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RESEARCH PAPER

A role for β,β-xanthophylls in Arabidopsis UV-B photoprotection

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Abstract

Plastidial isoprenoids, such as carotenoids and tocopherols, are important anti-oxidant metabolites synthesized in plastids from precursors generated by the methylerythritol 4-phosphate (MEP) pathway. In this study, we found that irradiation of *Arabidopsis thaliana* plants with UV-B caused a strong increase in the accumulation of the photoprotective xanthophyll zeaxanthin but also resulted in slightly higher levels of γ-tocopherol. Plants deficient in the MEP enzymes 1-deoxy-D-xylulose 5-phosphate synthase and 1-hydroxy-2-methyl-2-butenyl 4-diphosphate synthase showed a general reduction in both carotenoids and tocopherols and this was associated with increased DNA damage and decreased photosynthesis after exposure to UV-B. Genetic blockage of tocopherol biosynthesis did not affect DNA damage accumulation. In contrast, *lut2* mutants that accumulate β,β-xanthophylls showed decreased DNA damage when irradiated with UV-B. Analysis of *aba2* mutants showed that UV-B protection was not mediated by ABA (a hormone derived from β,β-xanthophylls). Plants accumulating β,β-xanthophylls also showed decreased oxidative damage and increased expression of DNA-repair enzymes, suggesting that this may be a mechanism for these plants to decrease DNA damage. In addition, *in vitro* experiments also provided evidence that β,β-xanthophylls can directly protect against DNA damage by absorbing radiation. Together, our results suggest that xanthophyll-cycle carotenoids that protect against excess illumination may also contribute to protection against UV-B.

Keywords: Isoprenoids, MEP pathway, UV-B damage, violaxanthin, xanthophylls, zeaxanthin.

Introduction

In natural environments, plants are continuously exposed to a variety of potentially damaging conditions. Sunlight reaching the earth's surface is composed of UV-B (280–315 nm), UV-A (315–400 nm), photosynthetically active (PAR, 400–700 nm), and infrared (>700 nm) radiation. In recent decades, the levels of UV-B radiation reaching the earth's surface have increased due to a decrease in the ozone layer as a result of the use of chlorofluorocarbons (McKenzie *et al.*, 2007). Despite the use of these compounds being forbidden in the Montreal Protocol the problem still continues, possibly because of the release of

greenhouse gasses (Newman *et al.*, 2001). Thus, whilst sunlight is required for photosynthesis and plant development, it also represents a major hazard. For example, too much light energy can overwhelm the photosynthetic capacity of plant cells and eventually cause photooxidative damage. In addition, excess UV-B levels can produce mutations in DNA. Given that plants are sessile, they have had to evolve different adaptation strategies to protect themselves against excessive PAR and UV-B levels (Li *et al.*, 1993; Stapleton and Walbot 1994; Landry *et al.*, 1995; Jansen *et al.*, 1998; Wargent *et al.*, 2011; Agati *et al.*, 2013; Baker *et al.*, 2017; Solovchenko and Neverov, 2017). In particular, carotenoids have been directly linked to photoprotection of the photosynthetic apparatus in plants. This ability is probably due to their function as efficient quenchers of high-energy shortwave radiation. Interestingly, xanthophylls, a category that includes all the oxygenated derivates of carotenes, absorb the shortest wavelength radiation within the light-harvesting complexes (Middleton and Teramura, 1993).

Among the several UV-B protection mechanisms that plants have developed, one of the most studied is protection by the products of secondary metabolism pathways, in particular the production of phenolic compounds in leaves (Li *et al.*, 1993; Stapleton and Walbot 1994; Landry *et al.*, 1995). Because DNA strongly absorbs UV-B, it is one of the most important sites of UV-B-induced damage (Britt, 1996). Phenolic compounds have been demonstrated to decrease such damage. For example, in maize, plants with increased flavonoid levels (primarily anthocyanins), have lower DNA damage after UV-B exposure than plants that are genetically deficient in these compounds (Stapleton and Walbot, 1994). Moreover, we have demonstrated that flavonols, a different class of flavonoids, are able to protect Arabidopsis plants from damage generated by exposure to UV-B radiation, including in the DNA (Emiliani *et al.*, 2013).

In addition to flavonoids, recent metabolomics studies have suggested that isoprenoids could also participate in UV protection in plants (Matus, 2016). Isoprenoids are a group of different biologically active compounds that participate in a wide range of biological functions, including photosynthesis, respiration, growth, defense, and adaptation to environmental conditions. Examples of isoprenoids are the photosynthetic pigments chlorophylls and carotenoids (including the xanthophylls), the hormones abscisic acid (ABA), gibberellins, cytokinins, and brassinosteroids, and tocopherols (Fig. 1A; Vranová *et al.*, 2013). Among them, carotenoids and tocopherols protect chloroplasts against high light levels, either by dissipating excess excitation energy as heat or by scavenging reactive oxygen species (ROS) and suppressing lipid peroxidation (Peñuelas and Munné-Bosch, 2005). All isoprenoids are derived by consecutive condensations of isopentenyl diphosphate (IPP) and its isomer dimethylallyl diphosphate (DMAPP). In plant plastids, both IPP and DMAPP are produced by the methylerythritol 4-phosphate (MEP) pathway (Fig. 1; Rodríguez-Concepción and Boronat, 2015). While the effect of flavonoids on filtering or absorbing UV-B is well known, the role of isoprenoid production in UV-B protection has not been clearly demonstrated. Therefore, we examined the role of different isoprenoids from the MEP pathway in UV-B protection in order to potentially expand our knowledge of plant metabolites with adaptive functions that allow survival under conditions of increased UV-B radiation. To achieve this, we used Arabidopsis transgenic and mutant lines deficient in the expression of different enzymes of the synthesis of isoprenoid derivatives from the MEP pathway. DNA damage accumulation, photosynthesis, oxidative stress, and parameters of isoprenoid accumulation patterns were analysed and compared using wild type and transgenic/mutant plants. The results presented here demonstrate that MEP-derived isoprenoids, and in particular carotenoids of the β,β-xanthophyll type, protect plants against damage induced by UV-B.

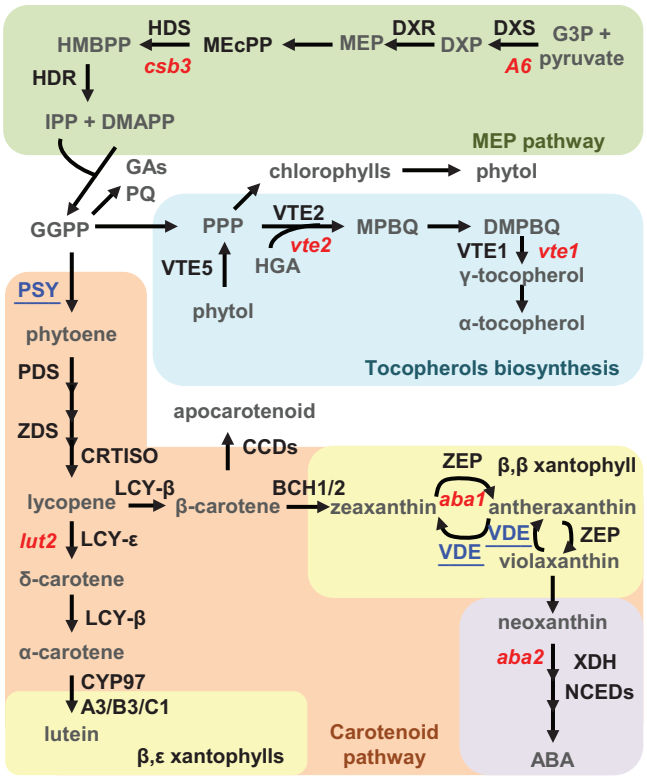

Fig. 1. Methylerythritol 4-phosphate (MEP) pathway in *Arabidopsis thaliana*. Enzymes catalysing steps regulating different branches are indicated. DXS, deoxyxylulose 5-phosphate synthase; DXR, 1-deoxy-D-xylulose 5-phosphate reductoisomerase; HDS, 1-hydroxy-2-methyl-2-butenyl 4-diphosphate synthase; HDR, 4-hydroxy-3-methylbut-2-enyl diphosphate reductase; PSY, phytoene synthase; PDS, phytoene desaturase; ZDS, zeta-carotene desaturase; CRTISO, carotenoid isomerase; LCY, lycopene cyclase; CYP97, carotene beta-ring hydroxylase/oxygen-binding; CCD, carotenoid cleavage dioxygenase; BCH, carotene beta-ring hydroxylase; ZEP, zeaxanthin epoxidase; VDE, violaxanthin de-epoxidase; NCED, 9-cis-epoxycarotenoid dioxygenase; XDH, xanthoxin dehydrogenase; VTE5, phytol kinase; VTE1, tocopherol cyclase; VTE2, homogentisate phytyltransferase. The HY5-regulated steps are underlined.

Materials and methods

Plant material, growth conditions, and UV-B treatments

Arabidopsis thaliana ecotype Columbia (Col-0) lines were used in the experiments. A list of the mutants and transgenic lines that were used is presented in Supplementary Table S1 at *JXB* online. Plants were sown directly in soil and placed at 4 °C in the dark for 3 d for stratification,

and were then transferred to a growth chamber at 22 °C with a 16/8 h light/dark regime under visible light at 100 μE m^{-2} s^{-1}. After 4 weeks, a group of plants were exposed to UV-B radiation for 4 h using UV-B lamps (Bio-Rad, Hercules, CA) mounted 30 cm above the plants giving a UV-B intensity of 2 W m^{-2} and a UV-A intensity of 0.7 W m^{-2}. The bulbs were covered with cellulose acetate filters (100-μm extra-clear cellulose acetate plastic; Tap Plastics, Mountain View, CA) to exclude wavelengths lower than 290 nm. The lamps had emission spectra from 290–310 nm, and a peak at 302 nm. A UV spectrum from a similar set-up has been shown previously in Casati and Walbot (2003). A different group of control plants were treated with the same bulbs covered with a polyester film (100-μm clear polyester plastic; Tap Plastics) that absorbed most UV-B radiation from the lamps (UV-B intensity 0.04 W m^{-2}, UV-A intensity 0.4 W m^{-2}). None of the plants used showed any visible phenotype after the UV-B treatments. Leaf samples were collected immediately after the treatments and stored at −80 °C. Experiments were repeated at least three times.

Extraction and quantification of soluble phenolics

For measurement of UV-absorbing phenolic pigments, 100 mg of frozen leaf samples in liquid nitrogen were ground to a powder with a mortar and pestle. The powder was extracted over 8 h with 0.6 ml of acidic methanol (1% v/v HCl in methanol), followed by a second extraction with 0.6 ml of chloroform and 0.3 ml of distilled water, as described by Emiliani *et al.* (2013). The extracts were vortexed, then centrifuged for 2 min at 3000 *g* and the upper aqueous phases were collected. The final pH was nearly 1; however, flavonoids and other phenolic compounds that absorb UV radiation are stable at this pH (Friedman and Jürgens, 2000). The absorbance of the aqueous phase solution was measured at 312 nm.

Measurement of maximum efficiency of photosystem II

Chlorophyll (Chl) fluorescence parameters were measured on dark-adapted leaves using a Qubit Systems pulse-modulated fluorometer (Qubit Systems Inc, Ontario, Canada). The minimum Chl fluorescence for open photosystem II (PSII) centers (F_o) was determined after 20 min in the dark using light (655 nm) at an intensity of 0.1 μmol m^{-2} s^{-1}. A saturation pulse of white light (2500 μmol m^{-2} s^{-1} for 0.8 s) was applied to determine the maximum Chl fluorescence for closed PSII centers in the dark (F_m). The parameters of light-adapted leaves (F_m' and F_t) were measured after 20 min of illumination at 100 μmol m^{-2} s^{-1}. Maximum efficiency (F_v/F_m) and quantum yield (φ_{PSII}) of PSII were calculated as $F_m - F_o/F_m$ and $F_m' - F_t/F_m'$, respectively (Baker and Rosenqvist, 2004; Ifuku *et al.*, 2005). Measurements were repeated three times for at least three different plants.

Analysis of DNA damage

For *in vitro* DNA damage analysis, samples of genomic DNA from Arabidopsis leaves (2 μg) were incubated with either 14 μg ml^{-1} violaxanthin purified from mango (*Mangifera indica*; see below for method), 100 μg ml^{-1} rutin, or without any additions for 30 min at 4 °C in a final volume of 100 μl before UV-B irradiation. Rutin (quercetin-3-β-D-rutinoside, Sigma-Aldrich) was used as a positive control as it protects against UV-B-induced DNA damage (Kootstra, 1994). Samples were irradiated at a UV-B intensity of 0.5 W m^{-2} and a UV-A intensity of 0.45 W m^{-2} in 96-well plates for 5, 10, or 15 min. Control samples were exposed for 15 min under the same UV-B lamps but covered with polyester filters to exclude UV-B radiation. After exposure, samples were transferred to tubes, the genomic DNA was denatured in 0.3 N NaOH for 10 min and dot-blotted on a nylon membrane. Levels of cyclobutane pyrimidine dimers (CPDs; a common product of UV-B-induced DNA damage) were determined using monoclonal specific antibodies from Cosmo Bio Co., Ltd. (TDM-2; Japan) as described by Emiliani *et al.* (2013). The experiments were repeated at least three times.

Measurement and quantification of isoprenoids

The isoprenoid pigment content of Arabidopsis leaf samples was determined using HPLC-DAD-FLD (diode array/fluorescence detection; Fraser *et al.*, 2000). All samples were protected from light and heat during all steps in the extraction procedure. A 4-mg sample of lyophilized leaf tissue was extracted with methanol (400 μl) with the addition of 1.2 mg of canthaxanthin as an internal standard. The suspension was mixed by agitation for 10 min at 4 °C and 400 μl of Tris-HCl (1 M, pH 7.5) was added, with further agitation at 4 °C for 10 min. After that, chloroform (800 μl) was added and mixed for 5 min at 4 °C. After centrifugation at maximum speed in a chilled bench-top microcentrifuge (Eppendorf 5418R, Hamburg, Germany) for 5 min at 4 °C, two different phases were produced. The upper phase was removed using a Pasteur pipette, and the lower phase, corresponding to the organic layer, was dried in a vacuum centrifuge (Eppendorf Concentrator Plus). Dried residues were resuspended in 200 μl ethyl acetate and filtered through a 0.2-μm polytetrafluoroethylene microfilter (Supelco). A 10-μl aliquot of each sample was injected onto an Agilent Technologies 1200 series HPLC system equipped with a diode array (Santa Clara, CA). A C30 reverse-phase column (YMC Carotenoid, 250 × 4.6 mm × 3 μm) was used, with three mobile phases consisting of methanol (A), water/methanol (20/80 v/v) containing 0.2% ammonium acetate (w/v) (B), and tert-methyl butyl ether (C). Metabolites were separated with the following gradient: 95% A, 5% B isocratically for 12 min, a step-up to 80% A, 5% B, 15% C at 12 min, followed by a linear gradient up to 30% A, 5% B, 65% C by 30 min. The flow rate was maintained at 1 ml min^{-1}. Isoprenoid pigments were monitored at 472 nm and 650 nm and compared to the retention times of chlorophyll and β-carotene authentic standards. Quantification of tocopherols was accomplished through fluorescence detection (excitation 290 nm, emission 330 nm) and comparison to a standard curve constructed with α-tocopherol. Peak areas of the standards were determined at the maximum absorbance wavelengths using the Waters Millennium32 software supplied.

Analysis of gene expression by RT-qPCR

Total RNA was isolated using 100 mg of leaf tissue using the TRIzol reagent (Invitrogen, Carlsbad, CA) and was then treated with DNase (Promega, Madison, WI). RNA was converted into first-strand cDNA using oligo-dT as a primer with SuperScript II reverse transcriptase (Invitrogen). The cDNA was used as a template for quantitative PCR amplification in a MiniOPTICON2 apparatus (Bio-Rad), using SYBR Green I (Invitrogen) as a fluorescent reporter and Platinum Taq Polymerase (Invitrogen). Primers were designed to generate unique 150–250-bp fragments with the PRIMER3 software (Rozen and Skaletsky, 2000). A list of primers used is given in Supplementary Table S2. Three biological replicates were performed for each sample. Gene expression was normalized to that of *CPK3*. Amplification conditions were as follows: 2 min denaturation at 94 °C; 40 to 45 cycles at 94 °C for 10 s, 57 °C for 15 s, and 72 °C for 20 s, followed by 5 min at 72 °C. Melting curves for each PCR product were determined by measuring the decrease of fluorescence with increasing temperature (65–95 °C).

Electrolyte leakage and content of thiobarbituric acid-reactive substances

Electrolyte leakage and content of thiobarbituric acid-reactive substances were determined as described by Emiliani *et al.* (2013).

Extraction and purification of violaxanthin

To obtain violaxanthin, a purification protocol adapted from Araki *et al.* (2016) was used with some modifications. A sample of 200 g of mango obtained from a fruit market was cut into small pieces, suspended in 300 ml of saturated aqueous $NaHCO_3$ and homogenized in a blender for 1 min. Then, 500 ml of acetone was added, the mixture was stirred for 5 min, and filtered through miracloth. The solid remaining on the filter was collected and mixed in 300 ml of CH_2Cl_2/acetone (2:1, v/v) for 15 min at room temperature to extract carotenoids. The mix was filtered again, but this time the filtrate

(600 ml) was collected and concentrated into a small volume to remove the CH_2Cl_2 and the acetone. Then, 200 ml of EtOAc and 200 ml of H_2O was added, without pH adjustment. The EtOAc layer was concentrated to dryness, then the carotenoid extract was saponified by the addition of 10 ml of a KOH solution (5 g of KOH/100 ml of 90% EtOH, v/v) and 5 ml of CH_2Cl_2. After this, 200 ml of EtOAc and 200 ml of H_2O were added and mixed. The EtOAc layer was collected and concentrated to dryness, obtaining a red oil. This oil was subjected to chromatography on a 20 × 20-cm aluminum plate of TLC silica gel 60 F254 (Merck) using hexane/acetone (3:1, v/v) as a mobile phase. Violoxanthin was separated as a yellow band with Rf=0.35, and it was collected by carefully scraping the plate with a spatula. The compound was extracted from the silica with methanol. The solvent was filtered, and evaporated, and the purified violaxanthin was concentrated to dryness.

Statistical analysis

Data were analysed using one- and two-way ANOVA, or by Student's *t* test. Minimum significant differences were calculated by the Bonferroni, Dunett, Turkey, or Duncan tests ($P<0.05$) using Sigma Stat 3.1 and Graphpad Prism 5.03 Software.

Factor Analysis was performed on data sets including mean values of isoprenoids and CPD levels relative to those in Col-0 control plants. Multivariate statistical analysis was carried out using the software package XLSTAT (Microsoft Excel).

Accession numbers

Sequence data from this study can be found at the Arabidopsis Genome Initiative under the following accession numbers: *AAA-TYPE ATPASE FAMILY PROTEIN-RELATED, AtAAA-ATPase*, At3g28580; *BON ASSOCIATION PROTEIN 1*, *BAP*, At3g61190; *FLOTILLIN-LIKE PROTEIN 3*, *FLOT*, At5g64870; *CHALCONE SYNTHASE*, *CHS*, At5g13930; *FLAVANONE 3-HYDROXYLASE*, *F3H*, At3g51240; *LONG HYPOCOTYL 5*, *HY5*, At5g11260; *UV-B RESISTANCE 8*, *UVR8*, At5g63860; *BETA-CAROTENE 3-HYDROXYLASE 1 BCH1*, At4g25700; *BETA-CAROTENE 3-HYDROXYLASE 2*, *BCH2*, At5g52570; *ZEAXANTHIN EPOXIDASE*, *ZEP*, At5g67030; *VIOLAXANTHIN DE-EPOXIDASE*, *VDE*, At1g08550; *ZETA-CAROTENE DESATURASE*, *ZEP*, At3g04870; *PHYTOENE SYNTHASE*, *PSY*, At5g17230; *PHOTOLYASE 1*, *UVR2*, At1g12370; *UV REPAIR DEFICIENT 7*, *UVR7*, At3g05210; *CALCIUM-DEPENDENT PROTEIN KINASE 3*, *CPK3*, At4g23650.

Results

The isoprenoid profile changes in Arabidopsis leaves exposed to UV-B

To investigate the possible involvement of plastidial isoprenoids derived from the MEP pathway in UV-B protection in Arabidopsis, we first analysed profile changes after exposure of wild-type (WT) plants of the Col-0 ecotype to UV-B for 4 h. Control treatments were performed using a polyester screen that absorbed most UV-B radiation. While some isoprenoids remained unchanged after the UV-B treatment, small but significant decreases in lutein, β-carotene, and chlorophylls were detected by HPLC analysis (Table 1; Supplementary Table S3). In addition, significant increases in both zeaxanthin (13-fold increase compared to control samples) and γ-tocopherol (1.88-fold increase; Table 1) were detected, suggesting that these particular plastidial isoprenoids could participate in UV-B protection in Arabidopsis. Interestingly, analysis of previously published transcriptomic data (GSE80111; Das *et al*, 2016) showed that some carotenoid biosynthetic genes were up-regulated in 4-week-old Arabidopsis plants 4 h after exposure to UV-B (1.31 kJ m^{-2}; Supplementary Fig. S1). In particular, the *BCH1* and *BCH2* genes, encoding enzymes involved in the production of zeaxanthin from β-carotene, were induced about 11-fold more in UV-B-irradiated plants. The gene encoding zeta-carotene epoxidase (*ZEP*), which transforms zeaxanthin into violaxanthin, was induced by about 2-fold more. The *VTE1* gene, involved in tocopherol biosynthesis, was similarly up-regulated (Supplementary Fig. S1). To determine whether some of these transcripts were also regulated by UV-B under our experimental conditions, we conducted qRT-PCR analysis using RNA samples extracted from plants irradiated under the same conditions as those used in the HPLC analysis. Both *BCH1* and *BCH2* were significantly induced by UV-B in our assays (Fig. 2), as were *PSY* and *ZDS*, which encode enzymes in the synthesis of carotenoids, xanthophylls, and carotenes. However, neither *ZEP* nor *VDE*, which encodes the enzyme that converts violaxanthin into zeaxanthin, showed changes in their expression under our experimental conditions (Fig. 2). Taken together, these results suggested that the observed changes in the isoprenoid profile of plants exposed to UV-B (i.e. higher zeaxanthin and γ-tocopherol accumulation but virtually unchanged levels of the other carotenoids, α-tocopherols, and chlorophylls) might have mainly been caused by changes in the levels (and hence activities) of the enzymes that produce these particular metabolites or transform them into downstream products.

Table 1. *Isoprenoid products from the MEP pathway, relative to levels Col-0 control plants.*

	Control					UV-B				
	Col-0	***csb3***	***lut2***	***aba2***	***aba1***	**Col-0**	***csb3***	***lut2***	***aba2***	***aba1***
Violaxanthin	1.00 ± 0.17	*0.54 ± 0.10**	**1.80 ± 0.27***	1.01 ± 0.49	N.D.	0.97 ± 0.12	*0.60 ± 0.06**	**1.77 ± 0.34***	0.84 ± 0.22	N.D.
Neoxanthin	1.00 ± 0.08	0.90 ± 0.05	*0.69 ± 0.07**	1.07 ± 0.02	N.D.	1.04 ± 0.07	1.06 ± 0.07	*0.70 ± 0.08**	1.07 ± 0.06	N.D.
Lutein	1.00 ± 0.08	*0.83 ± 0.03**	N.D.	1.10 ± 0.11	**1.24 ± 0.11***	*0.92 ± 0.08**	0.91 ± 0.05	N.D.	1.09 ± 0.08	**1.12 ± 0.06***
Zeaxanthin	1.00 ± 0.50	1.01 ± 0.06	**11.75 ± 4.16***	1.15 ± 0.11	**438.68 ± 22.33***	**13.05 ± 2.82***	**24.83 ± 1.88***	**15.00 ± 5.77***	**15.77 ± 3.25***	**423.94 ± 25.44***
β-carotene	1.00 ± 0.08	*0.70 ± 0.05**	1.08 ± 0.12	1.12 ± 0.09	**1.52 ± 0.35***	*0.90 ± 0.10**	0.90 ± 0.06	1.09 ± 0.10	1.08 ± 0.09	**1.31 ± 0.11***
Chlorophyll *b*	1.00 ± 0.08	*0.93 ± 0.03**	*0.78 ± 0.08**	1.02 ± 0.02	1.19 ± 0.24	*0.94 ± 0.07**	*0.87 ± 0.04**	0.80 ± 0.12	0.99 ± 0.05	1.04 ± 0.15
Chlorophyll *a*	1.00 ± 0.09	*0.85 ± 0.04**	*0.78 ± 0.10**	1.08 ± 0.03	1.20 ± 0.23	*0.93 ± 0.08**	*0.83 ± 0.05**	0.83 ± 0.12	0.95 ± 0.07	1.04 ± 0.13
γ-tocopherol	1.00 ± 0.37	*0.71 ± 0.07**	0.96 ± 0.37	**1.27 ± 0.19***	**3.18 ± 0.45***	**1.88 ± 0.42***	**1.39 ± 0.12***	1.16 ± 0.43	**1.52 ± 0.38***	**2.42 ± 3.38***
α-tocopherol	1.00 ± 0.15	*0.66 ± 0.03**	1.06 ± 0.12	0.91 ± 0.03	**21.24 ± 2.74***	1.03 ± 0.10	0.95 ± 0.06	0.99 ± 0.11	0.94 ± 0.04	**9.71 ± 1.42***
Phytoene	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
Antheraxanthin	1.00 ± 0.04	0.98 ± 0.05	**7.28 ± 0.67***	1.07 ± 0.02	N.D.	1.06 ± 0.03	1.08 ± 0.09	**7.72 ± 0.34***	1.03 ± 0.05	0.95 ± 0.03

* Significantly different to Col-0 control plants ($P<0.05$). **Bold** indicates higher levels than Col-0 control; *italics* indicate lower levels than Col-0 control; N.D., not detected.

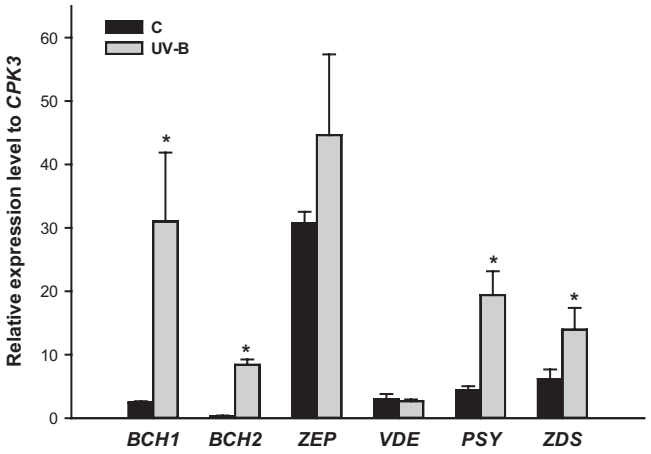

Fig. 2. Relative expression of methylerythritol 4-phosphate (MEP) pathway enzymes in Arabidopsis plants under control conditions (C) and after exposure to UV-B for 4 h. Data are means (±SE) of three biological replicates. Significant differences from the control were determined using Student's *t*-test: *P<0.05.

Arabidopsis leaves with decreased plastidial isoprenoid levels have increased DNA damage after exposure to UV-B

To investigate some of the *in vivo* roles of carotenoids and tocopherols in UV-B protection, we used transgenic Arabidopsis plants with decreased activities of MEP pathway enzymes. We first used plants that expressed an antisense construct against the *DXS* transcript (*A6*; Estévez *et al.*, 2001; Supplementary Table S1), which encodes the MEP pathway entry enzyme 1-deoxy-D-xylulose 5-phosphate synthase. These plants that were generated in the RLD background have decreased levels of *DXS* transcripts and DXS protein, and they have previously been demonstrated to have a general decrease in plastidial isoprenoids (Estévez *et al.*, 2001; Supplementary Table S4). Plants of the WT (RLD) and transgenic *A6* plants were grown in a greenhouse under very low UV-B (0.04 W m^{-2}, control conditions) for 4 weeks, and were then exposed to UV-B radiation at 2 W m^{-2} for 4 h. As a control, different sets of plants were irradiated with the same lamps covered with a polyester plastic that absorbs UV-B. Leaf samples were collected immediately after the end of the treatment. DNA was extracted and an immunological assay was used to compare the abundance of CPDs, which are indicators of UV-B-induced damage to DNA. Under control conditions, the steady-state levels of CPDs in WT and DXS-deficient plants were similar (Fig. 3A). After the UV-B treatment, CPDs were accumulated in all plants; however, the accumulation in *A6* plants was greater than that in the WT, suggesting that products of the MEP pathway protect plants against DNA damage by UV-B.

Decreased flux through the MEP pathway in DXS-deficient plants is expected to result in lower levels of the pathway intermediate methylerythritol cyclodiphosphate (MEcPP), a metabolite that has been demonstrated to mediate responses to stress (Xiao *et al.*, 2012). To test whether the decreased protection against UV-B irradiation in *A6* plants was a consequence of reduced levels of MEcPP or resulted from a decrease in production of downstream isoprenoids, we used the *constitutive subtilisin3* (*csb3*) mutant (Flores-Pérez *et al.*, 2008; Supplementary Table S1). Unlike DXS-defective lines, *csb3* plants show lower levels of MEP-derived plastidial isoprenoids but higher levels of MEcPP (Flores-Pérez *et al.*, 2008; González-Cabanelas *et al.*, 2015) due to reduced activity of the enzyme 1-hydroxy-2-methyl-2-butenyl 4-diphosphate synthase (HDS), which transforms MEcPP into downstream intermediates of the MEP pathway (Fig. 1). As shown in Fig. 3A, *csb3* plants showed an increase in DNA damage after UV-B irradiation compared to the WT. The similar pattern of results for the DXS- and HDS-defective lines in terms of UV-B protection argues against a role of MEcPP in this response and supports the conclusion that MEP-derived isoprenoids are required for the protection.

Decreased levels of carotenoids and tocopherols are associated with increased damage to photosystem II in plants irradiated with UV-B

To further investigate the effect of decreased levels of products of the MEP pathway on photoprotection to the photosynthetic machinery after UV-B exposure, the maximum efficiency of photosystem II (PSII, F_v/F_m) was assessed immediately after a 4- h UV-B treatment using the same plant genotypes as described above. Under control conditions, the maximum efficiency was similar in all plants analysed (Fig. 3B). After the UV-B treatment, WT plants in both genetic backgrounds, and the *A6* and *csb3* mutants showed a decrease in the maximum efficiency of PSII; however, this decrease was significantly more pronounced in the mutants than in the WT plants. Interestingly, WT RLD and *A6* plants showed similar levels of UV-B-absorbing phenolic pigments under control conditions and after the UV-B treatment (Fig. 3C), suggesting that compounds synthesized through the MEP pathway were required for UV-B protection in addition to phenolic compounds. Moreover, in the Col-0 background, the *csb3* mutants accumulated higher UV-B-absorbing phenolic compounds levels than WT plants, again demonstrating a role of products of the MEP pathway in UV-B protection.

Tocopherols are not required for protection against exposure to UV-B

To discriminate between the possible contributions of carotenoids and tocopherols, we next carried out a genetic approach based on examining the protection against UV-B of mutants lacking one or other of these two groups of metabolites. Carotenoid-deficient mutants show an albino seedling phenotype and are unable to survive. By contrast, mutants devoid of tocopherols are green and viable. In particular, Arabidopsis *vte1* and *vte2* mutants are deficient in tocopherol cyclase and homogentisate phytyltransferase activities, respectively (Fig. 1), and hence lack tocopherols (Sattler *et al.*, 2003; Maeda *et al.*, 2008). Both mutants showed CPD levels similar to WT plants after UV-B exposure (Fig. 4), supporting the conclusion that tocopherols do not have a major role in UV-B protection in Arabidopsis.

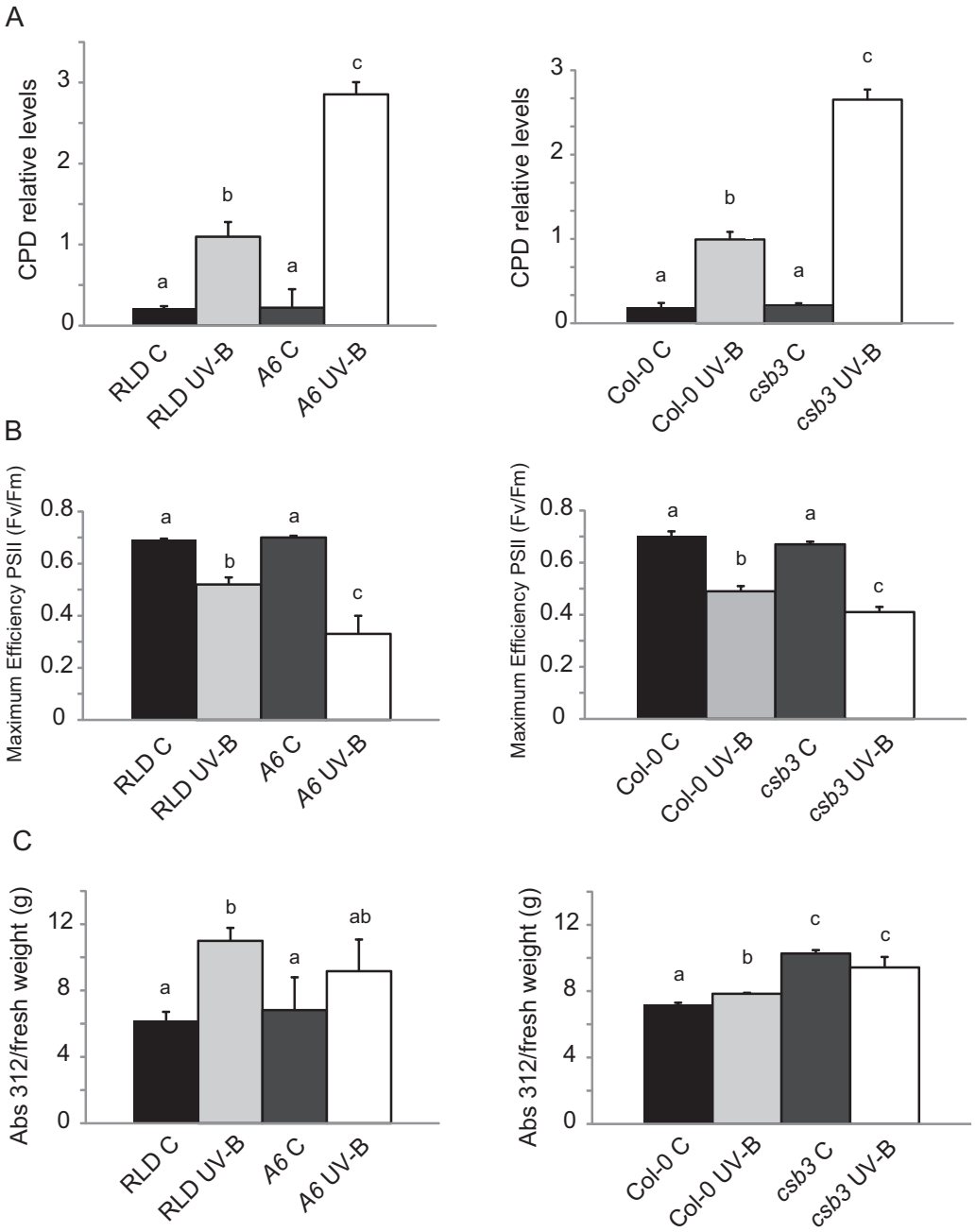

Fig. 3. UV-B effects in Arabidopsis *A6* and *csb3* mutant plants. (A) Levels of cyclobutane pyrimidine dimers (CPDs) in DNA of leaves under control (C) conditions without UV-B and after exposure to UV-B for 4 h. The *A6* mutant is in the Arabidopsis RLD background whilst the *csb3* mutant is in the Col-0 background. Experiments were carried out under conditions that allowed photorepair in the light. Samples of 2 µg of DNA were used. CPD levels are expressed as values relative to the wild-type in each case. (B) Maximum efficiency of photosystem II (PSII) in wild-type and *A6* and *csb3* mutant plants under control (C) conditions without UV-B and after UV-B exposure for 4 h. (C) Content of UV-B-absorbing compounds in leaves of wild-type and *A6* and *csb3* mutant plants under control (C) conditions without UV-B and after UV-B exposure for 4 h. Total UV-B-absorbing compounds were assayed by absorbance at 312 nm (see Methods). All data are means (±SEM) of leaf samples from six different plants. Different letters indicate significantly different values according to ANOVA and Tukey's test at $P<0.05$.

β,β-xanthophylls protect plants against DNA damage after UV-B exposure in Arabidopsis

To investigate whether particular carotenoids were more efficient than others in protecting plants against UV-B irradiation, we used Arabidopsis mutants with selected deficiencies. After desaturation and isomerization of phytoene to produce lycopene, the carotenoid pathway branches to carotenes and xanthophylls with either two β rings (such as β-carotene and derived β,β-xanthophylls) or with one β and one ε ring (such as lutein) (Fig. 1). Loss of function of *LUT2*, the only lycopene ε-cyclase found in Arabidopsis (Ruiz-Sola and Rodríguez-Concepción, 2012), results in no accumulation of the β,ε-xanthophyll lutein, but in increased levels of the β,β-xanthophylls: violaxanthin, zeaxanthin, and antheraxanthin (Table 1). Interestingly, *lut2*

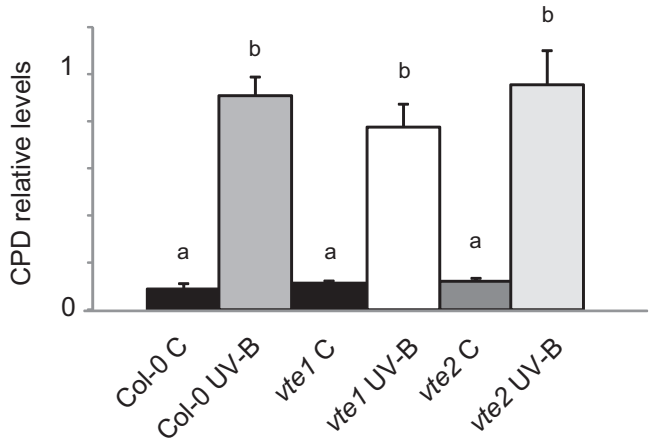

Fig. 4. Tocopherols do not protect Arabidopsis plants against UV-B exposure. Levels of cyclobutane pyrimidine dimers (CPDs) in DNA of leaves of wild-type (Col-0), *vte1*, and *vte2* mutant plants under control (C) conditions without UV-B and after exposure to UV-B for 4 h. Experiments were carried under conditions that allowed photorepair in the light. Samples of 2 µg of DNA were used. CPD levels are expressed as values relative to the wild-type in each case. Data are means (±SEM) of leaf samples from six different plants. Different letters indicate significantly different values according to ANOVA and Tukey's test at $P<0.05$.

mutants showed lower DNA damage after UV-B exposure than WT plants (Fig. 5A), suggesting that one or several of the β,β-xanthophylls that accumulated in these plants might have a role in UV-B protection in Arabidopsis.

Next, we assayed UV-B-triggered DNA damage in mutants defective in the *ABA1* gene, which encodes the only zeaxanthin epoxidase (ZEP) enzyme found in Arabidopsis (Ruiz-Sola and Rodríguez-Concepción, 2012). ZEP transforms zeaxanthin into violaxanthin via antheraxanthin (Fig. 1) and hence *aba1* mutants do not accumulate violaxanthin or antheraxanthin but have highly increased levels of zeaxanthin compared to WT plants (Table 1; Supplementary Table S3). Loss of violaxanthin and antheraxanthin, however, did not reduce the protection against UV-B in *aba1* mutants, as they showed similar CPD levels to WT plants after exposure (Fig. 5B). It is possible then that high zeaxanthin levels (which were 438-fold higher in *aba1* than in WT plants; Table 1) could compensate for the violaxanthin and antheraxanthin deficiency.

To further confirm our hypothesis, we performed a Factor Analysis (a multivariate statistical method) with the aim of determining the pattern of relationships among isoprenoid levels and DNA damage. Factor Analysis after varimax rotation indicated that the first two factors explain 68.91% of the total variance. In order to find the associations, factor-loading values were taken into account and those higher than 0.5 were considered significant. As shown in Supplementary Table S5, the second factor was positively related to lutein, γ-tocopherol, and DNA damage, while it was negatively related to violaxanthin and its precursor antheraxanthin. Thus, this statistical method validated our hypothesis that the xanthophylls violaxanthin and antheraxanthin can protect Arabidopsis against DNA damage caused by UV-B radiation.

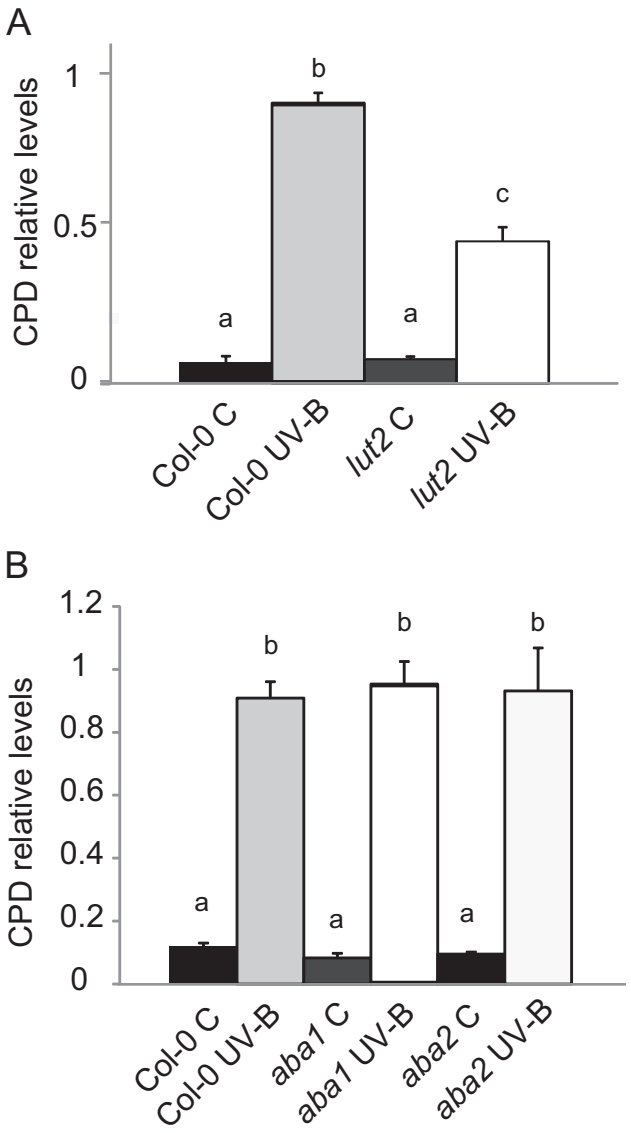

Fig. 5. β,β-xanthophylls protect Arabidopsis plants from UV-B damage. (A) Levels of cyclobutane pyrimidine dimers (CPDs) in DNA of leaves of wild-type (Col-0) and *lut2* mutant plants under control (C) conditions without UV-B and after UV-B exposure for 4 h. (B) CPD levels in DNA of Col-0, *aba1*, and *aba2* mutant plants under control (C) conditions without UV-B and after UV-B exposure for 4 h. Experiments were carried out under conditions that allowed photorepair in the light. Samples of 2 µg of DNA were used. Data are means (±SEM) of leaf samples from six different plants. Different letters indicate significant differences according to ANOVA and Tukey's test at $P<0.05$.

ABA is not involved in protection against DNA damage by UV-B in Arabidopsis

The results showed that β,β-xanthophylls contributed to the protection of Arabidopsis plants against UV-B exposure and suggested that zeaxanthin could partially compensate for the loss of violaxanthin and antheraxantin. As violaxanthin and its derived neoxanthin can be used for the production of ABA through the activity of NCED enzymes (Fig. 1), we next aimed to determine whether this hormone could also participate in UV-B protection. To this end, UV-B irradiation experiments were conducted using *aba2* mutants, which show WT levels of carotenoids

(including β,β-xanthophylls) but are deficient in ABA synthesis and accumulation (Table 1; González-Guzmán *et al.*, 2002). As shown in Fig. 5B, *aba2* mutants showed similar CPD accumulation as *aba1* and WT Col-0 plants, demonstrating that protection against UV-B damage is independent of ABA.

β,β-xanthophylls protect against oxidative damage after UV-B exposure in Arabidopsis

To better understand the role of β,β-xanthophylls in UV-B protection, we examined the expression of genes that participate in UV-B signaling and responses in WT plants, in *cbs3* mutants (deficient in β,β-xanthophylls), and in *lut2* plants (that accumulate β,β-xanthophylls). The expression of the UV-B photoreceptor *UVR8* was similar in all genotypes analysed, independent of the light conditions or the β,β-xanthophyll levels in the plants used (Fig. 6A). On the other hand, *HY5* (encoding a transcription factor that regulates UV-B responses), and *CHS* and *F3H* (encoding chalcone synthase and flavanone 3-hydroxylase, respectively, that participate in the biosynthesis of flavonoids, UV-B-absorbing phenolics in plants) were significantly increased after UV-B exposure in all plants (Fig. 6B–D). This was also true when transcript levels were analysed in *aba1* and *aba2* mutants (Supplementary Fig. S2 A-C). In the *lut2*, *aba1*, and *aba2* mutants, and in agreement with transcript expression analysis, UV-B-absorbing phenolics also increased after exposure (Supplementary Fig. S2D, E). However, none of these changes correlated with the DNA damage accumulated after UV-B exposure in the mutants (Figs 3, 5). Consequently, DNA protection against damage by UV-B mediated by β,β-xanthophylls

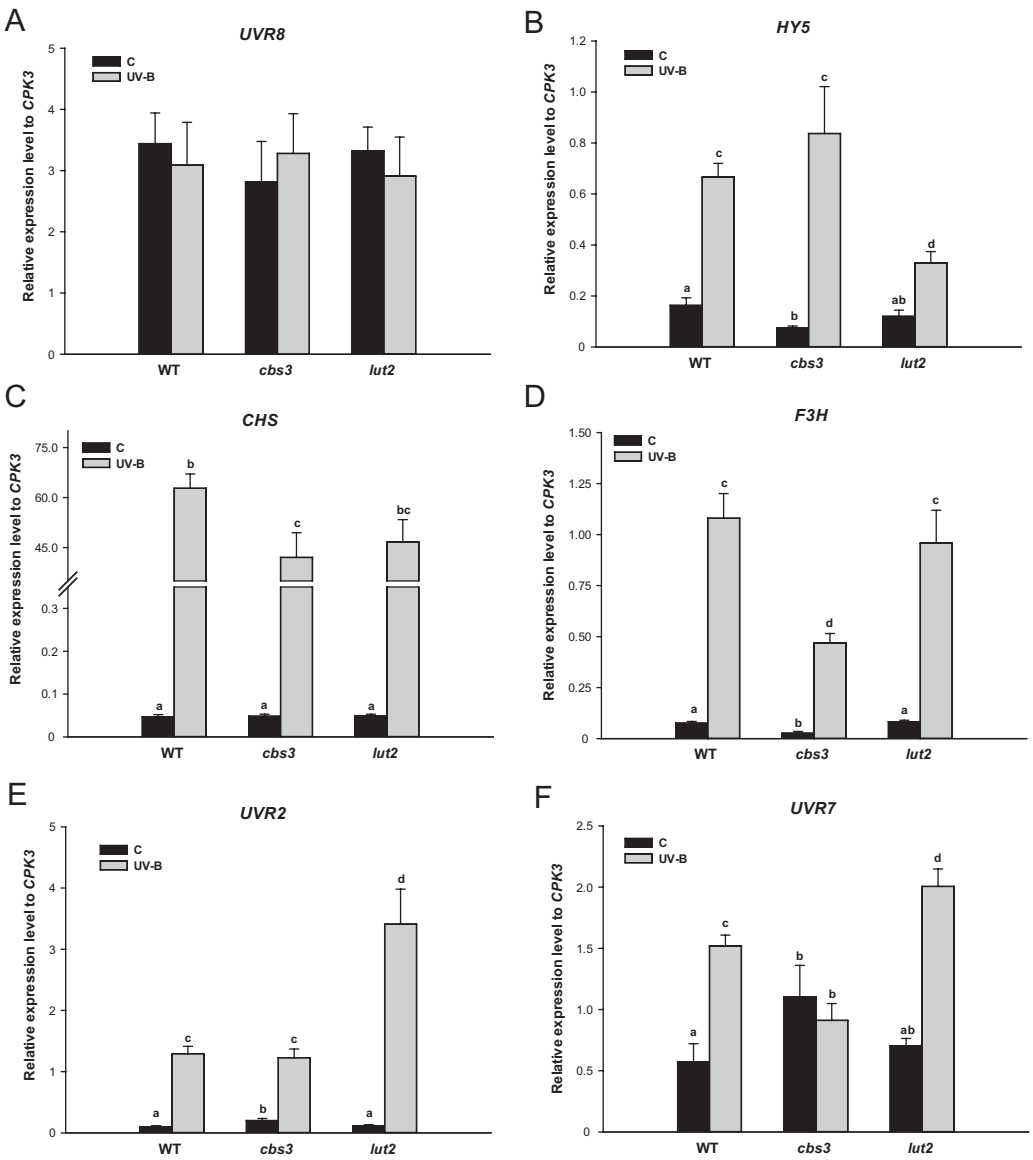

Fig. 6. Relative expression of UV-B-regulated genes determined by RT-qPCR in wild-type (WT), and *cbs3* and *lut2* mutant plants. Transcript levels were determined under control (C) conditions and after UV-B exposure for 4 h for (A) the *UVR8* photoreceptor, (B) the UV-B-regulated transcription factor *HY5*, (C) the flavonoid metabolism enzyme chalcone synthase (*CHS*), (D) the flavonoid metabolism enzyme flavanone 3-hydroxylase (*F3H*), (E) the DNA-repair photolyase *UVR2*, and (F) the DNA-repair endonuclease *UVR7*. Data are means (±SE) obtained from three biological replicates. Different letters indicate significant differences according to ANOVA and Tukey's test at $P<0.05$.

did not operate through changes in the expression of UV-B signaling and response genes, or through changes in the levels of UV-B-absorbing phenolics.

On the other hand, transcript levels of the DNA-repair enzymes *UVR2* (encoding a CPD photolyase) and *UVR7* (encoding ERCC1, a DNA excision-repair protein) were significantly and more highly increased after UV-B exposure in *lut2* than in WT plants, in agreement with the decreased CPD accumulation that was observed (Figs 5, 6). In the *cbs3* mutants, while *UVR2* levels were similar to those in WT plants after UV-B exposure, *UVR7* had reduced expression after irradiation, also in agreement with the DNA damage observed (Figs 3, 6). Thus, β,β-xanthophyll levels may affect the expression of DNA-repair enzymes and, as a consequence, the accumulation of DNA damage after UV-B exposure.

Carotenoids, including xanthophylls, are considered to be the first line of protection against singlet oxygen stress in plants (Ramel *et al.*, 2012). Therefore, we examined whether changes in transcript levels of singlet oxygen-responsive genes occurred after UV-B radiation. As shown in Fig. 7, singlet oxygen-responsive genes were significantly up-regulated after UV-B exposure, and they were also differentially expressed in the *cbs3* and *lut2* mutants in comparison to WT plants, both under control conditions and after UV-B exposure. While transcripts of *AAA-ATPase* (one of the six AAA-ATPases of the proteasome regulatory particle), *BAP1* (encoding a protein with a C2 domain, involved in defense and regulation of cell death), and *FLOT3* (encoding a protein involved in an endocytic pathway; Lee *et al.*, 2007) were significantly increased after UV-B exposure, the levels were lower in *lut2* than in

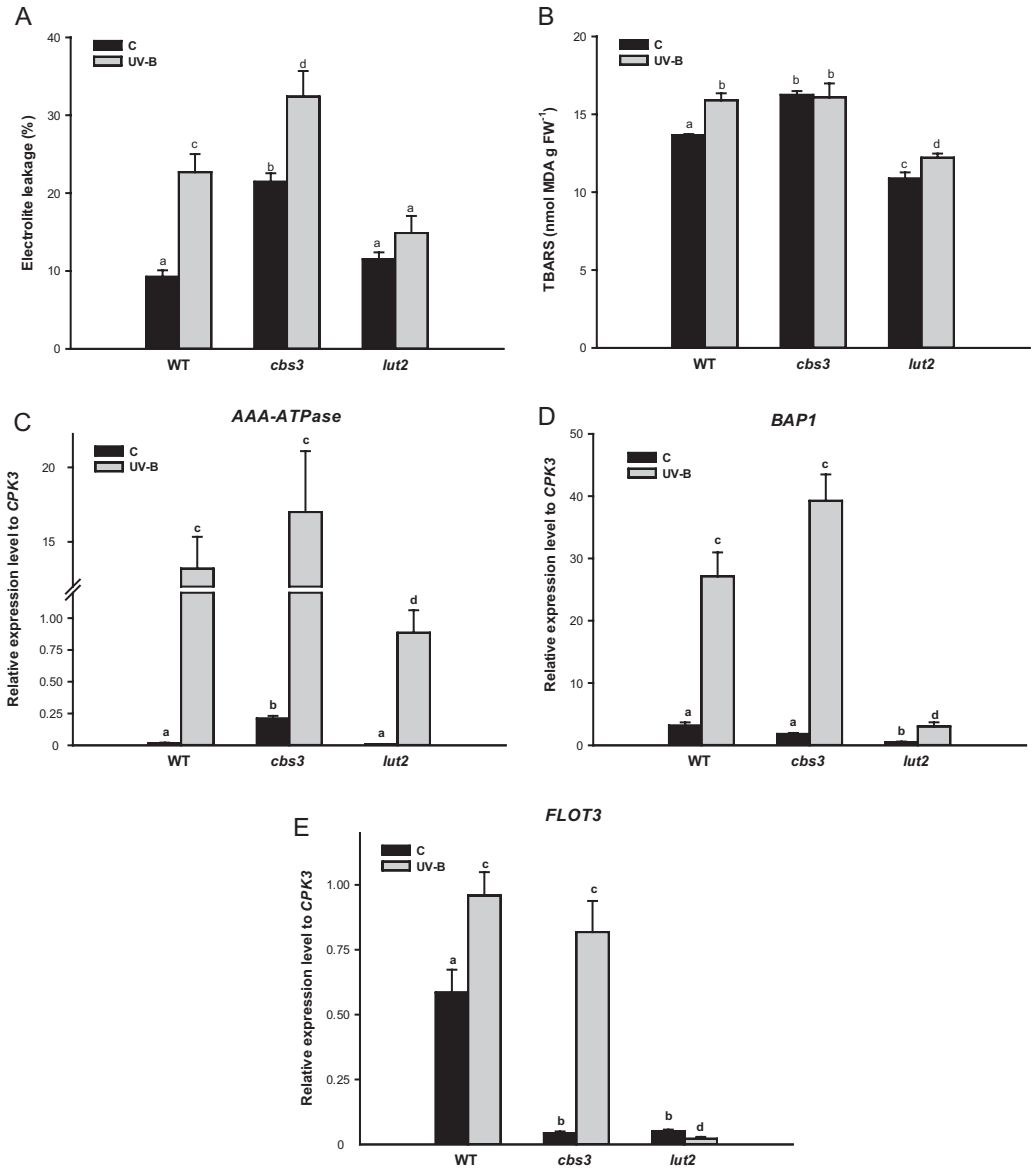

Fig. 7. Analysis of membrane injury, lipid peroxidation, and singlet oxygen-responsive genes in wild-type (WT), and *cbs3* and *lut2* mutant plants under control (C) conditions or after UV-B exposure for 4 h. (A) Electrolyte leakage and (B) content of thiobarbituric acid-reactive substances (TBARS). (C–E) Relative expression of transcripts of singlet oxygen-responsive genes as determined by RT-qPCR. Transcript levels of (C) *AAA-ATPase*, (D) *BAP1*, and (E) *FLOT3*. All data are means (±SE) from three biological replicates. Different letters indicate significant differences according to ANOVA and Tukey's test at $P<0.05$.

cbs3 and WT plants, suggesting that the oxidative stress (and in particular singlet oxygen stress) produced by exposure was lower in the *lut2* plants that accumulated β,β-xanthophylls. To further test this hypothesis, we examined the integrity of the cell membranes after UV-B exposure by measuring electrolyte leakage, which is a measure of oxidative damage. WT and *cbs3* plants showed a significant increase in electrolyte leakage in leaves after UV-B exposure, with *cbs3* plants showing higher values than WT plants; however, *lut2* plants exhibited no changes after UV-B exposure and had lower values than WT and *cbs3* plants (Fig. 7A). In addition, UV-B sensitivity was assayed though lipid peroxidation analysis (Blokhina *et al.*, 2003) by measuring the content of thiobarbituric acid-reactive substances (TBARS). TBARS were increased by UV-B in WT plants, while levels were high and similar under both control and exposure conditions in the *cbs3* mutant (Fig. 7B). In contrast, *lut2* plants showed lower TBARS than WT plants, both under control and UV-B conditions (Fig. 7B). Taken together, the results demonstrated that β,β-xanthophylls protected the plants against oxidative damage after UV-B exposure, probably through protection against singlet oxygen stress. It is possible then that the changes in the oxidative status of the cells may have affected the expression of not only singlet oxygen-responsive genes (Fig. 7); but also of DNA-repair enzymes (Fig. 6).

β,β-xanthophylls can protect against DNA damage after UV-B exposure in vitro

Finally, we examined whether the role of β,β-xanthophylls in protection against DNA damage by UV-B may be a consequence of direct absorption of the radiation. To this end, we analysed DNA damage *in vitro* after different UV-B exposure times using DNA purified from WT Arabidopsis plants in the presence or absence of violaxanthin. In addition, DNA damage was also analysed in the presence of rutin (quercetin 3-O-rutinoside) as a positive control, as quercetin is a UV-absorbing compound that has previously been demonstrated to protect against DNA damage (Emiliani *et al.*, 2013). As shown in Fig. 8, CPD accumulation increased after 5–15 min of UV-B exposure. However, when DNA was incubated in the presence of rutin or violaxanthin, the damage was significantly decreased. In contrast, when the exposures were carried out in the presence of other xanthophylls (neoxanthin and β-carotene), the damage to DNA was similar to that in the controls (Supplementary Fig. S3). Thus, although these experiments were done *in vitro*, our results suggested that β,β-xanthophylls, and violaxanthin in particular, could directly protect DNA against CPD formation by UV-B.

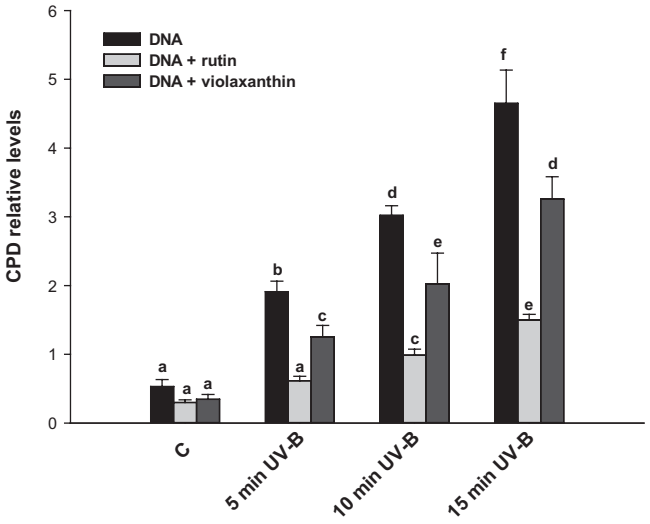

Fig. 8. Violaxanthin prevents UV-B-induced formation of cyclobutane pyrimidine dimers (CPDs) in *in vitro* assays. CPD levels in DNA under control (C) conditions (UV-B 0.02 W m^{-2} for 15 min) and after UV-B exposure (0.5 W m^{-2}) for 5, 10, or 15 min. The DNA was irradiated either on its own, or in the presence of rutin (quercetin 3-O-rutinoside, 100 μg ml^{-1}), a flavonol that absorbs UV-B (positive control), or violaxanthin (14 μg ml^{-1}). Samples of 2 μg of DNA were used. Data are means (±SEM) of six samples. Different letters indicate significant differences according to two-way ANOVA and Tukey's test at $P<0.05$.

Discussion

Isoprenoid derivatives are vital for all living organisms; in particular, plant isoprenoids participate in respiration, photosynthesis, membrane fluidity, and in the regulation of growth and development (Vranová *et al.*, 2013). Moreover, as specialized metabolites, they also have important roles in allelopathic and plant–pathogen interactions, and in the attraction of pollinators and seed-dispersing animals. A large group of isoprenoids participate in photosynthetic processes in plants, including light harvesting, energy conversion, electron transfer, and quenching of excited chlorophyll triplets (for a review see Vranová *et al.*, 2013). In particular, carotenoids (including carotenes and their oxygenated derivatives, the xanthophylls) are isoprenoids that quench excess excitation energy during light harvesting to protect the light-harvesting complex (for a review see Munné-Bosch *et al.*, 2013). Carotenoids are lipophilic antioxidants that perform essential roles in controlling oxidants when they are generated within the thylakoid membranes, such as singlet oxygen generated by PSII. In addition, they also participate in signal transduction pathways; for example, the oxidation of β-carotene leads to the production of signals that regulate gene expression or trigger cell death (Ramel *et al.*, 2012). In this case, the oxidation of β-carotene by singlet oxygen produces different volatile derivatives that regulate the expression of singlet oxygen-responsive genes.

In plant plastids, all isoprenoids, including carotenoids, are synthesized by the 5-C units IPP and DMAPP that are produced through the MEP pathway. In this study, we have demonstrated that MEP-derived isoprenoid metabolites participate in UV-B protection in plants. First, we have shown that after a UV-B treatment, there is an increase in the levels of specific plastidial isoprenoids, such as γ-tocopherol and the β,β-xanthophyll zeaxanthin (Table 1). Corresponding to this, plants deficient in the MEP pathway enzymes DXS (*A6*) or HDS (*cbs3*) have increased DNA damage after UV-B exposure and decreased photosynthesis (Fig. 3). Interestingly, after the UV-B treatment, *A6* and *csb3* plants showed a greater decrease in the maximum efficiency of PSII than WT plants (Fig. 3B): this may have been a consequence of an increase in oxidative stress in these plants after exposure, due to decreased levels of β-β xanthophylls (Figs. 6, 7). It has been reported previously

that the MEP intermediate MEcPP has an important role during stress responses (Flores-Pérez *et al.*, 2008). However, the fact that *A6* and *cbs3* accumulated lower and higher levels of this metabolite, respectively, but similar levels of CPDs demonstrates that, under our experimental conditions, MEcPP did not playing a major role in UV-B protection.

Second, a thorough genetic approach showed that an imbalance in the xanthophyll levels but not in tocopherol accumulation (Fig. 4) triggered a differential response in CPD accumulation after UV-B exposure. In particular, we demonstrated that plants that over-accumulate β,β-xanthophylls (violaxanthin, antheraxanthin, and zeaxanthin) but lack β,ε-xanthophylls (lutein), showed decreased DNA damage after UV-B exposure (Fig. 5). Remarkably, *in vitro* assays demonstrated that violaxanthin could protect against DNA damage by UV-B (Fig. 8), probably through direct absorbance. Although experiments *in planta* are required, it is possible that β,β-xanthophylls may have a role *in vivo* in absorbing UV-B for protection against DNA damage. However, the role of β,β-xanthophylls as screen pigments in the UV-B region might be very minor given their absorption spectra. On the other hand, CPD formation requires complex singlet/triplet excitation dynamics, where the triplet excited state of the pyrimidine dimer plays a key role before the ground-state CPDs are finally formed (Zhang and Eriksson, 2006). Therefore, if β,β-xanthophylls directly participate in DNA photoprotection by quenching the singlet/triplet excited state of the pyrimidine dimer to avoid CPD formation, they should be in close vicinity to the DNA molecules. The presence of xanthophylls in the nuclei has not been reported, but this possibility cannot be ruled out. Alternatively, or in addition, plants that accumulate higher levels of β,β-xanthophylls may express higher levels of DNA repair enzymes (Fig. 6), and hence increased repair may also be a mechanism that could explain the lower DNA damage observed in the *lut2* plants.

ABA levels were previously determined in *aba2* mutants by González-Guzmán *et al.* (2002) and in *A6* transgenic plants by Estévez *et al.* (2001). Although the *A6* transgenic plants with low ABA levels showed increased UV-B damage compared to WT plants, the *aba2* plants, which also have low ABA levels, did not show increased DNA damage after exposure. Thus, we provide evidence that this phenotype is specifically generated by the β,β-xanthophylls and is not mediated by their downstream product, the stress-response hormone ABA (Xiong and Zhu, 2003; Fig. 5). Interestingly, although the *lut2* mutants had significantly higher levels of β,β-xanthophylls that most of the other mutants examined in this work (violaxanthin, antheraxanthin, and zeaxanthin; Supplementary Table S3), they still had lower amounts of these compounds than *aba1* (by 2.4-fold), which showed similar DNA damage to the WT plants. However, the difference was mostly accounted for by zeaxanthin, which has a minor role in UV-B protection compared to violaxanthin and antheraxanthin, as shown by our Factor Analysis (Supplementary Table S5). According to these data, violaxanthin and antheraxanthin were the most important β,β-xanthophylls providing DNA damage protection, and the *aba1* mutants are deficient in these two compounds. Therefore, despite the fact that zeaxanthin can provide some UV-B shielding, high accumulation of this compound is not as effective in protecting plants as are high levels of violaxanthin and antheraxanthin.

The β,β-xanthophylls participate in the xanthophyll cycle, which has an important protection role in conditions of excess light energy. Under normal light conditions, the enzyme zeaxanthin epoxidase (ZEP) converts zeaxanthin into violaxanthin, thus maintaining it at elevated levels. However, when the light intensity is high, the enzyme violaxanthin de-epoxidase (VDE) is activated, converting violaxanthin back to zeaxanthin (Fig. 1). Thus, under high-light conditions plants maintain high levels of zeaxanthin, which is reported to be a better quencher than violaxanthin. Thus, whilst a number of studies have reported that zeaxanthin plays different photoprotective roles (Horton *et al.*, 1996; Baroli *et al.*, 2003; Holt *et al.*, 2005; Dall´Osto *et al.*, 2007), the function of violaxanthin as a direct photoprotector is more controversial. However, our results provide evidence that not only zeaxanthin but all β,β-xanthophylls can protect plants against excess exposure to UV-B, probably through the xanthophyll cycle.

A previous report using transgenic tobacco plants overexpressing β-carotene hydroxylase correlated an increase in zeaxanthin levels to a higher tolerance against UV radiation (Götz *et al.*, 2002). The authors also found an increase in violaxanthin levels after UV-B exposure in these transgenic plants. In our current study, analysis of the *aba1* mutant, which does not metabolize zeaxanthin into downstream xanthophylls and which accumulated CPD levels similar to those in WT plants after UV-B exposure, suggested the existence of an active role of violaxanthin and anteraxanthin in UV-B photoprotection. This was further validated by a Factor Analysis (Supplementary Table S5). On the other hand, Zhao *et al.* (2014) found that tobacco plants overexpressing Arabidopsis *β-carotene hydroxylase* and accumulating xanthophylls also displayed more tolerance to UV radiation, as shown by less leaf necrosis. Interestingly, the authors suggested that as more xanthophylls were accumulated in these plants, it could be possible that at least some of them may be free from photosystems and biologically active as free pigments, conferring protection under stress conditions. Therefore, violaxanthin might also have been responsible for the increased UV tolerance that was reported, in agreement with our results. Although carotenes do not directly absorb in the UV-B range of light, they have several times been demonstrated to protect against UV-induced photodamage, and they have been termed 'sun protectants' (Biesalski and Obermueller-Jevic, 2001). Thus, in animal epidermal cells, carotenoids can protect *in vivo* against UV-B-sensitized photochemical reactions, where excited species occur with exposure to UV-B light. Hence, carotenoid pigments have been proposed to have activity against skin cancer because they can quench excited species in epidermal cells exposed to UV-B (Mathews-Roth, 1986).

Carotenoids have been demonstrated to protect against singlet oxygen stress because they have the capacity to quench $^1O_2$ through a physical mechanism involving transfer of excitation energy followed by thermal deactivation, and also through a chemical mechanism involving their oxidation (Ramel *et al.*, 2012). In the presence of $^1O_2$, β-carotene, lutein, and zeaxanthin are oxidized to various aldehydes and endoperoxides, which are rapidly accumulated during high-light stress. This

accumulation parallels the degree of PSII photoinhibition and the expression of singlet oxygen marker genes. In our experiments, UV-B radiation also induced the expression of singlet oxygen marker genes and produced damage to membranes, both of which were reduced in plants accumulating higher levels of β,β-xanthophylls (Figs 6, 7). In this way, as well as affecting the expression of singlet oxygen marker genes, it is also possible that changes in the oxidative status of the cells regulated by β,β-xanthophylls may affect the expression of DNA-repair enzymes, and as a consequence impact on DNA damage levels.

It is interesting to note that the up-regulation of *PSY* and *VDE* by UV-B correlated with an increase in *HY5* transcript levels, in agreement with previous results reported by Toledo-Ortiz *et al.* (2014), who demonstrated that this transcription factor regulated the expression of these two genes. Therefore, the increase in zeaxanthin levels after UV-B exposure may have been a consequence of activation of metabolic pathways from precursors generated by the MEP pathway regulated by HY5.

Xanthophylls have been previously reported to confer tolerance to different stresses. For example, tobacco plants overexpressing the genes for lycopene *β*-cyclase or β-carotene hydroxylase show a significant increase in the levels of xanthophyll cycle pigments without the ABA content being affected, making the plants more tolerant to salt or drought stress, respectively (Zhao *et al.*, 2014; Jin *et al.*, 2015). Moreover, increased zeaxanthin levels as the result of overexpression of *β-carotene hydroxylase* in Arabidopsis have been shown to confer tolerance to high light and high temperature conditions (Davison *et al.*, 2002). These transgenic plants were shown to have decreased leaf necrosis, decreased content of anthocyanins, and reduced lipid peroxidation. Interestingly, the plants also over-accumulated violaxanthin. Therefore, co-operative effects on photoprotection by different xanthophyll species may exist. It is then possible that β,β-xanthophylls may have a similar protective role in plants after UV-B exposure.

Overall, the results presented here demonstrate that Arabidopsis plants accumulating higher levels of β,β-xanthophylls are more tolerant to UV-B damage. Violaxanthin, antheraxanthin, and zeaxanthin all participate in the xanthophyll cycle, and our data show that zeaxanthin levels significantly increase after UV-B exposure. Thus, in a similar way to what has already been demonstrated under excess white-light conditions, the xanthophyll cycle may have an important protection role under UV-B as well.

Supplementary data

Supplementary data are available at *JXB* online.

Fig. S1. Transcriptomic changes of the MEP, and carotenoid biosynthesis and degradation pathways in 4-week-old Arabidopsis plants after 4 h exposure to UV-B.

Fig. S2. Relative expression of the flavonoid metabolism enzymes chalcone synthase and flavanone 3-hydroxylase, and the UV-B regulated transcription factor *HY5* in wild-type, *aba1*, and *aba2* plants in response to UV-B exposure.

Fig. S3. CPD levels in DNA in response to UV-B exposure in the presence or absence of neoxanthin or β-carotene.

Table S1. Transgenic and mutant Arabidopsis plants used in this study.

Table S2. Primers used for RT-qPCR.

Table S3. Isoprenoid products from the MEP pathway in leaves of wild-type Col-0 and mutant plants in response to UV-B treatment.

Table S4. Isoprenoid products from the MEP pathway in leaves of RLD wild-type and *A6* transgenic plants in response to UV-B treatment.

Table S5. Results of Factor Analysis of data sets including mean values of isoprenoids and CPD levels relative to those in Col-0 control plants.

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