

Copyright is owned by the Author of the thesis. Permission is given for a copy to be downloaded by an individual for the purpose of research and private study only. The thesis may not be reproduced elsewhere without the permission of the Author.

Light-Induced Anthocyanin Pigmentation in Transgenic *Lc* Petunia

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

Master of Science in Plant Biology

at Massey University, Palmerston North, New Zealand

Nick William Albert

2006

Abstract

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

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

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

Acknowledgements

I thank my supervisors, Dr David Lewis, Dr Huaibi Zhang and Professor Paula Jameson. Without their guidance and encouragement I would not have enjoyed the successes this project has had. David, you have kept me on track and provided me with sound advice and friendship outside of study, especially during my illness, which I sincerely appreciate. Huaibi, your help has been invaluable. I have learned a lot from you, and your guidance and troubleshooting has enabled this research to progress trouble-free. Paula, you have always believed in my abilities, provided encouragement and enthusiasm, and helped me into post-graduate study. Your sincere empathy and understanding for me during my illness is something I am very grateful for.

I thank Crop & Food Research and the 'Plant Pigments Group' for providing me the project and support, and also Dr Kevin Davies and Dr Kathy Schwinn who have provided support to the project, particularly concerning transcription factors. Steve, Jan and Ian, your efforts make the whole lab run smoothly. I have made some wonderful friends, especially in the 'Molecular Biology' lab. To Steve, Nady, Lei and John – you make me laugh and smile every day.

I thank Massey University for providing me with the 'Massey Masterate Scholarship' during my thesis year.

To my parents, Denis and Jill, and to family and friends who have been my support team during my study – thank you. You have helped me through the hard times, especially during my illness, and your empathy, smiles and hugs made a huge difference.

I have special acknowledgements for Dr Jade Robertson and Dr Peter Snow, to whom I owe my health, without which I would have nothing. I would not have been able to finish this thesis or have quality of life, if it was not for their help. Sadly Dr Snow passed away before I could thank him, so I would like to extend to his family my sincere sympathy and gratitude.

Table of Contents

	Page
Abstract	i
Acknowledgements	ii
Table of Contents	iii
List of Figures	viii
List of Tables	x
Abbreviations	x
Chapter 1: Introduction	
1.1 Background	1
1.2 Petunia	2
1.3 Plant pigments	4
1.4 Flavonoid biosynthetic pathway	6
1.4.1 Induction of the flavonoid biosynthetic pathway	10
1.5 Regulation of the flavonoid biosynthetic pathway	11
1.5.1 bHLH transcription factors	12
1.5.2 Myb transcription factors	13
1.5.3 WD40 regulator	15
1.5.4 Myb-bHLH-WD40 regulatory complex	16
1.5.5 Light regulation of the early flavonoid biosynthetic genes	19
1.5.6 Light regulation of the flavonoid transcription factors	21
1.6 The transcription factor LEAF COLOUR (LC)	22
1.7 Project hypothesis and aims	24

Chapter 2: General materials and methods

2.1 Chemicals	26
2.2 Media	26
2.3 Buffers and solutions	26
2.4 Bacterial manipulation	27
2.4.1 General bacterial growth conditions	27
2.4.2 Chemically competent <i>E.coli</i> cells	27
2.4.3 Heat-shock transformation of <i>E.coli</i>	27
2.5 Plasmid DNA preparations	28
2.5.1 <i>E.coli</i> plasmid preparation using a standard alkaline lysis method	28
2.5.2 <i>E.coli</i> plasmid preparation – large volume	28
2.5.3 <i>E.coli</i> plasmid preparation – small volume	29
2.5.4 <i>A.tumefaciens</i> plasmid preparation using a standard alkaline lysis method	29
2.6 DNA manipulation	29
2.6.1 Restriction endonuclease digestion –plasmid	29
2.6.2 Agarose-gel electrophoresis	30
2.6.3 DNA quantification	30
2.6.3.1 Gel quantification	30
2.6.3.2 Spectrophotometric	31
2.7 DNA purification	31
2.7.1 Gel extraction and purification	31
2.7.2 PCR reaction clean-up	32
2.8 Hybridisation and probing of northern and Southern blots	32
2.8.1 Pre-hybridisation	32
2.8.2 Probing	32
2.8.3 Stringency washes	33
2.8.4 Autoradiography	33
2.8.5 Stripping membranes	33
2.9 Plant material and growth conditions	34

Chapter 3: Phenotypic and genotypic assessment of *Lc* petunia

3.1 Introduction	35
3.2 Materials and methods	36
3.2.1 Plant material and growth conditions	36
3.2.2 Genomic DNA extraction from petunia	37
3.2.3 Junction-fragment Southern blots	38
3.2.3.1 Genomic DNA restriction digest	38
3.2.3.2 Southern blotting	39
3.2.3.3 Probing	40
3.2.4 Kanamycin sensitivity	40
3.2.5 Zygoty of the 118C line	40
3.2.6 Northern blots	41
3.2.6.1 RNA extraction from petunia leaves	41
3.2.6.2 RNA quantification	41
3.2.6.3 Northern blotting	41
3.2.6.4 Probing	42
3.3 Results	43
3.3.1 Leaf colour phenotypes	43
3.3.2 Junction-fragment Southern blots – copy number of <i>Lc</i> tDNA	46
3.3.3 Kanamycin sensitivity	46
3.3.4 Zygoty of 118C line	50
3.3.5 Expression of <i>Leaf colour</i> (<i>Lc</i>) in transgenic lines	50
3.4 Discussion	51

Chapter 4: Light-induced anthocyanin pigmentation in *Lc* petunia

4.1 Introduction	56
4.2 Materials and methods	57
4.2.1 Plant material and growth conditions	57
4.2.2 Flavonoid extraction	58
4.2.2.1 HPLC analysis of anthocyanins and other flavonoids	58
4.2.3 Chlorophyll/carotenoid extraction	58
4.2.3.1 Spectrophotometric determination of total carotenoid	59

and chlorophyll concentration	
4.2.4 Northern blotting	59
4.2.4.1 Probing	60
4.3 Results	60
4.3.1 Anthocyanin pigmentation phenotypes	60
4.3.2 Pigment analysis	62
4.3.3 Northern analysis of flavonoid structural gene expression	66
4.4 Discussion	69

Chapter 5: Isolation of endogenous petunia Myb transcription factors

5.1 Introduction	79
5.2 Materials and methods	80
5.2.1 Plant material	80
5.2.2 Biolistic transformation of petunia leaves	80
5.2.2.1 DNA preparation	80
5.2.2.2 Growth and culture conditions	81
5.2.2.3 Gold preparation	81
5.2.2.4 Particle bombardment	81
5.2.2.5 Assessment	82
5.2.3 <i>Agrobacterium tumefaciens</i> -mediated transformation	82
5.2.3.1 Plant material	82
5.2.3.2 <i>Agrobacterium tumefaciens</i>	82
5.2.3.3 Transformation	83
5.2.4 Cloning endogenous petunia Myb transcription factors	84
5.2.4.1 Degenerate primer sequences to the conserved Myb domain	84
5.2.4.2 cDNA synthesis	84
5.2.4.3 Reverse-transcription-PCR (RT-PCR)	84
5.2.4.4 Cloning PCR products	85
5.2.4.5 3'Rapid amplification of cDNA ends (3'RACE)	86
5.2.4.6 Cloning 3'RACE products	86
5.2.4.7 Semi-quantitative RT-PCR of Myb gene expression	87
5.3 Results	87
5.3.1 Biolistic transformation	87

5.3.2 <i>Agrobacterium</i> -mediated transformation	89
5.3.3 Isolation of endogenous Myb transcription factors	90
5.4 Discussion	97
Chapter 6: General discussion and future directions	104
Appendices	
Appendix 1: Buffers, media and solutions	108
Appendix 2: The Wellburn equation	113
Appendix 3: Plasmid maps	114
Appendix 4: Partial nucleotide sequences of <i>MybI</i> and <i>MybII</i>	117
References	118

List of Figures

	Page
Figure 1.1	Pigmentation phenotypes of Mitchell and <i>Lc</i> petunia grown under high light. 3
Figure 1.2	The flavonoid biosynthetic pathway. 7
Figure 1.3	The flavonol and anthocyanidin structures. 8
Figure 1.4	Models for transcription factor complexes involved in regulating anthocyanin and flavonol biosynthetic genes. 18
Figure 3.1	Pigmentation phenotypes of the 118C <i>Lc</i> petunia line. 44
Figure 3.2	Pigmentation phenotypes of the 118B <i>Lc</i> petunia line. 45
Figure 3.3	Junction-fragment Southern blots of 118B and 118C <i>Lc</i> petunia lines. 47
Figure 3.4	Kanamycin sensitivity assay of 118B and 118C <i>Lc</i> petunia lines 49
Figure 3.5	Northern analysis of <i>Lc</i> expression in 118C <i>Lc</i> petunia grown under different lighting intensities. 52
Figure 4.1	Pigmentation phenotypes of Mitchell and <i>Lc</i> petunia grown under shade, ambient-greenhouse and high-light conditions. 61
Figure 4.2	Total flavonoid, and anthocyanin concentration of Mitchell and <i>Lc</i> petunia leaves, grown under shade, ambient-greenhouse and high-light conditions. 63
Figure 4.3	HPLC traces of flavonoids present in Mitchell and <i>Lc</i> petunia leaves. 64

Figure 4.4	HPLC traces of anthocyanins present in Mitchell and <i>Lc</i> petunia leaves.	65
Figure 4.5	Total chlorophyll and carotenoid concentration of Mitchell and <i>Lc</i> petunia leaves, grown under shade, ambient-greenhouse and high-light conditions.	67
Figure 4.6	Northern analysis of flavonoid structural gene expression in Mitchell and <i>Lc</i> petunia grown under shade, ambient-greenhouse and high-light conditions.	68
Figure 5.1	Biolistic transformation of <i>Lc</i> petunia leaves with <i>An2</i> .	88
Figure 5.2	<i>Agrobacterium</i> -mediated transformation of <i>Lc</i> petunia leaves with <i>Roseal</i> .	91
Figure 5.3	Nucleotide alignment of the R2R3 DNA binding domains of flavonoid-regulating, Myb transcription factors from dicotyledonous species.	92
Figure 5.4	RT-PCR of R2R3 Myb transcription factors from Mitchell and <i>Lc</i> petunia.	94
Figure 5.5	Nucleotide sequences of two partial Myb clones.	95
Figure 5.6	3'RACE amplification of endogenous petunia Myb genes.	96
Figure 5.7	Semi-quantitative RT-PCR of <i>MybI</i> and <i>MybII</i> in <i>Lc</i> and Mitchell petunia grown under shade and high light.	98
Figure 5.8	Alignment of the deduced amino acid sequence of <i>MybI</i> and <i>MybII</i> with anthocyanin-regulating Myb transcription factors from dicotyledonous species.	99

List of Tables

		Page
Table 3.1	Combined results for kanamycin sensitivity, junction-fragment Southern blots and pigmentation phenotype of transgenic <i>Lc</i> petunia plants.	48

List of Abbreviations

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

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

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

WT	wild type
YM	yeast-mannitol; bacterial growth media

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