American Journal of Botany 94(5): 784–790. 2007.

ATTENUATION OF INCIDENT LIGHT IN *GALAX URCEOLATA* (DIAPENSIACEAE): CONCERTED INFLUENCE OF ADAXIAL AND ABAXIAL ANTHOCYANIC LAYERS ON PHOTOPROTECTION[1]

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Although anthocyanin coloration in lower (abaxial) leaf cells has been documented for numerous species, the functional significance of this character has not been comprehensively investigated according to habitat or leaf orientation. Here, we demonstrate that abaxial anthocyanin may function as a photoprotectant, similarly to its purported role in upper (adaxial) cells, in leaves vulnerable to high irradiance incident on abaxial surfaces. Spectral scans were derived for *Galax urceolata* leaves with the following phenotypes: abaxial or adaxial anthocyanin only, abaxial and adaxial anthocyanin, and no anthocyanin. To determine whether anthocyanins conferred protection from photoinhibition, maximum photosystem II efficiencies of red (anthocyanic) and green (acyanic) surfaces were compared during and after exposure to photoinhibitory conditions. Leaves were either positioned with their adaxial surfaces facing the light source or inverted to expose abaxial surfaces. Spectral scans showed increased absorptance of 500–600 nm wavelengths by red surfaces (consistent with the absorbance spectrum of anthocyanin), regardless of whether that surface was abaxial or adaxial. Leaves with anthocyanin in either illuminated surface were also photoinhibited less than leaves lacking anthocyanin in that surface. These results suggest that anthocyanic layers reduce absorbed sunlight in the mesophyll not only for adaxial surfaces, but also for the abaxial. Adaxial/abaxial anthocyanin plasticity may therefore be adaptive in high-light environments or during light-sensitive developmental stages where leaf orientation and/or substrate albedo are variable.

Key words: abaxial; anthocyanin; *Diapensiaceae*; evergreen; *Galax*; leaf orientation; photoprotection, plasticity.

Anthocyanins have received considerable attention in the recent literature due to their proposed role as light-attenuators in leaves vulnerable to high light stress (Drumm-Herrel and Mohr, 1985; Landry et al., 1995; Smillie and Hetherington, 1999; Gould et al., 2000; Feild et al., 2001; Gould et al., 2002; Pietrini et al., 2002; Hoch et al., 2003; Neill and Gould, 2003; Manetas et al., 2003, Hughes et al., 2005; Karageorgou and Manetas, 2006). By absorbing blue-green light that may otherwise be absorbed by chlorophyll *b* in subjacent mesophyll cells, anthocyanins are thought to relieve potential damage to the photosystems when leaves are exposed to more light energy than can be effectively assimilated for photosynthesis or dissipated as heat. The physiological advantage that this confers has been evidenced in studies comparing light capture efficiency of anthocyanic and cyanic leaves under photoinhibitory conditions. When red and green leaf surfaces (typically adaxial) are exposed to high light stress, red leaves tend to be less photoinhibited than green leaves when other photoprotective mechanisms are equal (Feild et al., 2001; Hoch et al., 2003; Hughes et al., 2005). In the current study, we test whether abaxial anthocyanin may perform the same function in leaves where the abaxial surface is incident to high light rather than (or in addition to) the adaxial surface.

Exposure to high abaxial irradiance occurs under a broad range of conditions: in environments with high substrate albedos (e.g., snow and sand), among plants with inclined leaf angles that expose abaxial surfaces to ambient sunlight, and in species where developing juvenile leaves emerge with abaxial surfaces facing outwards (Drumm-Herrel and Mohr, 1985; Sherwin and Farrant, 1998; W. K. Smith, personal observation). However, little is known about adaptations of abaxial cells to withstand high irradiance, which is surprising given the increased sensitivity of abaxial surfaces relative to adaxial in dorsiventrally asymmetric leaves (Sun et al., 1996; Sun and Nishio, 2001). Although the synthesis of anthocyanin in abaxial cells in response to high light stress has been reported (Barker et al., 1997; Sherwin and Farrant, 1998; Pietrini et al., 2002; Hughes et al., 2005), and development of photostability has been shown to occur more quickly in seedlings with abaxial anthocyanin (Drumm-Herrel and Mohr, 1985), no studies have directly tested the prospect that these pigments decrease susceptibility to photoinhibition through light attenuation in abaxial cells.

Previous studies on abaxial anthocyanin have focused almost exclusively on understory species adapted to low light environments, where red and purple abaxial surfaces are also a common morphological character (Richards, 1952; Lee et al., 1979; Lee and Graham, 1986; Lee and Collins, 2001). Under these conditions, it has been suggested that abaxial anthocyanin functions to backscatter red wavelengths that enter the leaf from the adaxial surface, thereby increasing light capture efficiency in low intensity and poor light quality environments (Lee et al., 1979). However, this interpretation has not been well supported and seems insufficient to explain the appearance of anthocyanin in abaxial cells of leaves under high light stress (where an increase in light capture would likely exacerbate photoinhibition). Under such conditions, it is more likely that anthocyanins are functioning as light attenuators to reduce photoinhibition of abaxial cells.

[1] Manuscript received 14 June 2006; revision accepted 7 March 2007.

The authors thank Drs. L. Donovan and H. Neufeld for use of the spectroradiometric equipment, Dr. A. McCauley and the microscopy facility at WFU, C. Machalaba and S. Bissett for technical assistance, and the anonymous reviewers of this work, whose helpful suggestions greatly improved the quality of this manuscript. This work was supported financially by the graduate school at Wake Forest University.

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To determine whether abaxial anthocyanin functions to decrease susceptibility to photoinhibition similarly to adaxial anthocyanin, we took advantage of the phenotypic plasticity in the evergreen understory herb *Galax urceolata* (Poir.) Brummitt for synthesizing anthocyanin on either or both leaf surfaces receiving moderately high levels of irradiance (i.e., >750 $\mu mol \cdot m^{-2} \cdot s^{-1}$) during the winter. In its natural environment, *G. urceolata* leaves are typically oriented with adaxial surfaces facing canopy gaps, with leaf angles ranging from 0° to 90° (most commonly between 40° and 60°). Although highest irradiances are typically incident on adaxial surfaces (resulting in adaxial anthocyanin), abaxial surfaces of leaves with more vertical angles often experience relatively high irradiance from snow albedo and direct and diffuse light, resulting in anthocyanin synthesis in abaxial surfaces as well. Less frequently, leaf litter or debris will cause leaves to be entirely inverted, and only abaxial surfaces will appear red. By comparing the optical properties and ecophysiology of leaves with and without adaxial and abaxial anthocyanin, we demonstrate that anthocyanin located within abaxial tissues attenuates light in the same manner as anthocyanin located within adaxial cells, effectively reducing photoinhibition of abaxial leaf tissues vulnerable to high light stress.

MATERIALS AND METHODS

For comparing optical profiles of abaxially and adaxially anthocyanic leaves, spectral scans were derived for leaves with the following pigment patterns: entirely green (green adaxial and green abaxial), bicolored (either green adaxial with red abaxial or red adaxial with green abaxial), or entirely red (red adaxial, red abaxial) (Fig. 1). Three leaves of each type were removed from the field and stored under cold, moist conditions until analysis later that day. Spectral scans for reflectance and transmittance of photosynthetically active radiation (PAR), representing 400–700 nm, of both adaxial and abaxial surfaces were derived using a Li-Cor 1800 spectroradiometer with external integrating sphere (Li-Cor, Lincoln, Nebraska, USA). Measurements were taken at 1-nm intervals. Absorptance was calculated as (1 − reflectance − transmittance). The area tested was marked, and leaf thickness and pigment concentrations were subsequently quantified. Leaves were all first year leaves obtained from a wooded area in Jonas Ridge, North Carolina, USA (35°57′20″ N, 81°53′55″ W) in February 2006.

Leaf thickness was measured using digital calipers with 0.01 mm precision. Chlorophylls were extracted by placing three 0.33 cm^2 leaf discs in 3 mL *N*,*N*′-dimethylformamide in the dark for 24 h. Concentrations were determined using the equations described by Porra (2002). Anthocyanins were extracted by placing three discs in 3 mL 6M $HCl : H_2O :$ MeOH (7 : 23 : 70) in the dark at 4°C for 24 h. Following extraction, 1 mL of the solution was removed, and to it 1 mL water and 1 mL chloroform were added to separate anthocyanins (insoluble in chloroform) from chlorophylls. The solutions were centrifuged for 15 min at 2630 g, and 1 mL of the top layer (containing anthocyanins) was used for analysis. Anthocyanin concentration was determined as A_{530}, using an extinction coefficient of 30 000 1 $mol^{-1} \cdot cm^{-1}$ (Murray and Hackett, 1991). Prior to extraction, all leaf discs were submerged in liquid nitrogen to infiltrate cuticular waxes and disrupt cell membranes.

Chlorophyll *a* and *b* concentrations, ratios, total chlorophyll content, and leaf thickness of the four leaf groups were compared using a single factor ANOVA with Tukey–Kramer test for means difference, using JMP statistical software (SAS Institute, Cary, North Carolina, USA) (Zar, 1999). Anthocyanin concentrations were compared using a single factor ANOVA with planned contrast. Completely green leaves were predicted a priori to have significantly less anthocyanin than all other groups, and completely red leaves were predicted to have significantly more.

For microscopic imaging, leaves were sectioned into 50–100 μm sections using a vibratome. Sections were mounted on a Zeiss Axioplan upright microscope (Zeiss, Thornwood, New York, USA), viewed using differential interference contrast (DIC) optics, and images were captured using a Hamamatsu C5810 three-chip cooled color CCD camera (Hamamatsu Photonics; Hamamatsu City, Japan). Photographs of leaf sections were rotated and adjusted for brightness and image sharpness using Photoshop CS (Adobe Systems, San Jose, California, USA).

Another set of *G. urceolata* leaves was used to compare maximum light capture efficiency (F_v/F_m) of the leaf types during and following exposure to photoinhibitory conditions. Leaves were derived from the same plots described previously, also in February. Fluorescence measurements were taken using a Handy-PEA 1000 fluorescence analyzer (Hansatech Institute, Cambridge, UK), emitting a 2-s, 3 $mmol \cdot m^{-2} \cdot s^{-1}$ saturating pulse. Prior to measurement, plants were dark-adapted for 30 min using Handy-PEA leaf clips. Five leaves with red adaxial surfaces and five leaves with green adaxial surfaces (both having green abaxial surfaces) were used to test adaxial response to high light stress. Abaxial surfaces were tested in a separate experiment using five leaves for each of three phenotypes: green adaxial, green abaxial; red adaxial, red abaxial; red adaxial, green abaxial. Experiments differed only in the surface of the leaf exposed to the light source. Because results from abaxial experiments showed no effect of anthocyanin in the non-illuminated surface on photoprotection, it did not seem necessary to test all four combinations of anthocyanic layers in this study.

Leaves were collected from the field, and petioles were re-cut underwater and remained submerged until experimentation. To equalize starting F_v/F_m values, we placed all leaves in low stress environments for 4 d until no significant difference between F_v/F_m values of leaf groups was observed (for detailed description of why field F_v/F_m values of red and green leaves differ and why standardization of F_v/F_m is necessary, see Hughes et al., 2005). During this time, leaves with anthocyanic surfaces were kept at constant temperature (18°C) and exposed to 10 h of low light (175 $\mu mol \cdot m^{-2} \cdot s^{-1}$) provided by a 1000 W metal halide bulb equipped with water bath and neutral density shade cloth to obtain desired PPFDs, to allow F_v/F_m to recover to values similar to those of green leaves. Green leaves were placed under similar light conditions, but exposed to field temperatures (−10 to 15°C) to prevent F_v/F_m values from exceeding 0.6–0.7 (which red leaves could not recover to during the winter). Once values of F_v/F_m were no longer significantly different between the leaf groups, all leaves were placed in the outdoor, protected enclosure overnight. At 0700 hours the following morning, leaves were exposed to a high stress treatment, consisting of high light (1150 $\mu mol \cdot m^{-2} \cdot s^{-1}$ provided by a 1000 W metal halide bulb) and low temperatures (circulating outside air, with daily temperatures ranging from 0 to 10°C during the day, and −15 to 0°C during the night). High light stress was applied for 10 h per day over three consecutive days. On the fourth day, leaves were transferred into a low stress environment (same conditions as those described to pretreat red leaves) until F_v/F_m recovered to near starting values. Fluorescence readings were taken at 1700 hours each day throughout the experiment. A repeated measures MANOVA was used to compare fluorescence between leaf types during the high light treatments.

RESULTS

Levels of chlorophyll *a* and *b,* total chlorophyll content, chlorophyll *a* : *b,* and leaf thickness did not significantly differ between the four leaf phenotypes in *G. urceolata* ($P = 0.96$, 0.85, 0.85, 0.27, and 0.26, respectively) (Table 1). Leaves with anthocyanin occurring on both adaxial and abaxial surfaces had significantly higher anthocyanin content than the other three leaf types ($P < 0.01$), with two-fold higher anthocyanin concentration than bicolored leaves, and >seven-fold higher concentration than entirely green leaves (which contained virtually no anthocyanin). Adaxially and abaxially bicolored leaves did not significantly differ in anthocyanin concentration ($P = 0.86$), but did contain significantly more anthocyanin than green leaves ($P = 0.037$) (Table 1).

Leaves with anthocyanin in both surfaces absorbed the most blue-green to yellow (500–600 nm) light of the four leaf types, absorbing up to 20% more of the green wavelengths than entirely green leaves and 2–3% more than bicolored leaves (Fig. 2A, B). Absorption spectra of bicolored leaves were similar to those of entirely red leaves when light was shone on the anthocyanic surface, though absorption was reduced by roughly 10% when the acyanic surface was illuminated. However, absorptance of green wavelengths was still much

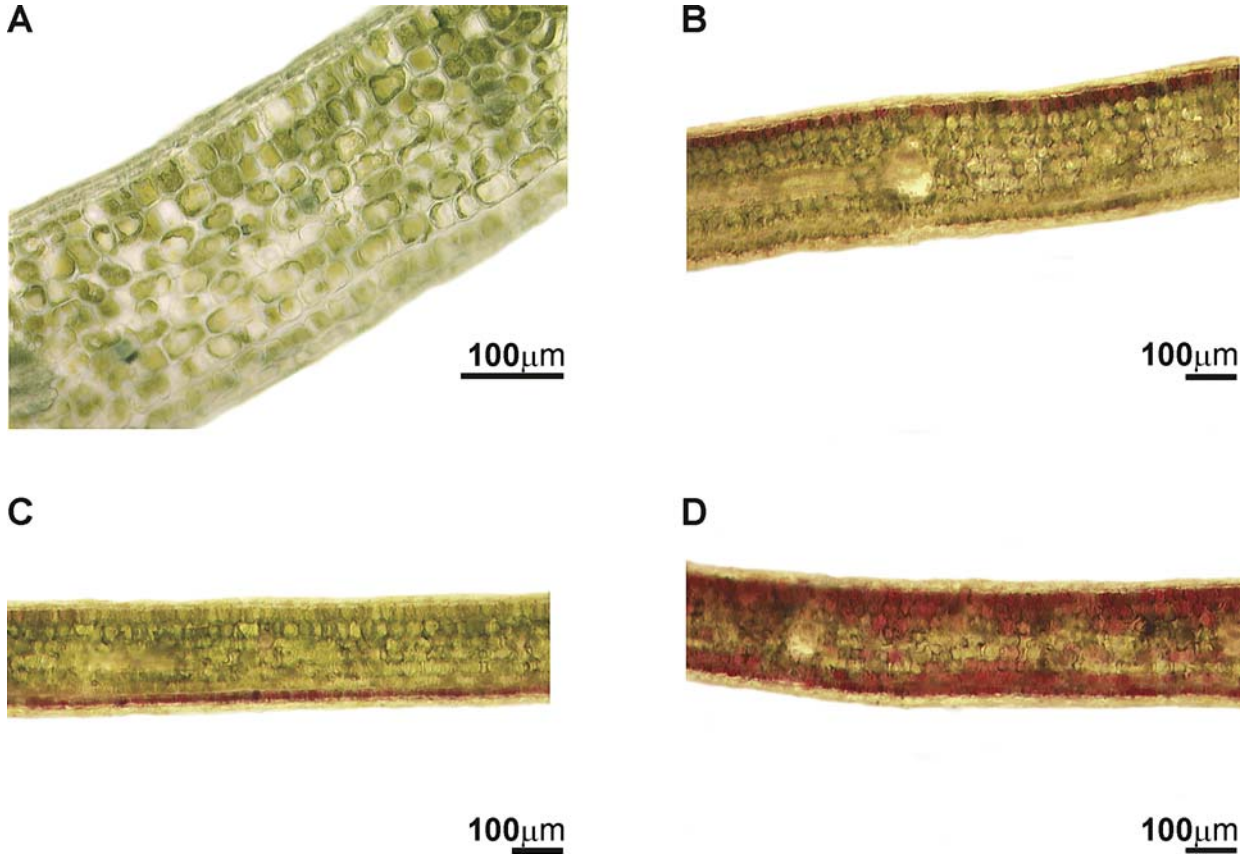

Fig. 1. Cross sections of leaves having the range of spatial distribution of anthocyanin seen in winter leaves of the evergreen herb *Galax urceolata*. (A) Acyanic adaxial and abaxial, (B) anthocyanic adaxial and acyanic abaxial, (C) acyanic adaxial and anthocyanic abaxial, and (D) anthocyanic adaxial and abaxial leaf surfaces with the adaxial surface facing upward.

higher (by up to 10%) than entirely green leaves. The increased absorptance of green light by anthocyanic tissues resulted in a dramatic decrease in transmittance of green wavelengths for anthocyanic leaves relative to entirely green leaves (Fig. 2C, D), with entirely red leaves reducing transmittance of blue-green light (500–550 nm) to 0%, and bicolored leaves to 2–3% (10% lower than green leaves). The increased absorptance of green light by anthocyanin also corresponded to a decrease in reflectance of these wavelengths when the red surface was illuminated (by up to 10%), though reflectance was not affected when light was incident on the green surface (Fig. 2E, F). In all leaves containing anthocyanin, roughly 1% more incident red light (600–675 nm) was reflected from the leaf surface compared to entirely green leaves, regardless of which surface was illuminated (acyanic or anthocyanic).

A comparison of spectral scans of abaxial and adaxial surfaces showed that leaves absorbed more light (5–7% between 450 and 600 nm wavelengths) when light was incident upon the adaxial surface, regardless of pigmentation (Fig. 2A, B). Furthermore, leaves reflected more light of all

TABLE 1. Pigment content and leaf thickness in the four leaf types of *Galax urceolata* tested in this study. Values represent means of three samples (±SE) for chlorophyll *a* (Chl *a*), chlorophyll *b* (Chl *b*), total chlorophyll (Total Chl), ratio of chlorophyll *a* to chlorophyll *b* (Chl *a*/*b*), anthocyanin, and leaf thickness. Row headings describe the coloration of the adaxial/abaxial surfaces. Green surfaces contain no visible anthocyanin; red surfaces contain visible anthocyanin.

Surface coloration (Adaxial/Abaxial)	Chl *a* (μg/cm^2)	Chl *b* (μg/cm^2)	Total Chl (μg/cm^2)	Chl *a*/*b*	Anthocyanin (μmol/cm^2)	Leaf thickness (mm)
Green/Green	35.9^a	13.5^a	49.4^a	2.64^a	0.0622^a	0.223^a
	(3.8)	(0.90)	(4.2)	(0.074)	(0.062)	(0.007)
Green/Red	35.4^a	15.0^a	50.4^a	2.36^a	11.2^b	0.273^a
	(3.3)	(0.93)	(4.2)	(0.099)	(5.9)	(0.022)
Red/Green	36.5^a	14.9^a	51.4^a	2.48^a	12.2^b	0.257^a
	(4.2)	(2.3)	(6.4)	(0.12)	(2.8)	(0.0033)
Red/Red	34.2^a	14.5^a	48.6^a	2.36^a	23.7^c	0.273^a
	(0.88)	(0.21)	(0.68)	(0.16)	(3.8)	(0.014)

Note: Means within a column followed by different letters are different at $P < 0.05$.

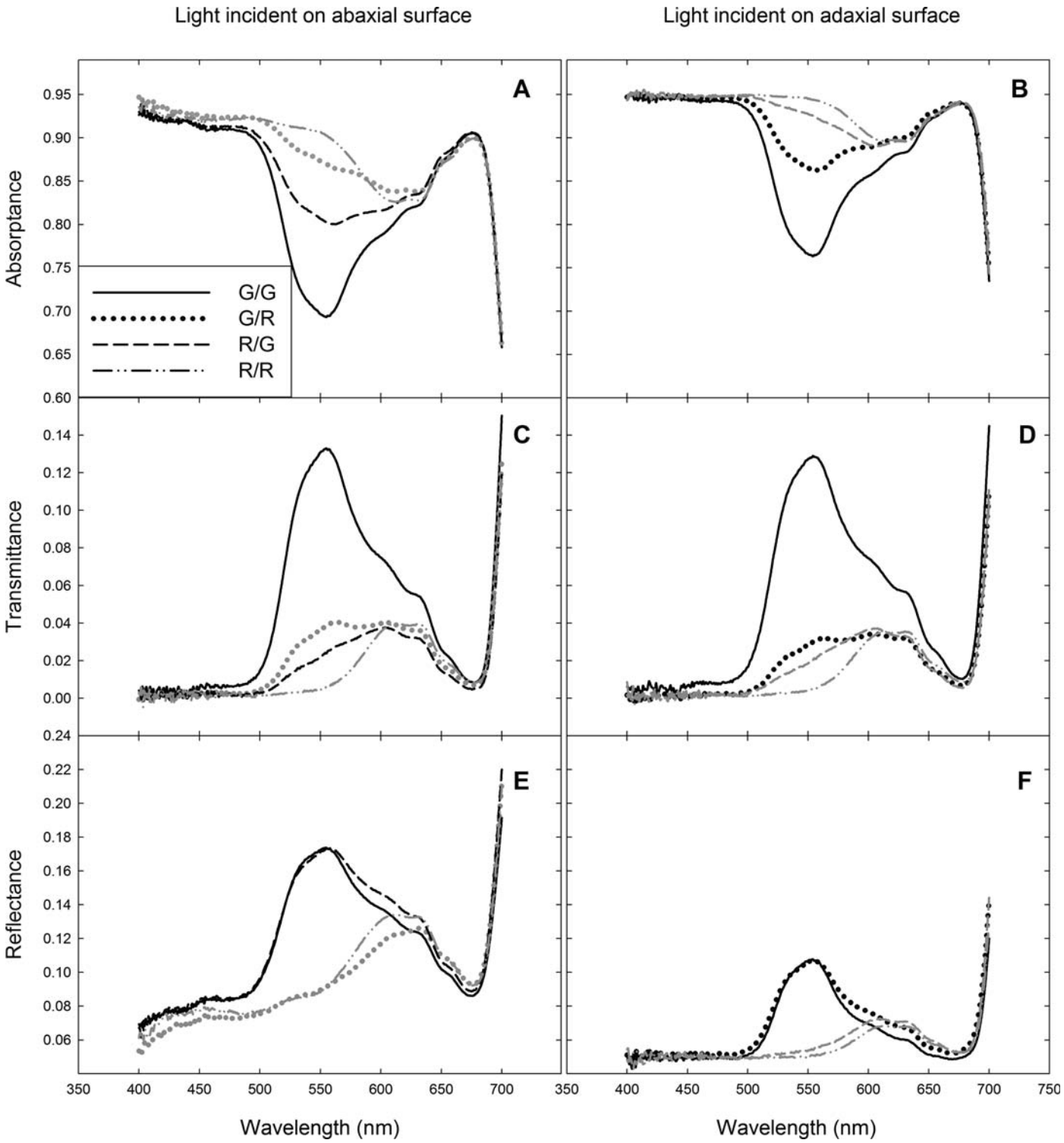

Fig. 2. Spectral scans of *Galax urceolata* leaves with four combinations of pigmented surfaces: green adaxial and green abaxial (solid line), green adaxial and red abaxial (dotted line), red adaxial and green abaxial (dashed line), and red adaxial and red abaxial (dashed and dotted line). Graphs in the left column represent (A) absorptance, (C) transmittance, and (E) reflectance curves of the leaves when light was shone on the lower surface; graphs in the right column represent (B) absorptance, (D) transmittance, and (F) reflectance curves of the leaves when light was shone on the upper surface. Lines are means of three leaves, and the color (black or gray) indicates the color of the surface that received experimental illumination: black represents green surfaces, gray represents red surfaces.

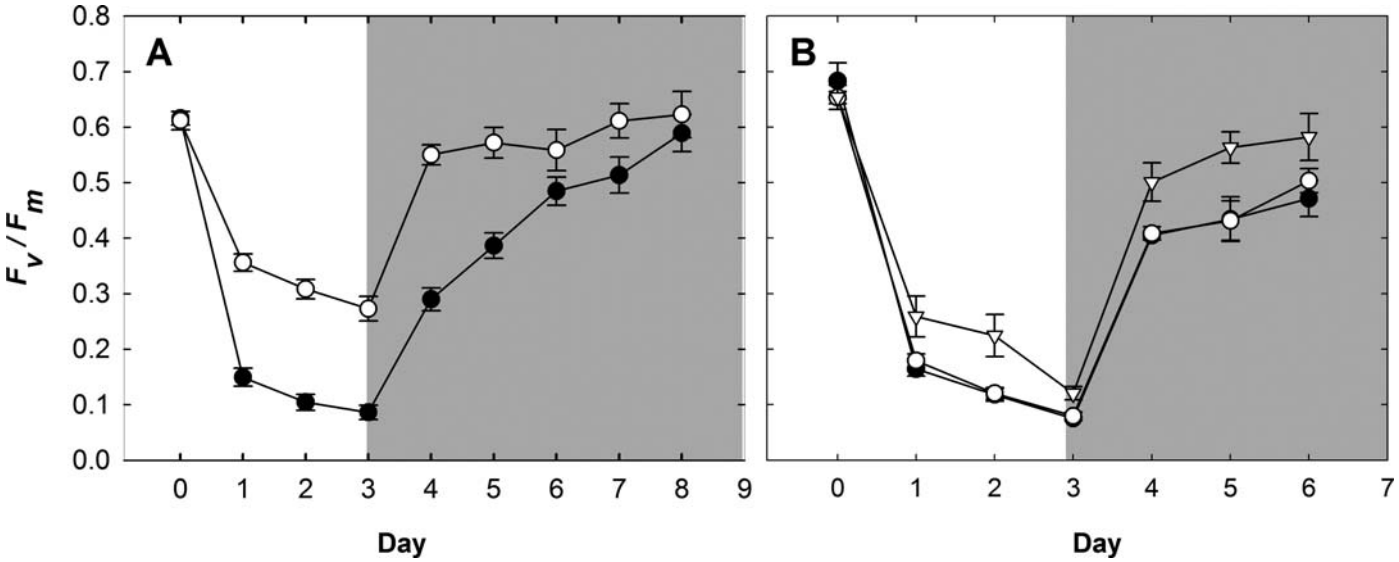

Fig. 3. Recovery of maximum photosystem II efficiency (F_v/F_m) for red and green *Galax urceolata* leaves. In (A), light was shone on adaxial surfaces only; in (B), only abaxial surfaces were illuminated. White areas represent high stress treatments, and gray areas represent recovery periods. Closed circles represent leaves with green adaxial and abaxial surfaces; open circles, leaves with red adaxial and green abaxial surfaces; open triangles, leaves with red adaxial and abaxial surfaces. Individual values are means ± SE of five replicates. Data in panel A and some in panel B are from Hughes et al. (2005).

wavelengths when the light was incident on the abaxial surface (Fig. 2D, E). However, transmittance across the leaf was not affected by the directionality of the light source (Fig. 2C, D).

Green leaf F_v/F_m declined by 86% compared to only 55% for red leaves following exposure to photoinhibitory conditions (Fig. 3A), a difference which was highly significant ($P < 0.0001$). This trend was also observed for abaxial surfaces (Fig. 3B, $P < 0.001$), where red undersides had a significantly lesser decline in F_v/F_m than did green undersides (regardless of whether the opposite surface was red or green). Photoinhibition of red abaxial cells was approximately 10% higher than that of red adaxial cells, though patterns of photoinhibition between all green surfaces (adaxial and abaxial) were similar. Recovery from photoinhibition also appeared to be affected by anthocyanin, because F_v/F_m values of red adaxial surfaces recovered to near-starting values after 1 d, while green adaxial surfaces required 5 d. This trend was also observed with red abaxial surfaces compared to green.

DISCUSSION

Analyses of leaf pigments and thicknesses (Table 1) showed that the leaf phenotypes measured here did not significantly differ in chlorophyll content or leaf thickness, only anthocyanin concentration. Therefore, any deviations in optical properties between leaf types were most likely due to the effect of anthocyanin.

Consistent with known spectral characteristics of anthocyanin, spectral scans indicated that anthocyanins affected leaf optical properties most apparently through their attenuation of blue-green to yellow (500–600 nm) light (Fig. 2), with peak absorptance occurring between 530–550 nm. When light was incident on red surfaces, absorptance of these wavelengths increased by up to 20%, regardless of whether that surface was abaxial or adaxial, or whether the opposite surface contained anthocyanin or not. This suggests that abaxial and adaxial anthocyanic layers may function independently to protect adjacent mesophyll cells or in concert to protect the leaf entire. Increased absorptance of blue-green to yellow light also corresponded with decreased transmittance (Fig. 2C, D) and reflectance (Fig. 2E, F) of these wavelengths. Because absorptance of blue-green light by anthocyanin has been shown to protect leaves from photoinhibition (Feild et al., 2001; Hughes et al., 2005), these data support the idea that anthocyanins on either leaf surface confer photoprotection.

Because leaves with high anthocyanin concentrations appear red, the photoprotective effect of anthocyanin may be misattributed to an increase in reflectance of red light. Because red light is preferentially absorbed by chlorophyll, increasing reflectance of these wavelengths would indeed be effective in curtailing photoinhibition. However, as show in Fig. 2E and F, the presence of anthocyanin does not necessarily increase the percentage of red light reflected by the leaf. Instead, leaves appear red due to the diminished reflectance of other wavelengths (primarily green and blue). As a result, the reflectance peak shifts from green to red as leaves accumulate anthocyanin, though the magnitude of reflectance of red wavelengths does not markedly change (i.e., greater than 1%). In addition, previous work found no difference in photoinhibition between red and green *G. urceolata* leaves illuminated with red light (Hughes et al., 2005). Therefore, any apparent increase in reflectance of red wavelengths afforded by anthocyanin does not convey a photoprotective effect in this species.

When the green surfaces of bicolored leaves were illuminated, anthocyanins still appeared able to intercept up to 10% of green wavelengths that would otherwise have been transmitted through the leaf (Fig. 2A, B). Because an increase in sunlight absorptance often translates into an increase in leaf

temperature, it has been proposed in previous studies that abaxial pigment layers may function to elevate leaf temperature (Smith, 1909; Lee et al., 1979, 1987). Although this function would seem adaptive in plants susceptible to low temperature photoinhibition, several studies of abaxially anthocyanic plants (including *G. urceolata*) have found no difference in leaf temperature between anthocyanic and acyanic leaves in the field (Lee et al., 1979, 1987; Hughes, 2004).

The effects of anthocyanin on maximum photosystem II efficiency (F_v/F_m) during photoinhibitory conditions are shown in Fig. 3. Leaves with red surfaces appeared less susceptible to low temperature photoinhibition (as evidenced by higher F_v/F_m) during the high stress treatment than did leaves with green surfaces, regardless of whether that surface was adaxial (3A) or abaxial (3B). Furthermore, anthocyanic surfaces recovered to starting F_v/F_m values more quickly than acyanic surfaces, suggesting a lesser degree of photodamage had been incurred. In a previous study on *G. urceolata* by the authors (Hughes et al., 2005), it was further shown that photoinhibition of red and green adaxial surfaces were similar under red light (which anthocyanins do not absorb), but under green light (which anthocyanins absorb strongly) red surfaces were significantly less photoinhibited than green. Therefore, we can presume in the case of abaxial anthocyanins as well, that any difference in F_v/F_m is due to the presence of anthocyanin, and not, for example, a difference in the pretreatments (which would have been evident under both red and green light conditions).

An additional trend that is apparent from these data is the increased susceptibility of red abaxial cells to photoinhibition compared to red adaxial cells. This is most likely due to lower anthocyanin content in abaxial relative to adaxial cells of leaves tested in this experiment (data not shown); natural leaf angles of this species generally cause adaxial surfaces to receive the greatest incoming radiation and thus to produce the most anthocyanins. Finally, it was also observed that the presence of anthocyanin in the non-illuminated surface did not appear to have an effect on F_v/F_m (3B), again suggesting an independent function of these layers on photoprotection of their respective surfaces.

Given these findings, which support the idea that abaxial anthocyanins function in light attenuation, what might be the function of abaxial anthocyanins in leaves of deeply shaded understory plants (where abaxial red coloration is most common)? As mentioned previously, although no studies have been able to provide a well-supported explanation, it is possible that abaxial anthocyanins are acting as light-attenuators in shade plants as well. For example, it is possible that abaxial anthocyanin protects the mesophyll from high ambient light (either diffuse or caused by sunflecks) striking the extremely dark-adapted abaxial surfaces. Another possibility, based on current research by the authors on *Begonia* spp., is that abaxial anthocyanins attenuate internally scattered green light (entering the leaf from the adaxial surface), thereby buffering internal light levels during high-intensity sunflecks. This function is consistent with the findings of Gould et al. (1995), in which abaxially red tropical understory plants had significantly higher F_v/F_m than did abaxially green conspecifics.

In conclusion, the results reported here for *Galax urceolata* support the idea that anthocyanins located in either adaxial or abaxial surfaces may confer a photoprotective advantage in tissues exposed to photoinhibitory conditions by effectively intercepting PAR incident on the respective surface. Moreover, the abaxial/adaxial anthocyanic plasticity observed in *G. urceolata* and numerous other species may be an important adaptation in photoinhibitory environments and/or during light-sensitive developmental stages where leaf orientation and/or substrate albedo are variable.

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