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Light-induced vegetative anthocyanin pigmentation in *Petunia*

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Abstract

The *Lc* petunia system, which displays enhanced, light-induced vegetative pigmentation, was used to investigate how high light affects anthocyanin biosynthesis, and to assess the effects of anthocyanin pigmentation upon photosynthesis. *Lc* petunia plants displayed intense purple anthocyanin pigmentation throughout the leaves and stems when grown under high-light conditions, yet remain acyanic when grown under shade conditions. The coloured phenotypes matched with an accumulation of anthocyanins and flavonols, as well as the activation of the early and late flavonoid biosynthetic genes required for flavonol and anthocyanin production. Pigmentation in *Lc* petunia only occurred under conditions which normally induce a modest amount of anthocyanin to accumulate in wild-type Mitchell petunia [*Petunia axillaris*×(*Petunia axillaris*×*Petunia hybrida* cv. 'Rose of Heaven')]. Anthocyanin pigmentation in *Lc* petunia leaves appears to screen underlying photosynthetic tissues, increasing light saturation and light compensation points, without reducing the maximal photosynthetic assimilation rate (A_{max}). In the *Lc* petunia system, where the bHLH factor *Leaf colour* is constitutively expressed, expression of the bHLH (*Lc*) and WD40 (*An11*) components of the anthocyanin regulatory system were not limited, suggesting that the high-light-induced anthocyanin pigmentation is regulated by endogenous MYB transcription factors.

Key words: Anthocyanin, bHLH, flavonol, *Lc*, *Leaf colour*, light, MYB, photosynthesis, vegetative pigmentation.

Introduction

Anthocyanins are plant pigments produced by the flavonoid biosynthetic pathway. They are often present in flowers, fruit, leaves, and stems and range in colour from orange/red to purple/blue. Anthocyanins function in flowers and fruits primarily to attract pollinators and seed distributors (Gould and Lister, 2006). The roles for vegetative pigmentation, however, are less clearly understood. Anthocyanins are often produced in vegetative tissues under stress conditions, such as high-light, cold temperature, nutrient deficiency or pathogen attack (Dixon and Paiva, 1995; Chalker-Scott, 1999). This induction suggests anthocyanins act as some form of protectant, but whether this is due to their antioxidant activities or their optical properties (or both) has yet to be resolved. There is evidence that anthocyanins

can protect photosynthetic tissues from photoinhibition by absorbing blue-green light and, thereby, reducing the amount of light reaching the chloroplasts (Feild *et al.*, 2001; Neill and Gould, 2003; Hughes *et al.*, 2005; Merzlyak *et al.*, 2008). Flavonoids, including anthocyanins, are potent antioxidants, raising the possibility they may scavenge reactive oxygen species (ROS) generated during photosynthesis, particularly under conditions of photoinhibition (typically high light and low temperature). Although anthocyanins are usually sequestered in vacuoles, colourless cytosolic anthocyanins may act to scavenge ROS generated by chloroplasts and mitochondria (Neill and Gould, 2003). In addition, ROS such as H_2O_2 diffuse rapidly through membranes, which may allow vacuolar anthocyanins to

scavenge ROS (Hatier and Gould, 2009). However, a major limitation for the investigation of anthocyanin functions in vegetative tissues has been the lack of an appropriate system to make physiological comparisons between anthocyanic and acyanic leaves. Most studies have relied on natural colour variants within species, or compared leaves of different developmental stages.

Flavonoid and anthocyanin biosynthesis is well characterized, and most of the biosynthetic genes have been identified and cloned (Fig. 1) (Grotewold, 2006). The three major anthocyanidins, pelargonidin (orange/red), cyanidin (pink), and delphinidin (violet/blue) differ in their hydroxylation pattern, which contributes to their optical and chemical properties. Other flavonoids such as flavonols are also produced by this pathway, and have specific roles distinct from anthocyanins. Flavonols often accumulate to high levels in leaves and flowers, providing protection from UV light (Li *et al.*, 1993; Middleton and Teramura, 1993). In addition, they are often associated with anthocyanins as co-pigments, forming complex inter-molecular interactions (Mol *et al.*, 1998; Aida *et al.*, 2000).

The regulation of flavonoid metabolism is achieved primarily through transcriptional regulation of the biosynthetic genes (Martin *et al.*, 2001; Davies and Schwinn, 2003), and several regulatory genes required for anthocyanin regulation have been identified, cloned, and character-ized in several species. These transcription factors principally belong to two classes, MYB and basic-Helix-Loop-Helix (bHLH), and together with a WD40 protein, are thought to regulate the anthocyanin biosynthetic genes co-operatively (Koes *et al.*, 2005). Flavonol regulation in *Arabidopsis* occurs by a separate system from anthocyanin regulation, which is independent of bHLH factors, and involves a subset of MYB factors distinct from those known to regulate anthocyanin synthesis. *At*MYB12 and two closely related transcription factors, *At*MYB11 and *At*MYB111, have been shown co-ordinately to regulate the early flavonoid biosynthetic genes *CHS*, *CHI*, and *F3H*, as well as the flavonol-specific gene *FLS*, in response to light for flavonol production in different tissues throughout the plant (Mehrtens *et al.*, 2005; Stracke *et al.*, 2007).

The ability of transcription factors to regulate an entire pathway has led to the use of anthocyanin-regulating transcription factors to alter anthocyanin production and pigmentation patterns in several plant systems (Boase *et al.*, 1998; Bradley *et al.*, 1998, 1999; Bovy *et al.*, 2002). *Leaf colour* (*Lc*) is a bHLH anthocyanin regulator from maize (Ludwig *et al.*, 1989) and, when expressed in petunia, resulted in enhanced anthocyanin production throughout the vegetative tissues (Bradley *et al.*, 1998). However, this enhanced pigmentation was dependent upon the environmental conditions (particularly light) to which the plants were exposed.

Ectopic expression of *Lc* in petunia (Bradley *et al.*, 1998), alfalfa (Ray *et al.*, 2003), tomato (Bovy *et al.*, 2002), and *Arabidopsis* (Lloyd *et al.*, 1992) enhanced anthocyanin production throughout the vegetative tissues, with each of these systems displaying some degree of light-induction. The mechanism responsible for determining these light-induced anthocyanin accumulation responses is unknown, especially with respect to the interaction between endogenous and transgenic transcription factors. Light-induced vegetative anthocyanin production is also a common feature of many non-transgenic plants under a range of environmental conditions, and developmental stages; particularly in seedlings, juvenile shoots at bud-burst, and stressed-plants. Our aims for this study were to characterize the light-induced anthocyanin pigmentation response in *Lc* and wild-type Mitchell petunia further, to understand better the regulation of the flavonoid pathway and possible interactions between transgenic and endogenous transcription factors, and to investigate the effect of intense anthocyanin accumulation upon photosynthesis in a near-isogenic system where anthocyanins could be induced to enhanced levels.

Fig. 1. A stylized diagram of the flavonoid biosynthetic pathway. CHS is the first committed step towards flavonoid production, leading to the production of the major flavonoids, flavonols, and anthocyanins. Abbreviations: PAL, phenylalanine ammonia lyase; CHS, chalcone synthase; CHI, chalcone isomerase; F3H, flavanone 3-hydroxylase; FLS, flavonol synthase; DFR, dihydroflavonol 4-reductase; ANS, anthocyanidin synthase; GT, glycosyltrans-ferases.

Materials and methods

Plant material and growth 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. Mitchell petunia (also known as W115) is *an2⁻ an4⁻*. The *Lc* petunia seed line 118C was derived from the primary transformant 118 described in Bradley *et al.* (1998), and is hemizygous for the transgene (Mitchell background). Seeds for both Mitchell and *Lc* petunia were sown and germinated under ambient summer greenhouse conditions (February 2004). Plants were grown under 50% shade cloth, to prevent the induction of pigmentation, until they had approximately 10 nodes. Axillary buds were removed to prevent a branched architecture which would result in self-shading. Five replicates of both Mitchell and *Lc* plants were transferred to either high-light or shade conditions. Plants were maintained under each lighting condition for 12 d. Plants were grown in bark:pumice (60:40 v/v) potting mix.

In a second experiment investigating the effects of anthocyanin pigmentation upon photosynthesis (January 2007), 10 homozygous (118C×118C) *Lc* petunia and 10 Mitchell plants were grown until they had reached seven nodes, at which time they were transferred to a growth cabinet set up as the low-light treatment.

Light treatments

In the first experiment, plants in the 'shade' condition were grown in the greenhouse under a tent of 50% shade cloth. The existing photoperiod was approximately 14 h, and the greenhouse was heated at 15 °C and vented at 25 °C. The 'high light' growth treatment was provided within a Contherm Cat 640 controlled environment growth cabinet and plants were arranged randomly within the cabinet and spaced to prevent shading. Plants were grown under a 14 h photoperiod, at a constant 22 °C and 65% humidity. White light in the growth cabinet was provided by 12 HPI-T metal halide bulbs (Philips). The light levels varied between 50–350 μmol m^{-2} s^{-1} in the shade treatment, depending on the time of day and weather, and were constant at 750 μmol m^{-2} s^{-1} in the high-light treatment.

In the second experiment for photosynthetic measurements, low-light and high-light conditions were provided in Contherm growth cabinets set at a 14 h photoperiod, 22 °C, 65% humidity, with either 80 μmol m^{-2} s^{-1} or 600 μmol m^{-2} s^{-1} lighting intensity, respectively. All light measurements were determined using a Li-Cor Li-250 light meter, using the LI-190SA quantum sensor.

Pigment extraction and analysis

Leaf tissue for pigment and RNA analysis was collected from nodes 7–12 from each plant and frozen immediately in liquid nitrogen. Sampling was performed at midday to standardize any circadian or diurnal effects on flavonoid biosynthetic gene transcript abundance. Flavonoids were extracted from 50 mg DW of ground leaf tissue in 2 ml methanol:acetic acid:water (70:3:27 by vol.) for 72 h at 4 °C. The supernatant was removed and the pellet re-extracted in 2 ml methanol:acetic acid:water (90:1:9 by vol.) overnight at 4 °C. The combined supernatants were dried under vacuum

and made up to 0.5 ml with methanol:acetic acid:water (80:2:18 by vol.). HPLC analysis of flavonoids was performed as described in Bradley *et al.* (1998). 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 by integrating peak areas.

Assessment of photosynthetic parameters

In experiment two, the plants were allowed to acclimatize for 24 h after transfer to the low-light treatment before photosynthetic measurements were taken. Five *Lc* and five Mitchell plants were measured, and then transferred to the high-light treatment. The same measurements were taken on the remaining *Lc* and Mitchell plants the following day, before these plants were returned to the low-light treatment. The plants were maintained under the different light regimes for 7 d, at which time their photosynthetic parameters were measured. Photosynthetic measurements were performed on the first fully expanded leaf. This leaf was marked at the beginning of the experiment and was measured again after the 7 d light treatment. The marked leaf and the next four initiated leaves were collected for pigment extractions.

Photosynthetic measurements

In experiment two, light response curves, were performed using a CIRAS-2 Infra-red gas analyser (PP systems, Hitchin, UK), coupled with a PLC6 leaf cuvette that maintained constant temperature (22±0.2 °C) and CO_2 (1200 μl l^{-1}), and excluded external light from the enclosed area of the leaf. A 15-point CO_2 saturated light response curve was then conducted, using a range of light levels appropriate to the physiological properties of the plants, low-light-grown plants at 0–800 μmol m^{-2} s^{-1}; high-light-grown plants at 0–1200 μmol m^{-2} s^{-1}. The maximal photosynthetic assimilation rate, A_{max}, quantum efficiency, QE, the light compensation point, and the light saturation levels were calculated for each plant using the 'Photosyn Assistant' software (Dundee Scientific, Dundee, UK).

Chlorophyll and carotenoids were extracted from 20 mg DW tissue with 2 ml acetone:methanol (7:3 v/v) containing 200 mg ml^{-1} CaCO$_3$. The extract was centrifuged at 10 000 g for 2 min, the supernatant removed, and the pellet re-extracted in 2 ml acetone:methanol (7:3 v/v). This procedure was repeated until the tissue was colourless. The combined supernatants were partitioned with equal volumes of diethyl ether and water, and the ether phase removed. The diethyl ether fractions were dried under O_2-free N_2 and the carotenoids and chlorophylls were dissolved in 1 ml ethyl acetate. Chlorophylls and carotenoids were quantified by measuring their absorbance at 480 nm, 648 nm, and 666 nm in chloroform, using a Jasco V-530 UV/Vis spectrophotometer (Jasco, Tokyo, Japan) and the pigment concentrations calculated using the equation described by Wellburn (1994). Flavonoids were extracted from 30 mg DW ground leaf tissue as described above. Total flavonoids were measured

spectrophotometrically at 350 nm (in methanol), and anthocyanins at 530 nm (in 0.1 N HCl methanol). Total flavonoids were determined as quercetin-3-*O*-rutinoside equivalents (ε=14300, M_r=610) and anthocyanins as cyanidin 3-*O*-glucoside equivalents (ε=22750, M_r=484.4).

RNA isolation and analysis

Total RNA was extracted from frozen ground leaf material, using a modified hot borate protocol (Hunter *et al.*, 2002). Northern blots were performed with 15 µg of total RNA separated on a 1.2% (w/v) denaturing agarose gel (1× MOPS, 0.66 M formaldehyde), and transferred to Hybond N$^+$ membrane (GE Healthcare) with 10× SSC by capillary action. RNA was cross-linked to the membrane by exposure to UV-C at 70 000 µl cm^{-2} (Hoefer, San Francisco, CA) for 1 min. Radiolabelled probes ([α-^{32}P]dCTP) were generated using the HighPrime® (Roche Applied Science) labelling kit, using inserts isolated from the following plasmids: pLc349 (*Lc*), pCGP701 (*CHSa*), pCGP62 (*CHIa*), pCGP481 (*FLS*), pCGP1402 (*DFRa*), pCGP1407 (*ANS*), and pTIP6 (25/26S rRNA). Hybridization and washing conditions were performed as described in Bradley *et al.* (1998).

Semi-quantitative RT-PCR

First strand cDNA was prepared from 5 µg total RNA from shade and high-light-grown Mitchell leaves using SuperscriptIII Reverse Transcriptase and oligo dT$_{12-18}$ (Invitrogen). Semi-quantitative reverse-transcription-PCR was performed for *An11* (primers K135 5′-AGCTGGTACCATGGAAA-ATTCAAGTCAAG-3′; K136 5′-CTGATCTAGATT-CAATC TTTCAATCACCT-3′), and Actin (NA22 5′-TTCAGCCACTTGTCTGTGAC-3′; NA23 5′-CGA-CATCACATTTCATGATGG-3′) from 1 µl of first strand cDNA with *Taq* polymerase. Cycling conditions were 94 °C for 2 min, then (94 °C for 30 s, 60 °C for 30 s, 72 °C for 45 s) with 23 or 30 cycles for Actin and *An11*, respectively. PCR reactions were run on 1.5% (w/v) agarose gels with ethidium bromide, and fluorescent images were captured using the FLA-5100 imaging system (Fujifilm).

Leaf disc transformation of petunia

Leaf discs of shade-grown (acyanic) *Lc* and Mitchell leaves were transformed by inoculation with *Agrobacterium tumefaciens* (LBA4404, Invitrogen) carrying the binary vector pLN83 (CaMV*35S*:*Rosea1*), expressing either an anthocyanin-regulating MYB gene from *Antirrhinum* (Schwinn *et al.*, 2006) or an ER-tagged GFP reporter construct pBIN-m-gfp5-ER (CaMV*35S*:*GFP*-ER) (Haselhoff *et al.*, 1997). Leaf discs were dipped into the bacterial culture, blotted, and transferred on to half-strength solid MS media (Murashige and Skoog, 1962). Leaf discs were cultured at 25 °C with a photoperiod of 16 h supplied by cool fluorescent tubes (25 µmol m^{-2} s^{-1}). Anthocyanin pigments were readily detectable by light microscopy after 3 d. Images were collected with an Olympus SZX12 light microscope and a 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).

Statistical calculations

Statistical significance for pigment and photosynthetic measurements was determined by two-way analysis of variance (ANOVA), using Genstat version 10 (2007) for Windows (VSN International Ltd, Hemel Hempstead, UK). For pigment measurements, light saturation and light compensation point, ANOVA was performed upon log$_{10}$ transformed means, as the variance was higher for treatments with higher means. Least significant difference (LSD) values are reported.

Results

High light induces vegetative anthocyanin pigmentation in petunia

The vegetative anthocyanin pigmentation phenotypes for Mitchell and *Lc* petunia plants grown under shade (50–350 µmol m^{-2} s^{-1}) and high-light (750 µmol m^{-2} s^{-1}) are shown in Fig. 2A and C, B and D, respectively. When Mitchell plants were grown under high-light conditions, weak anthocyanin pigmentation was observed, with purple anthocyanin pigmentation visible in the leaves, especially the veins (Fig. 2B). The stems of the plant were also purple with anthocyanin pigments. The modest amount of anthocyanin developed slowly over the light-treatment period. By contrast, the *Lc* plants exposed to high-light were dark purple with anthocyanin pigments throughout the leaves and stems (Fig. 2D), with the pigmentation visible within 12 h of exposure to high-light. Pigmentation intensity increased throughout the light treatment and, therefore, the older leaves appeared more intensely purple than newly exposed leaves at the apex.

Mitchell petunia plants grown under shade conditions (Fig. 2A), did not produce visible levels of anthocyanins, and *Lc* petunia appeared largely non-pigmented (Fig. 2C). Upon close inspection, shade-grown *Lc* petunia had a border, 1–2 cells wide, at the leaf margins that were pigmented with anthocyanins. Both Mitchell and *Lc* petunia grown under the shade conditions were taller than plants grown under high light.

Lc petunia grown under high-light conditions showed a large increase in anthocyanin concentration in leaf tissue up to approximately 3 mg g^{-1} DW (Table 1), while Mitchell plants grown under the same conditions exhibited much lower anthocyanin concentrations, (<0.5 mg g^{-1} DW). Three major anthocyanin peaks were detected by HPLC analysis of high-light Mitchell and *Lc* petunias (Fig. 3). These were identified as malvidin and petunidin glycosides, consistent with previous studies (Bloor *et al.*, 1998). The major anthocyanins with known structure are labelled. The same anthocyanin peaks detected in *Lc* petunia were present
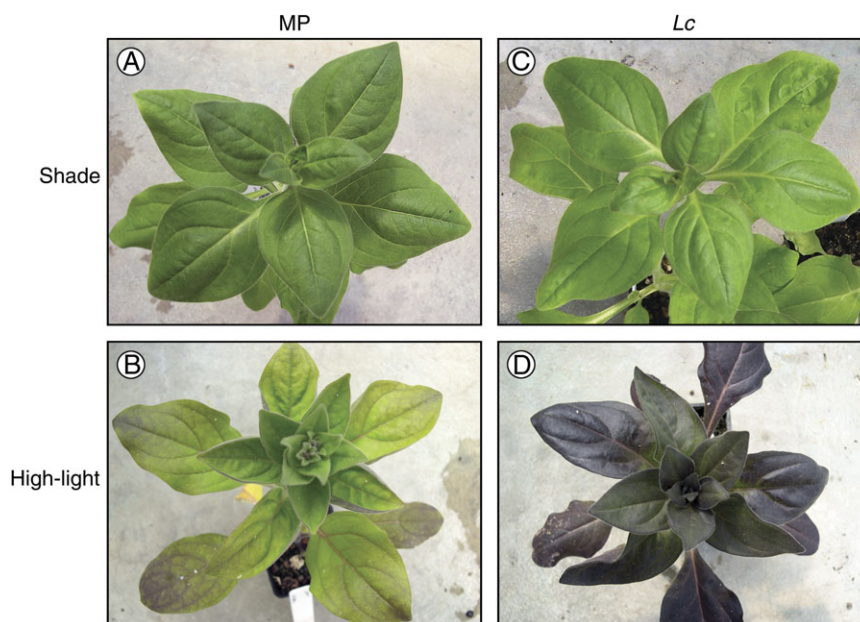
Fig. 2. Wild-type Mitchell and *Lc* petunia grown under shade and high-light treatments, showing vegetative pigmentation phenotypes. (A, C) Mitchell and *Lc* petunia grown under shade conditions (50–350 µmol m^{-2} s^{-1}). (B, D) Mitchell and *Lc* petunia grown under high-light conditions (750 µmol m^{-2} s^{-1}). The individual plants shown are representative of the five plants grown per treatment.

Table 1. Leaf anthocyanin and flavonoid content (mg g^{-1} DW) in Mitchell (MP) and *Lc* petunia grown under shade or high light

Data presented are the mean values for individual anthocyanins or flavonols as well as a total concentration (*n*=3). Individual compounds (A1-3 or F1-3) correspond to the labelled peaks for the chromatograms shown in Figs 3 and 4, respectively.

	Shade		High light		LSD
	MP	*Lc*	**MP**	*Lc*	
Anthocyanins					
Petunidin-3-rutinoside-5-glucoside, *p*-coumaric acid (A1)	ND[a]	ND	0.17	1.61	
Petunidin-3-rutinoside-5-glucoside, caffeic acid (A2)	ND	ND	0.03	0.39	
Petunidin-3-rutinoside-5-glucoside, 4-*O*-glucosyl-*p*-coumaric acid (A3)	ND	ND	0.15	0.40	
Other anthocyanins	ND	ND	0	0.54	
Total (mean)	ND	ND	0.35	2.94	
Log$_{10}$ total	–	–	(–0.46)	(0.47)	(0.32)[b]
Other flavonoids (flavonols)					
Quercetin-3-*O*-(caffeoyl diglucoside) (F1)	4.70	4.61	6.11	6.34	
Quercetin-3-*O*-(2-*O*-caffeoyl 6-*O*- malonyl diglucoside) (F2)	ND	ND	3.90	3.62	
Kaempferol 3-*O*-(feruloyl diglucoside) (F3)	0.50	0.51	3.18	4.37	
Other flavonols	1.89	1.99	9.26	10.82	
Total (mean)	7.09	6.79	22.45	25.15	
Log$_{10}$ total	(0.85)	(0.83)	(1.35)	(1.40)	(0.12)[b]

[a] ND, not detected.
[b] LSD reported is for the Log$_{10}$ total mean value only. LSD is at 5% significance level; residual df=8 for other flavonoids, df=4 for anthocyanins.

in wild-type Mitchell petunia, but at lower levels. Anthocyanins were not detected in Mitchell or *Lc* plants grown under shade conditions.

Flavonols unaffected by anthocyanin induction

Mitchell and *Lc* petunia plants had equivalent levels of other flavonoids (flavonols) in all light treatments (Table 1). Flavonol concentration was higher at increased light intensity, increasing from approximately 7 mg g^{-1} DW in shade-grown plants, to 25 mg g^{-1} DW in high-light plants. Mitchell and *Lc* petunia accumulated similar flavonoid compounds (Fig. 4), with the major peaks matching the various flavonols. The major flavonols with a known structure are labelled. A non-flavonoid compound was also present, and was identified as rosmarinic acid, after comparison with published data (Bloor *et al.*, 1998; Troncoso *et al.*, 2005). Rosmarinic acid was excluded from the calculations of other flavonoid total concentration (Table 1) as it is not derived from the flavonoid biosynthetic pathway.

Changes in flavonoid gene expression underpin induction of anthocyanin synthesis

Flavonoid gene expression in Mitchell and *Lc* plants grown under high-light and shade conditions is shown in Fig. 5. The flavonoid biosynthetic genes *CHS, CHI,* and *FLS* showed high transcript abundance in high-light Mitchell plants, while weak signals for *DFR* and *ANS* transcripts were also detected. *Lc* plants show enhanced transcript levels for *CHS* and *CHI* levels compared with Mitchell, while *FLS* transcripts were reduced, and strong signals for
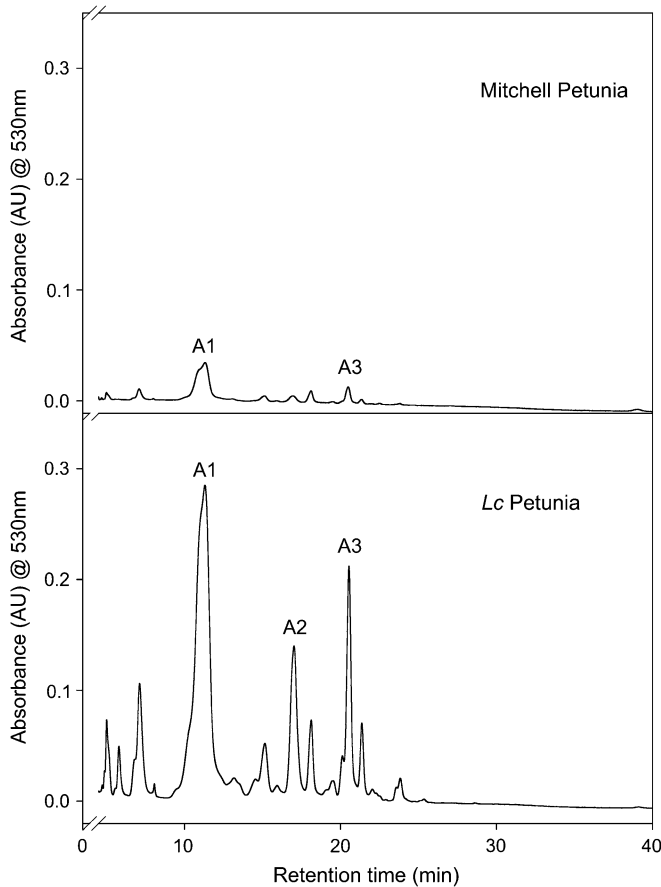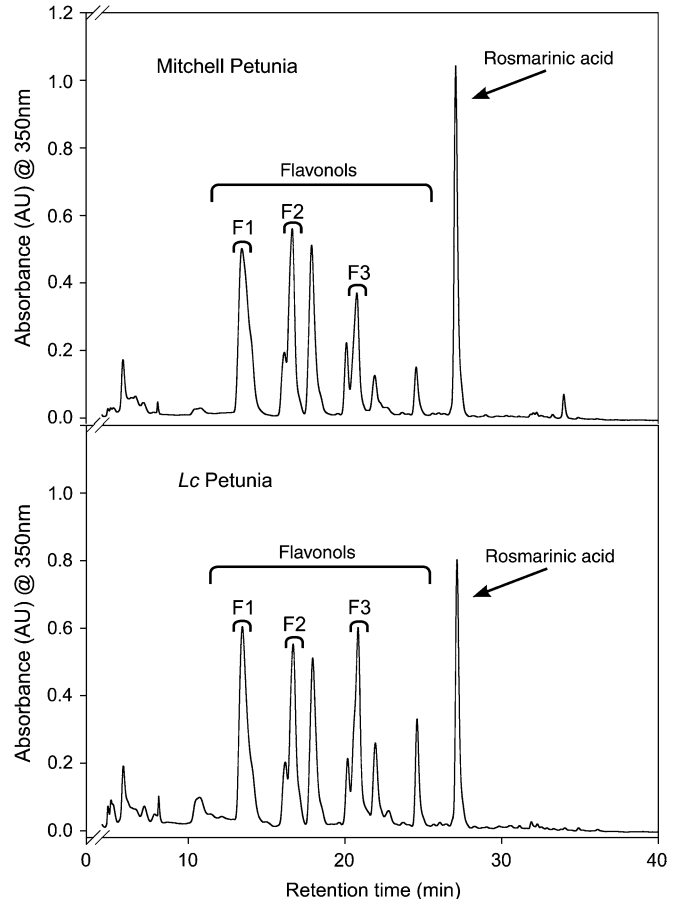
Fig. 3. HPLC chromatograms for leaf extracts from Mitchell and *Lc* petunia grown under high light. The absorbance was monitored at 530 nm to detect anthocyanins. The major anthocyanin peaks are indicated. The major anthocyanin peaks are petunidin-3-rutinoside-5-glucoside acylated with *p*-coumaric acid (A1), caffeic acid (A2), or 4-*O*-glucosyl-*p*-coumaric acid (A3).


Fig. 4. HPLC chromatograms for leaf extracts from Mitchell and *Lc* petunia grown under high light. Absorbance was monitored at 350 nm. The major flavonoids, the flavonols, and a non-flavonoid compound, rosmarinic acid, are indicated. F1, quercetin-3-*O*-(caffeoyl diglucoside); F2, quercetin-3-*O*-(2-*O*-caffeoyl 6-*O*-malonyl diglucoside); F3, kaempferol 3-*O*-(feruloyl diglucoside).

transcripts of the late biosynthetic genes *DFR* and *ANS* were clearly observed. Weak signals for *CHS*, *CHI,* and *FLS* were detected in Mitchell and *Lc* petunia plants grown under shade. Faint signals for *DFR* and *ANS* were detected in *Lc* petunia, but not in Mitchell.

Expression of a MYB anthocyanin regulator overcomes the high-light requirement for anthocyanin accumulation in Lc *petunia*

Light-induced anthocyanin pigmentation in *Lc* petunia raised questions about the regulation of the anthocyanin biosynthetic genes in petunia leaves. The current model for anthocyanin regulation links three components: bHLH, WD40, and MYB factors.

Expression of the bHLH *Lc* transgene in *Lc* petunia was shown to be insensitive to the light treatment (Fig. 6A), and therefore not responsible for the light-induced phenotype. A WD40 factor (*An11*) is required for floral anthocyanin regulation in petunia in addition to MYB and bHLH factors. Semi-quantitative RT-PCR showed similar transcript levels for *An11* in both Mitchell and *Lc* petunia leaves, in either shade or high-light conditions (Fig. 6B).

The anthocyanin-regulating MYB transcription factor *Rosea1*, introduced by *Agrobacterium*-mediated transformation into acyanic leaf discs of Mitchell and *Lc* petunia, was able to complement the requirement for high-light to induce pigmentation in *Lc* petunia. Transformation of Mitchell petunia with CaMV*35S*:*Rosea1* did not induce anthocyanin production (Fig. 7A), but scarlet anthocyanin-producing cells were clearly visible along the cut surface of the leaf disc in *Lc* petunia (Fig. 7B). Transformation with CaMV*35S*:*GFP* was performed as a negative control. Anthocyanins were not induced in Mitchell or *Lc* petunia (Fig. 7C, D) but GFP expression was clearly visible (Fig. 7E, F).

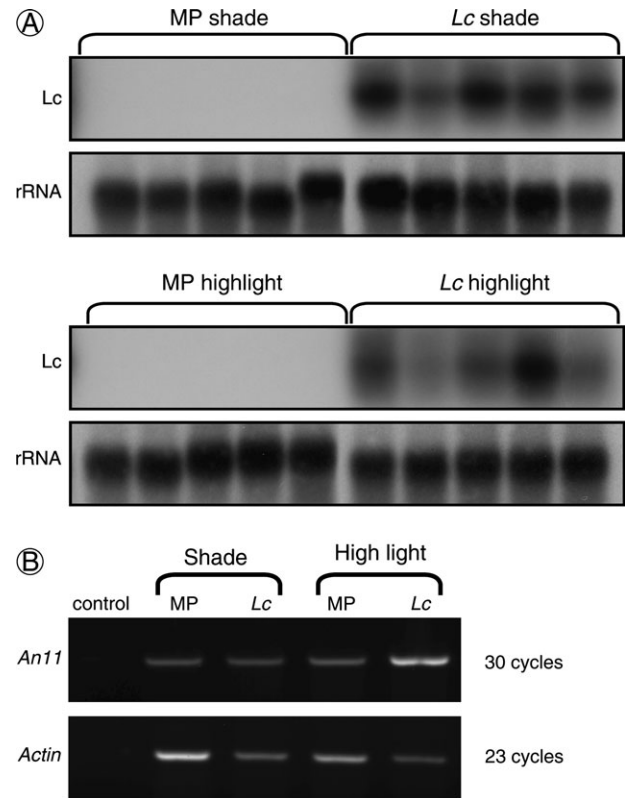*Vegetative anthocyanins screen photosynthetic tissues*

Lc and Mitchell plants were grown under different light conditions and leaf photosynthetic measurements were made to determine whether the accumulation of anthocyanin pigments in *Lc* petunia affected photosynthetic capacity.

Light response curves and photosynthetic CO_2 assimilation rates were determined for *Lc* and Mitchell plants

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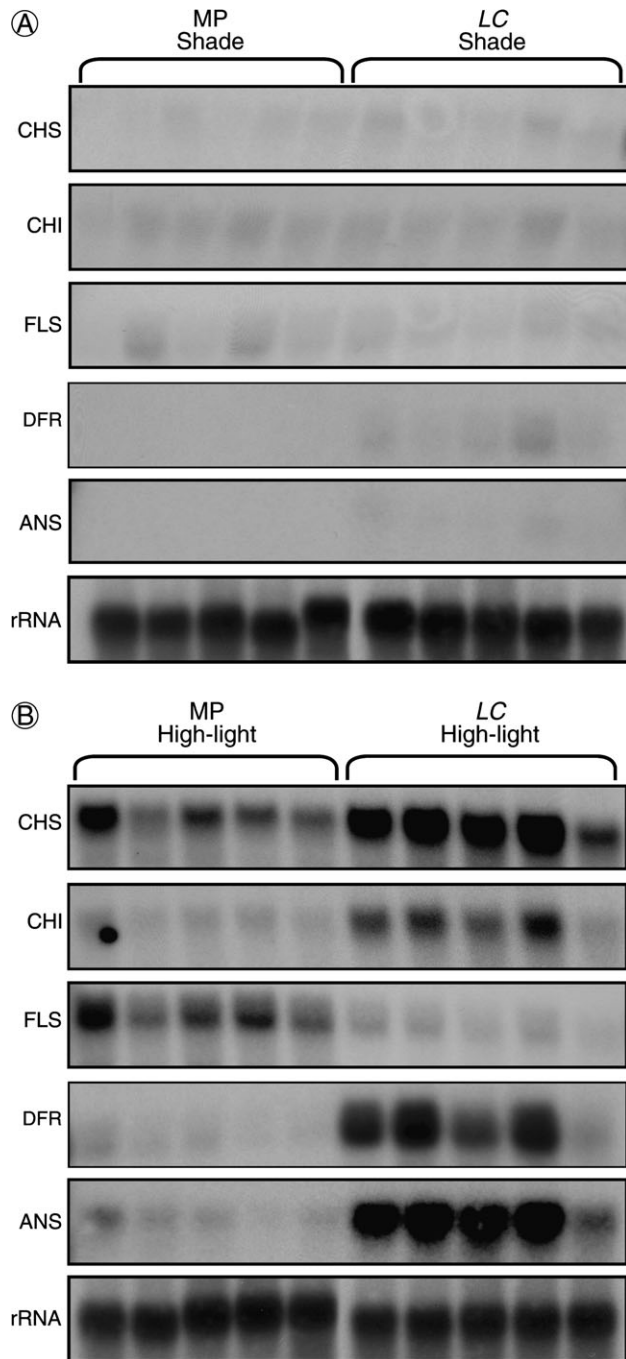
Fig. 5. Northern blot analysis of flavonoid structural gene expression in Mitchell (MP) and *Lc* petunia plants under (A) shade or (B) high-light treatments. Each lane represents a different individual plant within each treatment. 25/26S rRNA is shown as a loading control.
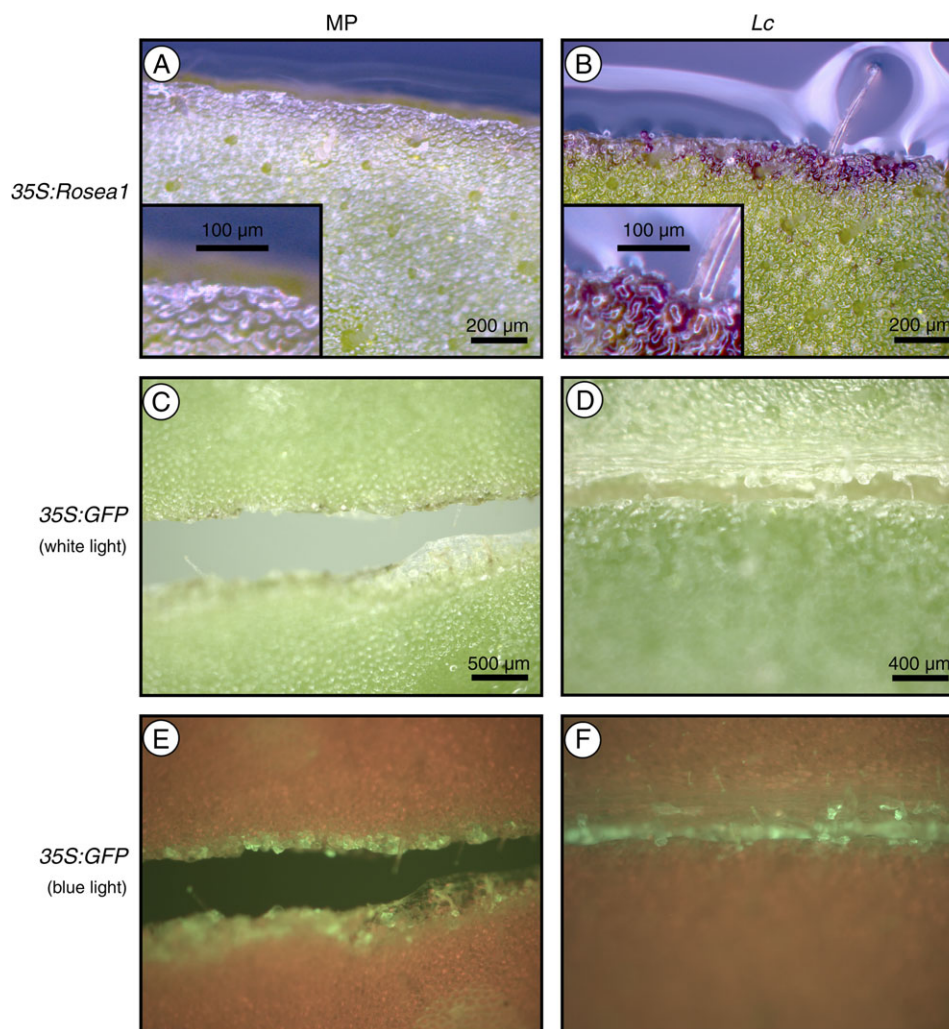
Fig. 6. Transcript abundance for anthocyanin regulation components. (A) Northern blot showing *Leaf colour* transcript abundance in *Lc* and Mitchell petunia (MP) grown under shade or high-light treatments. 25/26S rRNA is shown as a loading control. (B) Semi-quantitative RT-PCR of *An11* transcripts in shade and high-light-grown Mitchell and *Lc* petunia leaves. Actin was amplified as a cDNA loading control. PCR cycles are indicated.

compensation and saturation points, the maximum photosynthetic rate, A_{max}, was not significantly different between *Lc* and Mitchell plants, although it was higher in the high-light than low-light plants.

Photosynthetic pigment content

Vegetative pigment levels were also measured in the plants used to examine photosynthetic parameters. This included chlorophylls and carotenoids, as well as anthocyanins and flavonoids. No significant differences in pigment levels between *Lc* or Mitchell plants grown under low-light were noted (Table 3). Under high-light conditions, however, anthocyanins accumulated to 10-fold higher levels in *Lc* petunia and total flavonoid levels were slightly higher than in Mitchell (Table 3), consistent with the results from the first experiment (Table 1). Chlorophyll and carotenoid levels in Mitchell plants grown under high-light were lower than in the low-light treatment, and there was an increase in the chlorophyll *a/b* ratio. *Lc* petunia plants under high-light did not show the same reduction in chlorophyll and carotenoid levels or change in chlorophyll *a/b* ratio observed for the Mitchell plants.

before and after being subjected to either low- or high-light treatments. No differences in the CO_2 assimilation rates, light compensation points or light saturation points were observed in low-light plants (Table 2). However, once anthocyanins accumulation was induced by high-light in *Lc* petunia, the light saturation point was increased by 46% compared with Mitchell plants, and the light compensation point was raised by 68%. Despite the changes in light

Fig. 7. Light complementation experiments, utilizing *Agrobacterium*-mediated transformation of Mitchell (MP) and *Lc* petunia leaves. (A, B) MP and *Lc* leaf explants, respectively, transformed with CaMV*35S:Rosea1.* Inset images in (A) and (B) show higher magnification of cells at the cut surface of the leaves. MP and *Lc* leaf explants transformed with CaMV*35S*:GFP, viewed under white light (C, D) and blue light (E, F), respectively.

Table 2. Photosynthetic characteristics of Mitchell (MP) and *Lc* petunia after 7 d of low-light or high-light treatment, $n=5$

	A_{max} (μmol CO_2 m^{-2} s^{-1})	QE ($\times 10^{-2}$)	Light saturation point (μmol m^{-2} s^{-1})		Light compensation point (μmol m^{-2} s^{-1})	
	Mean	Mean	Mean	(Log$_{10}$)	Mean	(Log$_{10}$)
MP low light	12.2	6.11	216	(2.334)	15	(1.176)
Lc low light	14.2	6.89	225	(2.352)	18	(1.255)
MP high light	33.1	5.93	590	(2.771)	28	(1.447)
Lc high light	30.1	3.71	863	(2.936)	47	(1.672)
LSD[a]	4.3	0.77		(0.061)		(0.187)

[a] LSD is at the 5% significance level; residual df=16.

Discussion

Light-induced anthocyanin pigmentation

Light-induced vegetative anthocyanin pigmentation was confirmed in transgenic *Lc* petunia plants. Despite constitutive expression of the bHLH transgene *Lc*, increased pigmentation was only observed in the high-light treatment. This pigmentation was limited to those tissues which normally accumulate a modest amount of anthocyanin in Mitchell petunia (Fig. 2), in sub-epidermal cell layers overlying the photosynthetic cells in the leaves of both Mitchell and *Lc* petunia (Bradley *et al.*, 1998). Transgenic

Table 3. Leaf pigment analysis of Mitchell (MP) and *Lc* petunia plants grown under low or high light

Pigment concentrations are expressed as mg g^{-1} DW, $n=5$.

	Total flavonoids		Anthocyanins		Total chlorophylls		Chl*a*/Chl*b*		Total carotenoids	
	Mean	(Log$_{10}$)	Mean	(Log$_{10}$)	Mean	(Log$_{10}$)	Mean	(Log$_{10}$)	Mean	(Log$_{10}$)
MP low light	5.61	(0.749)	ND[a]	–	6.85	(0.836)	4.26	(0.629)	1.91	(0.281)
Lc low light	5.94	(0.774)	ND	–	6.04	(0.781)	4.08	(0.611)	1.85	(0.267)
MP high light	16.88	(1.227)	0.04	(–1.398)	1.93	(0.286)	8.16	(0.912)	0.74	(–0.131)
Lc high light	20.43	(1.310)	0.91	(–0.041)	3.05	(0.484)	7.02	(0.846)	1.02	(0.009)
LSD[b]		(0.077)		(0.130)		(0.072)		(0.086)		(0.055)

[a] ND, not detected.
[b] LSD is at the 5% significance level; residual df=16, except for anthocyanins; df=8.

alfalfa (*Medicago sativa*) (Ray *et al.*, 2003), tomato (*Solanum lycopersicum*) (Goldsbrough *et al.*, 1996), and *Arabidopsis* plants (Lloyd *et al.*, 1992) ectopically expressing *Lc* also showed light-induced anthocyanin pigmentation, and light has been shown to enhance proanthocyanidin (PA) production in lotus plants (*Lotus corniculatus*) ectopically expressing *Sn*, a bHLH transcription factor homologous to *Lc* (Paolocci *et al.*, 2005). These findings indicate that, in these systems, the bHLH transgene alone was insufficient to induce anthocyanin (or PA) biosynthesis, and it is acting with other endogenous regulatory factors in each of these systems to activate the biosynthetic genes, and that these endogenous factors are expressed in vegetative tissues during high-light or stress conditions.

Lc petunia under high-light conditions show enhanced expression of flavonoid biosynthetic genes required for anthocyanin production. The early genes *CHS* and *CHI*, as well as the late genes *DFR* and *ANS*, were induced in Mitchell petunia leaves, matching the modest accumulation of anthocyanin. Transcript levels for the early and late biosynthetic genes were greatly enhanced in *Lc* plants, matching the accumulation of anthocyanin, suggesting that LC was co-operatively activating the biosynthetic genes normally targeted by endogenous anthocyanin regulators. The specific anthocyanins which accumulated in *Lc* petunia were the same as those in Mitchell (Bloor *et al.*, 1998) (Fig. 3), indicating that the same genes, including those for anthocyanin modification (acylation, methylation, glycosylation) were being targeted. This suggests that the specificity of target gene recognition was provided by the endogenous regulators that act with LC.

The current model for anthocyanin regulation indicates MYB and bHLH transcription factors, together with a WD40 co-regulator, form a complex which activates the target anthocyanin biosynthetic genes (Koes *et al.*, 2005). The bHLH factor *Lc* is constitutively expressed in *Lc* petunia (Fig. 6A) and *An11* (WD40) was shown to be expressed in leaves irrespective of light treatment (Fig. 6B). The involvement of the endogenous petunia bHLH factors *Jaf13* and *An1* is unlikely to determine the light-induced phenotype, as the related bHLH *Lc* transgene was expressed at high levels throughout the plant, and bHLH factors involved in anthocyanin production cannot bind DNA on their own and act through their binding partners (Sainz *et al.*, 1997; Hernandez *et al.*, 2004; Koes *et al.*, 2005). Therefore, the possibility was investigated that MYB transcription factors may determine the light-induced anthocyanin biosynthesis in *Lc* petunia. Introduction of a known anthocyanin-regulating MYB transcription factor, ROSEA1, successfully complemented the requirement for high-light (Fig. 7B). It was hypothesized that endogenous MYB factors are induced by high light in vegetative tissues to control the production and distribution of anthocyanins to fulfil light-screening functions, and that the MYB(s) provide the specificity of target gene recognition. Mitchell petunia contains recessive alleles for the known anthocyanin-regulating MYB factors, *an2*$^-$ (null) and *an4*$^-$ (Quattrocchio *et al.*, 1993, 1998; Koes *et al.*, 2005), therefore, the hypothesized MYB determining light-induced vegetative pigmentation is a new MYB anthocyanin regulator.

Lc petunias grown under high-light conditions have higher transcript levels for *CHS* and *CHI,* as well as *DFR* and *ANS*, which suggests endogenous anthocyanin regulators may act upon both the early and late biosynthetic genes together with LC. The early anthocyanin biosynthetic genes are shared with multiple branches of the flavonoid pathway, including flavonols, indicating a requirement for multiple regulatory systems to act upon common genes to control the production of different flavonoid compounds. Such a system was demonstrated in *Arabidopsis*, where the promoters of the flavonoid genes *CHS, CHI,* and *F3H* contained multiple *cis*-elements conferring responsiveness to the anthocyanin regulators C1/SN (MYB/bHLH) or to light (light-regulated flavonol production) (Hartmann *et al.*, 2005). These elements consisted of a MYB-recognition element (MRE), a bHLH recognition sequence named the R-response-element (RRE), and an ACGT containing element (ACE), which are thought to bind MYB, bHLH, and bZIP transcription factors, respectively. Light-regulated expression of the early genes required both the MRE and ACE, while activation by C1/SN required the MRE and RRE, suggesting that both the flavonol regulators and the anthocyanin regulators may act upon the shared early biosynthetic genes, differentially and independently to regulate the production of different flavonoids. The observation that *myb11*$^-$ *myb12*$^-$ *myb11*$^-$ triple mutants do not

produce flavonols, yet still make anthocyanins is consistent with this (Stracke *et al.*, 2007), suggesting that the anthocyanin regulators can act redundantly upon the early biosynthetic genes (*CHS, CHI, F3H*), allowing expression of these genes in the absence of the flavonol regulators.

Light-regulated flavonol production occurs by a system distinct from anthocyanin regulation in petunia. Flavonols were induced with increasing light intensity in *Lc* and Mitchell petunia leaves, which have previously been identified as various acylated kaempferol and quercetin-glycosides (Bloor *et al.*, 1998). Transcripts for *CHS, CHI,* and *FLS* were induced by high light, in a manner that we believe is co-ordinated (Fig. 5). In *Arabidopsis*, co-ordinated expression of these genes is regulated by *At*MYB11, *At*MYB12, and *At*MYB111 for flavonol production in different tissues throughout the plant (Mehrtens *et al.*, 2005; Stracke *et al.*, 2007). It is possible that homologous regulators to *At*MYB12 regulate flavonol production in petunia, acting independently of anthocyanin regulators. Interestingly, *FLS* transcript levels were lower in high-light grown *Lc* petunia compared to Mitchell. It has been suggested that this was because the high level of anthocyanins in *Lc* petunia screened light from underlying cells, which reduced the light-induction of flavonol-related genes. A reduction in *CHS* and *CHI* transcripts was not observed, however, due to the activation of these genes by LC, together with endogenous anthocyanin regulators. Despite a reduction in the transcript levels for *FLS* in high-light *Lc* petunia, a reduction in flavonol content compared to Mitchell was not observed. *FLS* knock-down in Mitchell petunia flowers resulted in enhanced anthocyanin accumulation (Davies *et al.*, 2003), suggesting FLS strongly competes with DFR for di-hydroflavonol substrates. Increased flux through the early steps of the flavonoid pathway in high-light-grown *Lc* petunia may have elevated the pool of di-hydroflavonols, providing more substrate for FLS, allowing flavonols to accumulate to similar levels to Mitchell.

Physiological impact of anthocyanin pigmentation upon photosynthesis

The light-induced anthocyanin phenotype observed in *Lc* petunia allowed a comparison of photosynthetic capacity to be made in pigmented and non-pigmented leaves. Although anthocyanin pigmentation did not reduce the maximum photosynthetic rate (A_{max}), a screening role for anthocyanins was demonstrated. Under low-light conditions, when anthocyanins were not induced, Mitchell and *Lc* petunia had similar photosynthetic assimilation rates, light saturation, and light compensation points. However, once anthocyanins were induced in *Lc* petunia, the light-saturation point increased by 46% compared with Mitchell plants grown under the same conditions. This suggests that the anthocyanins in *Lc* petunia leaves were screening light from the underlying photosynthetic tissues. A light-screening role for anthocyanins was also supported by analysis of the photosynthetic pigments in these plants, which showed that *Lc* petunia leaves containing high levels of anthocyanin also had higher chlorophyll and carotenoid levels and a reduced chlorophyll *a/b* ratio. These changes observed in the purple *Lc* plants are generally associated with shade leaves, whereas the Mitchell plants grown in high light had the typical sun leaf characteristics of a lower chlorophyll content and higher *a/b* ratios (Huner *et al.*, 1998; Willows, 2004). Similar findings have been made in *Quercus coccifera*, comparing anthocyanic and acyanic leaves, where anthocyanin accumulation led to shade acclimation traits (Manetas *et al.*, 2003). The presence of anthocyanins in leaves of *Galax urceolata* reduced the photoinhibition caused by exposure to strong white or green light, but not to red wavelengths, demonstrating an *in vivo* light attenuation and photoprotection role for anthocyanins (Hughes *et al.*, 2005). While the absorption spectrum for anthocyanins peaks at around 550 nm *in vivo*, they also absorb wavelengths in the blue region, particularly when they accumulate to high levels (Merzlyak *et al.*, 2008). Together with our own findings, this indicates that foliar anthocyanins do screen light from underlying tissues.

Conclusions

Lc and Mitchell petunia have provided a unique, near-isogenic experimental system to examine the mechanism for vegetative light-induced anthocyanin pigmentation and the effect of pigmentation upon photosynthesis, whilst removing the variability associated with plants of differing genetic backgrounds or developmental stages. The enhanced pigmentation of *Lc* petunia has allowed us to demonstrate that sub-epidermal anthocyanins do act as a light screen, but without affecting the maximum photosynthetic rate. The fact that intense foliar pigmentation in *Lc* petunia occurred only when grown under high light points to the existence of a light-induced regulatory factor responsible for the activation of the anthocyanin pathway. Given that both the bHLH (*Lc*) and WD40 (*An11*) were constitutively expressed in *Lc* petunia, and that a MYB anthocyanin-regulator *Rosea1* was able to complement the high-light requirement, the endogenous regulatory factor appears likely to be a MYB protein.

Acknowledgements

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