

Examining the photoprotection hypothesis for adaxial foliar anthocyanin accumulation by revisiting comparisons of green- and red-leafed varieties of coleus (*Solenostemon scutellarioides*)

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Received: 5 January 2015 / Accepted: 23 March 2015 / Published online: 11 April 2015
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Abstract Although plants rely on light to drive energy production via photosynthesis, excess light can be harmful. Plants have evolved photoprotective mechanisms to mitigate this threat, including thermal energy dissipation, the most common form of which involves de-epoxidized constituents of the xanthophyll cycle facilitating the conversion of excess excitation energy to heat. A role in photoprotection has also been proposed for red anthocyanins when they accumulate near the adaxial leaf surface. Here, we compared the response to experimental light stress of a red-leafed (anthocyanin rich) and a green-leafed variety of coleus [*Solenostemon scutellarioides* (L.) Codd], examining chlorophyll fluorescence emission and pigment composition. After experimentally imposed intense white light, red- and green-leafed coleus exhibited manifestations of light stress (decreased photosystem II quantum efficiency) of a similar magnitude. This, considered alone, could be interpreted as evidence that anthocyanins do not serve a photoprotective role. However, during excess light exposure, the green-leafed variety employed a greater level of thermal energy dissipation and possessed correspondingly higher xanthophyll cycle pool sizes and de-epoxidation states. During exposure to red light, which anthocyanins absorb very poorly, levels of thermal energy dissipation did not differ between coleus varieties. Taken together, our findings suggest that adaxial anthocyanins minimize stress associated with excess light absorption and

that the green-leafed variety of coleus compensated for its much lower levels of adaxial anthocyanins by invoking higher levels of energy dissipation. Thus, anthocyanin accumulation should be considered alongside the suite of photoprotective mechanisms employed by photosynthetic tissues.

Keywords Anthocyanins · Coleus · Light stress · Photoprotection · *Solenostemon scutellarioides* (L.) Codd · Xanthophyll cycle

Introduction

Many plant species accumulate anthocyanins in the vacuoles of adaxial epidermal or palisade parenchyma cells of leaves during exposure to intense light in combination with environmental stresses (e.g. chilling temperatures or low soil nutrient availability) and/or during leaf expansion or senescence (reviewed in Gould 2010; Solovchenko 2010). Environmental stress generally limits photosynthetic light utilization and can thus increase the absorption of light in excess of the needs of photosynthesis (reviewed in Logan et al. 1999; Logan 2006). Similarly, during the assembly and disassembly of the photosynthetic apparatus during juvenile leaf expansion and senescence, respectively, photosynthetic light use is often compromised and excess light stress is exacerbated as a consequence. The correlation between the appearance of adaxial anthocyanin accumulation and the potential for acute excess light stress has led to the hypothesis that adaxial anthocyanins protect underlying chlorophyllous cell layers from reactive oxygen species (ROS)-mediated damage by lowering the light intensities they experience. Many studies support a photoprotective role for anthocyanin accumulation, demonstrating, for

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example, that red leaves, when compared to green leaves, experience less photoinactivation of photosystem II (PSII) during high light stress (Gould et al. 1995; Field et al. 2001; Manetas et al. 2002, 2003; Pietrini et al. 2002; Hughes et al. 2005, 2007; Neill and Gould 2003; Close and Beadle 2003; Liakopoulos et al. 2006; Hughes and Smith 2007; Steyn et al. 2009; Gould et al. 2010; Zhang et al. 2010; Neilsen and Simonsen 2011; Hughes et al. 2012; Hatier et al. 2013). However, authors of some studies do not implicate foliar anthocyanins in photoprotection (Burger and Edwards 1996; Dodd et al. 1998; Kyparissis et al. 2007; Esteban et al. 2008; Zeliou et al. 2009; Nikiforou and Manetas 2010; Liakopoulos and Spanorigas 2012). For example, Burger and Edwards (1996) reported that the quantum yield for photosynthetic oxygen evolution decreased to a similar degree after experimentally imposed high light stress in leaf discs of red and green varieties of *Solenostemon scutellarioides* (coleus). They concluded that anthocyanins may be protective against ultraviolet light but not against visible light. Their study is especially compelling since its design avoids some of the challenges confronted by many comparative studies, which include the fact that green and red leaves may differ in ontogenic state (mature versus juvenile or senescing) or growth environment (shade versus sun exposed), with incumbent differences in a broad range of leaf features, not simply anthocyanin content.

Leaf photoprotection has been the subject of intense research over recent decades. It is now well appreciated that plants protect their leaves from excess light-mediated PSII photoinactivation and other forms of damage via mechanisms that operate at all levels of the organism hierarchy, including steep branch and leaf angles (Mooney et al. 1977; Givnish 1988; Valladares and Pearcy 1998), reflective leaf surface features (Ehleringer and Björkman 1978; Barker et al. 1997) and aspects of chloroplast position, ultrastructure and biochemistry (Haupt and Scheuerlein 1990, Brugnoli and Björkman 1992; Grace and Logan 1996; Logan et al. 1998, 2014; Williams et al. 2003). All wild-type plants ever subjected to study have been shown to employ thermal energy dissipation (reviewed in Demmig-Adams and Adams 2006; Demmig-Adams et al. 2012; Jahns and Holzwarth 2012; Ruban et al. 2012). This ubiquitous photoprotective mechanism de-excites chlorophyll (Chl), converting the excitation energy to heat, which harmlessly radiates across the leaf lamina. Thermal energy dissipation reduces ROS-mediated cell damage by shortening the lifetime of excited Chl, thereby proactively reducing the probability of ROS formation. Thermal energy dissipation can be measured as non-photochemical quenching (NPQ) of Chl fluorescence emission (Bilger and Björkman 1990) and generally involves de-epoxidized xanthophylls,

typically zeaxanthin (and possibly antheraxanthin) of the xanthophyll cycle (also known as the violaxanthin cycle).

A rich understanding of photoprotection requires that one assess the possible role of adaxial anthocyanins in the context of other key photoprotective mechanisms. With that in mind, and recognizing the expanding literature on the diversity of photoprotective mechanisms and the functional consequences of foliar anthocyanin accumulation, here, we revisit the experimental system employed by Burger and Edwards (1996)—red and green coleus varieties—using analyses of Chl fluorescence emission, which enabled us to assess excitation energy partitioning to thermal energy dissipation and photosynthesis during exposure to light stress. We couple this to characterizations of the size and conversion state of the xanthophyll cycle and other aspects of leaf pigment composition.

Materials and methods

Study species and growth conditions

Two varieties of coleus [*S. scutellarioides* (L.) Codd] were obtained from local nurseries, green-leafed ‘Wizard Jade’ and red-leafed ‘Black Dragon’, which accumulate anthocyanins in the vacuoles of upper epidermal cells (Burger and Edwards 1996). The green and red varieties examined in Burger and Edwards (1996), Green Rainbow and Red Dragon, respectively, were not available. Like those varieties chosen by Burger and Edwards (1996), Wizard Jade and Black Dragon did not differ significantly in leaf chlorophyll content (described in detail below). Red-leafed Black Dragon possessed ~125-fold higher anthocyanin contents when compared with green-leafed Wizard Jade, which exhibited no noticeable red colouration. This is a larger difference in leaf anthocyanin content between varieties than that reported by Burger and Edwards (1996).

Plants were grown in a controlled environment growth chamber (Conviro, Winnipeg, Canada) at 25 °C under a combination of fluorescent and incandescent light sources that yielded photosynthetically active radiation (PAR) of 300–400 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ at the leaf surface with a 10-h diurnal photoperiod. Plants were provided with replete water every other day and fertilized weekly with Scotts 21-5-20 Multi-Purpose Fertilizer (Scotts-Sierra Horticultural Products Company, Marysville, OH, USA). Plant positions in the chamber were randomized routinely. Emergent inflorescences were removed along with the occasional removal of some aboveground vegetative material. All analyses were performed on fully expanded upper canopy leaves that had developed under the growth conditions described above.

Chlorophyll fluorescence analyses

With the exception of red actinic light exposures (described below), analyses of Chl fluorescence emission were performed using an FMS2 pulse-modulated fluorometer (Hansatech Instruments, King's Lynn, UK). For lengthy light stress exposures, attached leaves of plants that had been dark-acclimated for a minimum of 30 min were fitted into the leaf clip of the fluorometer and subjected to the following exposure routine: 5 min each at 50, 200 and 1000 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ (in that order), 90 min at 2000 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$, 5 min each at 1000, 200 and 50 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ (in that order) and 30 min at low photosynthetically active radiation (PAR; $>2 \mu\text{mol photons m}^{-2} \text{s}^{-1}$). Actinic, mixed-wavelength ('white') light was generated by the halogen light source integrated into the FMS2. This light source exhibits a broad spectral output from ~ 380 to 730 nm with peak output between 535 and 705 nm (Hansatech Instruments, *pers. comm.*). Maximal (F_m) and minimal (F_o) fluorescence emission from leaves in the dark-acclimated state were measured immediately before the light exposure and at the end of the 30 min period of dark acclimation after the exposure. At the end of exposure to each irradiance, as well as at 5, 30 and 60 min of exposure to the highest PAR, steady state (F_s), maximal (F'_m) and minimal (F'_o) fluorescence emission were measured. Maximal fluorescence was measured during a 0.8-s exposure to a $\sim 3000 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ saturating pulse of light generated by the fluorometer. Minimal fluorescence was measured during transient exposure to weak far-red illumination generated by the FMS2. Fluorescence parameters were used to calculate the following: maximum quantum efficiency of PSII in the dark-acclimated state (F_v/F_m , where $F_v = F_m - F_o$), PSII quantum efficiency during illumination (ΦPSII ; $[F'_m - F'_s]/F'_m$), NPQ ($[F_m/F'_m] - 1$) and the reduction state of PSII ($[F_s - F'_o]/[F'_m - F'_o]$; also referred to as $1 - qP$) (Schreiber et al. 1986; Bilger and Björkman 1990; Melis 1999; Baker 2008).

To assess the induction of NPQ, dark-acclimated plants (>30 min) were subjected to a 10-min exposure to 1000 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ generated by the halogen light source of the FMS2. F_m was measured immediately before the imposition of light stress and F'_m each minute thereafter. The induction of NPQ was also assessed during exposure to red light using a Li-6400 Portable Photosynthesis Analyzer (Li-Cor Biosciences, Lincoln, Nebraska, USA). Leaves of dark-acclimated plants were fitted into the leaf chamber fluorometer cuvette (LI6400F) programmed to deliver only red (peak wavelength = 630 nm) actinic light from the light emitting diode (LED) source designed into the instrument (i.e., the

blue LED source was turned off). Leaves were otherwise handled as described above.

Leaf pigment extractions and analyses

Leaf discs (0.4 cm^2) were detached immediately after the conclusion of the 10 min exposures to the actinic white light generated by the FMS2, frozen in liquid N_2 and stored at $-80 \text{ }^\circ\text{C}$ until extraction. Leaf chlorophylls and carotenoids were analysed by high-performance liquid chromatography. Samples were extracted according to the protocol of Adams and Demmig-Adams (1992) using a ball mill modified to accept 2 mL microcentrifuge tubes. Pigments were separated and quantified according to the solvent system described in Gilmore and Yamamoto (1991), using an Agilent 1100 Series (Agilent Technologies, Palo Alto, CA, USA) equipped with an Agilent 1100 diode array detector and a YMC CarotenoidTM C₃₀ reverse phase column (5 μm particle size, 250 mm \times 4.6 mm I.D.; Waters Corporation, Milford, MA, USA, protected by a Phenomenex C₁₈ guard column; Phenomenex, Torrance, CA, USA). Anthocyanin content was measured according to Gould et al. (2000) from extracts in a 1:3:16 3 M HCl:H₂O:MeOH solution at 563 nm using a Beckman DU 520 spectrophotometer (Beckman Coulter, Inc, Indianapolis, IN, USA).

Statistical analyses

All statistical analyses were performed using SPSS (IBM, Armonk, NY, USA). Results of chlorophyll fluorescence analyses were analysed by repeated measures one-way analyses of variance (rmANOVA). When rmANOVA yielded significant effects of coleus variety, post hoc two-tailed t tests were employed to examine the effect of variety at each individual point during exposures. Aspects of leaf pigment composition were examined by unpaired two-tailed t tests. Differences between varieties were considered significant when $p < 0.05$.

Results

Lengthy light stress exposures

The experimentally imposed, lengthy high light stress exposures, which included 90 min at 2000 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ of white light, resulted in, on average, a 17 % decline in the maximum quantum efficiency of PSII in the dark-acclimated state (F_v/F_m Fig. 1); however, there was no significant difference in the magnitude of the decline experienced by red-leafed versus green-leafed coleus

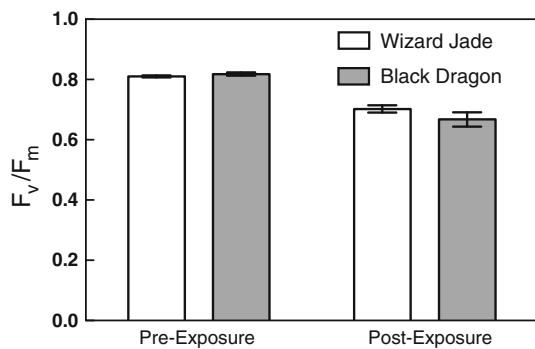


Fig. 1 The maximum quantum efficiency of photosystem II (F_v/F_m) of Wizard Jade (open bars) and anthocyanin-rich Black Dragon (grey bars) leaves before and after exposure to the lengthy white light stress regime (post-exposure measurements were preceded by a 30-min period of acclimation to very low PAR). The experimental light exposure significantly reduced F_v/F_m ; however, no statistically significant differences between varieties were observed. Data are mean \pm SEM; $N = 7-8$

varieties (rmANOVA, $p = 0.4$). Over the course of exposures, light intensity had a statistically significant, inverse effect on Φ_{PSII} ($p < 0.001$; Fig. 2, upper panel), but no statistically significant differences were observed between the two coleus varieties ($p = 0.32$). In contrast, the level of NPQ was greater at higher irradiances ($p < 0.001$) in both the varieties. Wizard Jade, the green-leafed variety, possessed significantly higher levels of NPQ than red-leafed Black Dragon over the course of the exposure ($p < 0.001$; Fig. 2, middle panel). At the highest irradiances, NPQ of Wizard Jade was 28–42 % higher than that of Red Dragon. *t* tests performed post hoc to examine the effect of variety at each time-point in the light stress exposure showed that Wizard Jade possessed significantly higher NPQ than Black Dragon at every instance when measurements were collected during exposure to 1000 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ or greater. Black Dragon possessed higher NPQ at the end of the initial exposure to 50 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$, when, overall, values for NPQ were very low. Statistically significant effects of exposure PAR on the reduction state of PSII were observed ($p < 0.001$; Fig. 2, lower panel); however, the PSII reduction states of Wizard Jade and Black Dragon did not differ significantly ($p = 0.38$).

NPQ induction

Both the red- and green-leafed coleus rapidly induced NPQ upon abrupt exposure to 1000 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ of white or red light (Fig. 3). During exposure to white light, green-leafed coleus possessed significantly higher NPQ than red-leafed coleus ($p = 0.02$; Fig. 3, upper panel), which was evident at every time-point by post hoc analysis. During exposure to red actinic light, no statistically

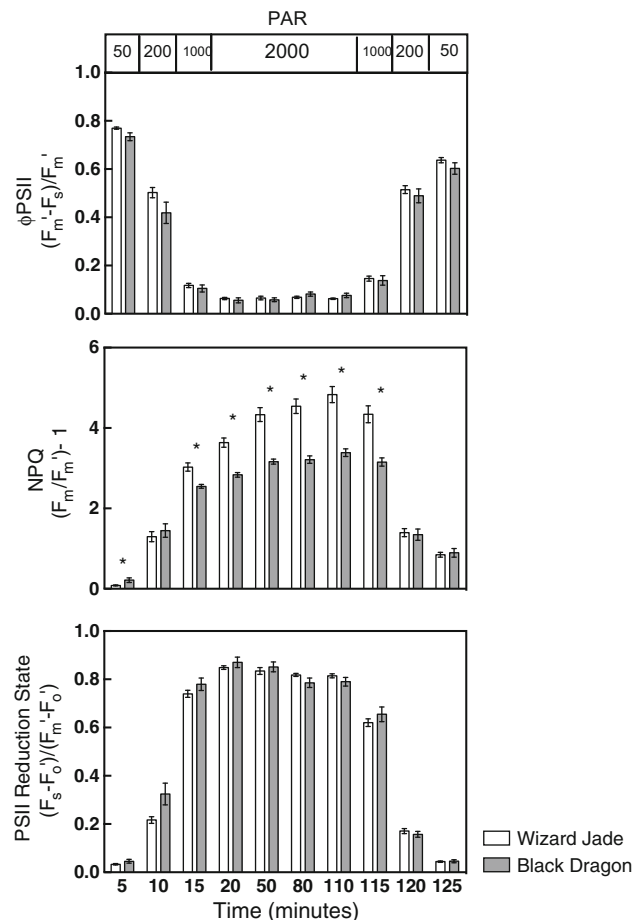


Fig. 2 The photosystem II efficiency during illumination (Φ_{PSII} ; upper panel), non-photochemical quenching (NPQ; middle panel) and photosystem II reduction state (lower panel) of Wizard Jade (open bars) and anthocyanin-rich Black Dragon (grey bars) leaves during the lengthy white light stress exposure. Photosynthetically active radiation (PAR) is noted at the top of the figure. Note that time is not plotted linearly. * indicates significant differences between the varieties as revealed by post hoc analyses as described in the materials and methods. Data are mean \pm SEM; $N = 7-8$

significant difference was observed between the NPQ of the two coleus varieties ($p = 0.53$; Fig. 3, lower panel).

Leaf chlorophyll and carotenoid composition

Leaf Chl and xanthophyll cycle compositions were assessed from tissue collected immediately following the 10 min exposure to 1000 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ of white light. Although green-leafed Wizard Jade and red-leafed Black Dragon did not differ in Chl *a + b* contents ($p = 0.12$; Fig. 4A), Black Dragon possessed significantly lower ratios of Chl *a* to Chl *b* ($p < 0.001$; Fig. 4B). Leaves of Wizard Jade possessed significantly larger xanthophyll cycle pools when expressed per unit Chl ($p = 0.005$; Fig. 4C). Wizard Jade had also converted a higher

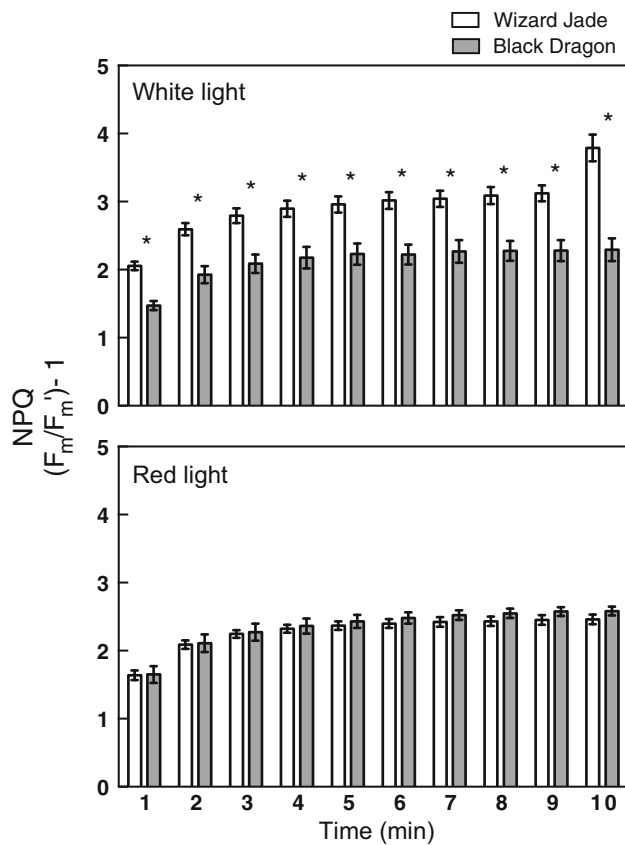


Fig. 3 Non-photochemical quenching (NPQ) of Wizard Jade (*open bars*) and anthocyanin-rich Black Dragon (*grey bars*) leaves during a 10-min exposure to 1,000 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ of white light (*upper panel*) or red light (*lower panel*; peak wavelength = 630 nm). * indicates significant differences between the varieties as revealed by post hoc analyses as described in the Materials and Methods. Data are mean \pm SEM; $N = 6$

proportion of its xanthophyll cycle pool to the de-epoxidized forms, antheraxanthin and zeaxanthin ($p = 0.003$; Fig. 4D) at the moment of collection.

Discussion

Burger and Edwards (1996) reported that the magnitude of reductions in the quantum yield of photosynthetic oxygen evolution caused by experimental exposure to intense visible light did not differ significantly between red- and green-leaved varieties of coleus. On the basis of this evidence, they concluded that foliar anthocyanins do not reduce PSII photoinactivation (which they refer to as photoinhibition) by screening visible light. Like Burger and Edwards (1996), we found that red- and green-leaved coleus varieties experienced similar reductions in the maximum quantum efficiency of PSII (F_v/F_m) induced by exposure to intense white light (Fig. 1). However, our

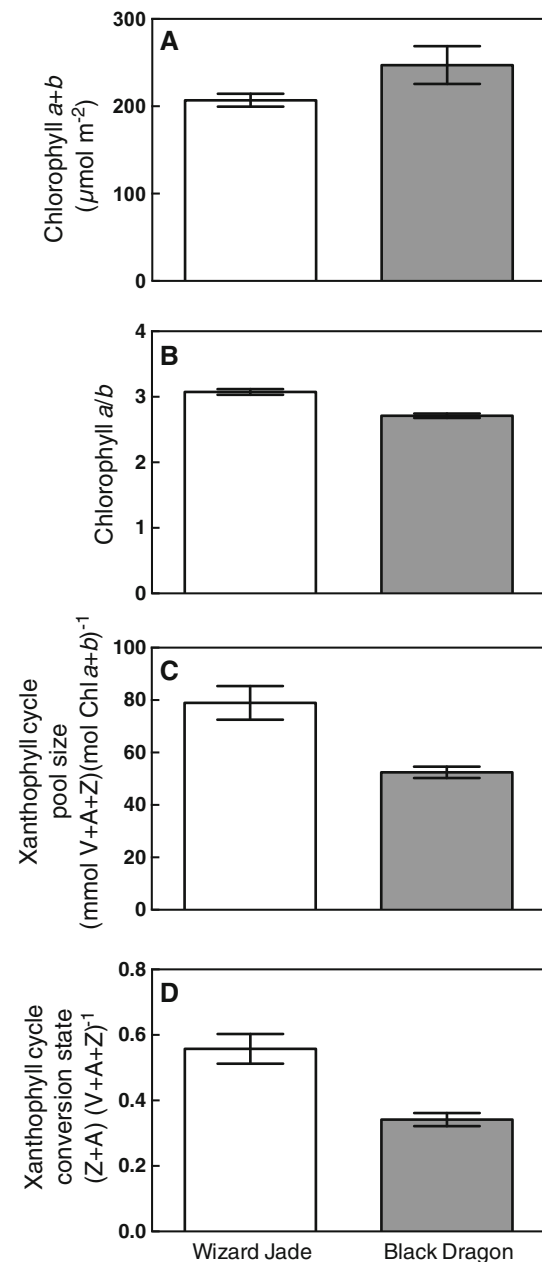


Fig. 4 Chlorophyll $a + b$ content (A) Chlorophyll a/b ratio (B) xanthophyll cycle pool size (C) and xanthophyll cycle conversion state (D) of Wizard Jade (*open bars*) and anthocyanin-rich Black Dragon (*grey bars*), collected after the tenth minute of exposure to 1000 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ of white light. Statistically significant differences between varieties were observed in all parameters except chlorophyll $a + b$ content (A). Data are mean \pm SEM; $N = 5$

analyses of chlorophyll fluorescence emission enabled us to examine the partitioning of absorbed light energy during high light exposure, which revealed the compensatory nature of photoprotective strategies and a role for adaxial anthocyanins in photoprotection. During high light exposure, green-leaved Wizard Jade engaged significantly higher levels of thermal energy dissipation, measured as

NPQ, than red-leafed Black Dragon (Figs. 2, 3). Thus, our data suggest that, by maintaining higher levels of thermal energy dissipation, green-leafed coleus compensated for the absence of light screening by adaxial anthocyanins and achieved levels of protection against PSII photoinactivation similar to those exhibited by red-leafed coleus.

Photosystem II is more vulnerable to photoinactivation when it is actively undertaking the electron transfer events of charge separation because of the increased risk of double reduction, the generation of dangerous radicals and ensuing macromolecular damage (Melis 1999). Thus, the PSII reduction state, an estimate of the instantaneous proportion of PSII reaction centres processing a photon, serves as an informative measure of light stress. During experimental exposure to intense visible light, the PSII reduction states of green- and red-leafed coleus varieties increased relative to values recorded at lower light intensities but did not differ from each other significantly (Fig. 2). This is consistent with our observation that exposure to intense white light led to appreciable PSII photoinactivation whose magnitude did not differ significantly between varieties (Fig. 1). Our data suggest that greater levels of thermal energy dissipation (measured as NPQ) observed in green- versus red-leafed coleus compensated for the absence of visible light screening by an adaxial anthocyanin layer to yield equivalent levels of photoprotection, which were, nonetheless, insufficient to fully protect leaves from PSII photoinactivation during experimentally imposed, lengthy high light stress exposures.

At biologically relevant pHs, anthocyanins absorb maximally in the green region of the visible spectrum and absorb red light very poorly. Thus, an adaxial anthocyanin layer is optically transparent to incident red light. During exposure to intense red light, red- and green-leafed varieties of coleus maintained similar levels of thermal energy dissipation (measured as NPQ) (Fig. 3).

If adaxial anthocyanin accumulation contributes to photoprotection by absorbing some fraction of incident visible light, underlying chloroplasts of red-leafed coleus should be experiencing less intense growth light environments when compared with chloroplasts of green-leafed coleus raised in the same environment. Our observations of the leaf Chl and carotenoid compositions of green-leafed in comparison to red-leafed coleus varieties are consistent with this. The pigment composition of red-leafed coleus was significantly 'shadier' in character, with a lower Chl *a:b* ratio and a smaller xanthophyll cycle pool size (Demmig-Adams and Adams 1992; Thornber et al. 1993; Grace and Logan 1996; Logan et al. 1996, 1998), as has been reported previously for other species (e.g., Gould et al. 1995; Manetas et al. 2002, 2003; Lee et al. 2003; Hughes et al. 2007; Kyparissis et al. 2007; Zeliou et al. 2009; Henry et al. 2012) (Fig. 4). In addition, during exposure to saturating white light, red-

leafed coleus converted a significantly smaller proportion of its xanthophyll cycle to antheraxanthin and zeaxanthin, the de-epoxidized cycle constituents known to facilitate thermal energy dissipation. This is consistent with observed lower levels of NPQ and suggests that red-leafed coleus chloroplasts experienced less intense light stress during high light exposure (Demmig-Adams and Adams 1992; Grace and Logan 1996; Logan et al. 1998) (Fig. 4).

Conclusions

In the present study, we have demonstrated a role for adaxial anthocyanin accumulation in the photoprotection of coleus. However, this role did not result in reduced manifestations of high light stress on PSII quantum efficiency because green-leafed coleus achieved similar susceptibilities to light stress, when compared with red-leafed coleus, by maintaining greater levels of thermal energy dissipation, an alternative photoprotective mechanism. Our findings underscore the value of examining the partitioning of absorbed light during high light exposure and also the need to use caution when comparing green versus red tissues with regards to the aftereffects of high light stress alone (on, e.g., F_v/F_m). We conclude that adaxial anthocyanin accumulation should be considered alongside the suite of photoprotective mechanisms employed by photosynthetic tissues.

Acknowledgments This work was supported by the Bowdoin College, a Howard Hughes Medical Institute Summer Research Fellowship [to W.C.S.], United States Department of Agriculture [MER-2002-04818], and the National Science Foundation [DUE 0088517]. We thank an anonymous reviewer for helpful comments on our original submission.

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