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# The influence of ascorbate on anthocyanin accumulation during high light acclimation in *Arabidopsis thaliana*: further evidence for redox control of anthocyanin synthesis.

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**ABSTRACT**

Ascorbate and anthocyanins act as photoprotectants during exposure to high light (HL). They accumulate in Arabidopsis leaves in response to HL on a similar time-scale, suggesting a potential relationship between them. Flavonoids and related metabolites were identified and profiled by LC-MS/MS. The ascorbate deficient mutants *vtc1, vtc2* and *vtc3* accumulated less anthocyanin than wild-type during HL acclimation. In contrast, kaempferol glycoside accumulation was less affected by light and not decreased by ascorbate deficiency, while sinapoyl malate levels decreased during HL acclimation. Comparison of six Arabidopsis ecotypes showed a positive correlation between ascorbate and anthocyanin accumulation in HL. mRNA-Seq analysis showed that all flavonoid biosynthesis transcripts were increased by HL acclimation in wild-type. RT-PCR analysis showed that *vtc1* and *vtc2* were impaired in HL induction of transcripts of anthocyanin biosynthesis enzymes, and the transcription factors PAP1, GL3 and EGL3 that activate the pathway. Abscisic acid and jasmonic acid, hormones that could affect anthocyanin accumulation, were unaffected in *vtc* mutants. It is concluded that HL induction of anthocyanin synthesis involves a redox-sensitive process upstream of the known transcription factors. Because anthocyanins accumulate in preference to kaempferol glycosides and sinapoyl malate in HL, they might have specific properties that make them useful in high light acclimation.

*Key-words*: ascorbate, cyanidin, abscisic acid; jasmonic acid; kaempferol glycosides; light stress; metabolite profiling; mRNA-Seq; LC-MS/MS; oxidative stress.

## INTRODUCTION

Accumulation of purple anthocyanin pigments is one of the most obvious biochemical changes in *Arabidopsis thaliana* leaves exposed to high light intensity (HL). Anthocyanins are flavonoid compounds synthesised, along with the flavonols (kaempferol and quercetin), from phenylalanine and malonyl-CoA (Winkel-Shirley 2002). As well as being light-responsive, anthocyanin accumulation is inducible by sucrose (Teng *et al.* 2005), hormones such ABA and jasmonates (Loreti *et al.* 2008; Shan *et al.* 2009), low temperature (Leyva *et al.* 1995), nutrient deficiency (Rubin *et al.* 2009) and wounding (Chalker-Scott 1999). Anthocyanins have various roles, most obviously in attracting pollinators and seed dispersers. Another function in some species is anti-herbivore defence (Karageorgou & Manetas 2006). Abiotic stress-induced accumulation in leaves and stems implies that flavonoids have a protective function. Two roles are proposed: UV-B screening and a direct antioxidant effect. Flavonoids have an absorption maximum in the UV-B region of the spectrum and Arabidopsis mutants impaired in flavonoid biosynthesis are UV-B sensitive (Landry *et al.* 1995; Li *et al.* 1993; Ormrod *et al.* 1995). The other main group of UV-B-absorbing phenolic compounds in Arabidopsis are the phenylpropanoids. A coniferyl aldehyde 5-hydroxylase mutant, *fah1*, that lacks sinapic acid and sinapoyl malate, the predominant sinapoyl ester in Arabidopsis leaves (Chapple *et al.* 1992), is even more UV-B sensitive than flavonoid mutants (Landry *et al.* 1995). There is strong evidence that anthocyanin accumulation in leaves protects against photoinhibitory damage caused by high irradiance (Gould *et al.* 2010; Havaux & Kloppstech 2001; Zeng *et al.* 2010; Zhang *et al.* 2010). This could be attributed to their visible and UV-B screening effect or to an antioxidant effect. Excess excitation energy can result in increased production of reactive oxygen species (ROS) and increases in antioxidants (Foyer & Noctor 2011; Galvez-Valdivieso & Mullineaux 2010; Murchie & Niyogi 2011). It has been suggested that flavonoids could act as

antioxidants (Grace & Logan 2000; Hernandez *et al.* 2009). They can react with hydrogen peroxide in peroxidase-catalysed reactions (Yamasaki *et al.* 1997) as well as superoxide and peroxynitrite (Rahman *et al.* 2006). Some flavonoids chelate iron and could therefore act as antioxidants by preventing hydroxyl radical formation through the Fenton reaction (Perron & Brumaghim 2009). It is difficult to distinguish between UV-B screening and more direct antioxidant effects *in vivo* because UV-B itself causes oxidative stress (Landry *et al.* 1995), so this role remains to be fully established.

Like anthocyanins, ascorbic acid (AsA) accumulates when Arabidopsis plants are transferred from low to HL conditions (Bartoli *et al.* 2006; Golan *et al.* 2006; Smirnoff 2000; Zechmann *et al.* 2010). It is presumed that AsA accumulation is related to its role in photosynthesis and photoprotection. AsA and ascorbate peroxidase (APX) are involved in removing hydrogen peroxide produced by oxygen photoreduction and photorespiration (Foyer & Noctor 2011). Cytosolic APX (cAPX) is required for modulating the hydrogen peroxide that leaks from chloroplasts and peroxisomes and is involved in signalling processes (Davletova *et al.* 2005; Galvez-Valdivieso *et al.* 2009). AsA may also reduce tocopheroxyl radicals produced as a result of singlet oxygen-induced lipid peroxidation in PSII (Havaux 2003). Thylakoid lumen AsA is involved in the xanthophyll cycle as a cofactor for violaxanthin de-epoxidase. AsA-deficient mutants are impaired in non photochemical quenching (Muller-Moulé *et al.* 2003; Smirnoff 2000). It is also possible that lumenal AsA can protect PSII by acting as an emergency electron donor when the oxygen evolving complex is inactivated by stress, particularly UV-B and high temperature (Toth *et al.* 2009). AsA-deficient *vtc* mutants are more susceptible to transfer to HL although they can acclimate to long term exposure to some extent (Muller-Moulé *et al.* 2004). This proposal takes on significance if inactivation of the oxygen evolving complex turns out to be a primary cause of HL damage to PSII (Takahashi & Badger 2011; Takahashi *et al.* 2010).

Previously, it has been noted that AsA deficient Arabidopsis *vtc2-1* accumulates much less anthocyanin under high irradiance (Giacomelli *et al.* 2007; Giacomelli *et al.* 2006). In contrast, a mutant in cAPX accumulates more anthocyanin in response to low temperature (Asai *et al.* 2004) or HL (Miller *et al.* 2007). Manipulation of hydrogen peroxide scavenging capacity by reducing catalase (*CAT2*) expression in Arabidopsis decreases both the expression of flavonoid biosynthesis genes and anthocyanin accumulation (Vanderauwera *et al.* 2005). The results therefore suggest a role for photosynthetically-produced ROS and antioxidants in HL-induced anthocyanin accumulation.

The flavonoid biosynthesis pathway appears to be co-ordinately controlled at the transcriptional level. Under conditions that induce flavonoid synthesis, the genes encoding most of the enzymes from phenylalanine ammonia lyase (PAL), through to the transferases that decorate the flavonoid and anthocyanin rings, are induced (Vanderauwera *et al.* 2005; Winkel-Shirley 2002). Flavonoid biosynthesis enzymes may also form complexes to channel pathway intermediates (Burbulis & Winkel-Shirley 1999). Three of the enzymes involved in flavonoid biosynthesis are 2-oxoglutarate-dependent dioxygenases (2-ODDs): flavanone 3-hydroxylase (F3H), flavonol synthase (FLS) and leucoanthocyanidin dioxygenase (LDOX) (Fig. 2). 2-ODDs generally require AsA for efficient activity. AsA prevents enzyme inactivation by reducing over-oxidised $Fe^{IV}$ to $Fe^{II}$ in the active site (Lukacin & Britsch 1997; Martens *et al.* 2010; Prescott & John 1996). 2-ODD activity in *vtc* mutants could therefore be limited by AsA supply.

Given the decreased anthocyanin accumulation in *vtc2-1* (Giacomelli *et al.* 2007; Giacomelli *et al.* 2006) and the observation that anthocyanin accumulation in leaves exposed to HL is on a similar time-scale (days) to that of AsA, the relationship between these processes merits further investigation. Therefore, the aim of the

experiments reported here was to assess whether a link exists between AsA and anthocyanins during acclimation to HL by making use of AsA-deficient mutants, as well as natural variation in AsA in various *A. thaliana* accessions.

## MATERIALS AND METHODS

### Plant material and growth conditions

*vtc* mutants were obtained from Patricia Conklin (State University of New York, Cortland) and were all in the Col-0 background (Conklin *et al.* 2000). The Col-0, GOT1, HR-5, Is-0, NFE1 and Old-2 ecotypes were obtained from The European Arabidopsis Stock Centre (NASC, Nottingham, UK). Surface sterilised seeds were cold treated for 3 days at 4 °C, then sown onto Levington F2 compost (Scotts, Marysville, USA) (4:1, compost:vermiculite). Plants were grown in controlled environment growth rooms under short day conditions (8 h light, 16 h dark) at a photosynthetic photon flux density (PPFD) of 100 µmol $m^{-2}$ $s^{-1}$, 23 °C and 65% relative humidity (RH) for 7 weeks, resulting in large rosettes but preventing bolting. After this time, plants were acclimated to long days (16 h light, 8 h dark) in a controlled environment growth cabinet (23 °C, 65% RH, PPFD 100 µmol $m^{-2}$ $s^{-1}$) for 4 days (Microclima 1000E, Snijders, Tilburg, Netherlands). High light (HL) treatments were performed in the same growth cabinets by subjecting these plants to a PPFD of 550-650 µmol $m^{-2}$ $s^{-1}$, 16 h light period for 4 days. Control LL plants were grown concurrently in the same cabinet at an irradiance of 100 µmol $m^{-2}$ $s^{-1}$.

### Ascorbate assay

Three fully expanded rosette leaves were excised, weighed, then flash frozen in liquid nitrogen. Leaf samples were ground in 1 ml 1% metaphosphoric acid (MPA). The homogenate was centrifuged for 5 min at 15,500 x g, 4 °C. The supernatant was retained and 125 µl of each sample combined with an equal volume of either 1% MPA (to assay reduced AsA) or 20 mM tris(2-carboxyethyl)phosphine hydrochloride (TCEP) in 1% MPA (to assay AsA + dehydroascorbate (DHA) – referred to as "total AsA"). DHA was reduced to AsA within 30 min under the conditions used and the AsA remained stable for at least 24 h at 21 °C. Assay mixes were passed through 0.2 µm PVDF syringe filters (Chromacol, Welwyn Garden City, UK) and AsA was measured by HPLC. Mobile phases consisted of 95% water, 5% acetonitrile, 0.1% formic acid (A) or 95% acetonitrile, 5% water, 0.1% formic acid (B). 20 µl of sample was injected onto a Phenomenex Luna C18 column (10 µm particle size, 250 x 4.6 mm) and subjected to the following gradient using a Dionex DX500 HPLC system (Dionex, Sunnyvale, USA): 0 min – 0% B; 4 min – 40% B; 7 min – 100% B; 9 min – 100% B; 10 min – 0% B; 2 min post time. Flow rate was maintained at 1 ml $min^{-1}$ and the assay was carried out at 21 °C. AsA was detected using a SPD-10A dual wavelength detector (Shimadzu, Japan) at 265 nm and 280 nm, and had a retention time of approximately 3.5 min. Data were analysed with Chromeleon software (Dionex, Sunnyvale, USA). The peak purity of AsA was determined by monitoring the 265/280 nm signal ratio, which is ~10 for pure AsA. Interfering compounds were not found under the chromatographic conditions used. AsA was quantified by comparison with external standards using the peak areas at 265 nm.

**Flavonoid, phenylpropanoid and hormone measurements**

Total anthocyanins were determined in the 1% MPA extracts prepared for AsA. Samples were diluted 1:1 with 1% MPA and absorbance was measured at 530 nm

and 657 nm using an Infinite M200 plate reader (Tecan, Switzerland). [A530 – A657] values were normalised for tissue weight.

For metabolite profiling, 10 mg freeze dried leaf powder was extracted in 0.8 ml 10% methanol containing1% acetic acid. After centrifugation (10 min at 16,100 x g, 4 °C), the samples were filtered through a 0.2 µm (PVDF) syringe filter (Chromacol, Welwyn Garden City, UK). For LC-MS/MS analysis of anthocyanins and hormones, 10 mg freeze dried leaf powder was extracted in 0.8 ml 10 % methanol +1% acetic acid containing deuterated standards (Forcat *et al.* 2008). Metabolite profiling was performed using a QToF 6520 mass spectrometer (Agilent Technologies, Palo Alto, USA) coupled to a 1200 series Rapid Resolution HPLC system. 5 µl of sample extract was loaded onto a Zorbax StableBond C18 1.8 µm, 2.1 x 100 mm reverse phase analytical column (Agilent Technologies, Palo Alto, USA). Mobile phase A comprised 5% acetonitrile with 0.1% formic acid in water and mobile phase B was 95% acetonitrile with 0.1% formic acid in water. The following gradient was used: 0 min – 0% B; 1 min – 0% B; 5 min – 20% B; 20 min – 100% B; 25 min – 100% B; 26 min – 0% B; 9 min post time. The flow rate was 0.25 ml $min^{-1}$ and the column temperature was held at 35 °C for the duration. The source conditions for electrospray ionisation were as follows: gas temperature was 350 °C with a drying gas flow rate of 11 l $min^{-1}$ and a nebuliser pressure of 55 psig. The capillary voltage was 3.5 kV in both positive and negative ion mode. The fragmentor voltage was 115 V and skimmer 70 V. Scanning was performed using the autoMS/MS function at 3 scans $sec^{-1}$ with a sloped collision energy of 3.5 V/100 Da with an offset of 5 V.

Hormone and anthocyanin quantitative analysis was performed using an Agilent 6420B triple quadrupole (QQQ) mass spectrometer (Agilent Technologies, Palo Alto, USA). The HPLC system was the same as that used for QToF analyses. 40 µl of sample extract were loaded onto a Zorbax Eclipse Plus $C_{18}$ 3.5 µm, 2.1 x 150 mm

reverse phase analytical column (Agilent Technologies, Palo Alto, USA). The following gradient was used: 0 min – 0% B; 1 min – 0% B; 5 min – 20% B; 20 min – 100% B; 25 min – 100% B; 27 min – 0% B; 7 min post time. QQQ source conditions were as follows: gas temperature 350°C, drying gas flow rate 9 l $min^{-1}$, nebuliser pressure 35 psig, capillary voltage ±4 kV. The fragmentor voltage and collision energies were optimised for each compound. Flavonoids were detected in positive ion mode using multiple reaction monitoring (MRM). The MRMs used for flavonoid analyses are shown in Table 1. These were based on previous MS/MS identification of flavonoids in Arabidopsis (Stobiecki *et al.* 2006; Tohge *et al.* 2005) and confirmed by accurate mass QToF analyses. In the absence of authentic standards, the flavonoids were quantified by peak area. Accurate quantification of abscisic acid (ABA) and jasmonic acid (JA) in negative ion mode used previously determined MRMs and deuterated internal standards added during sample extraction (Forcat *et al.* 2008).

**Identification of phenylpropanoids**

Phenylalanine and the phenylpropanoids were identified from their accurate masses and MS/MS spectra measured in negative ion mode. The data were extracted using MassHunter software (Agilent Technologies, Palo Alto, USA). Theoretical m/z values, isotope abundances and product ion m/z values for the identified compounds are in brackets. MS/MS spectra were compared with ESI-QToF-MS/MS spectra of known compounds from the MassBank database (Horai *et al.* 2010) (http://www.massbank.jp/en/database.html). Phenylalanine: retention time (RT) = 2.77 min; m/z 164.0716 (164.0717); isotope 1 abundance 7.83% (10.29%), isotope 2 abundance 1.41%, (0.89%); product ions m/z 147.0456 (147.0458). Sinapoyl malate: RT = 8.99 min; m/z 339.0709 (339.0722); isotope 1 abundance 16.35% (16.74%), isotope 2 abundance 0.32% (0.37%); product ions m/z 223.0598 (223.0596),

208.0366 (208.0370), 164.0468 (164.0467), 149.0235 (149.0235), 115.0030 (115.0032), 71.0468 (71.0153). 5-Hydroxyferulic acid: RT = 7.87 min; m/z 209.0441 (209.0455); isotope 1 abundance 12.17% (11.11%), isotope 2 abundance 2.64% (1.57%); product ion m/z 165.0581 (165.0557- calculated from ferulic acid + 1 oxygen atom).

**Determination of transcript levels by semi-quantitative RT-PCR and mRNA-Seq**

RNA was extracted from three biological replicates which were then pooled for PCR analysis. Frozen tissue was ground in Z6 buffer (8 M guanidine hydrochloride, 20 mM MES, 20 mM EDTA, pH 7.0), then mixed with phenol:chloroform to remove DNA and protein. RNA was precipitated, then re-suspended in nuclease-free water. cDNA synthesis was carried out using the RevertAid™ cDNA Synthesis system (Fermentas, Vilnius, Lithuania). Reactions contained 2 µg total RNA in a total volume of 25 µl, with conditions as described in the manufacturer's protocol.

PCR reactions were carried out with 1 µl cDNA template in a total volume of 20 µl, using *ACT2* (At3g18780) as a control – 22 cycles with forward primer 5'-GCAGGAGATGGAAACCTCAAAG-3' and reverse primer 5'-CTGCTGGAATGGAGATCCACAT-3'. Genes of interest were amplified as follows: *CHS* (At5g13930) – forward 5'-GCTGGTGCTTCTTCTTTGGATG-3' and reverse 5'-CTGACTTCCTCCTCATCTCGTCTAG-3' for 29 cycles; *EGL3* (At1g63650) – forward 5'-CCGACACCGAGTGGTACTACTTAG-3' and reverse 5'-CACAGATGATGATCGCTTCCACC-3' for 33 cycles; *FLS* (At5g08640) – forward 5'-CACAACATTCCGAGGTCCAAC-3' and reverse 5'-GCTTGCGGTAACTGTAATCCTTG-3' for 29 cycles; *GL3* (At5g41315) – forward 5'-GCTGCGGTTAAGACAGTGGTTTGC-3' and reverse 5'-CATCTCTGGCTTCTGGTGAGTCC-3' for 33 cycles; *LDOX* (At4g22880) – forward 5'-

GTCCTCAAGTTCCCACAATCG-3' and reverse 5'-CACATTTTGCAGTGACCCATTTGC-3' for 29 cycles; *PAP1* (At1g56650) – forward 5'-GGCACCAAGTTCCTGTAAGAGC-3' and reverse 5'-CCCTTTTCTGTTGTCGTCGC-3' for 26 cycles; *TTG1* (At5g24520) – forward 5'-GTCACATACGACTCACCATATCCAC-3' and reverse 5'-CAATCCAATCAGGCTGCGAAG-3' for 26 cycles. Band intensities were quantified using ImageJ software and normalised to *ACT2* intensities. Inspection of mRNA-Seq data confirmed that this gene is not light responsive. It should be noted that since semi-quantitative RT-PCR cycle number varied between amplifications, relative expression values between genes are not directly comparable.

For mRNA-Seq, leaf tissue was pooled from four biological replicates and RNA extracted as above for semi-quantitative RT-PCR. RNA quality was assessed using the Agilent 2100 BioAnalyser (Agilent Technologies, Palo Alto, USA), with high quality samples concentrated and re-quantified before library preparation. Library preparation was carried out according to the Illumina mRNA-Seq manual, with the following changes included: centrifugation steps involving spin-columns were performed at a slower speed of 6,000-10,000 x g; during spin-column purifications, columns were incubated with EB buffer for 15 min at room temperature; agarose was replaced with polyacrylamide for gel matrices; excised polyacrylamide gel slices were incubated in 0.3 M sodium acetate, 2 mM EDTA pH 8.0 at 37 °C overnight, then subjected to an ethanol precipitation to recover DNA. Samples were run on an Illumina GAIIx DNA sequencer (Illumina Inc., San Diego, USA) to generate 76-bp single-end reads. Raw reads were trimmed to remove the typical variable sequence over the first 12 bases, and then filtered to remove reads containing the adaptor sequence. Alignment of reads to the *A. thaliana* genome (TAIR9 release) was performed with Tophat 1.0.13 and Bowtie 0.12.5 – only reads with a mapping quality of Q>30 were accepted. Resulting files were processed with HTSeq to extract gene

level counts, which were subsequently analysed with DESeq (Anders & Huber 2010) to obtain gene level differential expression calls. Differential expression data were visualised using MapMan 3.5.1 (Thimm *et al.* 2004).

**Statistical analysis**

All experiments (except mRNA-Seq) were repeated at least twice and data from a representative experiment are shown. Metabolite data were subjected to Student's *t*-test or analysis of variance (ANOVA) using SPSS v. 16 (IBM, Chicago, USA). Where significant effects ($p<0.05$) were found by ANOVA, the treatment means were compared with the Tukey HSD test.

## RESULTS

### AsA and anthocyanin accumulation during acclimation to high light

It has previously been shown that foliar AsA pool size increases in response to HL intensity in Arabidopsis (Bartoli *et al.* 2006; Dowdle *et al.* 2007; Smirnoff 2000). We investigated how AsA and anthocyanin accumulation were affected by smaller adjustments in light intensity over a 4-day period. At the beginning of the time course, there was no difference in the foliar AsA content of any of the plants (Fig. 1a). On all subsequent days, those leaves that had received the highest light intensity (730 µmol $m^{-2}$ $s^{-1}$) had significantly more AsA than control plants (70 µmol $m^{-2}$ $s^{-1}$). The AsA content of plants grown at intermediate intensities was not statistically different from the control plants, although a general irradiance-dependent increase was observed supporting the hypothesis that AsA pool size is tightly linked to light intensity. Anthocyanins started to accumulate after transferring Arabidopsis plants from LL to HL conditions. Initially they accumulated in the lower epidermis, followed by the

epidermal cells above the vascular bundles from where accumulation spread to the epidermal layers above the mesophyll cells. Accumulation was more extensive in fully expanded leaves than in young leaves, the former being sampled in these experiments. Plants grown at the three highest light intensities visibly accumulated more anthocyanins than those plants grown at the two lowest intensities. At 540 and 730 µmol $m^{-2}$ $s^{-1}$, leaves had a larger anthocyanin pool after 24 h of HL treatment compared to control plants. Similarly to AsA pool size, foliar anthocyanin content tended to increase in line with incremental changes in light intensity.

Transcript levels of genes related to flavonoid and phenylpropanoid metabolism were determined by mRNA-Seq from leaves acclimated to LL and HL for 4 days (Fig. 2 and supplementary Table S1). This showed that, with the exception of cinnamate 4-hydroxylase (C4H), the transcript levels of all the genes involved in flavonoid biosynthesis were increased by HL acclimation. Phenylpropanoids are synthesised from coumaroyl-CoA. Transcripts of genes in the first three steps of this pathway were not light responsive although some of the transcripts encoding enzymes of the later stages of the pathway showed modest increases (Fig. 2). In contrast to flavonoids, transcripts of enzymes in the D-mannose/L-galactose AsA biosynthesis pathway, with the exception of VTC2 (GDP-L-galactose phosphorylase) did not increase after HL acclimation (supplementary Table S1).

**High light induced anthocyanin accumulation is impaired in *vtc* mutants**

Nine anthocyanins based on cyanidin and five flavonol (kaempferol) glycosides were identified. Their identities were based on previous analysis of the major flavonoids in Arabidopsis (Tohge *et al.* 2005), along with accurate mass and MS/MS spectra determined by LC-QToF-MS/MS (Table 1). The relative concentrations of these flavonoids were then determined by LC-QQQ-MS/MS using multiple reaction

366 monitoring (MRM). All 9 anthocyanins showed an identical pattern of accumulation
367 across the *vtc* mutants after transfer to HL (Fig. 3). Each compound accumulated in
368 WT and all were significantly lower in the *vtc* mutants ($p<0.001$). Anthocyanin
369 accumulation in *vtc3-1* was intermediate between WT and the other mutants, being
370 significantly different from both ($p<0.001$). In strong contrast to the anthocyanins, the
371 five kaempferol glycosides showed no significant difference between HL and LL or
372 between WT and *vtc* mutants (Fig. 4). It is therefore clear that anthocyanin
373 accumulation is far more responsive to light than the kaempferol glycosides and the
374 *vtc* mutants are collectively impaired in anthocyanin accumulation. The AsA
375 concentrations in the *vtc* mutants were significantly lower than WT and, unlike WT,
376 did not increase significantly in HL (Fig. 5). The results show a close relationship
377 between AsA and anthocyanin accumulation, both following a very similar time
378 course in WT plants after transfer to HL (Fig. 1).
379
380 Both flavonoids and the other major group of phenolic compounds in Arabidopsis, the
381 phenylpropanoids, are synthesised from phenylalanine (Fig. 2). To determine if
382 phenylpropanoid synthesis is also affected in the *vtc* mutants, several
383 representatives of this class of compounds were identified from a LC-QToF-MS/MS
384 metabolite profiling experiment and their relative concentrations determined (Fig. 6).
385 Phenylalanine concentration was the same in all strains and was significantly lower in
386 HL ($p<0.01$) only in the case of *vtc2-1*. Sinapoyl malate was significantly lower than
387 WT in *vtc1* and *vtc2-1* but not *vtc3-1*. Sinapoyl malate was also significantly
388 decreased in HL in all strains except *vtc2-1*. 5-Hydroxyferulic acid was identified in
389 WT and was significantly decreased in HL. It was below the limit of detection in the
390 *vtc* mutants. Sinapoyl malate is the major phenylpropanoid in Arabidopsis (Chapple
391 *et al.* 1992; Lorenzen *et al.* 1996; Nair *et al.* 2004), and in contrast to anthocyanins,
392 its accumulation was generally decreased by HL. The inability to detect 5-

hydroxyferulic acid in the *vtc* mutants suggests that phenylpropanoid accumulation is limited by AsA deficiency to some extent.

**AsA and anthocyanin accumulation correlate across Arabidopsis ecotypes**

Natural variation present in *Arabidopsis* was used to help establish whether a relationship existed between AsA and anthocyanin pool size. Ecotypes with an altered AsA content compared to Col-0 were identified (Fig. 7). Is-0 and Old-2 had significantly less AsA under both LL and HL, while HR5 showed a signification reduction under HL only. GOT1 and NFE1 had wild-type AsA levels under both light conditions. No differences in the redox state of AsA were observed across ecotypes. The anthocyanin profiles of these ecotypes were subsequently analysed through targeted LC-MS. Under LL conditions, six anthocyanins were detected in Col-0 (A1, A2, A3, A4, A6 and A9), albeit at very low concentrations (Table 2). The GOT1, HR5, Is-0 and Old-2 ecotypes accumulated relatively large amounts of A6, while Old-2 accumulated a relatively large amount of A9, compared to Col-0. A1, A2, A3 and A4 were less abundant in the test ecotypes compared to Col-0. In leaves exposed to HL, anthocyanins were substantially more abundant, with nine detected in each of the ecotypes. Accumulation was highest in Col-0 for all anthocyanins, while levels in the test ecotypes were markedly reduced in comparison. Old-2 had the least total AsA and the least total anthocyanins after HL treatment, highlighting a strong correlation between the two (Fig. 8).

**ABA and JA in *vtc* mutants**

Both ABA and JA have been implicated in mediating stress-induced anthocyanin accumulation. Therefore, the effect of light intensity on the concentrations of these hormones was measured in WT and *vtc* mutants (Fig. 9). ABA concentration was

unaffected in the *vtc* mutants compared to WT and was generally not significantly increased by HL. Similarly, JA concentrations were not significantly different between strains and light treatments.

### High light induction of genes involved in anthocyanin biosynthesis is impaired in *vtc* mutants

Semi-quantitative RT-PCR analysis was used to determine whether AsA content affected the expression level of genes involved in anthocyanin biosynthesis. Prior to HL treatment when anthocyanin accumulation was not directly observable, the expression of biosynthetic genes (*CHS*, *FLS* and *LDOX*) and signalling genes (*EGL3*, *GL3*, *PAP1* and *TTG1*) was low in WT and *vtc* mutant plants (Fig. 10). With the exception of *TTG1*, expression of these genes was greatly increased after HL treatment in WT leaves, consistent with a deep-purple leaf colour. Transcript levels also tended to increase in the *vtc* mutants, but remained substantially lower than WT, corresponding to a lower anthocyanin pool (Fig. 3). The expression of *TTG1* showed little change in any of the lines after HL treatment. These results are consistent across *vtc* mutants and show that AsA deficiency strongly decreases the expression of anthocyanin-related genes.

## DISCUSSION

### High light-induced AsA and anthocyanin accumulation

AsA and anthocyanins accumulated progressively in mature Arabidopsis leaves in an irradiance-dependent manner in the four days following the transfer from LL. A close correspondence between AsA and anthocyanin accumulation was found in three different *vtc* mutants. VTC1 and VTC2 are involved in AsA biosynthesis (Conklin *et*

449 *al.* 1999; Laing *et al.* 2007; Linster *et al.* 2007), while the function of VTC3 is not
450 established. The results establish that AsA deficiency, rather than any other effects
451 the mutations may have, is the cause of impaired anthocyanin accumulation. The
452 fine tuning of this relationship is illustrated by the correlation between AsA and
453 anthocyanin across six Arabidopsis ecotypes. Furthermore, it was apparent that
454 anthocyanin accumulation varies in different leaves and inspection of the raw data
455 shows that within a genotype, samples with high anthocyanin tended to have high
456 AsA (data not shown). The close correspondence between irradiance and
457 concentration suggests that there is a light sensing and signal transduction
458 mechanism that finely adjusts the levels of these metabolites during acclimation to
459 the prevailing irradiance level and, importantly, that this is influenced by AsA. UV-B,
460 blue light (cryptochrome) and red light (phytochrome) photoreceptors have been
461 proposed to act as the sensors for light-induced anthocyanin accumulation at low
462 irradiances (Chatterjee *et al.* 2006; Shin *et al.* 2007). However, it is currently not clear
463 if these photoreceptors are involved in adjusting anthocyanin synthesis during HL
464 acclimation. It is possible that chloroplast-derived signals that result from the sensing
465 of excess excitation energy, *via* changes in redox state of photosynthetic
466 components or ROS production, could be involved, as for example occurs with HL
467 induction of *APX2* (Galvez-Valdivieso *et al.* 2009).
468 The light responsiveness of AsA in Arabidopsis and other species is well-known
469 (Bartoli *et al.* 2006; Grace & Logan 1996; Muller-Moulé *et al.* 2004; Smirnoff 2000).
470 AsA concentration is finely tuned to irradiance but, as in the case of anthocyanins,
471 the mechanism is not fully understood. The redox state of the photosynthetic electron
472 transport chain has been proposed as a signal for both ascorbate and anthocyanin
473 accumulation (Das *et al.* 2011; Jeong *et al.* 2010; Yabuta *et al.* 2007). The transcript
474 levels and enzyme activity of VTC2 (GDP-L-galactose phosphorylase), the first
475 dedicated enzyme in the AsA biosynthesis pathway *via* L-galactose, are rapidly
476 increased by transfer from low to HL while the other pathway enzymes show little or

no change (Dowdle *et al.* 2007; Muller-Moulé 2008). Transcriptome analysis by mRNA-Seq showed that *VTC2* is the only gene in the mannose/L-galactose pathway with higher transcript levels after HL acclimation (supplementary Table S1). *VTC2* could therefore be a target for the irradiance-dependent transcriptional regulation of AsA biosynthesis. The pattern of accumulation supports the proposed roles of AsA and anthocyanins in photoprotection (see Introduction).

**The effect of high light and AsA deficiency on phenylpropanoid and flavonoid metabolism**

LC-MS/MS analysis enabled the identification and profiling of 5 flavonols (kaempferol glycosides) and 9 cyanidin-based anthocyanins. The anthocyanins all accumulated in a similar manner during HL acclimation in wild-type plants. Accumulation was greatly decreased in the *vtc* mutants with no differences in the profiles across the strains. In contrast to anthocyanins, the kaempferol glycosides tended to increase in HL in all the strains although the response was not significant. In strong contrast to the anthocyanins, the levels of kaempferol glycosides were identical in WT and mutants. HL acclimation therefore specifically increases anthocyanins, even though there is a strong increase in transcript levels of genes encoding enzymes in the early part of the flavonoid biosynthesis pathway and in the flavonol and anthocyanin branches (Fig. 2). The pathway for phenylpropanoid biosynthesis branches off from the flavonoid pathway at coumaroyl-CoA, leading to the synthesis of sinapoyl malate, the major sinapate ester in Arabidopsis (Chapple *et al.* 1992). HL acclimation decreased sinapoyl malate and 5-hydroxyferulic acid, a related metabolite. Although transcripts of some of the genes encoding sinapoyl malate had modest increases in HL, the initial stages of the phenylpropanoid pathway were unaffected. The results are consistent with a diversion from sinapoyl malate synthesis to anthocyanins under HL. This could arise from direct competition between the pathways for coumaroyl-CoA

505 and/or incorporation of sinapic and coumaric acids into the anthocyanins. All of the
506 nine anthocyanins analysed contain coumaroyl or sinapoyl residues. Although both
507 sinapoyl malate and flavonoids act as UV-B screens (Landry *et al.* 1995; Li *et al.*
508 1993), the preferential accumulation of anthocyanins over flavonols and sinapoyl
509 malate suggest that the former may have specific properties that are advantageous
510 for acclimation to HL across the biologically-active spectrum (Gould *et al.* 2010;
511 Gould *et al.* 2002; Havaux & Kloppstech 2001; Zhang *et al.* 2010).
512
513 AsA deficiency specifically affects anthocyanin but not flavonol accumulation. There
514 was however, also a significant decrease in sinapoyl malate in *vtc1* and *vtc2-1* while
515 5-hydroxyferulic acid, a low abundance metabolite in WT was not detectable in the
516 *vtc1* mutants. Low AsA therefore reduces phenylpropanoid accumulation, although
517 this effect is much less than on anthocyanin accumulation. Phenylalanine, the
518 precursor of all the phenolics was not significantly lower in the *vtc* mutants, so the
519 supply of this amino acid is unlikely to be limiting for flavonoid and phenylpropanoid
520 synthesis. A number of reasons for decreased anthocyanin accumulation can be
521 envisaged. Firstly, AsA could stabilise anthocyanins against oxidative degradation;
522 AsA occurs in vacuoles and could inhibit oxidative degradation by peroxidases.
523 Secondly, anthocyanin biosynthesis employs a number of 2-oxoglutarate-dependent
524 dioxygenases (Fig. 2), which as noted above require AsA to prevent over-oxidation of
525 the active centre iron. However, this class of enzyme is involved in both flavonol and
526 anthocyanin synthesis so differential AsA requirement or availability would need to be
527 proposed. The final possibility, discussed below, is that AsA deficiency disrupts the
528 signalling processes required for anthocyanin accumulation.
529
530 **AsA influences signalling processes required for HL-induced anthocyanin**
531 **accumulation**
532

Acclimation to HL increased expression of almost all the known enzymes and transcription factors involved in anthocyanin synthesis (Vanderauwera *et al.* 2005). In contrast, it is clear that transfer to HL failed to increase the transcripts of selected transcription factors and enzymes involved in flavonoid biosynthesis in *vtc1*, *vtc2-1* and *vtc2-2*, while a large increase occurred in WT plants. The decreased expression of transcription factors and their target enzymes strongly suggests that the decreased anthocyanin production by the *vtc* mutants is primarily caused by a lesion in the processes involved in the sensing or signalling of HL. In this respect, it is already known that *vtc* mutants are more resistant to biotrophic pathogens, most likely through activation of salicylic acid-mediated defence responses (Barth *et al.* 2004; Colville & Smirnoff 2008; Mukherjee *et al.* 2010; Pastori *et al.* 2003; Pavet *et al.* 2005). Therefore, AsA deficiency affects redox status so as to prime pathogen defences. Increased hydrogen peroxide (Mukherjee *et al.* 2010) or changes in the amount or redox state of glutathione (Pavet *et al.* 2005) have been proposed to mediate the effect of AsA status. Previously, it has been demonstrated that Arabidopsis plants with 7% of normal catalase activity, as a result of RNAi suppression, are strongly impaired in HL-induced anthocyanin accumulation and also show decreased expression of most of the genes encoding flavonoid biosynthesis enzymes and transcription factors (Vanderauwera *et al.* 2005). It appears that the increased hydrogen peroxide in these plants somehow suppresses the induction of anthocyanin synthesis. The reason why HL-induced accumulation of kaempferol glycosides is not affected in *vtc* mutants, even though chalcone synthase (common to both pathways) and flavonol synthase (specific to kaempferol synthesis) expression are decreased requires further investigation. The small HL-induced kaempferol glycoside accumulation must require a slow rate of synthesis that is therefore less limited by enzyme capacity. Also, if the kaempferol glycosides are more stable than the anthocyanins, an even lower synthesis rate would be required to maintain pool size. Catalase deficiency also has major effects on glutathione

561 synthesis and redox state (Queval *et al.* 2007; Smith *et al.* 1984). These results are
562 consistent with AsA and catalase deficiency affecting hydrogen peroxide or
563 glutathione-mediated redox signals that are upstream of transcription factors such as
564 PAP1. Glutathione deficient Arabidopsis, produced by antisense suppression of γ-
565 EC synthetase, also has decreased HL-induced anthocyanin accumulation (Xiang *et*
566 *al.* 2001). This result was interpreted as indicating a requirement for transport of
567 anthocyanin into vacuoles as GSH conjugates. However, it is becoming clear that
568 anthocyanin transport may not exclusively involve GSH conjugates (Poustka *et al.*
569 2007; Zhao & Dixon 2010), leaving open the possibility that the altered GSH levels
570 affect anthocyanin biosynthesis more directly. However, this simple interpretation is
571 complicated by results from APX mutants. Increased anthocyanin accumulation
572 occurs in an *apx1/tapx* double mutant in response to HL (Miller *et al.* 2007) and in
573 response to low temperature in an *apx1* mutant (Asai *et al.* 2004). Since these
574 mutants are expected to have increased hydrogen peroxide levels, the results are
575 currently not easily reconciled with the opposite effect of decreased catalase activity.
576
577 ABA and JA have both been implicated in mediating sucrose-induced anthocyanin
578 accumulation (Loreti *et al.* 2008; Shan *et al.* 2009). ABA hypersensitive
579 *ELONGATOR* mutants have increased expression of anthocyanin genes and
580 accumulate more anthocyanin (Zhou *et al.* 2009). It is therefore possible that AsA
581 deficiency affects hormone production or action. Leaf ABA and JA levels were not
582 significantly different between wild-type and *vtc* mutants and ABA was marginally
583 higher in HL. ABA was previously reported to be higher in *vtc1* (Pastori *et al.* 2003).
584 However, despite extensive ABA measurements additional to those shown here,
585 higher levels have never been found in *vtc* mutants (N. Sultana and N. Smirnoff,
586 unpublished results). The unchanged ABA and JA levels show that AsA status does
587 not affect synthesis of these hormones but does not rule out decreased sensitivity.
588

## CONCLUSIONS

The ability to accumulate anthocyanins in Arabidopsis is finely tuned by AsA status. Comparison of this response with catalase and glutathione deficient mutants, in which anthocyanin synthesis is also impaired in HL, supports the hypothesis that the early signalling events controlling the expression of the transcription factors that co-ordinately regulate the whole flavonoid biosynthesis pathway are redox sensitive. Transcriptional regulation of the flavonoid pathway is complex, with signals from light and carbohydrate status requiring integration with negative regulation *via* nitrogen status (Rubin *et al.* 2009). The targets and mechanism of the proposed redox regulation remain to be discovered. It is seemingly paradoxical that oxidative conditions should suppress accumulation of a class of molecules that are potential antioxidants or photoprotectants. Over-oxidation of a thiol/disulphide-dependent signalling component in the mutant plants could provide an explanation.

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## Supplementary material

The following supplementary material is available for this article online:

**Table S1**. The effect of high light acclimation on the transcript levels of flavonoid, phenylpropanoid and ascorbate biosynthesis genes in WT Arabidopsis determined by mRNA-Seq.

This material is available as part of the online article from http://www.blackwell-synergy.com

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**Table 1.** Flavonoids identified in *Arabidopsis thaliana* leaf tissue by liquid chromatography–electrospray ionisation–tandem mass spectrometry (ESI-LC-MS/MS) analysis. Product ions in bold font were used for quantification. Cy = cyanidin, Glc = glucose, Xyl = xylose, Cou = *p*-coumaroyl moiety, Mal = malonyl moiety, Sin = sinapoyl moiety, Km = kaempferol, Rha = rhamnose.

| Peak | $R_t$ (mins) | Precursor Ion (*m/z*) | Product Ion (*m/z*) | Compound |
|---|---|---|---|---|
| A1 | 11.19 | 889 $[M]^+$ | **287 $[Cy]^+$**<br>449 $[Cy + Glc]^+$<br>727 $[Cy + Glc + Xyl + Cou]^+$ | Cyanidin 3-O-[2''-O-(xylosyl) 6''-O-(p-coumaroyl) glucoside] 5-O-glucoside |
| A2 | 10.04 | 949 $[M]^+$ | **287 $[Cy]^+$**<br>449 $[Cy + Glc]^+$ | Cyanidin 3-O-[2''-O-(2'''-O-(sinapoyl) xylosyl) glucoside] 5-O-glucoside |
| A3 | 11.46 | 975 $[M]^+$ | **287 $[Cy]^+$**<br>535 $[Cy + Glc + Mal]^+$<br>727 $[Cy + Glc + Xyl + Cou]^+$ | Cyanidin 3-O-[2''-O-(xylosyl)-6''-O-(p-coumaroyl) glucoside] 5-O-malonylglucoside |
| A4 | 10.23 | 1051 $[M]^+$ | **287 $[Cy]^+$**<br>449 $[Cy + Glc]^+$<br>889 $[Cy + 2Glc + Xyl + Cou]^+$ | Cyanidin 3-O-[2''-O-(xylosyl)-6''-O-(p-O-(glucosyl)-p-coumaroyl) glucoside] 5-O-glucoside |
| A5 | 11.45 | 1095 $[M]^+$ | **287 $[Cy]^+$**<br>535 $[Cy + Glc + Mal]^+$<br>975 $[Cy + 2Glc + Xyl + Cou + Mal]^+$ | Cyanidin 3-O-[2''-O-(2'''-O-(sinapoyl) xylosyl) 6''-O-(p-coumaroyl) glucoside] 5-O-glucoside |
| A6 | 10.46 | 1137 $[M]^+$ | 287 $[Cy]^+$<br>**535 $[Cy + Glc + Mal]^+$**<br>889 $[Cy + 2Glc + Xyl + Cou]^+$ | Cyanidin 3-O-[2''-O-(xylosyl) 6''-O-(p-O-(glucosyl) p-coumaroyl) glucoside] 5-O-[6'''-O-(malonyl) glucoside] |
| A7 | 11.72 | 1181 $[M]^+$ | **287 $[Cy]^+$**<br>535 $[Cy + Glc + Mal]^+$<br>933 $[Cy + Glc + Xyl + Sin + Cou]^+$ | Cyanidin 3-O-[2''-O-(2'''-O-(sinapoyl) xylosyl) 6''-O-(p-O-coumaroyl) glucoside] 5-O-[6''''-O-(malonyl) glucoside] |
| A8 | 10.68 | 1257 $[M]^+$ | **287 $[Cy]^+$**<br>449 $[Cy + Glc]^+$<br>1095 $[Cy + 2Glc + Xyl + Cou + Sin]^+$ | Cyanidin 3-O-[2''-O-(2'''-O-(sinapoyl) xylosyl) 6''-O-(p-O-(glucosyl) p-coumaroyl) glucoside] 5-O-glucoside |
| A9 | 10.90 | 1343 $[M]^+$ | 287 $[Cy]^+$<br>**535 $[Cy + Glc + Mal]^+$**<br>1095 $[Cy + 2Glc + Xyl + Cou + Sin]^+$ | Cyanidin 3-O-[2''-O-(6'''-O-(sinapoyl) xylosyl) 6''-O-(p-O-(glucosyl)-p-coumaroyl) glucoside] 5-O-(6''''-O-malonyl) glucoside |
| F1 | 11.48 | 433 $[M + H]^+$ | **287 $[Km + H]^+$** | Kaempferol 3-O-rhamnoside |
| F2 | 11.28 | 565 $[M + H]^+$ | **287 $[Km + H]^+$**<br>433 $[Km + Rha + H]^+$ | Kaempferol [(pentoside)-rhamnoside] |
| F3 | 11.47 | 579 $[M + H]^+$ | **287 $[Km + H]^+$**<br>433 $[Km + Rha + H]^+$ | Kaempferol 3-O-rhamnoside 7-O-rhamnoside |
| F4 | 11.12 | 595 $[M + H]^+$ | **287 $[Km + H]^+$**<br>433 $[Km + Rha + H]^+$ | Kaempferol 3-O-glucoside 7-O-rhamnoside |
| F5 | 10.48 | 741 $[M + H]^+$ | **287 $[Km + H]^+$**<br>433 $[Km + Rha + H]^+$<br>595 $[Km + Rha + Glc + H]^+$ | Kaempferol 3-O-[6''-O-(rhamnosyl) glucoside] 7-O-rhamnoside |

**Table 2.** Foliar anthocyanin profiles of six *Arabidopsis thaliana* ecotypes acclimated to LL (100 µmol $m^{-2}$ $s^{-1}$) or HL (550-650 µmol $m^{-2}$ $s^{-1}$) for 4 days. Means ±1 SEM (n =3), nd = not detected. Anthocyanins A1-A9 (see Table 1) were quantified by LC-ESI-QQQ MS/MS.

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| | **Low Light (Peak Area)** | | | | | | **High Light (Peak Area x $10^3$)** | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Peak** | **Col-0** | **GOT1** | **HR5** | **Is-0** | **NFE1** | **Old-2** | **Col-0** | **GOT1** | **HR5** | **Is-0** | **NFE1** | **Old-2** |
| A1 | 91 ± 63 | nd | nd | nd | nd | 40 ± 21 | 32.9 ± 3.2 | 18.4 ± 3.1 | 14.2 ± 1.7 | 11.3 ± 0.5 | 3.2 ± 0.3 | 10.2 ± 6.0 |
| A2 | 18 ± 18 | nd | nd | nd | nd | nd | 7.7 ± 0.2 | 6.0 ± 0.3 | 4.9 ± 0.5 | 3.5 ± 0.3 | 4.2 ± 0.3 | 1.6 ± 0.4 |
| A3 | 291 ± 190 | 58 ± 29 | nd | 25 ± 25 | 48 ± 48 | 21 ± 21 | 362.1 ± 14.7 | 177.7 ± 30.3 | 197.2 ± 17.3 | 78.3 ± 4.2 | 28.8 ± 3.3 | 56.2 ± 16.4 |
| A4 | 179 ± 99 | 51 ± 30 | nd | nd | nd | nd | 58.3 ± 3.2 | 29.6 ± 2.0 | 16.5 ± 0.9 | 15.4 ± 1.0 | 8.7 ± 1.5 | 5.7 ± 2.7 |
| A5 | nd | nd | nd | nd | nd | nd | 58.0 ± 13.1 | 34.2 ± 4.6 | 29.3 ± 4.9 | 17.8 ± 2.1 | 34.5 ± 3.9 | 10.6 ± 5.9 |
| A6 | 18 ± 18 | 201 ± 201 | 106 ± 86 | 66 ± 66 | nd | 449 ± 390 | 182.2 ± 7.1 | 115.6 ± 7.1 | 108.7 ± 3.0 | 123.4 ± 7.3 | 40.2 ± 6.7 | 20.1 ± 6.4 |
| A7 | nd | nd | nd | nd | nd | nd | 62.8 ± 3.4 | 43.1 ± 5.2 | 42.4 ± 1.2 | 16.5 ± 2.3 | 19.2 ± 3.6 | 17.0 ± 5.9 |
| A8 | nd | nd | nd | nd | nd | nd | 69.2 ± 6.5 | 33.7 ± 2.4 | 23.9 ± 3.5 | 13.7 ± 1.7 | 35.7 ± 2.9 | 6.7 ± 1.8 |
| A9 | 27 ± 27 | nd | 40 ± 40 | 54 ± 54 | 20 ± 20 | 314 ± 229 | 190.5 ± 4.5 | 106.9 ± 7.6 | 118.0 ± 14.7 | 78.0 ± 8.8 | 148.1 ± 7.9 | 25.2 ± 5.4 |
| Total | 1312 | 821 | 333 | 434 | 206 | 1343 | 1023.7 | 565.2 | 555.1 | 357.9 | 285.6 | 153.3 |

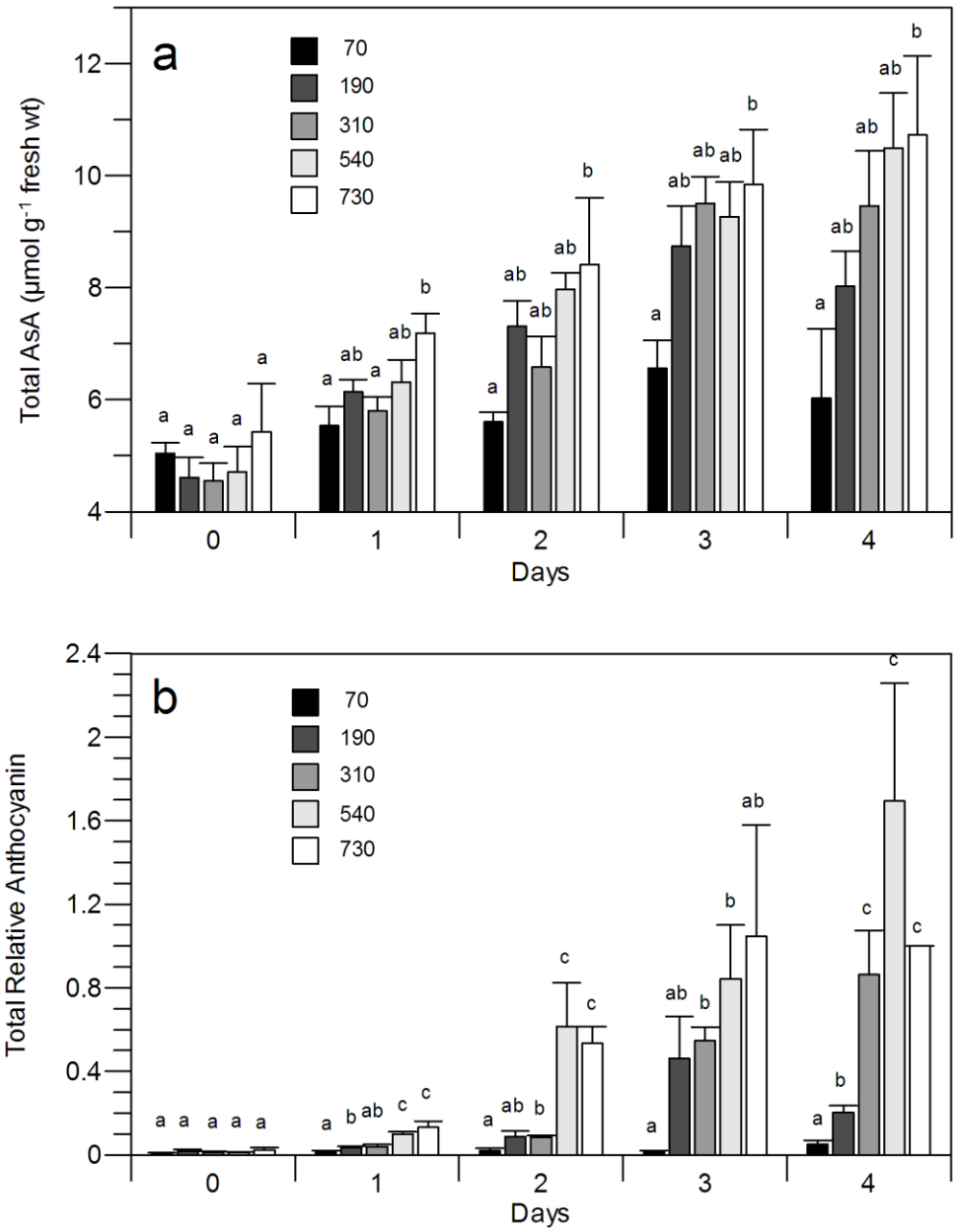


**Figure 1.** The effect of light intensity on ascorbate and anthocyanin accumulation. (a) Total foliar ascorbate accumulation in wild-type *Arabidopsis thaliana* plants during a 4-day light treatment. Plants were subjected to five different light intensities (µmol $m^{-2}$ $s^{-1}$) and sampled every 24 h (on day 0, all plants had previously been grown at 70 µmol $m^{-2}$ $s^{-1}$ and were sampled for ascorbate before transferring to the stated light intensities). Error bars represent +1 standard error of the mean (SEM, n = 4), lower-case letters represent significant differences on each day ($p<0.05$) calculated using Student's *t*-test. (b) Foliar anthocyanin content in the same plants over the same 4-day light treatment. Absorbance values were normalised for tissue weight, then made relative to the day 4, 730 µmol $m^{-2}$ $s^{-1}$ value. Error bars represent +1 SEM, n = 3, lower-case letters represent significant differences on each day ($p<0.05$) calculated using Student's *t*-test.

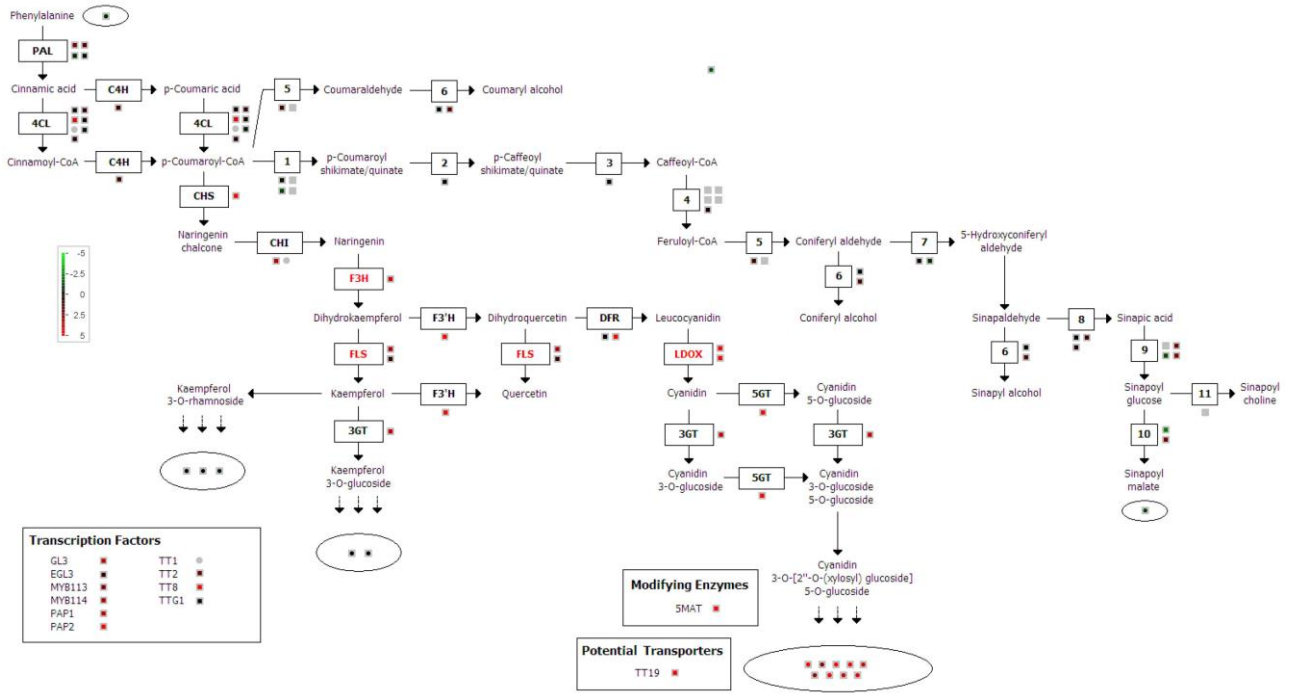


**Figure 2.** Phenylpropanoid and flavonoid biosynthesis pathway showing changes in gene expression and phenylpropanoid/flavonoid accumulation in response to HL. *Arabidopsis thaliana* plants were subjected to 4 days of LL (100 µmol $m^{-2}$ $s^{-1}$) or HL (600 µmol $m^{-2}$ $s^{-1}$). Each spot represents mRNA-Seq differential expression data for one gene. Genes for which raw counts were too low to permit reliable inferences are denoted by grey squares. Pathway products/intermediates are shown in oval boxes. Differential expression/accumulation values are on a $\log_2$ fold-change scale. Enzymes shown in red font are 2-oxoglutarate-dependent dioxygenases which may require AsA to maintain their activity. Abbreviations: PAL - phenylalanine ammonia lyase; 4CL - 4-coumarate CoA ligase; C4H - cinnamate-4-hydroxylase; CHS - chalcone synthase; CHI - chalcone flavanone isomerase; F3H - flavanone 3-hydroxylase; F3'H - flavonoid 3' hydroxylase; FLS - flavonol synthase; DFR - dihydroflavonol 4-reductase; LDOX - leucoanthocyanidin dioxygenase; 3GT - anthocyanidin 3-O-glucosyltransferase 5GT - anthocyanidin 5-O-glucosyltransferase; GL3 - glabra 3; EGL3 - enhancer of glabra 3; PAP - production of anthocyanin pigment; TT - transparent testa; TTG1 - transparent testa glabra 1; 5MAT - malonyl coA:anthocyanidin 5-O-glucoside-6''-O-malonyltransferase; 1 - hydroxycinnamoyl-CoA: shikimate/quinate hydroxycinnamoyltransferase; 2 - p-coumaroyl shikimate/quinate 3'-hydroxylase; 3 - hydroxycinnamoyl CoA:shikimate/quinate hydroxycinnamoyltransferase; 4 - caffeoyl-CoA O-methyltransferase; 5 - cinnamoyl-CoA reductase;

6 - sinapyl/coniferyl/cinnamyl alcohol dehydrogenase; 7 - coniferyl aldehyde 5-hydroxylase; 8 - sinapaldehyde dehydrogenase; 9 – sinapate 1-glucosyltransferase; 10 - sinapoylglucose:malate sinapoyltransferase; 11 - sinapoylglucose:choline-O-sinapoyltransferase.

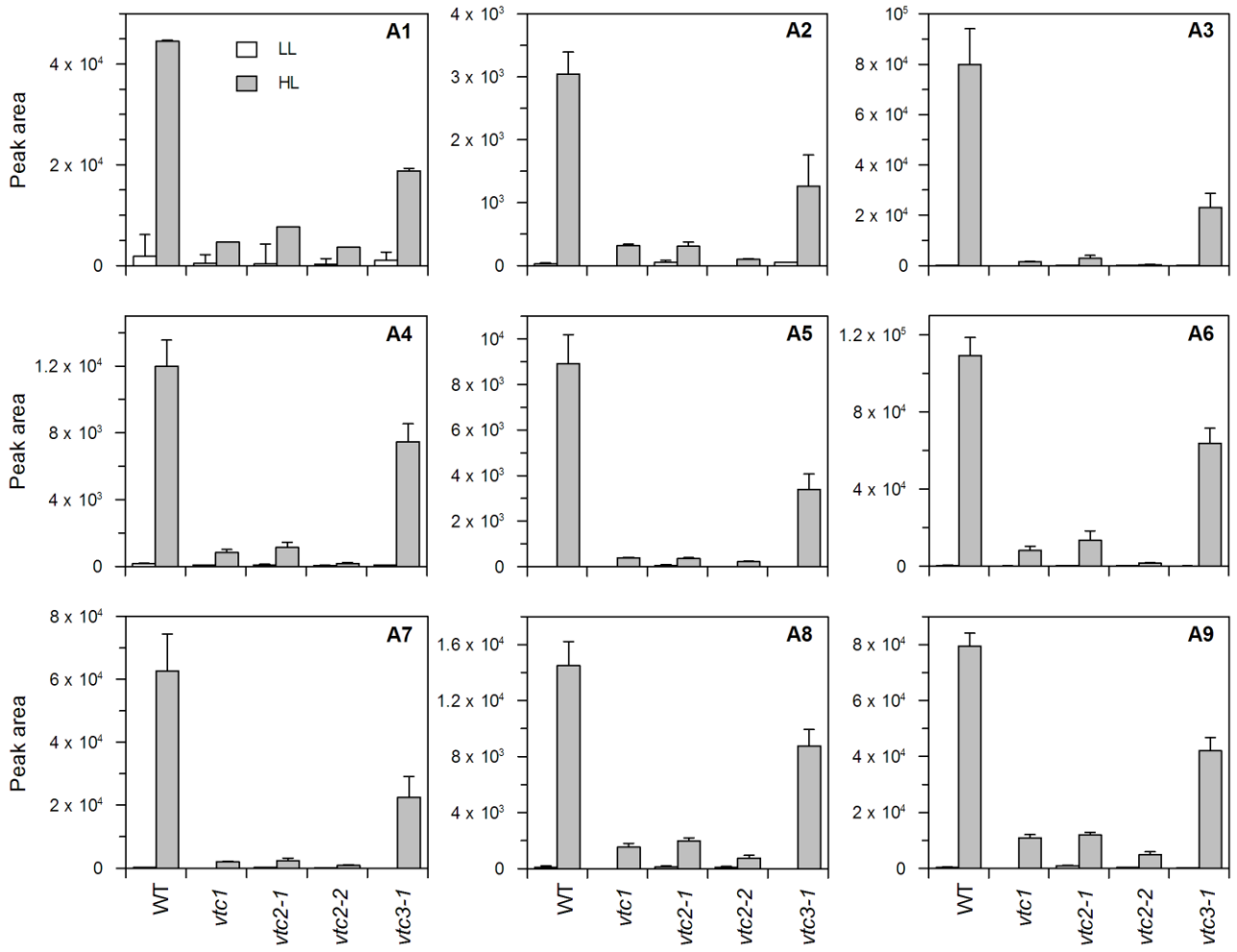


**Figure 3.** Foliar anthocyanin profiles of *Arabidopsis thaliana* WT and *vtc* mutants acclimated to LL (100 µmol $m^{-2}$ $s^{-1}$) or HL (550-650 µmol $m^{-2}$ $s^{-1}$) for 4 days. Anthocyanins A1-A9 (see Table 1) were quantified by LC-ESI-QQQ MS/MS. Mean values +1 SEM (n = 4) are shown. Columns labelled “a” are significantly different ($p<0.05$) from the LL treatment for the same strain. Columns labelled “b” are significantly different ($p<0.05$) from the corresponding wild type treatment. Significant effects are only shown for Anthocyanin 1 and all other anthocyanins followed the same pattern.

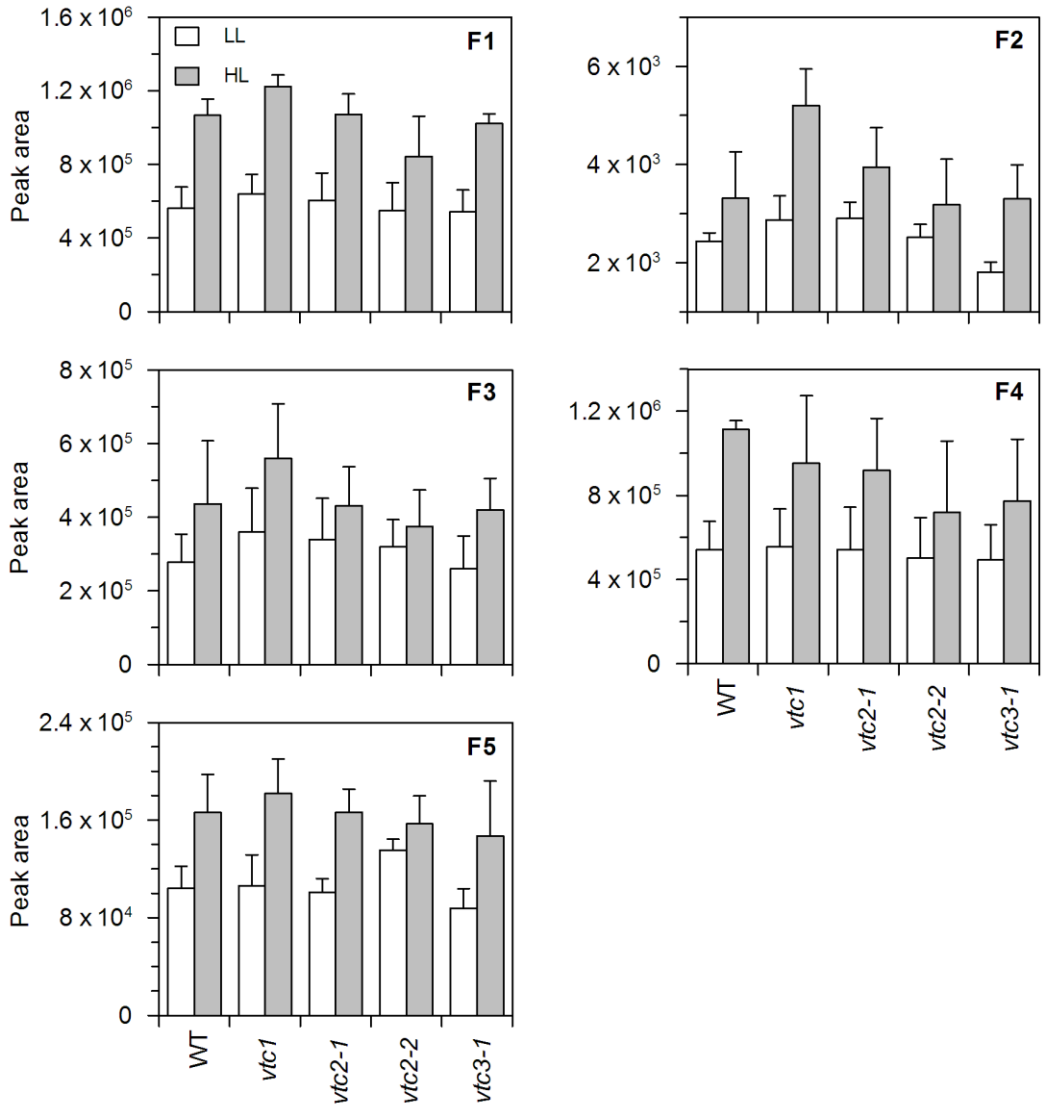


**Figure 4.** Foliar flavonol glycoside profiles of *Arabidopsis thaliana* WT and *vtc* mutants acclimated to LL (100 µmol $m^{-2}$ $s^{-1}$) or HL (550-650 µmol $m^{-2}$ $s^{-1}$) for 4 days. Flavonols F1-F5 (see Table 1) were quantified by LC-ESI-QQQ MS/MS. Mean values +1 SEM (n = 4) are shown. Light treatments had no significant effect within strains and within light treatments there were no significant differences between strains.

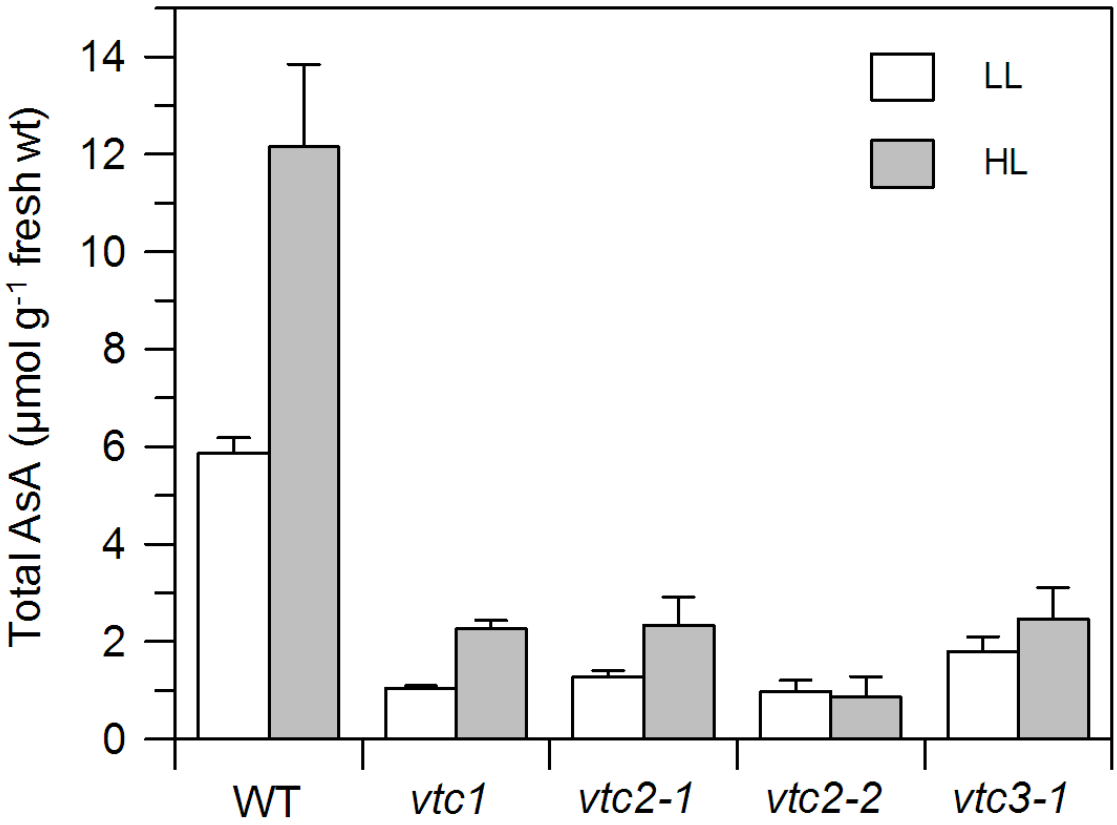


**Figure 5.** Total foliar ascorbate concentration in *Arabidopsis thaliana* WT and *vtc* mutants acclimated to LL (100 µmol $m^{-2}$ $s^{-1}$) or HL (550-650 µmol $m^{-2}$ $s^{-1}$) for 4 days. Mean values +1 SEM (n = 4) are shown. Columns labelled "a" are significantly different ($p<0.05$) from the LL treatment for the same strain. Columns labelled "b" are significantly different ($p<0.05$) from the corresponding WT treatment.

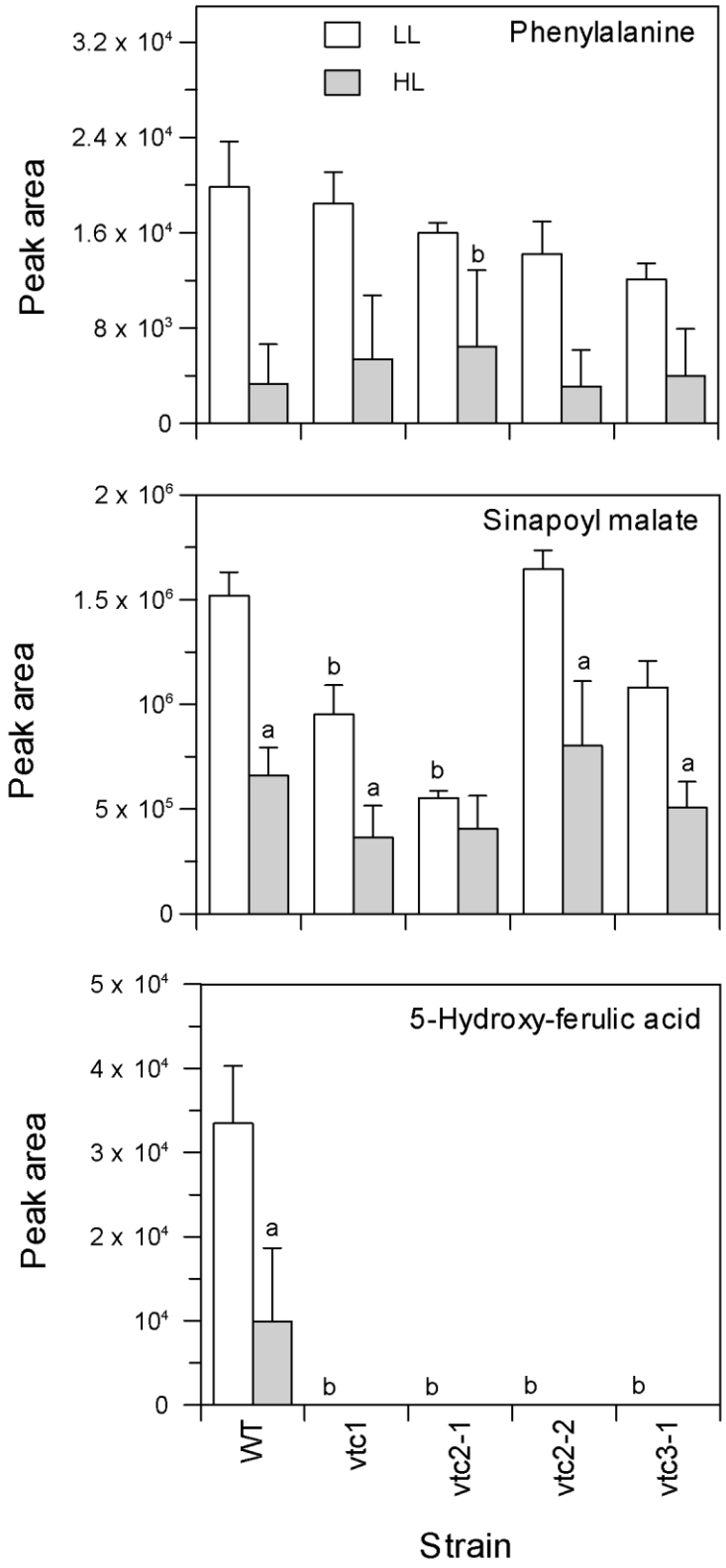


**Figure 6.** Foliar phenylalanine, sinapoyl malate and 5-hydroxyferulate profiles of *Arabidopsis thaliana* WT and *vtc* mutants acclimated to LL (100 µmol $m^{-2}$ $s^{-1}$) or HL (550-650 µmol $m^{-2}$ $s^{-1}$) for 4 days. The compounds were identified and quantified by LC-ESI-QToF MS/MS. Mean values +1 SEM (n = 3) are shown. Columns labelled “a” are significantly different ($p<0.05$) from the LL treatment for the same strain. Columns labelled “b” are significantly different ($p<0.05$) from the corresponding WT treatment.

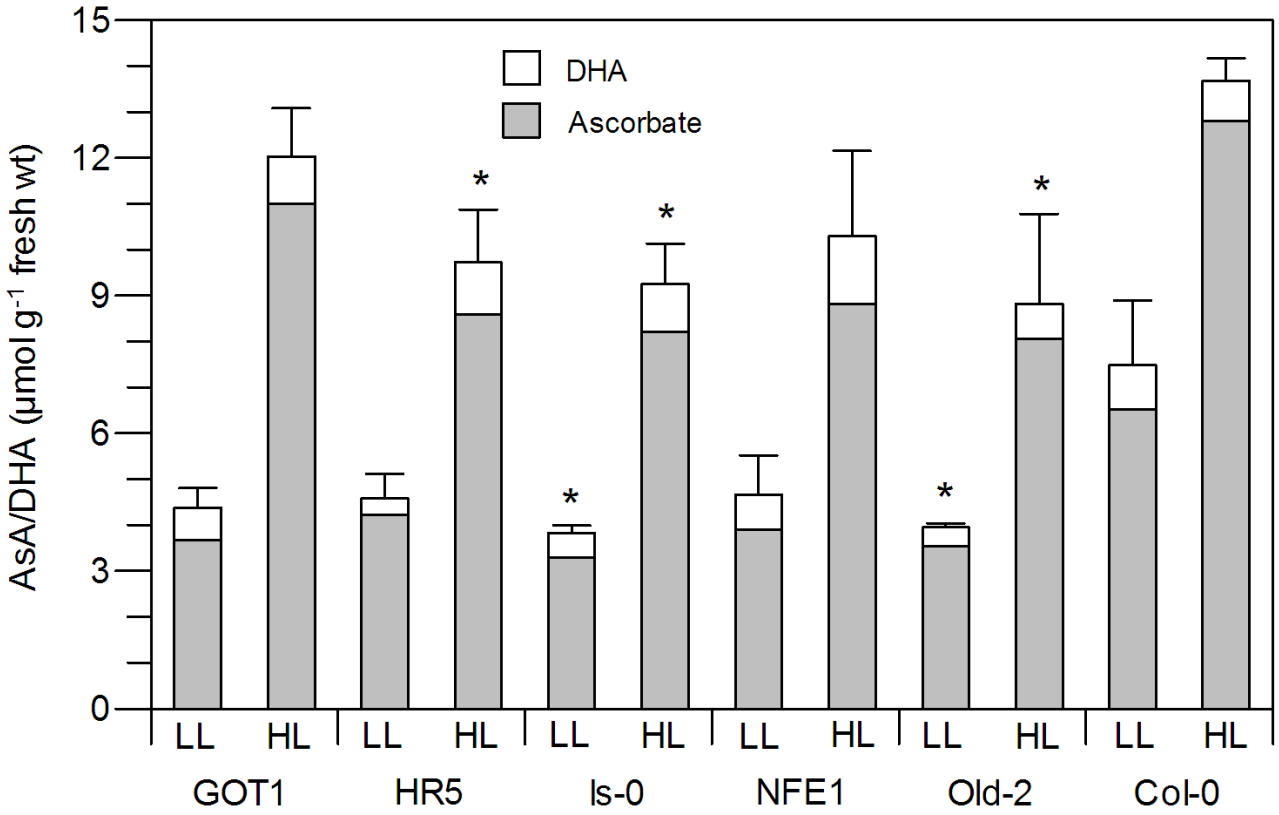


**Figure 7.** Foliar ascorbate and dehydroascorbate in Col-0 and five other *Arabidopsis thaliana* ecotypes acclimated to either to LL (100 µmol $m^{-2}$ $s^{-1}$) or HL (600 µmol $m^{-2}$ $s^{-1}$) for 4 days. Error bars represent +1 SEM of the total AsA, n = 3, * = significant difference vs. Col-0 ($p<0.05$) using Student's *t*-test (LL and HL data analysed separately).

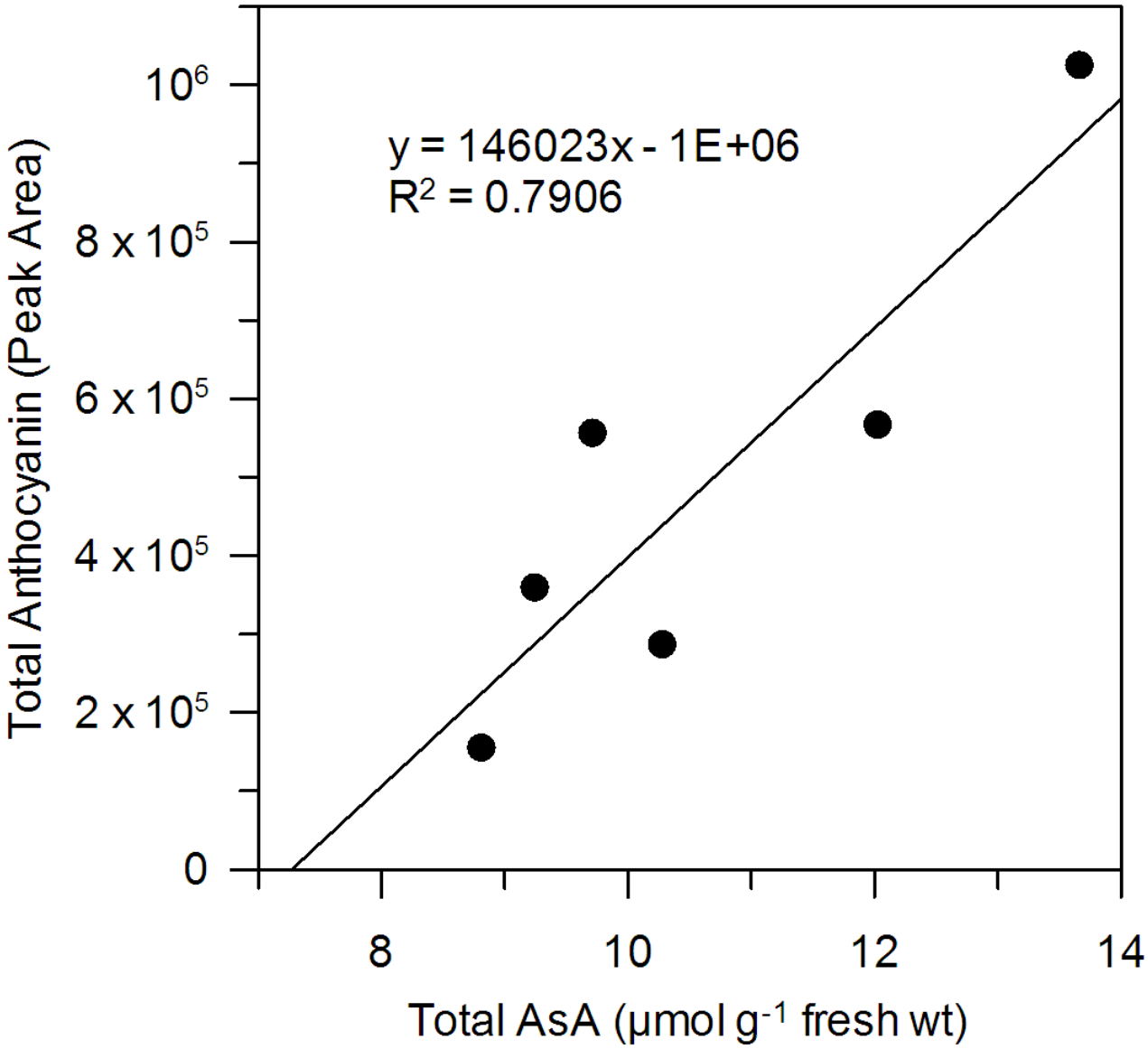


**Figure 8.** The relationship between HL-induced AsA and anthocyanin pools in leaves of six *A. thaliana* ecotypes. Mean values for each strain are taken from Figure 7 and Table 2. r = 0.8892, p<0.02.

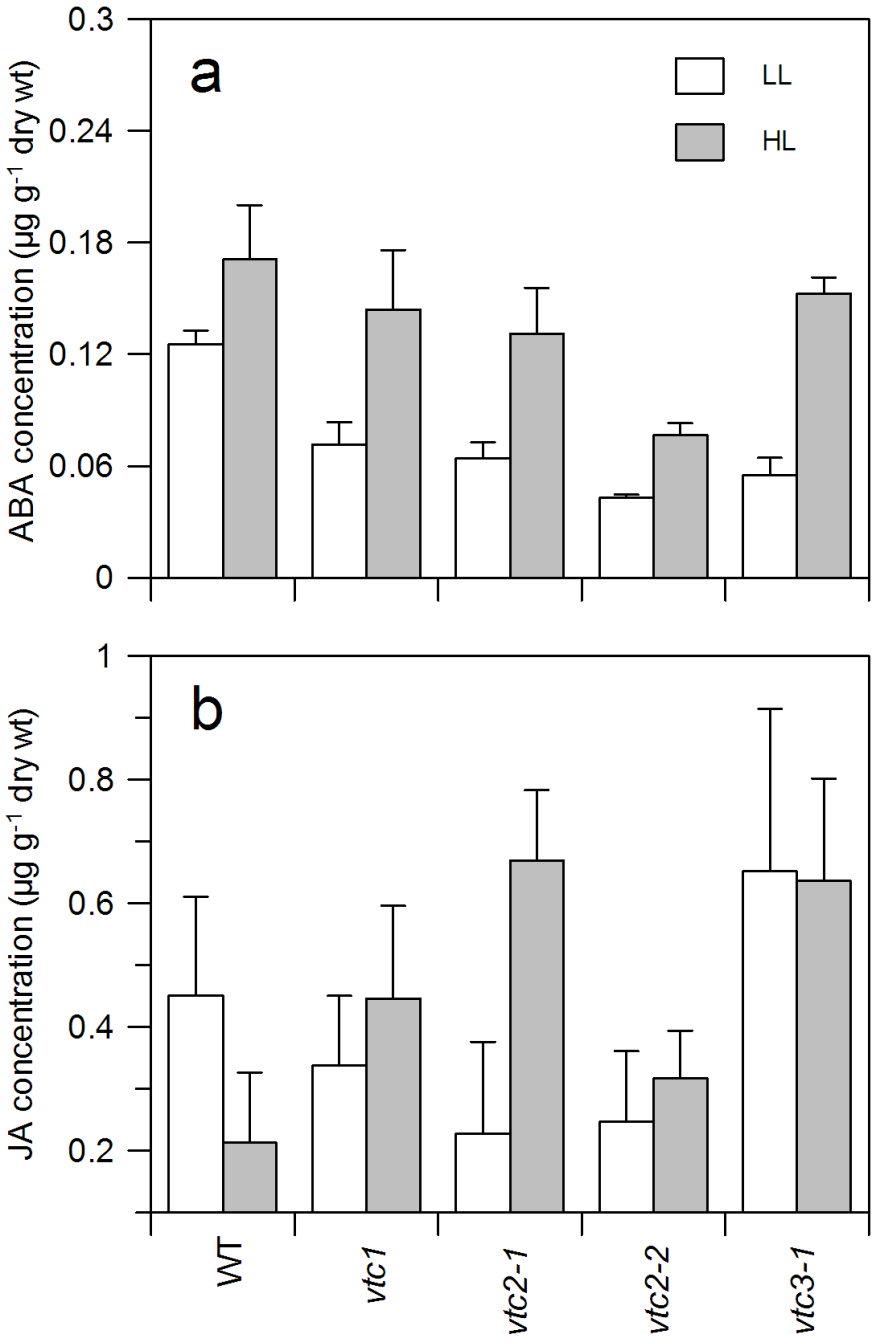


**Figure 9.** Foliar abscisic acid (a) and jasmonic acid (b) concentration in *Arabidopsis thaliana* WT and *vtc* mutants acclimated to LL (100 µmol $m^{-2}$ $s^{-1}$) or HL (550-650 µmol $m^{-2}$ $s^{-1}$) for 4 days. Mean values +1 SEM (n = 4) are shown. Light treatments had no significant effect within strains and within light treatments there were no significant differences between strains.

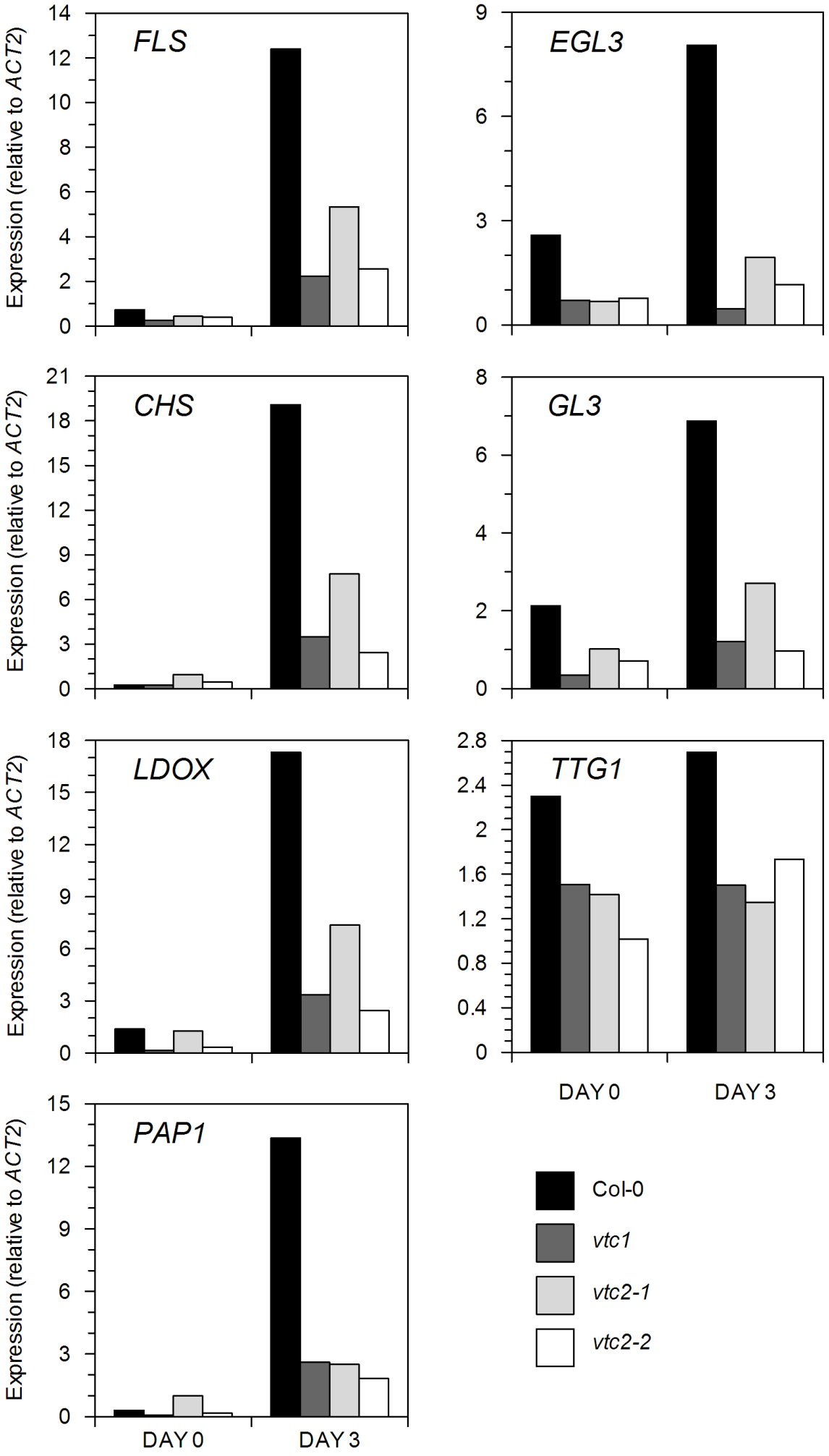
FLS
EGL3
CHS
GL3
LDOX
TTG1
PAP1
Expression (relative to ACT2)
DAY 0
DAY 3
Col-0
vtc1
vtc2-1
vtc2-2

**Figure 10.** Relative transcript levels of anthocyanin-related genes in leaves of *Arabidopsis thaliana* WT and *vtc* mutants acclimated to LL (100 μmol $m^{-2}$ $s^{-1}$) on day 0 and after 3 days acclimation to HL (550-650 μmol $m^{-2}$ $s^{-1}$). Transcript levels were determined by semi-quantitative RT-PCR and staining intensities of PCR products normalised against *ACT2*. Abbreviations: FLS - flavonol synthase; CHS - chalcone synthase; LDOX - leucoanthocyanidin dioxygenase; GL3 - glabra 3; EGL3 - enhancer of glabra 3; PAP1 - production of anthocyanin pigment 1; TTG1 - transparent testa glabra 1.

**Supplementary data**

**Table S1.** The effect of high light acclimation on the transcript levels of flavonoid, phenylpropanoid and ascorbate biosynthesis genes determined in WT Arabidopsis determined by mRNA-Seq.