albert, et al. 2009, de Vetten *et al.* 1997, Morita *et al.* 2006, Walker *et al.* 1999). Tobacco plants that express the bHLH *Delila* and MYB *Rosea1* constitutively from *CaMV35S* promoters produce anthocyanin in all cell types throughout stems and leaves (Professor Cathie Martin, unpublished), suggesting the WDR component is expressed ubiquitously. If this is the case, then the vascular ray pigmentation observed in the *PHZ* OE lines may be due to the expression of a bHLH partner that PHZ, but not DPL, is able to interact with to activate anthocyanin synthesis. Similar vascular associated pigmentation has also been observed in $35S_{pro}$*:PAP1* (*AtMYB75*) tomato plants, though the mechanisms for this spatial distribution remain unknown (Zuluaga *et al.* 2008).

Petunia has two known bHLH factors, AN1 and JAF13, which are thought to be involved in anthocyanin regulation. The role for JAF13 is unclear, as it cannot compensate for the loss of AN1 activity in flowers (Spelt *et al.* 2000). *JAF13* is expressed in vegetative tissues (Chapter 4, Figure 4.1B), and it is possible that it may contribute to the vegetative pigmentation patterns. Specificity for bHLH partners has been suggested for the MYB ROSEA2 in *Antirrhinum*, which appears to act with the bHLH factor MUTABILIS, but not DELILA, while the other MYB factors ROSEA1 and VENOSA can function with either bHLH (Schwinn, et al. 2006).

Ectopic expression of *DPL* or *PHZ* alone was capable of activating anthocyanin synthesis in vegetative tissues in petunia. The model for anthocyanin regulation suggests a transcriptional activation complex consisting of MYB, bHLH and WDR factors is required to activate the anthocyanin biosynthetic genes. The strong pigmentation phenotypes achieved by over-expressing *DPL* or *PHZ* alone, therefore, raises questions about which transcription factor partners are required to activate anthocyanin synthesis in these petunia lines. Previously *An11*, the WDR component, was shown to be expressed in leaves (Chapter 4, Figure 4.1B) (Albert, et al. 2009, de Vetten, et al. 1997). The involvement of the bHLH factors *JAF13* and *An1* is less clear. *JAF13* appears to be expressed constitutively throughout the plant, including leaves (Chapter 4, Figures 4.1, 4.2, 4.3) (Quattrocchio, et al. 1998). However, its involvement in anthocyanin regulation has not been equivocally established. *An1* is absolutely required for anthocyanin regulation in floral tissues (Spelt, et al. 2000), and it was shown to be induced by high light treatment in leaves, correlating to the modest accumulation of anthocyanin in wild type plants (Chapter 4, Figure 4.1). Interestingly, in $35S_{pro}$*:An2* petunias, *An1* was ectopically expressed in leaves, suggesting hierarchical or feed-forward interactions were occurring between MYB and bHLH factors (Quattrocchio, et al. 1998). Therefore, the involvement of *JAF13*, *An1* and *An11* for determining the pigmentation phenotypes in the *DPL* and *PHZ* OE petunia lines is examined in Chapter 7.

Chapter 7

Combinatorial and hierarchical control of anthocyanin synthesis in *Petunia*

7.0 Introduction

The current model for anthocyanin regulation suggests MYB transcription factors form a complex with a WDR protein and with one or more bHLH transcription factors to regulate anthocyanin synthesis (MBW; refer to Chapter 1, Figure 1.3). The co-regulators that the MYB factors *DPL* and *PHZ* act with to regulate anthocyanins are unknown. However, the *$35S_{pro}$:DPL* and *$35S_{pro}$:PHZ* petunias over-express MYB anthocyanin regulators, resulting in ectopic anthocyanin pigmentation in leaves. This suggests the both DPL and PHZ interact with endogenous bHLH and WDR factors, cooperatively, to activate anthocyanin synthesis.

Transcript abundance for transcription factors involved in anthocyanin regulation was determined in the leaves of *$35S_{pro}$:DPL* and *$35S_{pro}$:PHZ* petunia lines. Defined mutants for *an1* (bHLH) and *an11* (WDR) were examined to further address the question about which transcription factor partners are required by *DPL* and *PHZ* to regulate anthocyanins. Crosses between the *MYB* over-expression lines into these mutant backgrounds were made to determine whether there is an absolute requirement for these regulators by DPL or PHZ to activate anthocyanin synthesis.

Hierarchy between transcription factors has been suggested to play a role in anthocyanin regulation. The bHLH factor *An1* in petunia was ectopically expressed in leaves of petunias over-expressing the MYB *An2*, yet it is not known if AN2 directly activates *An1* (Quattrocchio et al., 1998). It was previously shown that the putative repressor of anthocyanin synthesis, PhMYB27, was directly regulated by AN1 (bHLH) in petunia flowers (Spelt et al., 2000). As bHLH factors involved in anthocyanin regulation have only ever been shown to function with MYB factors, it appears likely that PhMYB27

may be regulated by both AN1 (bHLH) and an R2R3 MYB activator, such as AN2, DPL or PHZ. The bHLH factor TT8 in *Arabidopsis*, for example, directly regulates its own expression in a positive feedback loop in combination with the MYB factors TT2 and PAP1 (Baudry et al., 2004; Baudry et al., 2006).

The stable *$35S_{pro}$:DPL* and *$35S_{pro}$:PHZ* petunia lines provide a system to examine possible hierarchical interaction between R2R3 MYB activators, bHLH, WDR, R3 MYB repressors and R2R3 MYB repressors. Leaves do not normally produce large amounts of anthocyanin and, therefore, the ectopic over-expression of the *MYB* factors *DPL* and *PHZ* may have altered the expression of other anthocyanin regulators. Determining the transcript abundance for transcription factors involved in anthocyanin regulation in the leaves of *$35S_{pro}$:DPL* and *$35S_{pro}$:PHZ* petunia lines also allowed the possible hierarchical interactions to be investigated. The promoters of regulatory genes that were activated as a result of *MYB* over-expression were cloned. Promoter activation assays using a range of MYB-bHLH combinations were conducted, to determine if the regulatory genes were direct targets of the MYB factors DPL, PHZ and AN2.

Several lines of evidence suggest that the MYB transcription factors *An2*, *DPL* and *PHZ* differ in their ability to activate anthocyanin production. The vegetative and floral pigmentation phenotypes of *$35S_{pro}$:An2*, *$35S_{pro}$:DPL* and *$35S_{pro}$:PHZ* petunia plants are very different (Chapter 6) (Quattrocchio et al., 1998). Differences in the pigment intensity, and spatial localisation of anthocyanins in *DPL* and *PHZ* over-expression lines suggest these factors may function with different bHLH partners and the genes they activate. Floral pigmentation phenotypes of the *An2*, *DPL*, *PHZ* OE petunia lines (MP background) show striking differences: *An2* OE resulted in a pink flower, with anthocyanin restricted to the flower limb (Quattrocchio et al., 1998); DPL OE resulted in purple flower; PHZ OE resulted in a very pale mauve flower (nearly white). These differences are likely to reflect differences in the target biosynthetic genes the MYB factors activate. This differential regulation is also likely to be under combinatorial control, where different combinations of MYB and bHLH factors have different activation activity and/or affinity for different target gene promoters. To test this hypothesis directly, transient promoter activation assays using *Agrobacterium*-mediated transformation and a dual luciferase system were established.

7.1 Materials and methods

7.1.1 Quantitative reverse transcription PCR (qRT-PCR)

First strand cDNA was prepared (Section 4.1.3) from RNA extracted from pooled leaf material (Section 6.1.4) for each independent line: untransformed controls (MP/UC/3 and MP/UC/4), *35S$_{pro}$:DPL* (MP/12/5, MP/12/6, MP/12/8 and MP/12/9) and *35S$_{pro}$:PHZ* (MP/14/1, MP/12/5 MP/14/6 and MP/14/8). Quantitative reverse transcription PCR was subsequently performed for *An1, An11, JAF13, DPL, PHZ, MYBx, PhMYB27, Act2*, *HisH4* and *EF1α*, as described in Section 4.1.3. Relative transcript abundance was determined by comparative quantification to the geometric mean of the most stable housekeeping genes (ACT2 and EF1α), as described in Section 4.1.3.

7.1.2 Mutant crosses

7.1.2.1Mutant petunia lines

cDNAs for *an11*$^{-}$ from W134 were cloned by RT-PCR from mixed stage flower bud RNA with primers K135/K136. The product was cloned into pGEMteasy and sequenced. A 5 bp direct repeat present in the W134 *an11*$^{-}$ allele, generated during the excision of the *dTph1* transposon, resulted in a frame-shift of the coding sequence (de Vetten et al., 1997).

cDNAs for *an1*$^{-}$ from W225 were cloned by RT-PCR from mixed stage flower bud RNA with primers K356/K357. The product was cloned into pGEMteasy and sequenced. The cDNA contained a 7 bp insertion, causing a frame-shift in the coding sequence. This 7 bp insertion corresponded to an intron position in the *An1* gene, and the 7 bp matched sequence from the intron, indicating mis-splicing. The region surrounding the intron was cloned from genomic DNA of W225 and MP by PCR using the primers K370/K371, to determine the molecular basis for the W225 mutation to allow PCR genotyping. The product was cloned into pGEMteasy and sequenced. The genomic clone of W225 *an1*$^{-}$ contained a 7 bp insertion, consisting of a 5 bp direct repeat separated by 2 bp, representing the excision footprint of the transposon *dTph1* (Spelt et al., 2002).

7.1.2.2 Crosses to mutant lines

Flowers of MP/12/9 (*35S$_{pro}$:DPL*) and MP/14/8 (*35S$_{pro}$:PHZ*) MYB over-expression lines were pollinated with W225 (*an1$^-$ an1$^-$*) or W134 (*an11$^-$ an11$^-$*) petunia mutants. Stage five flower buds were opened and anthers removed (pollen acceptor). Anthers with pollen (dehisced) were collected from the mutant lines (pollen donor) and rubbed on the stigma of the transgenic flower. The pollinated flower was covered until the corolla abscised. Seed was collected from mature seed pods. The seed from the first cross was sown and plants containing the transgene were selected (visually, due to their purple leaves) and grown on until flowering. The F1 generation were back-crossed with the W225 (*an1$^-$ an1$^-$*) or W134 (*an11$^-$ an11$^-$*) mutants, to generate plants containing the over-expression construct in background lacking either *an1* or *an11*. The F2 seed from the resulting crosses were sterilised for 10 min in 15% v/v Janola™ bleach (0.63 % (w/v) sodium hypochlorite, 15 mM NaOH) and a few drops of Tween20, followed by three washes with sterile water. The seed was imbibed overnight in 100 mg mL^{-1} GA_3. The GA_3 solution was removed and the seed was suspended in sterile 0.1% (w/v) agarose and dispensed on media #2 (Appendix 1.5) containing 200 mg L^{-1} kanamycin to select for transgenic seedlings (Deroles and Gardner, 1988). Plants that remained healthy were confirmed to contain the transgene by PCR. Seedlings containing the transgene were grown on, exflasked, and grown in the GMO greenhouse.

7.1.2.3 Genotyping F2 transformants

The resulting transformants were screened by PCR to ensure they contained the transgenic *MYB* over-expression cassette (NWA12 *DPL* NA34/NA35; NWA14 *PHZ* NA24/NA25). Genotyping the F2 transformants for the mutant or wild type alleles of *an1$^-$/An1$^+$* or *an11$^-$/An11$^+$* was performed by amplifying a portion of the gene containing the transposon footprints, and determining the genotype on the basis of size, as the mutant alleles will generate a product 5 or 7 bp larger for *an1$^-$* and *an11$^-$*, respectively, than the wild type alleles. The PCR products were fluorescently labelled with 6-FAM, using a 6-FAM labelled M13 primer based on the method described by Schuelke (2000), and separated by capillary electrophoresis (ABI3730 Genetic Analyzer, Applied Biosystems Inc) at the Allan Wilson Centre, Massey University.

Genotyping reactions were performed with the 6-FAM M13 primer and the gene specific primers NA145/NA146 for *an11*$^{-}$/*An11*$^{+}$ and NA147/NA148 for *an1*$^{-}$/*An1*$^{+}$.

Each genotyping reaction contained:

1 µL	template (gDNA)	(~50-100 ng)
0.4 µL	10 µM sense 6-FAM M13 primer	(0.16 µM)
0.1 µL	10 µM sense primer	(0.04 µM)
0.4 µL	10 µM antisense primer	(0.16 µM)
0.5 µL	10 mM dNTPs	(200 µM, each)
2.5 µL	10 × PCR buffer	
19.9 µL	sterile H_2O	
0.2 µL	***Taq*** polymerase	
25 µL		

Genomic DNA was extracted from leaf material of the F2 population, and from Mitchell, W134 and W225 petunia lines (Section 2.9.2). Positive and negative controls were always performed.

Initial denaturation	94℃	2 min	
Denaturation	94℃	30 s	× 35
Annealing	58℃	30 s	
Extension	72℃	30 s	
Final extension to Promote A-tailing	72℃	30 min	
Hold	12℃	Hold	

The final extension at 72°C was performed to favour non-template A-tailing of PCR products by *taq* polymerase, to provide a homogenous population of PCR products for capillary separation. An aliquot of the PCR reactions were run on agarose gels to ensure amplification, before 1 µL of diluted PCR product (*An1* 1:2, *An11* 1:2) was sent for capillary separation. Separation of the wild type (Mitchell) and mutant (W134 or W225) amplification products enabled a simple comparison for genotyping, in addition to molecular size standards.

7.1.3 Activation assays

7.1.3.1 Plant material and growth conditions

Mitchell petunia plants were grown under ambient greenhouse conditions, during February-March (summer). Young plants were grown until they had branched from their axillary buds, at which time young expanding leaves at each apex were used for *Agrobacterium* infiltration. Plants were well watered prior to infiltration, to ensure stomata were open to allow efficient infiltration of the *Agrobacterium* broth.

7.1.3.2 Effector constructs

A cDNA for the bHLH factor *JAF13* was amplified from mixed stage flower cDNA (Mitchell petunia), by reverse-transcription-PCR using the primers NA48/NA49. The product was directionally cloned into pART7 with *Eco*RI/*Xho*I, generating the plasmid pNWA28. *JAF13* contained an internal *Eco*RI site, so the amplified *JAF13* cDNA was fully digested with *Xho*I, before partial digestion with *Eco*RI.

Effector constructs over-expressing cDNAs of petunia anthocyanin transcription factors from a *35S* promoter were generated by cloning the *NotI* cassette from the pART7 based vectors pPN73 (*$35S_{pro}$:An2*), pKES14 (*$35S_{pro}$:An1*), pNWA28 (*$35S_{pro}$:JAF13*) and pPN79 (*$35S_{pro}$:An11*) into the *Not*I site of the pART27 binary vector. The resulting binary vector plasmids were named pNWA29 (*$35S_{pro}$:An2*), pNWA30 (*$35S_{pro}$:An1*) and pNWA31 (*$35S_{pro}$:JAF13*). These binary constructs, along with pNWA12 (*$35S_{pro}$:DPL*) and pNWA8 (*$35S_{pro}$:PHZ*) (Section 6.1.1) were transformed into *Agrobacterium* GV3101 by electroporation (Section 2.4.4).

7.1.3.3 Promoter:Luciferase reporter constructs

The promoters for *CHS-A*, *CHS-J*, *Hf1* (F3′5′H), *Hf2* (F3′5′H), *DFR-A*, *An1* (bHLH), and *MYBx* (R3 MYB) from petunia, were amplified with primers and template listed in Table 7.1. These included the restriction sites *Xho*I and *Nco*I, which enabled the promoter to be cloned in front of the firefly luciferase gene in the reporter vector pGreenII 0800-LUC (Appendix 3). The promoter sequences for *CHS-A*, *CHS-J*, *DFR-A*

and *An11* were obtained from Genbank (Accessions X14591; X14597; X79723; U94748 respectively), and primers were designed based upon this. The promoters for *Hf1*, *hf1-1*, *Hf2*, *An1* and *MYBx* were isolated by genome walking, as described in Section 2.12. The positive control pNWA38 was generated by cloning the the double *35S* promoter from pDAH2 into pGreenII 0800-LUC with *Pst*I/*Nco*I, placing a double *35S* promoter in front of the firefly luciferase reporter gene. pGreenII 0800-LUC also has a $35S_{pro}$*:REN* luciferase construct to serve as an internal control, to allow normalisation for infection efficiency.

Table 7.1 Primers and template for amplifying promoters of petunia flavonoid biosynthetic and regulatory genes.

Promoter	**Gene-specific primers**	**Source of template DNA**
Biosynthetic genes		
***CHS-A*$_{pro}$**	NA117/NA118	V26 petunia*
***CHS-J*$_{pro}$**	NA119/NA120	Mitchell petunia
***Hf1*$_{pro}$**	NA123/NA124	V26 petunia*
***hf1-1*$_{pro}$**	NA165/NA124	Mitchell petunia
***Hf2*$_{pro}$**	NA125/NA126	Mitchell petunia
***DFR-A*$_{pro}$**	NA121/NA122	Mitchell petunia
Regulatory genes		
***An1*$_{pro}$**	NA115/NA116	Mitchell petunia
***MYBx*$_{pro}$**	NA133/NA112	Mitchell petunia

* V26 was chosen as the template, because Mitchell petunia contains the ***hf1-1*** allele at the ***Hf1*** locus in a homozygous form, which is only expressed in the flower tube, while V26 has fully functional copies for ***Hf1***. ***CHS-A*** was amplified from V26 as amplification from Mitchell petunia was unsuccessful. Primers for the ***CHS-A*** promoter were based upon sequence data obtained from the hybrid petunia background V30, which is genetically more similar to V26.

7.1.3.4 Agrobacterium infiltration

Agrobacterium-infiltration was performed as described by Hellens et al. (2005). *A. tumefaciens* carrying reporter or effector plasmids were grown on LB-agar with appropriate antibiotic selection. For strains carrying the reporter plasmids (pGreenII 0800-Luc based + pSOUP helper) selection was 50 mg L^{-1} kanamycin, 20 mg L^{-1} gentamicin and 50 mg L^{-1} rifampicin. A loop (10 µL approx.) of freshly grown confluent bacteria was re-suspended in 10 mL of infiltration media (10 mM $MgCl_2$, 500 µM acetosyringone), and left at room temperature without shaking for 2-4 h prior to infiltration.

For each assay, 100 µL of the *Agrobacterium* culture containing the reporter construct (*promoter:LUC*) was mixed with 450 µL of *Agrobacterium* culture containing an effector construct (*$35S_{pro}$:TF*) and 450 µL of *Agrobacterium* culture containing a second effector (*$35S_{pro}$:TF*) or the pART27 empty vector. This enabled the proportion of *Agrobacterium* containing the reporter construct and each effector to be the same for each combination. Positive and negative controls were always performed: *$35S_{pro}$:LUC* (positive), pGreenII 0800-LUC (*LUC* background, *REN* positive) and each reporter promoter construct with pART27 empty vector (background activity of promoter).

The *Agrobacterium* mixture was infiltrated into the abaxial surface of young petunia leaves (200-500 µL) with at least two biological replicates (separate plants). Plants were allowed to recover overnight in a dim, cool room before being returned to the greenhouse. The dual luciferase assay was performed three days post infiltration.

7.1.3.5 Dual luciferase assay

Leaf discs (3 mm) were made from the infiltrated area (at least three replicates) of each leaf from each of the biological replicates, and each disc was placed into a well of a white 96-well plate (Optiplate) containing 40 µL of 1× PBS (Appendix 1.8.3) containing 1 mM DTT. DTT was included to prevent oxidation of phenolic compounds present in petunia leaves. The DualGlo™ Assay System (Promega) was used: 40 µL of DualGlo™ luciferase reagent was added to each well, the sample was incubated for 10 min at 25°C before luciferase activity was measured (20 s measurement) in a BMG

PolarStar fluorescence/luminescence plate reader (25°C). Subsequently, the firefly luciferase activity was quenched, and Renilla activity activated by adding 40 μL of DualGlo™ Stop and Glo reagent to each well, and incubating for 10 min at 25°C, before Renilla activity was measured (20 s, 25°C). The Luciferase values were normalised to Renilla.

7.2 Results

7.2.1 Requirement for anthocyanin co-regulators

Petunia lines containing null alleles for the WDR factor *An11* and the bHLH factor *An1* were used for experiments to test whether these particular factors are required by the MYB factors *DPL* and *PHZ* to regulate anthocyanin synthesis.

The mutant line W134 (*an11*$^{-}$) completely lacks anthocyanin. The flowers are not pigmented and anthocyanin pigmentation in the vegetative tissues was never observed (Figure 7.1A). The pedicel is green, yet this is usually highly coloured with anthocyanin in most petunia varieties. Mitchell, V26, V30 and W59 petunia lines grown under the same conditions produced anthocyanins in the stems on the sun-exposed side, and the pedicels were always coloured. The gene structure for *an11*^{W134} is shown in Figure 7.1A, showing the insertion of a 5 bp footprint, left after the excision of the *dTph1* transposon. The presence of this footprint results in a frame-shift, rendering the allele null.

The mutant line W225 (*an1*$^{-}$) completely lacks anthocyanin. The flowers are not pigmented (Figure 7.1B), although, in the two years this line has been grown, two flowers have shown pale pink, partial revertant sectors in the petal limb (out of hundreds of flowers). Anthocyanin pigmentation in vegetative tissues is never observed and the pedicels or vegetative stems are never coloured. The gene structure for *an1*^{W225} is shown in Figure 7.1B, showing the presence of a 7 bp footprint left from the excision of the *dTph1* transposon, adjacent to the splice site at the 3′ end of intron six. The presence of this insertion is known to severely reduce transcript accumulation (Spelt et

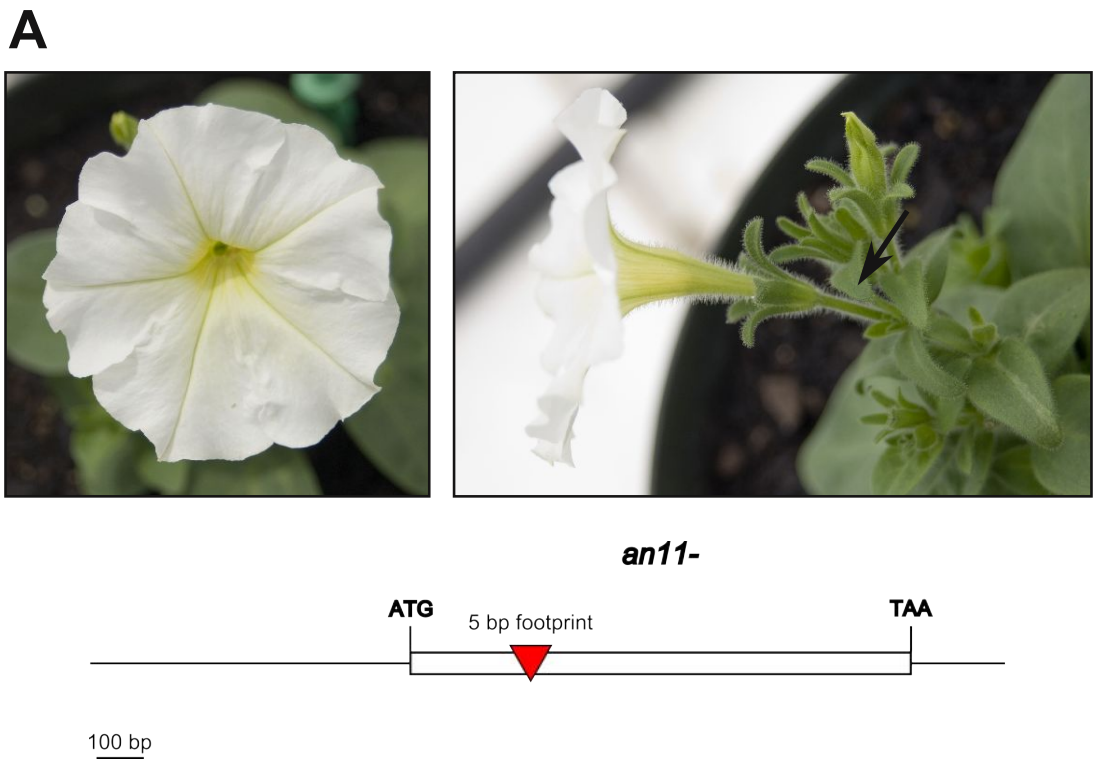


Figure 7.1 Pigmentation phenotypes for W134 (*an11*$^{-}$) (**A**) and W225 (*an1*$^{-}$) (**B**) petunia lines. Arrows indicate the lack of anthocyanin on the pedicel. The molecular basis for the mutant alleles is shown below phenotypes: start and stop codons are indicated; boxes represent coding sequence; green lines represent introns; red triangles represent transposon footprints.

al., 2002). cDNAs for the W225 allele of *an1* were cloned, and found to contain a 7 bp frame-shift, rendering any accumulated transcript null.

The *MYB* OE lines (*DPL* MP/12/9; *PHZ* MP/14/8) were crossed into *an1*$^{-}$ (W225) and *an11*$^{-}$ (W134) mutant lines (Figure 7.2) to determine the requirement for *An1* and *An11* by *DPL* and *PHZ*. Coloured F1 seedlings were selected and grown on, and these were then backcrossed to the mutant background (either W225 or W134). The resulting F2 seed was germinated on media with kanamycin selection. The seedlings were scored for their kanamycin sensitivity and pigmentation phenotypes (Table 7.2). A 1:1 ratio (approx.) of coloured (anthocyanin positive) to non-coloured seedlings was observed for the kanamycin resistant F2 progeny of the *DPL/an11*$^{-}$, *PHZ/an11*$^{-}$ and *DPL*/*an1*$^{-}$ crosses. The kanamycin resistant F2 progeny of the *PHZ* /*an1*$^{-}$ cross were all coloured.

Representative progeny are shown in Figure 7.3. The homozygous mutant *an1*$^{-}$ and *an11*$^{-}$ plants over-expressing *DPL* lacked anthocyanin, and the homozygous mutant *an11*$^{-}$ plants over-expressing *PHZ* also lacked anthocyanin. The presence of the transgenic cassette was confirmed and progeny were genotyped for the mutant and wild type alleles. Genotyping was performed based upon the presence of small bp differences between the mutant alleles, containing transposon footprints, and the Mitchell alleles. The regions containing the allelic differences were amplified by PCR, and the alleles were separated by capillary electrophoresis and detected by fluorescence, as is routinely performed for microsatellite analyses.

There were problems genotyping the F2 progeny from the (*35S$_{pro}$:PHZ* ×W225) × W225 cross. The progeny segregated 1:1 for full vegetative colour, and colour restricted to the vasculature in vegetative tissues. However, genotyping suggested all the progeny tested were heterozygous for *An1*/*an1*$^{-}$. The progeny were phenotypically the result of an F2 cross, as flower petal fading segregated at approximately 1:1, which is expected as *Fading* is a dominant trait from Mitchell petunia (not shown). It was thus prudent to test for the presence of the *an11*$^{-}$ (transposon footprint) allele in these plants, in case pollen sources were mixed up during the crossing (since both W225 (*an1*$^{-}$) and W134 (*an11*$^{-}$) are phenotypically identical). The presence of the *an11*$^{-}$ allele could not be detected in the *PHZ* /*an1* progeny. It appears *An1*$^{+}$ (Mitchell petunia) is linked to the T-DNA insertion (containing *35S$_{pro}$:PHZ*) in the transgenic line MP/14/8. New crosses

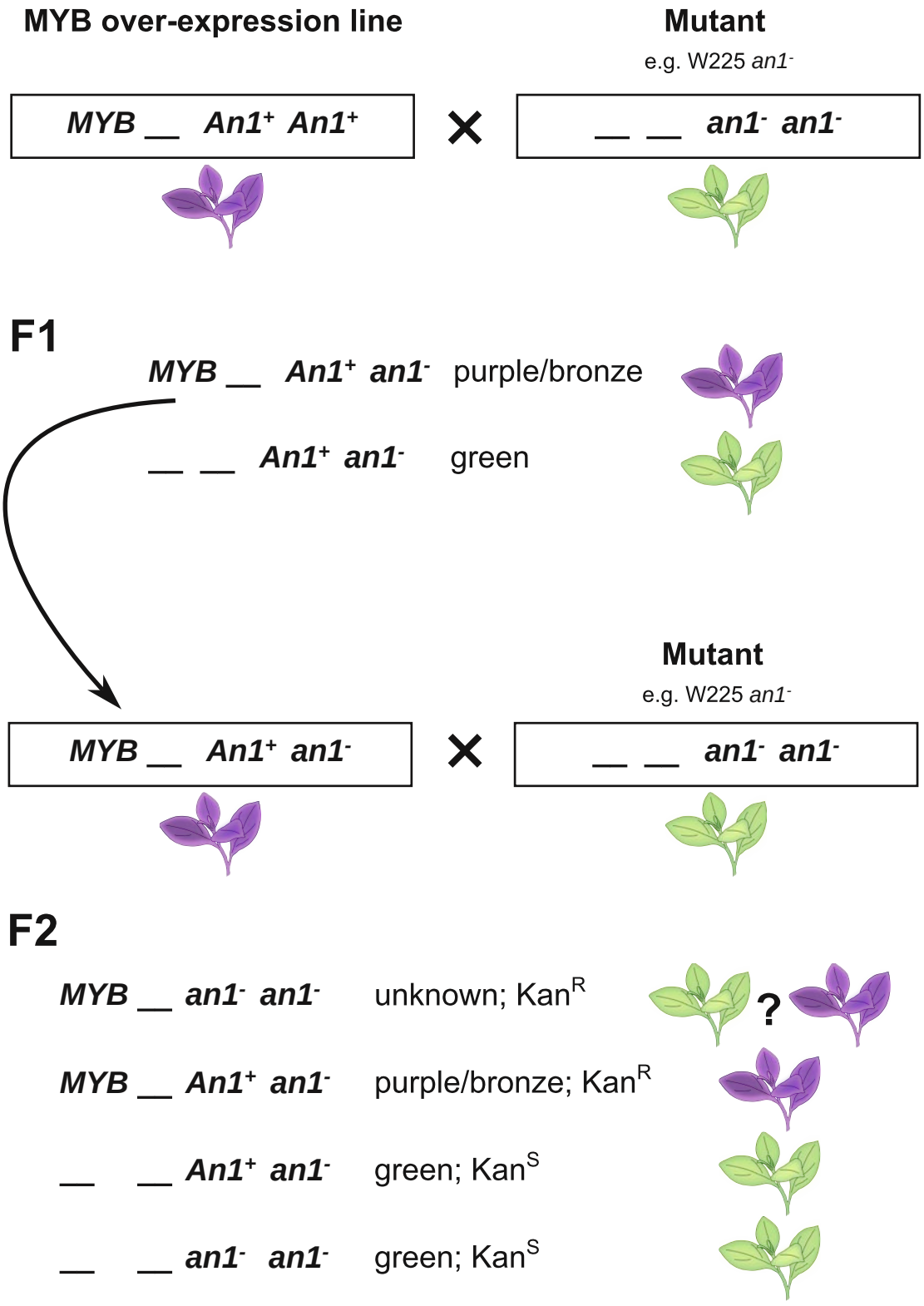


Figure 7.2 Diagramatic depiction of the strategy for introducing MYB over-expression constructs into mutant *an1*$^{-}$ or *an11*$^{-}$ backgrounds (W225/*an1*$^{-}$ is shown as an example). MYB represents the transgene containing the over-expression construct and kanamycin resistance. This is based upon a single transgene locus, although this has not been determined. Predicted pigmentation phenotypes are indicated, as are sensitivity or resistance to kanamycin (Kan^{S} or Kan^{R}).

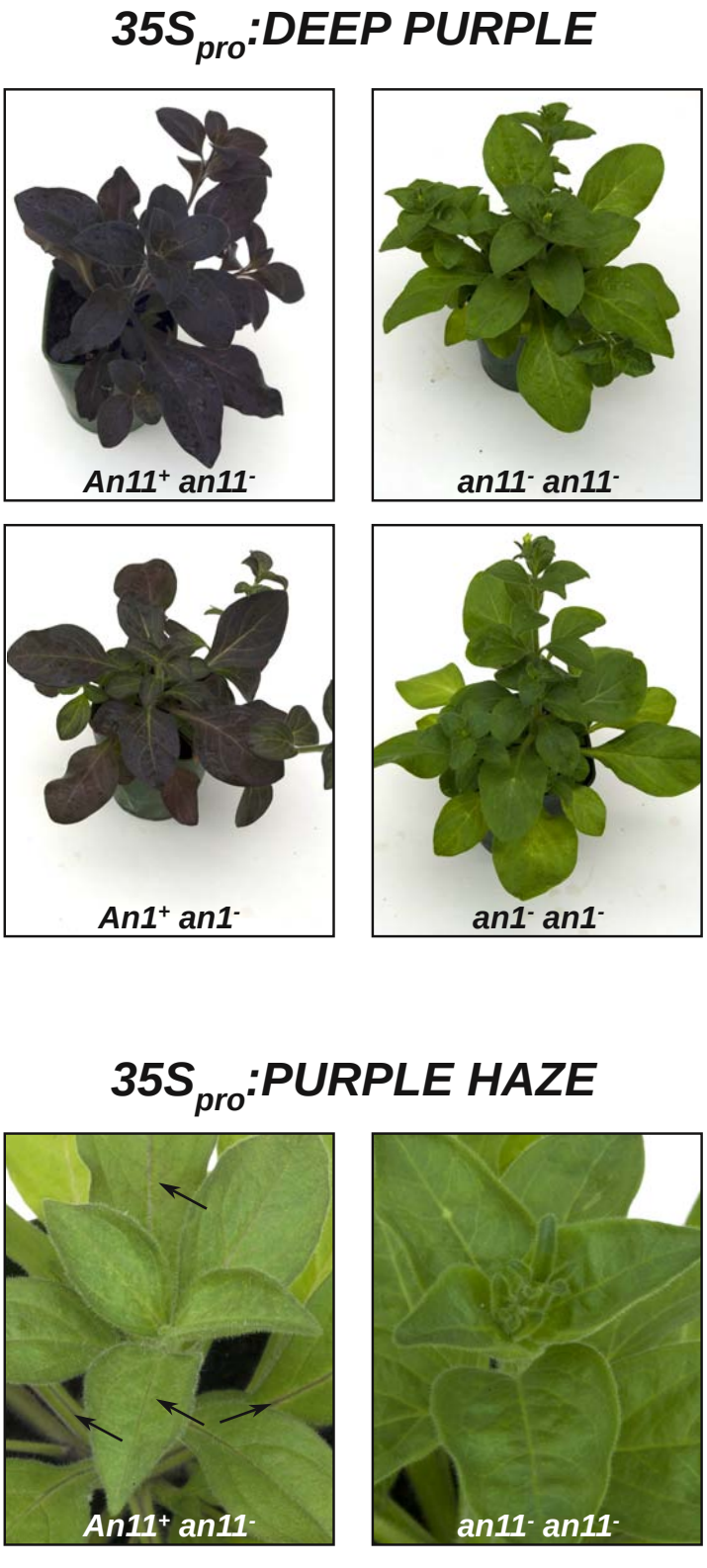


Figure 7.3 Vegetative pigmentation phenotypes of F2 progeny of *35S$_{pro}$:DEEP PURPLE* or *35S$_{pro}$:PURPLE HAZE* crossed into W134 petunia (*an11^{-}*) or W225 petunia (*an1^{-}*). Arrows indicate vascular associated anthocyanin pigmentation. Plants were confirmed to contain the transgene by PCR, and were genotyped (*An1^{+}/an1^{-}* or *An11^{+}/an11^{-}*).

have been set up with different transgenic lines (MP/14/1 and MP/14/6), which will be analysed in a future investigation once F2 seed has been collected, but this is not part of this current PhD investigation.

Table 7.2 Segregation of F2 generation. Representative data from a single cross is shown.

Line	Vegetative pigmentation phenotype	Kanamycin sensitivity Sensitive S; Resistant R	Number	Proportion
***DPL*/*an1*⁻**				
(MP/12/9 × W225) × W225				
	Dead (bleached)	S	34	0.36
	Green	R	33	0.35
	Purple	R	28	0.29
		Total	95	
***DPL*/*an11*⁻**				
(MP/12/9 × W134) × W134				
	Dead (bleached)	S	8	0.10
	Green	R	31	0.40
	Purple	R	38	0.49
		Total	77	
***PHZ*/*an1*⁻**				
(MP/14/8 × W225) × W225				
	Dead (bleached)	S	21	0.40
	Green	R	0	0.00
	Purple	R	31	0.60
		Total	52	
***PHZ/an11*⁻**				
(MP/14/8 × W134) × W134				
	Dead (bleached)	S	24	0.39
	Green	R	15	0.24
	Purple	R	23	0.37
		Total	62	

7.2.2 Transcript abundance of anthocyanin regulators in MYB OE petunia lines

Transcript abundance for transcription factors involved in anthocyanin synthesis were determined in leaves of untransformed Mitchell, *35S$_{pro}$:DPL* (*DPL* OE) and *35S$_{pro}$ PHZ* (*PHZ* OE) petunias (Figure 7.4). Representative vegetative phenotypes are shown in Figure 7.4A. High levels of transcripts were detected for *DPL* and *PHZ* in the *DPL* OE and *PHZ* OE lines, respectively. The transcript levels of *DPL* in the *DPL* OE lines was >10 fold higher than the levels of *PHZ* in the *PHZ* OE lines.

Transcript levels for the bHLH factor *JAF13* were unchanged in the MYB OE lines. *An1* transcripts were not detected in the leaves of untransformed Mitchell petunia, but were highly activated in the MYB OE lines. *DPL* lines had higher transcript levels for *An1* than *PHZ* lines. *MYBx* transcripts were also highly activated in MYB OE lines compared to the untransformed Mitchell controls.

MYB27 and *An11* transcript levels were slightly elevated in the MYB OE lines. *MYB27* showed a two-fold increase in both *DPL* and *PHZ* lines compared to the Mitchell controls, while *An11* showed a smaller induction (≤ two fold).

7.2.3 Promoter activation assays – Transcription factors

The enhanced transcript levels for *An1*, *MYBx* and *MYB27* in the leaves of the *MYB* OE lines (Figure 7.4) suggested *DPL* and *PHZ* may directly activate these genes. The hypothesis that DPL, PHZ and AN2 directly regulate *An1* and *MYBx* was tested using promoter activation assays. The promoters for *An1* and *MYBx* were isolated by genome-walking. The activity of the MYB AN2 was compared to DPL and PHZ, and pairwise combinations with the two known bHLH regulators, AN1 and JAF13 were performed.

The *An1* promoter failed to be strongly activated by any of the transcription factor combinations tested (Figure 7.5), especially when compared to Figure 7.6, which shows

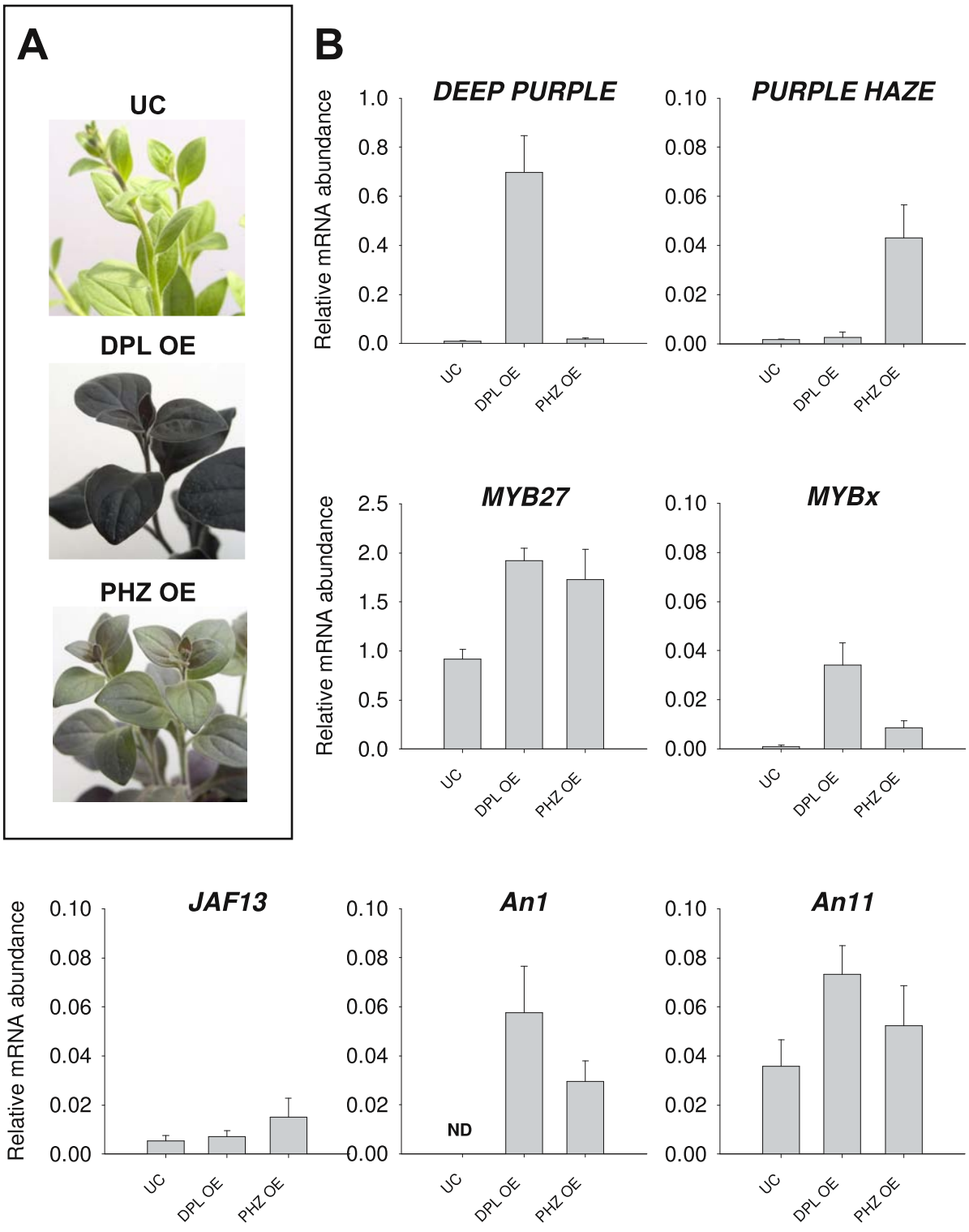


Figure 7.4 A. Vegetative phenotypes of Untransformed controls (UC), *35S$_{pro}$:DEEP PURPLE* (*DPL* OE) and *35S$_{pro}$:PURPLE HAZE* (*DPL* OE) petunia lines. **B.** Transcript abundance for transcription factors involved in anthocyanin regulation in leaves of UC, DPL OE and PHZ OE. Means of independent lines are shown ± SE, *n*=2 UC, *n*=4 *DPL* OE, *n*=3 *PHZ* OE. Line MP/14/5 was excluded from the *PHZ* OE mean as this line does not express the transgene. Relative transcript abundance was determined by qRT-PCR, normalised to the geometric mean of *ACT2* and *EF1α*. ND = not detected.

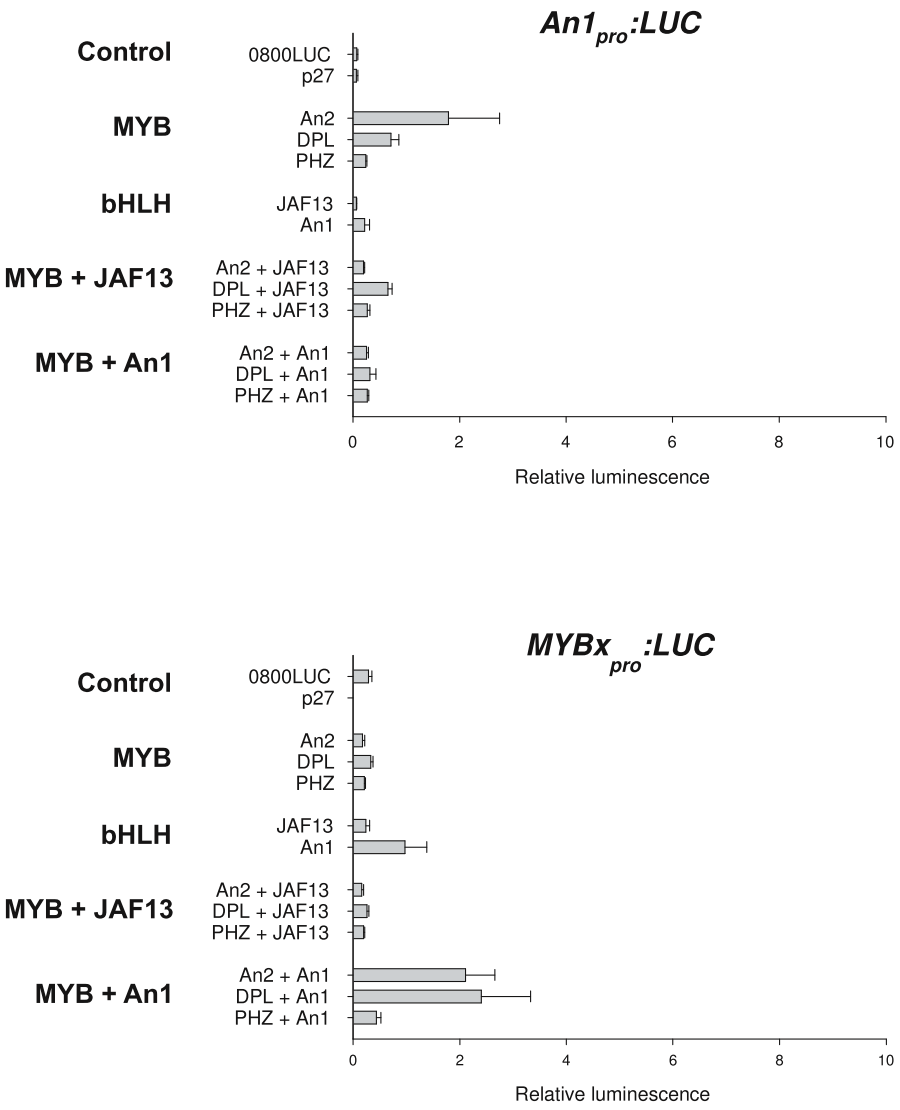


Figure 7.5 Activation assays upon the *An1* and *MYBx* promoters, using a dual luciferase reporter system and *Agrobacterium* infiltration of Mitchell petunia leaves. Effector constructs expressing MYB (AN2, DPL, PHZ) and bHLH (JAF13, AN1) factors were tested alone, and in pairwise combinations. p0800LUC is the empty vector control, p27 is the promoter without effectors (background promoter activity). Means ± SE, *n*=6 are reported.

strong activation by MBW combinations. The variable, low level activity may be the result of weak activation by endogenous transcription factors, which has occurred as an indirect consequence of anthocyanin accumulation.

The *MYBx* promoter was weakly activated in leaves co-transformed with MYB and bHLH transcription factors (Figure 7.5). AN2 or DPL co-transformed with AN1 resulted in activated *MYBx* promoter activity. The infiltrated region of leaves transformed with AN2+AN1 or DPL+AN1 became highly coloured with anthocyanin. Other combinations also accumulated anthocyanin, but to a lesser extent.

7.2.4 Promoter activation assays – Biosynthetic genes

Activation assays upon the promoters of flavonoid biosynthetic genes were performed to examine whether the MYB gene family members had target gene, or bHLH partner preferences. Two genes encoding chalcone synthase in petunia, *CHS-A* and *CHS-J*, have previously been reported to differ in their responses to the anthocyanin regulators. *CHS-A*, which accounts for 90% of CHS transcripts in flowers, was reported to be regulated independently of the anthocyanin regulators, while *CHS-J* is thought to be a direct target. The promoter of *DFR-A* was also chosen for investigation, as this is the committed step towards anthocyanin synthesis, and it has been used in previous studies examining anthocyanin regulation by *An2*, *An1* and *JAF13* (Quattrocchio et al., 1998; Spelt et al., 2000).

The *CHS-A* promoter was activated by AN2, DPL and PHZ without the addition of a bHLH partner, and DPL gave the highest activation (Figure 7.6). The addition of JAF13 did not increase the activation in any MYB-bHLH combination. The addition of AN1 did increase the activation of the *CHS-A* promoter when combined with AN2 or PHZ, but not DPL.

The *CHS-J* promoter was activated by AN2, DPL and PHZ (Figure 7.6). This was enhanced if JAF13 or AN1 were co-transformed. Combinations that included DPL

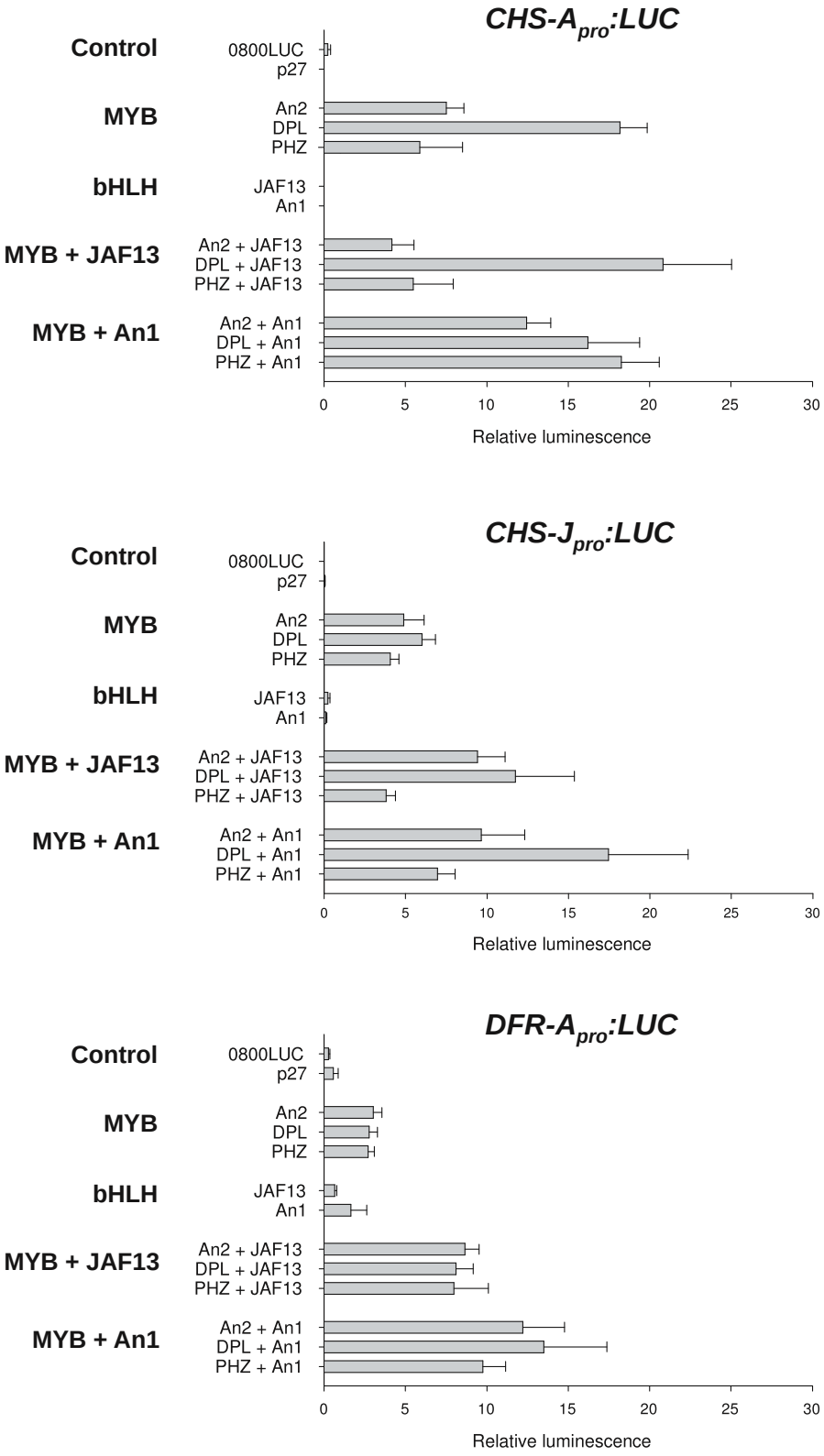


Figure 7.6 Activation assays upon the *CHS-A*, *CHS-J* and *DFR-A* promoters, using a dual luciferase reporter system and *Agrobacterium* infiltration of Mitchell petunia leaves. Effector constructs expressing MYB (AN2, DPL, PHZ) and bHLH (JAF13, AN1) factors were tested alone, and in pairwise combinations. p0800LUC is the empty vector control, p27 is the promoter without effectors (background promoter activity). Means ± SE, *n*=6 are reported.

consistently showed slightly higher activation than the other MYB-bHLH combinations tested.

The *DFR-A* promoter was activated by all MYB factors equally (Figure 7.6). The addition of JAF13 or AN1 further enhanced activation compared to the MYB factors alone.

The floral pigmentation phenotypes for *$35S_{pro}$:An2* (pink), *DPL* (purple) and *PHZ* (pale mauve) petunia lines suggested that these MYB factors may have different activation activity for genes encoding F3′5′H. Two genes encoding F3′5′H are present in petunia, *Hf1* and *Hf2*. *Hf1* is expressed in the flower tube and limb, while *Hf2* expression is limited to the flower limb (Tornielli et al., 2008), which may suggest these genes are differentially regulated by different MYB family members. Mitchell petunia harbours the *hf1-1* allele, which is expressed predominantly in the flower tube, but only weakly in other parts of the flower (Tornielli et al., 2008). The *hf1-1* allele is derived from the wild progenitor species *Petunia axillaris*, yet the molecular basis of the *hf1-1* allele is unknown.

The promoter for *Hf1* (V26 petunia) and *hf1-1* (MP) were cloned by gene-walking up from the coding sequence. These two sequences share approximately 380 bp of highly conserved sequence, upstream of the start codon, but then diverge completely. The region of conservation includes the putative TATA box, and is thought to contain the basal promoter. Two insertions lie upstream of the putative basal promoter in the MP *hf1-1* promoter, compared to the fully functional V26 *Hf1* allele (Figure 7.7A). The *Hf2* promoter was also cloned from MP, although it is unknown whether MP contains a functional *Hf2* allele or not.

The *Hf1* promoter (V26) was activated by MYB-bHLH combinations and DPL or AN2 alone, but not PHZ (Figure 7.7B). The addition of the bHLH partner JAF13 combined with PHZ resulted in activation, but did not enhance the activation by AN2 or DPL. The inclusion of the bHLH factor AN1 combined with the MYB gave the highest activation, particularly when combined with DPL.

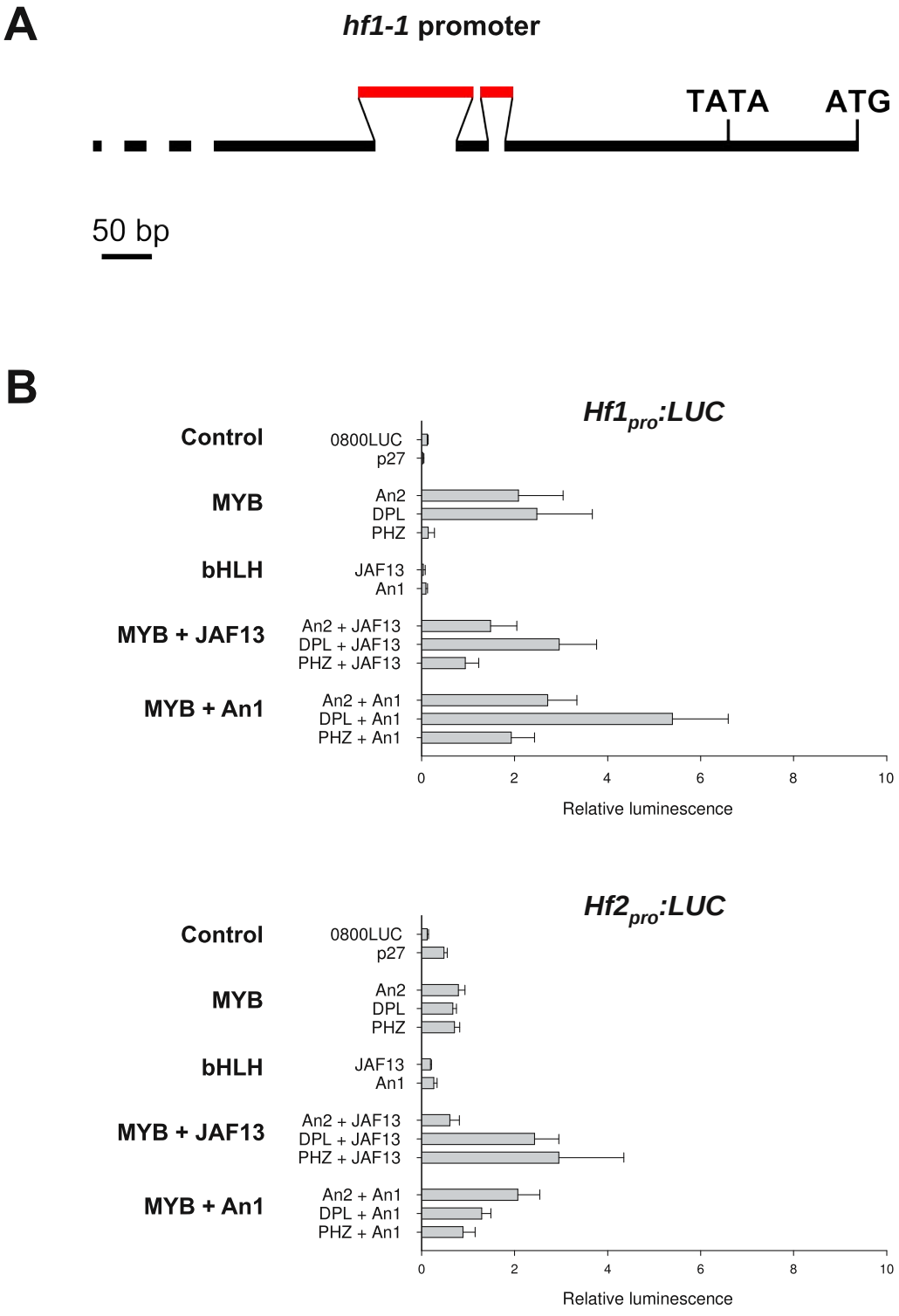


Figure 7.7 A. The promoter structure of the *hf1-1* allele from Mitchell petunia, containing two insertions upstream of the putative TATA box. **B.** Activation assays upon the promoters for genes that encode F3′5′H, *Hf1* (V26 allele) and *Hf2* (MP allele), using a dual luciferase reporter system and *Agrobacterium* infiltration of Mitchell petunia leaves. Effector constructs expressing MYB (AN2, DPL, PHZ) and bHLH (JAF13, AN1) factors were tested alone, and in pairwise combinations. p0800LUC is the empty vector control, p27 is the promoter without effectors (background promoter activity). Means ± SE, *n*=6 are reported

The *Hf2* promoter (MP) was responsive to MYB-bHLH combinations (Figure 7.7B). Expression of the MYBs AN2, DPL or PHZ alone gave activity slightly above that of the background promoter activity (p27 negative control). The addition of JAF13, combined with DPL or PHZ, but not AN2, enhanced promoter activation. The addition of AN1 combined with AN2 activated the *Hf2* reporter. The addition of AN1 did not enhance the activation by PHZ, and only slightly enhanced the activation by DPL alone.

7.3 Discussion

7.3.1Transcription factor partners

In this chapter, the combinatorial control of anthocyanin synthesis in petunia was examined. The mutant lines for the bHLH anthocyanin regulator AN1 and WDR co-regulator AN11 never produce anthocyanins. This suggests that both these proteins are required for the activation of the anthocyanin biosynthetic genes in all organs and tissues throughout the plant. The current model for anthocyanin regulation includes these factors in a complex with a MYB type transcription factor. Over-expression lines of *DEEP PURPLE* and *PURPLE HAZE* were crossed into these mutant backgrounds to determine if the pigmentation phenotypes generated by over-expression required AN1 or AN11. The WDR protein AN11 was absolutely required for *DPL*- and *PHZ*-dependent anthocyanin production. AN1 was also absolutely required for *DPL*-dependent anthocyanin production. These results are consistent with the model that the MYB anthocyanin regulators require WDR and bHLH partners, specifically AN11 and AN1, respectively.

The requirement for AN1 by PHZ was not determined, due to problems introgressing the transgene into a homozygous $an1^-$ background. It appears that recombination suppression prevented introgression of $35S_{pro}$*:PHZ* into $an1^- an1^-$ petunia. The crosses between MP/14/8 ($35S_{pro}$*:PHZ*) line and W225 ($an1^-$) failed to generate homozygous $an1^-/an1^-$ plants that also contained the transgene. The crosses were performed correctly, and all sources of error were excluded. The results indicate that the T-DNA (in line MP/14/8) and $An1^+$ (from Mitchell petunia) were linked, and failed to segregate.

Mitchell petunia is a doubled haploid from an interspecific cross (*Petunia axillaris* × [*P. axillaris* × *P. hybrida*]), while W225 is an inbred *P. hybrida* cultivar. The differences in genetic background may have prevented homologous (or homeologous) recombination from occurring.

Suppressed recombination is known to occur, particularly with wide crosses. For example, Robbins et al. (1995) investigated the segregation of T-DNA insertions to phenotypic markers in petunia, and provided evidence that recombination was suppressed between T-DNAs and phenotypic markers located on the same chromosome. Physical localisation of the T-DNAs and mapping markers using fluorescent *in situ* hybridisation (FISH) on petunia chromosomes confirmed this suppression effect (ten Hoopen et al., 1996). Suppressed meiotic recombination was also shown to occur in two very closely related petunia species (*P. axillaris* and *P. axillaris parodii*) (Galliot et al., 2006). It is possible, therefore, that the T-DNA insertion containing the $35S_{pro}$*:PHZ* transgene was located on chromosome VI, which contains the *An1* locus. If recombination between homeologous chromosomes did not occur, then *An1* would be linked to the transgene. Consistent with this hypothesis is that crossing this same line into the $an11^{-}$ $an11^{-}$ background was successful: *An11* is located on chromosome IV, and therefore would segregate independently of the transgene. New crosses between alternative $35S_{pro}$*:PHZ* containing lines and W225 have been set up, which are likely to be successful provided the T-DNA insertion is not located on chromosome VI. It is anticipated that AN1 will be required for $35S_{pro}$*:PHZ* mediated vegetative pigmentation, based on the observations that anthocyanins are completely absent in W225 ($an1^{-}$) petunias.

In terms of mechanisms, suppressed recombination between homologous or homeologous chromosomes can occur for a range of reasons. Changes in genome organisation such as chromosome rearrangements (inversions, deletions, translocations) or regions of genomic instability (transposon rich regions) may suppress recombination (Li et al., 2007). Chromosome rearrangements involving the translocation of *CHSa* has been shown to have occurred in Mitchell and V26 petunia (located on the distal end of chromosome III) compared to V30 petunia (located on chromosome V) using FISH (Fransz et al., 1996), suggesting genomic rearrangements may contribute to recombination suppression between some cultivars of *Petunia*. It is likely that

structural differences between homeologous chromosomes, originating from different wild species, do not align correctly, preventing recombination (Robbins et al., 1995). Indeed, cytological observations of various *P. hybrida* cultivars often report variable pairing of homologous chromosomes, and pairing between chromosomes of visibly different lengths (Strommer et al., 2008). These findings suggest that structural differences between different parental *P. hybrida* genomes are a major source of recombination suppression.

7.3.2 Hierarchy between transcription factors

At least three components are required for regulating anthocyanin synthesis. The WDR proteins are ubiquitously expressed, but MYB and bHLH transcription factors are often only expressed in tissues when anthocyanin pigmentation is required. This raises questions about the expression of these two factors, and whether hierarchical interactions occur between MYB and bHLH factors. The recent identification of proteins that repress anthocyanin synthesis, through active (MYB27) or competitive (MYBx) activities raises questions about how these factors are regulated in response to anthocyanin accumulation.

In this thesis, *An1* was shown to be absolutely required for anthocyanin synthesis, even in lines which over express *DPL* (and presumably also for *PHZ* OE lines). The MYB OE lines express only a MYB factor, and *An1* transcripts are not normally detectable in leaves unless plants were stressed (Chapter 4, Figure 4.1), yet the MYB OE lines were highly pigmented with anthocyanin. A hierarchy between MYB and bHLH transcription factors may exist. The findings from this study, where over-expression of *DPL* or *PHZ* results in ectopic expression of *An1* in leaves, are consistent with such a hypothesis (Figure 7.4). *An1* was also ectopically expressed in the leaves of petunias over-expressing *An2* (MYB) (Quattrocchio et al., 1998), suggesting a similar mechanism leading to ectopic *An1* expression is common to *DPL*, *PHZ* and *An2* over-expressing petunias. The expression of *An1* in anthers, for regulating anthocyanins, requires *An4* activity (MYB homologous to DPL) (Spelt et al., 2000), suggesting hierarchical interactions between MYB and bHLH factors occur normally during

development in non-transgenic systems. Promoter activation assays showed that *An2*, *DPL* and *PHZ* were not able to activate the *An1* promoter, with or without a bHLH partner (Figure 7.5). This suggests the MYB factors are not direct regulators of *An1*, but that they may regulate the expression of an endogenous intermediate transcriptional activator. This contrasts with *Arabidopsis*, where the bHLH *TT8* (homologous to *An1*) auto-activates its own expression, directly, with the MYB transcription factors involved in pro-anthocyanidin (*TT2*) and anthocyanin regulation (*PAP1*) (Baudry et al., 2006).

In terms of the small R3 MYB repressor *MYBx*, the evidence suggests that it is a competitive inhibitor of anthocyanin synthesis. Earlier transcript expression studies had shown *MYBx* to be induced in light-stressed leaves (Chapter 4, Figure 4.1), and during flower development (Figures 4.2, 4.3) (Kroon, 2004). The MYB OE petunias, which are highly pigmented, ectopically express *MYBx* in their leaves (Figure 7.4). The level and timing of transcript accumulation indicated that this MYB repressor could provide feedback repression upon anthocyanin synthesis or accumulation. MYBx is proposed to inhibit anthocyanin synthesis by competing with R2R3 MYB activators for bHLH partners (Chapter 4, Figure 4.6B), and *MYBx* over expression was shown to inhibit anthocyanin synthesis in petunia flowers (Kroon, 2004).

Further, *MYBx* provides feedback repression for anthocyanin synthesis. This feedback regulation may occur by two mechanisms: *i) MYBx* is directly targeted by the MBW complex that activates anthocyanin synthesis, or *ii)* that *MYBx* is expressed in response to anthocyanin accumulation. The promoter activation assays suggested that the *MYBx* promoter was unlikely to be a direct target of the MBW complex. Previously, Kroon (2004) demonstrated that *MYBx* transcripts were reduced in the flowers of *an1*$^{-}$ (bHLH) or *an11*$^{-}$ (WDR) petunia lines, which implicated these anthocyanin regulators as potential activators of *MYBx*. However, *MYBx* transcripts were not reduced in mutants of *an2*$^{-}$, the floral MYB partner , and constitutive expression of *An1* or *An2* from a 35S promoter did not result in ectopic expression of *MYBx* (Kroon, 2004), which is consistent with the findings from the promoter activation assays presented here (Figure 7.5).

The *MYBx* promoter activity was highest in those MYB-bHLH combinations that were most highly pigmented with anthocyanin. Transformation of petunia leaves with the

MYB activators (DPL, PHZ, AN2) alone also resulted in anthocyanin accumulation, yet the *MYBx* promoter failed to be highly activated. The luciferase activity for a target of the MBW complex typically shows above background activity when the MYB alone is transformed, which is further enhanced with the addition of a bHLH partner (e.g. Figures 7.6. and 7.7). It is probable that *MYBx* is regulated in response to anthocyanin accumulation by an unknown mechanism. It would be interesting to measure the levels of *MYBx* transcripts in $35S_{pro}$*:DPL* or $35S_{pro}$*:PHZ* petunias in a mutant background that has a biosynthetic block upon anthocyanin synthesis.

Finally, transcripts for the putative active repressor of anthocyanin synthesis, *MYB27*, were enhanced in the MYB OE petunias. The mechanism for these increased levels of *MYB27* transcripts is unknown. The *MYB* OE petunias accumulate high levels of anthocyanins (Chapter 6, Figure 6.7), which can cause internal shading in leaves (Albert et al., 2009). *MYB27* is normally expressed to high levels in shaded leaves (Chapter 4, Figure 4.1B), and so it is possible that transcripts for *MYB27* have increased due to this. There is evidence *MYB27* is a direct target of the bHLH factor AN1 in flowers, as *MYB27* transcripts were activated by an AN1-GR fusion protein in the presence of cyclohexamide (Spelt et al., 2000). It is possible that MYB27 is a direct target of the MBW complex, and that the anthocyanin MYBs directly regulate *MYB27* expression. However, AN1 is known to interact with MYB factors involved in processes other than anthocyanin synthesis, such as PH4 which regulates vacuolar acidification in petals. The regulation of *MYB27* warrants further investigation.

7.3.3 Differential activity of MYB-bHLH combinations on flavonoid biosynthetic genes

The activities of the different MYB-bHLH combinations upon flavonoid biosynthetic genes were also examined. It is known that some flavonoid biosynthetic gene family members have different spatial expression patterns within flowers. The MYB gene family members in petunia are thought to be regulated in spatially distinct patterns, which may provide a basis for the expression patterns for the flavonoid biosynthetic genes. The MYB factor *Venosa*, from *Antirrhinum*, appears to be a relatively weak

regulator of DFR, compared to *Rosea1* or *Rosea2* (Schwinn et al., 2006). *Rosea2* also appears to have bHLH partner specificity for *Delila* (Schwinn et al., 2006). This suggests that even highly related MYB factors can have different specificities for target genes and transcription factor partners.

The *CHS-A* promoter is regulated by MYB anthocyanin regulators. *CHS-A* was activated by the MYB factors AN2, DPL and PHZ (Figure 7.6), with DPL transformation consistently resulting in the highest promoter activation. These findings suggest DPL has higher affinity for the *CHS-A* promoter than AN2 or PHZ alone. However, the addition of AN1 resulted in enhanced activation when combined with AN2 and PHZ, but not DPL. This suggests AN2 and PHZ have partner specificity to regulate the *CHS-A* promoter, while DPL may use endogenous bHLH factors distinct from AN1 to regulate *CHS-A*.

In the activation experiments, the addition of the bHLH partner JAF13 with the MYB factors had no additive effect on the activation of *CHS-A*. This result can be interpreted in two ways: *i)* the MYBs act with the endogenous JAF13 to activate the promoter or *ii)* the MYBs can bind the promoter without a bHLH partner. While R2R3 MYB anthocyanin regulators have been shown capable of binding DNA in the absence of a bHLH partner *in vitro* (Sainz et al., 1997; Espley et al., 2009), it is thought *in planta* they bind as a MYB-bHLH-WDR complex (Grotewold et al., 2000; Baudry et al., 2004; Hernandez et al., 2004). The R2R3 anthocyanin MYB factors AN2 and AN4 (paralogous to DPL) require a bHLH to regulate anthocyanins, and can physically interact with both AN1 and JAF13 in yeast (Kroon, 2004). *JAF13* is constitutively expressed throughout plant organs (Chapter 4, Figures 4.1B, 4.2A, 4.3A; Quattrocchio et al., 1998; Albert et al., 2009) which makes this bHLH a good candidate for an endogenous transcription factor, that can act as a co-regulator of flavonoid biosynthetic genes with the MYBs in the promoter activation assays. JAF13 activity cannot replace AN1, as *an1*$^{-}$ plants completely lack anthocyanins (Spelt et al., 2000) Figure 7.1B. However, JAF13 has been shown to consistently activate the promoters of flavonoid and anthocyanin genes in transient assays (Figures 7.6, 7.7; Quattrocchio et al., 1998; Spelt et al., 2000). It is likely that JAF13 is unable to activate one or more genes that AN1 regulates, preventing anthocyanin synthesis, transport or storage.

The activation of *CHS-A* by MYB anthocyanin regulators shown in this thesis contrasts to earlier reports. Previously, the *CHS-A* promoter was shown to be relatively non-responsive to AN2 + JAF13 (Quattrocchio et al., 1998). The results presented here differ somewhat, as all MYB activators, including AN2, resulted in activation of the *CHS-A* promoter. However, strongest activation of *CHS-A* was only observed when AN2 was combined with AN1, a combination that was not previously tested by other researchers. Differences in the allele of the *CHS-A* promoter (V26 petunia this study, V30 petunia Quattrocchio et al., 1998), the reporter constructs (1200 bp promoter this study or 800 bp Quattrocchio et al., 1998), or the assay system used may account for these differences. Mutant analysis experiments show that *CHS-A* transcripts were not severely affected in petunias that lack *an1*, *an2* or *an11*, which suggested that these genes were regulated independently of these loci (Quattrocchio et al., 1993). Evidence from *Arabidopsis* suggests the flavonol regulators (MYBs) normally regulate the early biosynthetic genes (*CHS, CHI, F3H* and *FLS*) independently of the anthocyanins regulators (Hartmann et al., 2005; Mehrtens et al., 2005; Stracke et al., 2007). However, in the absence of the flavonol regulators (*Atmyb11*$^{-}$, *Atmyb12*$^{-}$, *Atmyb111*$^{-}$ triple mutants) anthocyanins can still be made, which suggests the anthocyanin regulators can act redundantly upon these genes (Stracke et al., 2007). In transgenic systems, where transcription factors have been used to enhance anthocyanin synthesis, the early biosynthetic genes often show enhanced expression (Chapter 6, Figures 6.10, 6.11; Bradley et al., 1998; Bovy et al., 2002; Tohge et al., 2005; Albert et al., 2009), supporting a redundant role for regulating these genes.

In addition to *CHS-A, CHS-J* is also activated by the anthocyanin regulators. AN2, DPL and PHZ were all able to activate the *CHS-J* promoter, which was enhanced if a bHLH factor was co-transformed. Some small differences between the MYB-bHLH combinations were observed; DPL consistently gave the highest activation, followed by AN2 and then PHZ. The addition of a bHLH factor increased promoter activity for all combinations. These findings are consistent with previous promoter assays where the *CHS-J* promoter was activated by AN2 + JAF13 (Quattrocchio et al., 1998). This is further supported by genetic evidence, as *CHS-J* expression is abolished in petunia flowers that lack *an2*, *an1* or *an11* activity (Quattrocchio et al., 1993). Interestingly, this also suggests that *CHS-J* is not regulated by MYB12-like flavonol regulators.

In terms of the late biosynthetic genes, DPL and PHZ activate the *DFR-A* promoter. DFR is the first committed step towards anthocyanin synthesis (also proanthocyanidins), and activation of this gene by DPL and PHZ provides further evidence that they regulate anthocyanin synthesis. These findings are consistent with previous studies, which have shown that AN2 can activate *DFR-A* with either JAF13 or AN1 with similar efficacy (Quattrocchio et al., 1998; Spelt et al., 2000). The similar *DFR-A* promoter activation levels for all the MYBs tested suggests these transcription factors all normally regulate *DFR* expression, and that differences in activation observed for the other promoters tested are likely to reflect real differences in partner and target gene specificity.

The floral pigmentation phenotypes of $35S_{pro}$*:An2* (pink), $35S_{pro}$*:DPL* (purple) and $35S_{pro}$*:PHZ* (pale purple) petunias (Mitchell background) were very different suggesting differential activity upon genes that encode F3′5′H. Two genes encode F3′5′H in petunia, *Hf1* and *Hf2*. The *Hf1* promoter (V26 petunia) was differentially regulated by different combinations of MYB and bHLH factors. DPL consistently activated the *Hf1* promoter to a greater extent than combinations of AN2 or PHZ.

The activity of AN2, DPL and PHZ upon genes encoding F3′5′H may not solely account for the floral pigmentation phenotypes in the MYB OE petunias. The pink flowers of $35S_{pro}$*:An2* (Quattrocchio et al., 1998) are likely to contain a mixture of both delphinidin and cyanidin based pigments. AN2 may have higher activity upon genes encoding F3′H than DPL or PHZ. Two genes encode F3′H in petunia, *Ht1* (tube and limb) and *Ht2* (tube), which have distinct spatial expression patterns in flowers, similar to *Hf1* (tube and limb) and *Hf2* (limb) which encode F3′5′H (Tornielli et al., 2008). These hypotheses could be tested in future studies.

Mitchell petunia contains the *hf1-1* allele, which is expressed more weakly, and is restricted to the flower tube. The *hf1-1* promoter region contains two insertions, upstream of the putative basal promoter, which may have displaced *cis* regulatory elements and enhancers that are important for full expression of this gene. Promoter activation assays upon the *hf1-1* promoter would provide further insight into the differential regulation of this gene by a family of related MYB transcription factors. It is possible that the insertions in the *hf1-1* promoter may have reduced the overall

promoter strength, and that as DPL has a higher activation potential upon *Hf1* (shown for the fully functional V26 allele) it may allow for activation of this *F3'5'H* gene above a threshold level, allowing delphinidin-based anthocyanin pigments to be produced, such as in flowers of *$35S_{pro}$:DPL* petunia, (Chapter 6, Figure 6.3). This hypothesis is consistent with the reports that the *hf1-1* allele is most active in the flower tube (for venation pigmentation) (Tornielli et al., 2008), where *DPL* is believed to regulate anthocyanin synthesis in a venation pattern.

Activation assays upon the Mitchell allele of *Hf2* promoter showed this promoter was responsive to the MYB-bHLH combinations tested. The largest activations were achieved with DPL or PHZ combined with JAF13. Interestingly, AN2 combined with JAF13 did not significantly activate the *Hf2* promoter, but AN2 combined with AN1 did. It appears there is partner specificity for the MYBs tested for either JAF13 or AN1. The promoter activity was low, and it is unknown whether Mitchell contains a fully active allele of *Hf2*. The activity of *Hf2* is restricted to the flower limb (determined phenotypically), while the *Hf1* gene is expressed in the flower tube and limb (Tornielli et al., 2008). These differences in spatial expression may arise through the spatial expression patterns of particular MYB and bHLH factors. Further information about the spatial localisation of *JAF13* in floral tissues may provide insight into the spatial regulation of anthocyanin biosynthetic genes and pigmentation patterning.

Mutant analyses, transcript expression studies of anthocyanin regulators in the MYB OE lines and promoter activation studies have reinforced the MYB-bHLH-WDR regulatory complex for anthocyanin synthesis. Differences were observed between MYB and bHLH combinations for regulating different flavonoid biosynthetic genes. Hierarchy between MYB and bHLH transcription factors occurs in the vegetative tissues of the MYB OE lines, and explains how over-expression of a MYB transcription factor alone was capable of resulting in such intense pigmentation and accumulation of anthocyanin. The hierarchical interactions are not direct, however, and occur through unknown mechanisms. The interactions with repressors extend the limited understanding of how pigmentation patterning is controlled.

Chapter 8
General discussion

The overall aim of this thesis was to characterise two novel MYB transcription factors, *DEEP PURPLE* (*DPL*) and *PURPLE HAZE* (*PHZ*), and investigate their roles in determining anthocyanin pigmentation patterns in *Petunia*. Full length cDNAs for *DPL* and *PHZ* were cloned, and shown to encode MYB transcription factors homologous to known anthocyanin regulators.

The expression of both *DPL* and *PHZ* was linked to pigmentation patterns. *PHZ* expression was tightly linked to light-induced pigmentation patterns in vegetative tissues, and also in developing flower buds resulting in a 'blush' pattern. *DPL* expression was localised to the vasculature in vegetative tissues. The loss of flower tube venation in W59 petunia was attributed to the insertion of an LTR transposon within *DPL*, linking the expression of *DPL* to floral venation pigmentation patterns.

Functional experiments showed *DPL* and *PHZ* regulate anthocyanin synthesis. *DPL* and *PHZ* were both able to complement the *rosea*^{dorsea} phenotype in *Antirrhinum*, and activate anthocyanin synthesis in petunia. In addition to over-expression studies, attempts to silence *DPL* and *PHZ* by RNA interference (RNAi) in stable transgenic petunia lines were also attempted. Silencing was not sufficient to give a phenotype, even though transcripts were reduced to 50% wild type, which may reflect the naturally low transcript abundance for these genes. The GUS localisation patterns for the promoter activities of *DPL* and *PHZ*, and the identification of the *DPL::LTR* insertion allele in W59 petunia provided alternative approaches to establish roles as anthocyanin regulators, which complemented the results from the over-expression studies.

DPL and *PHZ* are members of a small gene family in petunia that control discrete pigmentation patterns in vegetative and floral tissues. *Anthocyanin2* (*An2*) was the first identified member of this gene family, and is primarily responsible for full petal colour in flowers (Quattrocchio et al., 1999; Hoballah et al., 2007) as depicted in Figure 8.1.

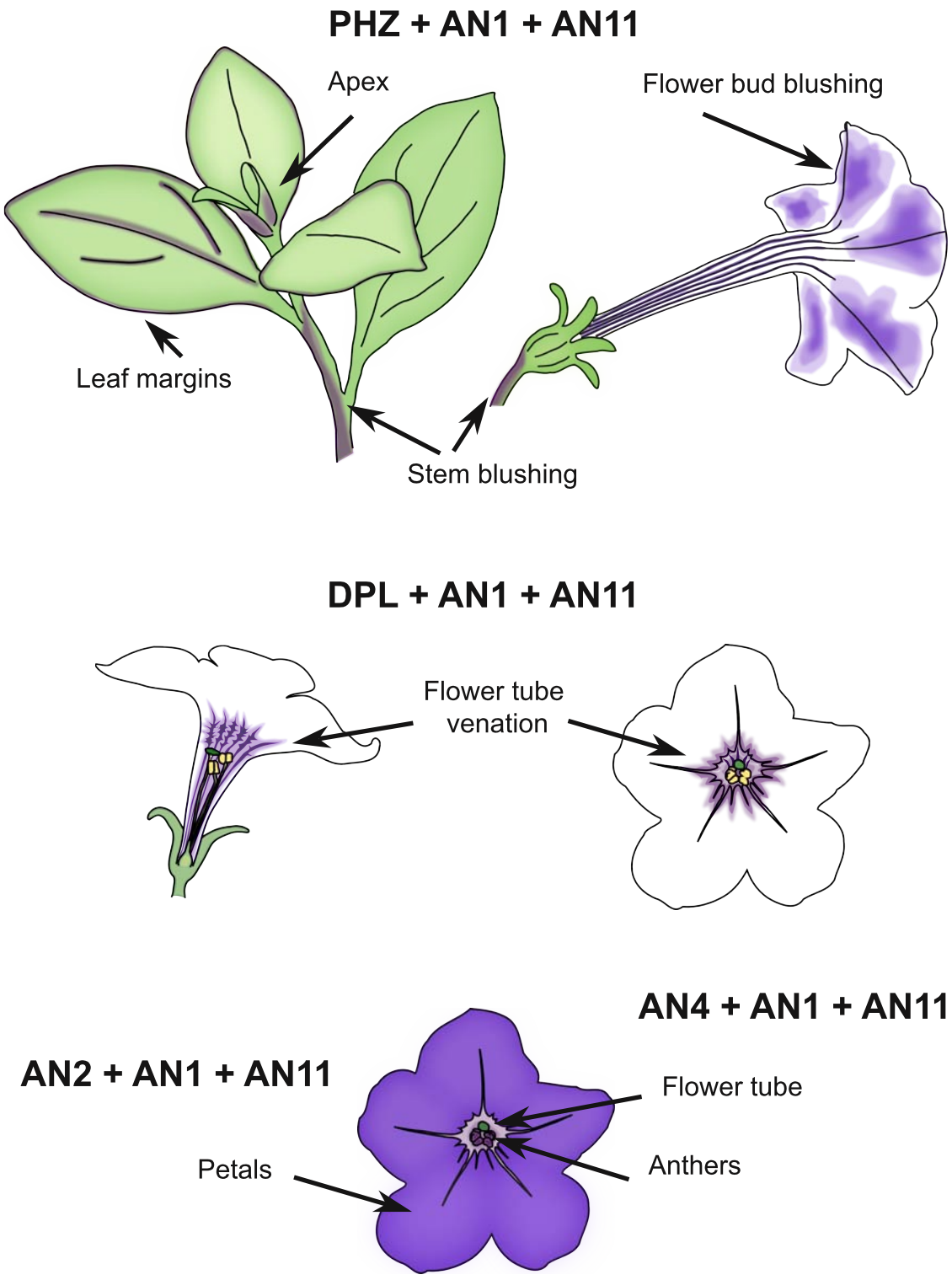


Figure 8.1 Models for pigmentation patterns controlled by distinct MYB family members. Pigmentation patterns are indicated, along with the transcription factors involved: AN2, AN4, PHZ, DPL (MYB), AN1 (bHLH) and AN11 (WDR).

The *An4* locus controls anthocyanin synthesis in anthers, and also contributes to flower tube pigmentation. Recently a MYB clone was identified that segregated with the *An4* locus (Kroon, 2004), confirming earlier reports that this locus contained a MYB anthocyanin regulator. *DPL* is expressed throughout floral and vegetative tissues, and is associated with the vasculature. *DPL* is thought to control anthocyanin synthesis in the flower tube, in a reticulated venation pattern. The roles for *DPL* in non-pigmented tissues are unclear. *PHZ* is expressed in response to light-stress in vegetative tissues, and controls floral bud-blushing. Figure 8.1 depicts how the anthocyanin pigmentation patterns in petunia may be determined by different members of the MYB family.

The different spatial and temporal expression patterns for individual MYB family members appear to be the molecular basis of pigmentation patterning. A MYB family in *Antirrhinum* controls floral and vegetative anthocyanin patterning. *Rosea1* controls full petal colour, *Rosea2* weak petal colour, and *Venosa* controls vein-associated anthocyanin patterning in the petals (Schwinn et al., 2006). In addition to these known MYB family members, a third MYB located at the *Rosea* locus is hypothesised, that may control light-induced vegetative anthocyanin synthesis, and the weak anthocyanin bud-blushing observed in $rosea^{dorsea}$ genotypes (Chapter 6, Figure 6.1A). The similarities between *Antirrhinum* and *Petunia* suggest complex pigmentation patterns are derived from conserved mechanisms.

Anthocyanins are distributed throughout the plant kingdom, from ferns to gymnosperms, and angiosperms (Cooper-Driver, 2001). The occurrence of anthocyanins in ferns and gymnosperms suggests vegetative anthocyanin pigmentation is an ancient trait, which was subsequently incorporated in floral and fruit tissues and functioned as visual cues for pollinators and seed distributors in angiosperms. The proposed roles for vegetative anthocyanins are diverse and debated, although anthocyanins appear to offer protection during stress conditions and photoprotective roles are well established (Hughes et al., 2005; Albert et al., 2009; Hatier and Gould, 2009). *PHZ* is the MYB factor in petunia that regulates anthocyanin synthesis in response to stress (particularly light stress) in vegetative tissues. *PHZ* also regulates a light-induced bud blush phenotype in flowers. Such a pigmentation pattern may have provided an advantage for pollination. Subsequent gene duplications and selection for flowers with more highly coloured flowers may explain why a family of related MYB

factors control distinct pigmentation patterns. The weaker pigmentation patterns, such as bud blushing and venation, are often masked by the more intense 'full colour' phenotypes. The evolution of full petal colour in *Antirrhinum* is thought to have evolved from floral venation patterns (Y. Shang ***et al.,*** manuscript in preparation; Professor Cathie Martin *Pers. Comm.*).

It is interesting to note, that after acquiring full floral pigmentation, some species of *Petunia* have since lost full floral pigmentation due to mutations in *An2* (Quattrocchio et al., 1999; Hoballah et al., 2007) . These mutations have resulted in distinct pollination syndromes – *Petunia axillaris* (white, $an2^{-}$ $an4^{-}$) is pollinated by hawkmoths, while *Petunia integrifolia* is bee-pollinated (Stuurman et al., 2004). Introduction of a functional *An2* allele into a pale coloured $an2^{-}$ petunia backgrounds was able to change pollinator preferences (Hoballah et al., 2007), demonstrating the strong selective pressure upon pigmentation phenotypes by pollinators.

The bHLH factor AN1, and WDR protein AN11 are absolutely required for regulating anthocyanin synthesis in petunia. Null mutations in either of these genes prevent anthocyanin synthesis, even when MYB anthocyanin regulators are constitutively expressed.

DPL has an absolute requirement for the bHLH partner AN1 to regulate anthocyanins (Chapter 7). *DPL* is expressed associated with the vasculature throughout vegetative tissues. The spatial separation of AN1 from DPL in non-stressed plants explains why vascular-associated anthocyanins are not normally produced in leaves. In flowers, *An1* is co-expressed with *DPL*, and anthocyanins are synthesised in a venation pattern. The bHLH factor JAF13 is also expressed in leaves, yet it wasn't able to substitute for AN1, even in petunias ectopically expressing *DPL*. This result was interesting, because promoter activation assays suggested JAF13 was capable of activating anthocyanin biosynthetic genes with a MYB partner. The lack of anthocyanin synthesis may be due to a single gene, essential for anthocyanin synthesis or transport, escaping regulation by JAF13 with a MYB partner.

DPL may have additional roles to regulating anthocyanin synthesis. Promoter activation assays also showed DPL had very high activity upon the *CHS-A* promoter. It

is possible DPL controls the expression of *CHS-A*, and perhaps other flavonoid genes, together with JAF13, associated with the vasculature in vegetative tissues. This is still unclear, and the contribution JAF13 makes to flavonoid regulation requires further investigation.

The MYB-bHLH combinations tested had different responses upon the promoters of anthocyanin biosynthetic genes. DPL was particularly active upon the *CHS-A* promoter, a gene that has previously been reported to be regulated independently of MYB-anthocyanin regulators. The related MYB transcription factors AN2, DPL and PHZ differentially activated the promoters of genes encoding CHS and F3′5′H, but activated the *DFR-A* promoter equally. The activation of *DFR-A* is further evidence these related proteins all activate anthocyanin synthesis, and the differences in activating other flavonoid biosynthetic genes indicates they do have subtle target gene specificities. Interestingly, the bHLH included in these assays also affected the level of promoter activation, suggesting that target site recognition occurs through interactions with both MYB and bHLH partners.

Anthocyanin synthesis is fine-tuned by MYB repressors, in addition to the family of MYB activators that promote anthocyanin synthesis. In vegetative tissues, where light capture and carbon fixation are the primary function, anthocyanin synthesis is actively repressed by an R2R3 MYB repressor, MYB27. Accumulation of anthocyanin in vegetative tissues at an inappropriate time would reduce photon capture. Under stress conditions, however, when the amount of light exceeds that which can be used, *MYB27* is itself repressed, and *PHZ* is induced, allowing the activation of the anthocyanin biosynthetic genes, and anthocyanin accumulation. This induction by light stress also induces the expression of the bHLH *An1*, which is absolutely required for activating anthocyanin synthesis. It is unclear whether the *An1* promoter itself responds to the light stress signal, or if it is induced, indirectly, as a result of *PHZ* expression. The competitive inhibitor *MYBx* is induced in response to anthocyanin accumulation, which provides feedback repression, to allow fine control of anthocyanin synthesis.

The roles for the repressive MYB transcription factors in petunia petals are unclear, since their primary function is to attract pollinators. A similar situation occurs in strawberries, where FaMYB1 actively represses anthocyanin synthesis, and is most

highly expressed in ripe fruit, once anthocyanins have accumulated (Aharoni et al., 2001). The presence of MYB repressors does provide a system to limit anthocyanin production to a particular level, even in tissues and organs that have a functional requirement to become coloured. The involvement of repressive MYB factors for anthocyanin regulation requires further investigation

Further experiments investigating the requirement by PHZ for AN1 are underway. The issues encountered with recombination suppression meant new crosses between alternative *35S$_{pro}$:PHZ* transgenic lines and W225 (*an1*$^{-}$) petunia needed to be established. Unfortunately the time required to reach the F2 generation prevented inclusion in this thesis.

DPL may have additional roles regulating flavonoids distinct from anthocyanins. The vascular-associated expression of *DPL* in non-pigmented vegetative tissues, and strong activation of the *CHS-A* promoter raise questions about the function of *DPL* in vegetative tissues. The transgenic *35S$_{pro}$:DPL* lines in the homozygous *an1*$^{-}$ *an1*$^{-}$ background provide an ideal system to investigate this, since it eliminates the hierarchical effects of *An1* activation which would lead to anthocyanin synthesis. Constitutive expression of *DPL* in this background would ensure *DPL* and *JAF13* were co-expressed. Transcript analysis for flavonoid biosynthetic genes may provide further insight into the role of *DPL* in vegetative tissues, and may identify genes that escape regulation by *JAF13*, which would account for the lack of functional redundancy with *An1*.

DPL and *PHZ* are both anthocyanin regulators, involved in the control of floral and vegetative pigmentation patterns. *PHZ* is light induced, and appears to be the MYB required for vegetative pigmentation. Specificities for target anthocyanin biosynthetic genes and bHLH partners by DPL, PHZ and AN2 were demonstrated, but these interactions were intricate, demonstrating that a network of MYB transcriptional activators and repressors determine the complex vegetative and floral pigmentation patterns observed in *Petunia*.

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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, 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.

Terrific broth (TB)

TB media contains 12 g L^{-1} bactotryptone, 24 g L^{-1} yeast extract, 4 mL glycerol, 2.31 g L^{-1} KH_2PO_4, 12.54 g L^{-1} K_2HPO_4, dissolved in 900 mL water, made up to 1 L with water, then autoclaved.

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 ($MgSO_4.7H_2O$), 0.05% (w/v) di-potassium hydrogen orthophosphate (K_2HPO_4) 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 and 1% (w/v) SDS made up to required volume with water, and stored at room temperature.

Solution III – Neutralisation solution

Solution II contains 3 M potassium acetate and 11.5 % (v/v) glacial acetic acid made up to required volume with water 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 × MOPS buffer

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

RNase decontamination solution

1× RNase decontamination solution contained 11 mM NaClO, 130 mM NaOH, 15 mM potassium citrate and 3.5 mM SDS.

1.3.2 Sample preparation

10 × loading dye

10 × loading dye contained 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 x 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

Antibiotic salts were dissolved in an appropriate amount of milliQ water to make 100 mg mL^{-1} stocks: ***ampicillin*** (sodium salt of ampicillin), ***streptomycin*** (streptomycin sulphate), ***spectinomycin*** (spectinomycin dihydrochloride), ***kanamycin*** (kanamycin sulphate), ***gentamicin*** (gentamicin sulphate). The antibiotic solutions were filter sterilised (0.2 µm) and stored at 4°C, except ampicillin which was stored at -20°C.

Rifampicin solution (50 mg mL^{-1}) was made by dissolving rifampicin crystals in an appropriate volume of DMSO, and stored in foil-wrapped tubes at -20°C.

Tetracycline solution (10 mg mL^{-1}) was made by dissolving tetracycline hydrochloride in 50% (v/v) ethanol. The solution was filter sterilised (0.2 µm) and stored in foil-wrapped tubes at -20°C.

1.5 Tissue culture/Transformation media

Virulence induction media (#1003)

Liquid virulence induction media contained 1 ×AB salts (1 g L^{-1} NH_4Cl, 0.3 g L^{-1} $MgSO_4$, 0.15 g L^{-1} KCl, 0.01 g L^{-1} $CaCl_2$), 240 mg L^{-1} NaH_2PO_4, 10 g L^{-1} glucose, 14.693 g L^{-1} morpholinoethane sulfonic acid. Media was supplemented with 200 µM acetosyringone after sterilisation.

Co-cultivation media (#1828)

1 × MS micro, 0.5 ×MS macro, 50 µM Ferric sodium EDTA ($C_{10}H_{12}FeN_2NaO_8 3H_2O$), 1 × B5 vitamins, 5 mg L^{-1} $CuSO_4$, 0.2 mg L^{-1} IAA, 3 mg L^{-1} BAP, 30 g L^{-1} sucrose, 7.5 g L^{-1} Davis Agar, 200 µM acetosyringone Supplemented with, or without, 150 mg L^{-1} kanamycin sulphate as required.

Shoot regeneration media (#2251)

1 × MS micro, 0.5 ×MS macro, 50 µM Ferric sodium EDTA ($C_{10}H_{12}FeN_2NaO_8 3H_2O$), 1 × B5 vitamins, 5 mg L^{-1} $CuSO_4$, 0.2 mg L^{-1} IAA, 3 mg L^{-1} BAP, 500 mg L^{-1} cefotaxime, 30 g L^{-1} sucrose, 7.5 g L^{-1} Davis Agar. Supplemented with, or without, 150 mg L^{-1} kanamycin sulphate as required.

Root regeneration media (#1609)

1 × MS micro, 0.5 × MS macro, 50 μM Ferric sodium EDTA ($C_{10}H_{12}FeN_2NaO_8 3H_2O$), 1 × B5 vitamins, 10 mg L^{-1} $CuSO_4$, 500 mg L^{-1} cefotaxime, 30 g L^{-1} sucrose, 7.5 g L^{-1} Davis Agar. Supplemented with, or without, 100 mg L^{-1} kanamycin sulphate as required.

Media #2

Media #2 is half-strength solid MS (Murashige and Skoog, 1962). For selecting transgenic seedlings, the media was supplemented with 200 mgL^{-1} kanamycin.

1.6 Nucleic acid extraction, blotting and hybridisation reagents

1.6.1 Guanidine nucleic acid extraction buffer

Guanidine extraction buffer contained 4 M guanidine thiocyanate, 25 mM sodium citrate, 0.5 % (w/v) sodium lauryl sarcosinate and was adjusted to pH 7 if required. β-mercaptoethanol was added to guanidine extraction buffer prior to use at a rate of 8 μL per 1 mL of buffer.

1.6.2 Un-buffered phenol

Solid phenol crystals (250 g) were dissolved with 100 mL sterile H_2O and 0.25 g of 8-hydroxy quinoline. Once dissolved, additional sterile water was added to completely saturate the phenol, leaving a layer of water on top. Phenol was protected from light and stored at 4°C.

1.6.3 Tris-buffered phenol

Solid phenol crystals (250 g) were dissolved with 100 mL sterile H_2O and 0.25 g of 8-hydroxy quinoline. The phenol was washed with 50 mL1M tris buffer (pH 8), shaken, and the phases left to separate. The aqueous buffer overlying the phenol was removed. This process was repeated twice more, followed by two further washes with 0.1 M tris (pH 8). A layer of 0.1 M tris was left overlying the tris-buffered phenol. Phenol was protected from light and stored at 4°C.

1.6.4 Phenol:[Chloroform:IAA]

Tris-buffered phenol (Appendix 1.6.3) was combined in equal volumes with chloroform:IAA (24:1 v:v).

1.6.5 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 to 10 × and 2 × as required.

1.6.6 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.

1.6.7 TE buffer

TE buffer contains 10 mM TrisHCl (pH 7.5) and 1 mM EDTA.

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.

1.8 Miscellaneous buffers

1.8.1 Phosphate buffer pH 7 (50 mM)

Phosphate buffer (50 mM, pH 7) was prepared from 30.5 mL 0.2 M $Na_2HPO_4.2H_2O$ and 19.5 mL 0.2 M $NaH_2PO_4.1H_2O$ made up to 200 mL with sterile water.

1.8.2 X-gluc staining solution

The staining solution contained 0.63 mM X-gluc (5-bromo-4-chloro-3-indolyl-beta-D-glucuronic acid, cyclohexylammonium salt), 10 mM EDTA, 0.1% (v/v) TritonX-100 in 50 mM pH 7 phosphate buffer: methanol 80:20 (v:v). X gluc was dissolved in DMF (10 μL DMF per mg X-gluc) prior to adding phosphate buffer: methanol. The staining solution was protected from light, and stored at 4°C.

1.8.3 Phosphate buffered saline (PBS)

Phosphate buffered saline (1 ×) contained 137 mM NaCl , 2.7 mM KCl, 10 mM $Na_2HPO_4.7H_2O$ and 1.76 mM KH_2PO_4 and pH was adjusted to 7.4 with HCl.

Appendix 2: Primer sequences

Primer	Sites	S/AS	Sequence 5′ → 3′
NA10		S	CACAAGGATGAATATAGCAAAC
NA12		AS	TTACATGGGAATCAAAATGG
NA13		S	GATAAAGATGGAAGTAACAATG
NA15		AS	TTTAATGAATGGATTCTCTTATTC
NA16		AS	CATTGTTACTTCCATCTTTATC
NA17		AS	GAGATCTACTTCATCCTCAGAG
NA18		AS	GTTTGCTATATTCATCCTTGTG
NA19		AS	CACTTCCTCCGGACAAAAG
NA26	***Kpn***I	S	gg**ggtacc** TGGAAAGGGCAAAACTAAC
NA27	***Kpn***I	S	gg**ggtacc** ATGTGTTCACAGCTACTTGAG
NA30	***Bam***HI	AS	cg**ggatcc** TTTAATGAATGGATTCTCTTATT
NA38	***Bam***HI	AS	cg**ggatcc** TTACATGGGAATCAAAATGG
NA48	***Xho***I	S	at**ctcgag** ATGGCTATGGGATGCAAAGAC
NA49	***Eco***RI	AS	at**gaattc** TCAAGATTTCCAGACTACTCG
NA50	***Cla***I	S	cc**atcgat** ATGGTGCAGCCTCATGTCAT
NA51	***Bam***HI	AS	cg**ggatcc** TTAAAACCCTTTGGCAACATCATC
NA52	***Cla***I	S	cc**atcgat** ATGGCAGGTGAAGTAGCAAAAC
NA53	***Bam***HI	AS	cg**ggatcc** TTAAGCAGTTTCCATGAGGAACT
NA54	***Cla***I	S	cc**atcgat** ATGGAGAATGAGATGAAGCACT
NA55	***Bam***HI	AS	cg**ggatcc** CTATGTAGTCGAGACCTTAGC
NA56	***Eco***RI	S	g**gaattc** ATGACAGGCAAAACCGCC
NA57	***Xba***I	AS	ctc**tagagc** CTAGGAGAGACGCCTGCAAAG
NA58	***Eco***RI	S	at**gaattc** ATG CCC CTT CAC CTC CG
NA59	***Xba***I	AS	at**tctaga** CTAGACTTCAACATTGCTTAACAT
NA60		AS	CTTCAGCCCATGCTCCTTTCCTCAGTA
NA61		AS	GTCAAAAAGATCTCAAGTAGCTGTGAACAC
NA66		AS	GATGCCGGCCGGTAGTTTTTGAAATAAG
NA67		AS	GCACCAAGCTCAGTAAGTAGCATCATGAC
NA76		AS	GAGACAAGACCATGACTAGGCGTTGG
NA77		AS	CTGCTGGGGAAGTCAAGTAATGTGAAG
NA78	***Nhe***I	S	ata**gctagc** TCACGCCCAATCAGTGATC
NA79	***Nco***I	AS	tat**ccatgg** ATATATCACGCGATGAATTG
NA81	***Nco***I	AS	tat**ccatgg** ATATGGGGGGTCACACTG

NA82		S	GCCGCATTGCCGTGGGTAG
NA83		AS	GGGATTGGGTTTAGGGTTAGGGTTTC
NA84		S	TCTGCCGGCGAATCAAATCAA
NA85		AS	GTCTGTACGCGGGCACTCTTAGC
NA86		S	ACGGATGATAATATGAGTAACGGTGTGC
NA87		AS	CTTGATGGTCTAGTGGGGCAGGC
NA88		S	TAGCTAACTGCAACGAAAATGATGAAACA
NA89		AS	TCCTCGTGCAACAAACTCGTAACTT
NA90		S	GAACGGCAAACGATGTGAAAAACTACTG
NA91		AS	GCATTCTTGGCCATGGTCCTAATTTC
NA98		AS	GATATGGGGGGTCACACTGCATATGACTCC
NA99		AS	CTTTCCTCACTAGTCCAGACGACTTGGGAATAG
NA100		AS	GAAGTGCTCCGATCACCGGCCACCCTCTTG
NA101		AS	GGAGACGCCGGCCGTTAGTTATAGAAAGAAG
NA102	***Xba*I**	S	atc**tctaga** TAGAGCCAGTGTCGGTGG
NA103		S	GTGGCTCCTCGGATGTTAGTTTCA
NA104		AS	GACCACCTCTCGCCAACCAAATTA
NA105		S	GATGGACTTCAATGGTGGGCCAAT
NA106		AS	CGATGGTGCTGTTTCCTCATGCAA
NA112	***Nco*I**	AS	tat**ccatgg** ATTAAGTTTGAAAGACTAGTAGTG
NA113	***Xho*I**	S	at**ctcgag** GATCTGTCTCGAAAACCCTTCATTA
NA114	***Nco*I**	AS	tat**ccatgg** TTTGATGAATTGAAAGTTGTGTG
NA115	***Xho*I**	S	at**ctcgag** GGTCCGAGAAAAGTTAGGCAG
NA116	***Nco*I**	AS	tat**ccatgg** TATTATTTGTACTGCAACCAGAAAC
NA117	***Xho*I**	S	at**ctcgag** GGCTGCTTTAGGTAGGAAGAC
NA118	***Nco*I**	AS	tat**ccatgg** TTTTGCTTGAAAAAAGTTTGGTATT
NA119	***Xho*I**	S	at**ctcgag** GCATTCTTGCCACTACAACAAATC
NA120	***Nco*I**	AS	tat**ccatgg** TGTTGCCAACAAAAAAAAAAGGATA
NA121	***Xho*I**	S	at**ctcgag** CCTGTTGAACGTGTTAGGGC
NA122	***Nco*I**	AS	tat**ccatgg** TATCAGATATGGAAGTTGGAAAG
NA123	***Xho*I**	S	at**ctcgag** GAACTTCAGCCCACAAAGATTAAAG
NA124	***Nco*I**	AS	tat**ccatgg** GACTAAAGGAAAACGTATATGGC
NA125	***Xho*I**	S	at**ctcgag** TATCCACTTTAATAGGAGTAACATA
NA126	***Nco*I**	AS	tat**ccatgg** TGTTTAAAGCCAGATGAGCTGG
NA129		AS	GATGTCTTTCCTGAAACTAACATCCGAGGAGC
NA130		AS	CCACCACATCTTGAGAATCAGCCGGAGTATTTAC
NA133	***Xho*I**	S	at**ctcgag** GTGCATATTCCAAATCAGAAGCAC
NA145	M13	S	**gacgttgtaaaacgacggcc** ACTTACCCAATTTACTCCATGG
NA146		AS	ACTTCCCATAAACGCAAATAATC
NA147	M13	S	**gacgttgtaaaacgacggcc** CTTAAAGAAGGGGTAGAGTGTATG
NA148		AS	TGAACAAACGGGTCCATAGTATC

NA165	***Xho***I	S	at**ctcgag** TTCGCGACAAGGTGGGAGTG
NA166	***Xho***I	S	at**ctcgag** CTATAGGAATCTATCGCTATTCC
NA167	***Nco***I	AS	tat**ccatgg** TCTATATGATTTTCTGGTTTGT
PhACT2 F		S	CCTGATGAAGATCCTCACCGA
PhACT2 R		AS	CAAGAGCCACATAGGCAAGCT
PhEF1α F		S	TGTTCTCTGCCTTGTATGTCTGG
PhEF1α R		AS	TCAAAAGAGGCAGGCAGACAG
PhHisH4 F		S	ATACGCTTGCACCCACCCCTA
PhHisH4 R		AS	GGAGGAGCTAAACGACACCG
K135		S	AGCTGGTACCATGGAAAATTCAAGTCAAG
K136		AS	CTGATCTAGATTCAATCTTTCAATCACCT
K356	***Eco***RI	S	agcta**gaattc** ATGCAGCTGCAAACCATGTTA
K357		AS	AGCTATCTGAGTTAAACTCTAGGGATTAACTG
GUS AS		AS	TGTAACGCGCTTTCCCACCAAC
CMF		S	ATGTGATATCTCCACTGACG
6-FAM M13	6-FAM	S	**6FAM**-GACGTTGTAAAACGACGGCC

Appendix 3: Plasmids

Plasmid name	Source	Construct details	Base vector
pNWA			
pNWA1	***Petunia***: MP	***35S$_{pro}$***: ***DEEP PURPLE***: OCS	pART7
pNWA2	***Petunia***: MP	***35S$_{pro}$***: ***PURPLE HAZE***: OCS	pART7
pNWA3	***Petunia***: MP	***DEEP PURPLE*** full cDNA	pGEMteasy
pNWA4	***Petunia***: MP	***PURPLE HAZE*** full cDNA	pGEMteasy
pNWA9	***Petunia***: MP	Actin	pGEMteasy
pNWA12	***Petunia***: MP	***35S$_{pro}$***: ***DEEP PURPLE***: OCS	pART27
pNWA14	***Petunia***: MP	***35S$_{pro}$***: ***PURPLE HAZE***: OCS	pART27
pNWA17	***Petunia hybrida***	***An3***/F3H	pBluescript
pNWA18	***Petunia hybrida***	***HF1***/F3′5′H	pBluescript
pNWA19	***Petunia hybrida***	***An9***/GST	pBluescript
pNWA20	***Petunia hybrida***	UF3GT	pBluescript
pNWA21	***Petunia***: MP	Genomic ***DEEP PURPLE***	pGEMteasy
pNWA22	***Petunia***: MP	Genomic ***PURPLE HAZE***	pGEMteasy
pNWA23	***Petunia hybrida***: V26	***35S$_{pro}$***:***3RT*** (***Rt***): OCS	pART7
pNWA24	***Petunia hybrida***: V26	***35S$_{pro}$***: ***AT***: OCS	pART7
pNWA25	***Petunia hybrida***: V26	***35S$_{pro}$***: ***UF5GT***:OCS	pART7
pNWA26	***Petunia hybrida***: V26	***35S$_{pro}$***: ***MT***:OCS	pART7
pNWA27	***Petunia hybrida***: V26	***35S$_{pro}$***: ***DFRa***: OCS	pART7
pNWA28	***Petunia***: MP	***35S$_{pro}$***: ***JAF13***: OCS	pART7
pNWA29	***Petunia hybrida***	***35S$_{pro}$***: ***An2*** : OCS	pART27
pNWA30	***Petunia hybrida***	***35S$_{pro}$***: ***An1*** : OCS	pART27
pNWA31	***Petunia***: MP	***35S$_{pro}$***: ***JAF13*** : OCS	pART27
pNWA32	***Petunia hybrida***	***35S$_{pro}$***: ***An11*** : OCS	pART27
pNWA33	***Petunia***: MP	***DEEP PURPLE$_{pro}$***: ***iGUS***: OCS	pDAH3
pNWA34	***Petunia***: MP	***PURPLE HAZE$_{pro}$***: ***iGUS***: OCS	pDAH3
pNWA35	***Petunia***: MP	***DEEP PURPLE$_{pro}$***: ***iGUS***: OCS	pART27
pNWA36	***Petunia***: MP	***PURPLE HAZE$_{pro}$***: ***iGUS***: OCS	pART27
pNWA37	CaMV	2 × ***35S$_{pro}$***: ***iGUS***: OCS	pART27
pNWA38	CaMV	2 × ***35S$_{pro}$***: ***Luciferase***: CaMV ***35S$_{pro}$***: ***Renilla***: CaMV	pGreenII 0800-LUC
pNWA39	***Petunia hybrida***: V26	***CHSa$_{pro}$***: ***Luciferase***: CaMV ***35S$_{pro}$***: ***Renilla***: CaMV	pGreenII 0800-LUC
pNWA40	***Petunia***: MP	***CHSj$_{pro}$***: ***Luciferase***: CaMV ***35S$_{pro}$***: ***Renilla***: CaMV	pGreenII 0800-LUC
pNWA41	***Petunia***: MP	***DFRa$_{pro}$***: ***Luciferase***: CaMV ***35S$_{pro}$***: ***Renilla***: CaMV	pGreenII 0800-LUC

pNWA42	***Petunia hybrida***: V26	***HF1$_{pro}$***: ***Luciferase***: CaMV ***35S$_{pro}$***: ***Renilla***: CaMV	pGreenII 0800-LUC
pNWA43	***Petunia***: MP	***HF2$_{pro}$***: ***Luciferase***: CaMV ***35S$_{pro}$***: ***Renilla***: CaMV	pGreenII 0800-LUC
pNWA44	***Petunia***: MP	***An1$_{pro}$***: ***Luciferase***: CaMV ***35S$_{pro}$***: ***Renilla***: CaMV	pGreenII 0800-LUC
pNWA45	***Petunia***: MP	***An11$_{pro}$***: ***Luciferase***: CaMV ***35S$_{pro}$***: ***Renilla***: CaMV	pGreenII 0800-LUC
pNWA46	***Petunia***: MP	***MYBx$_{pro}$***: ***Luciferase***: CaMV ***35S$_{pro}$***: ***Renilla***: CaMV	pGreenII 0800-LUC
Others			
pDAH2	N/A	2 × ***35S$_{pro}$***: RNAi cloning vector	
pDAH			
pKES14	***Petunia hybrida***	***35S$_{pro}$***: ***An1*** : OCS	pART7
pPN73	***Petunia hybrida***	***35S$_{pro}$***: ***An2*** : OCS	pART7
pPN79	***Petunia hybrida***	***35S$_{pro}$***: ***An11*** : OCS	pART7
pPN296	***Petunia hybrida***	PAL	pBluescript
pPN297	***Petunia hybrida***	CHSa	pBluescript
pPN299	***Petunia hybrida***	ANS	pBluescript
pPN300	***Petunia hybrida***	CHIa	pBluescript
pPN301	***Petunia hybrida***	F3'H	pBluescript
pPN302	***Petunia hybrida***	FLS (***Fl***)	pBluescript
pTIP6	***Asparagus officinalis***	18S rRNA	pBluescript
pSOUP	N/A	Helper plasmid for pGreenII	
pGreenII 0800-LUC	Firefly, ***Renilla***	Dual Luciferase reporter binary plasmid	pGreenII

Appendix 4: Nucleic acid sequences

See attached compact disc (CD)

Appendix 5: Vegetative phenotypes of transgenic V26 petunia lines

Appendix 5 Vegetative pigmentation phenotypes of representative independent transgenic V26 petunia lines transformed with *35S$_{pro}$:DEEP PURPLE* or *35S$_{pro}$:PURPLE HAZE*.