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Research

Functional role of anthocyanins in high-light winter leaves of the evergreen herb *Galax urceolata*

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Summary

• High-light leaves of the evergreen herb *Galax urceolata* exhibit a striking color change from green to red during winter months due to anthocyanin synthesis in outermost mesophyll cells. Here we investigate three possible functions of this color change.

• To test the hypothesis that anthocyanins function as light attenuators, maximum photosystem II efficiency (F_v/F_m) of red and green leaves was measured during and after exposure to wavelengths either strongly or poorly absorbed by anthocyanin. To determine whether anthocyanins elevate radical-scavenging capacity, antioxidant activity of red and green leaves was assessed using the α,α-diphenyl-β-picrylhydrazyl assay. Nonstructural carbohydrate levels were analyzed to test the hypothesis that anthocyanins function as a carbon sink.

• Declines in F_v/F_m under white and green light were significantly greater for green than red leaves, but were comparable under red light. Anthocyanin content positively correlated with antioxidant activity. Although levels of anthocyanins did not appear to be related to nonstructural carbohydrate concentration, high levels of sugars may be necessary for their photoinduction.

• Results suggest that anthocyanins function as light attenuators and may also contribute to the antioxidant pool in winter leaves.

Key words: anthocyanin, antioxidant, carbohydrate, evergreen herb, *Galax urceolata*, photoinhibition.

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Introduction

Galax urceolata is an evergreen understory herb native to the Appalachian Mountains. When exposed to extended periods of cold temperatures and high light, *Galax* leaves produce anthocyanins in their outermost mesophyll cells, causing leaves to turn a deep shade of red. Leaves that remain shaded, however, stay green. In the spring, anthocyanins dissipate with the onset of warm temperatures and return of the deciduous canopy. Leaves may subsequently persist for up to three additional growing seasons (McCarron, 1995). Because the appearance of anthocyanins coincides predictably with cold temperatures and high light, one might suspect that these pigments are performing some type of regulated function within *Galax* leaf tissues. Indeed, recent studies have provided much support for the prospect that anthocyanins play more than a merely benign role in leaf tissues, suggesting instead that they may confer a significant degree of protection against photooxidative damage in light-stressed plants by acting as antioxidants and/or light attenuators (Landry *et al.*, 1995; Smillie & Hetherington, 1999; Gould *et al.*, 2000; Merzlyak & Chivkunova, 2000; Feild *et al.*, 2001; Hoch *et al.*, 2001; Neill *et al.*, 2002; Pietrini *et al.*, 2002; Neill & Gould, 2003). With this known, it is surprising that so few studies have been conducted on functional roles of anthocyanins in temperate evergreen plants, as these plants must seasonally endure the stress of excess irradiance compounded by low temperatures (Parker, 1962; Kaku *et al.*, 1992; Grace *et al.*, 1998; Gould *et al.*, 2000). The purpose of this study was to examine the apparent effects of anthocyanins on leaf biochemistry and

ecophysiology of the broadleaf evergreen herb *G. urceolata*, in light of both traditional and less-explored hypotheses of color change, as described below.

One hypothesis that has received much attention in the recent literature proposes that anthocyanins located within the epidermal, palisade, and/or peripheral mesophyll layers of leaves exposed to high irradiances act as light attenuators, absorbing blue-green light that could otherwise be absorbed by chlorophyll *b* in the subjacent mesophyll (Gould *et al.*, 1995; Feild *et al.*, 2001; Hoch *et al.*, 2001; Lee & Gould, 2002; Neill & Gould, 2003). Steyn *et al.* (2002) describe the adaptive value of a green light-absorbing accessory pigment in their review, suggesting that absorbance of these wavelengths would afford protection from a highly abundant, highly energetic and deeply penetrating wavelength of the solar spectrum, without interfering with the function of red and blue light-mediated photoreceptors. Interception of these photons would be especially advantageous to plants that absorb more light than can be effectively converted to chemical energy via photosynthesis, as is the case for many high-light overwintering species.

Because light-harvesting reactions occur essentially independently of temperature (Baker, 1994), an increase in light will result in an increase in energy absorbed by the photosystems, regardless of ambient temperature. However, the rates of biochemical reactions in the Calvin cycle are retarded by cold temperatures, and as temperature decreases, so will the rate of carbon fixation. This imbalance between energy capture and assimilation may lead ultimately to a decline in available open reaction centers and, subsequently, increasing amounts of absorbed energy that are transferred to nonchlorophytic molecules such as xanthophyll pigments and, more deleteriously, oxygen in the surrounding tissue (Demmig-Adams & Adams, 1996; Logan *et al.*, 1998). The transfer of energy to molecular O_2 may then drive the formation of biologically damaging reactive oxygen intermediates (ROIs) including singlet oxygen ($^1O_2$); superoxide (O_2^-); hydrogen peroxide (H_2O_2); and hydroxyl (OH^-) radicals (Ort, 2001; Mittler, 2002). Such an increase in ROIs may lead to tissue damage and even cell death, as the reactive molecules oxidize proteins, peroxidize lipids, inhibit enzymes and damage DNA and RNA (Mittler, 2002).

In addition to absorbing light energy, anthocyanins have demonstrated an additional photoprotective function through their role as antioxidants (Grace *et al.*, 1998; Mittler, 2002; Neill *et al.*, 2002). Classified with ascorbic acid and other flavonoids as low molecular-weight antioxidants (LMWAs), anthocyanins have been shown to scavenge the reactive oxygen molecules H_2O_2, O_2^-, $ONOO^-$, and possibly OH^- and $^1O_2$, and have exhibited roughly four times greater antioxidant capacity than α-tocopherol and ascorbic acid (Lee & Gould, 2002; Mittler, 2002; Neill *et al.*, 2002). Their precursor, chlorogenic acid, possesses an antioxidant activity even greater than this, contributing more to the LMWA pool than any other LMWA in some species (Grace *et al.*, 1998). For these reasons, it has been thought that anthocyanins, when located within photosynthetic mesophyll cells, may be functioning to neutralize excess ROIs as they are produced by organelles during times of stress (Gould *et al.*, 2002). During periods of environmental stress, it is not unusual for plants to increase their production of antioxidants to accommodate increases in ROIs (Grace & Logan, 1996; Mittler, 2002; Neill *et al.*, 2002), which may be another reason why *Galax* produces anthocyanins under high-light, cold-temperature conditions.

An additional hypothesis, which has received significantly less experimental attention than the above, is the possibility that anthocyanins occur as a product of increased carbon flow through the phenylpropanoid pathway when source carbohydrate levels exceed their utilization, and/or transport of these compounds to the rhizome or other storage organs is impeded (such as by freezing of the soils and/or cessation of growth). Because phenylpropanoids are built on a carbon skeleton, with anthocyanins containing an additional glucose molecule, these compounds could conceivably be acting as a temporary carbon sink, remaining sequestered within the vacuole until transport mechanisms are restored. Furthermore, as this theory would predict, when the sink becomes no longer limiting (such as during periods of new growth or temperature increase), anthocyanins diminish. Several studies have observed anthocyanin synthesis to be initiated in response to increases in leaf carbohydrate content (Onslow, 1925; Jeanette *et al.*, 2000), and recent studies have further reported that genes for chalcone synthase (*CHS-A*), dihydroflavonol reductase (*dfr*), and UDPG-flavonoid 3-O-glucosyl transferase (*Bz1*) are sugar-inducible (Tsukaya *et al.*, 1991; Jeanette *et al.*, 2000; Gollop *et al.*, 2002). However, no studies have been conducted to test the possibility that plants which seasonally produce anthocyanins do so as a means of alleviating source : sink imbalances.

The objective of this study was to determine whether any of the above functions might be ascribed to anthocyanin synthesis in high-light winter leaves of *G. urceolata.* Individual hypotheses were tested using a combination of observational and experimental procedures to assess quantitatively the assumptions of each hypothesis in turn, as described below.

Materials and Methods

Plant material

Sun and shade *Galax urceolata* (Poir.) Brummitt used in this study were obtained from the understory of temperate deciduous forests on Long Arm Mountain in Jonas Ridge, NC, USA (35°57′20″ N, 81°53′55″ W). Leaves were derived either directly from plants in the field, or from plants that had been potted and cultivated in a glasshouse 4 months before experimentation. Field plants were located in five separate plots within

a 1-km radius on Long Arm Mountain. Each individual plot contained both sun leaves (red during winter) and shade leaves, with shade being provided either by *Rhododendron*, *Tsuga* or *Kalmia* spp. Plants to be potted were transplanted in July, and were derived from a localized plot on Long Arm Mountain. Potted plants consisted of a rhizome at least 7.5 cm long, and two to three leaves. Potting mix was *c.* 2 : 1 : 1 pine bark : peat : Perlite, with Osmocote Plus 15-9-12 used as a fertilizer. All experiments were conducted on first-year leaves, as previous studies have shown declines in photosynthetic processes of *Galax* leaves as they age (McCarron, 1995). First-year leaves were tagged in the spring to mark leaf age.

Environmental monitoring

Photosynthetic photon flux density (PPFD) was measured at 1-min intervals using an LI-250 light meter equipped with a 190SA quantum sensor (Li-Cor, Inc., Lincoln, NE, USA) connected to a 21x datalogger (Campbell Scientific Inc., Logan, UT, USA). Total daily PPFD was also measured using a microvolt integrator (Delta-T Devices, Cambridge, UK).

Quantification of pigments and total nonstructural carbohydrates

A standard hole puncher was used to excise four 0.28-cm^2 discs from leaves. Discs were submerged in liquid nitrogen for 5 min before extraction, to perforate the waxy cuticle and disrupt cell membranes, before being placed in plastic vials containing 2.5 ml 6 M HCl : H_2O : MeOH (7 : 23 : 70) to extract in the dark at 4°C for 24 h. Anthocyanin content was determined spectrophotometrically as $A_{530} - 0.24A_{653}$ (Murray & Hackett, 1991).

Chlorophyll content was determined using tissue from three leaf discs derived from the same leaves as above. Discs were placed in 3 ml *N*,*N*′-dimethylformamide to extract for 24 h. Solutions were zeroed at 720 nm, and absorbances measured at 664 and 647 nm using a UV-VIS spectrophotometer (Shimadzu Scientific Instruments, Columbia, MD, USA). Chlorophyll pigment concentrations were calculated using the equations described by Porra (2002).

For quantification of total nonstructural carbohydrates (TNCs), freshly cut leaves were immersed in liquid nitrogen, then oven-dried at 50–60°C for at least 24 h. Starch and soluble sugar concentrations were determined enzymatically by the UV method using the Boehringer Mannheim kit (R-Biopharm, Inc., Marshall, MI, USA). Starch and soluble sugars were extracted from 25-mg samples using a solution of dimethylsulfoxide/HCl. Following neutralization and filtration, starch and soluble sugars were assayed according to kit instructions using amyloglucosidase to hydrolyze starch into glucose. Results were reported in units of mg glucose equivalents g^{-1} d. wt.

Leaf optics

Red and green leaf absorbance spectra were derived using leaves that had been removed from plants in the morning and kept on ice in a wet paper towel until measurement later that day. A Li-Cor 1800 spectroradiometer with external integrating sphere was used to measure reflectance and transmission of PPFD at 2-nm intervals for adaxial leaf surfaces. Absorbance was calculated as (1 − transmittance − reflectance). The amount of energy absorbed, reflected and transmitted by the leaves at each wavelength was calculated by multiplying each value by the standard energy contained at each wavelength in ambient sunlight.

Light effects on pigments and biomolecules

To quantify the effects of light intensity on pigment and carbohydrate concentrations, shade structures (0.25 m^2) constructed from PVC pipe and neutral density cloth, providing either 80, 60, 40, 20 or 0% shade, were placed randomly within a single, large (20 m^2), naturally occurring high-light *Galax* plot. A second series of shade treatments was also established using potted plants in a location completely free of overstory. The shade treatments were set in place during October 2003 before leaves had begun to turn red. Each shade treatment contained at least 10 healthy green leaves. On 16 December 2003, leaves of potted plants were harvested for quantification of pigments and TNCs using the protocols described previously; leaves from the natural plot were harvested and analyzed on 13 January.

To determine whether UV light was necessary to induce anthocyanin synthesis, 1-m^2 PVC shade structures covered with either mylar (to exclude UV) or Teflon (UV-transparent) were placed over both naturally occurring high-light plots and potted plants in the autumn, before color change. Presence or absence of anthocyanins was determined in the winter by assessing visually whether leaves exhibited red coloration.

Chlorophyll fluorescence

Chlorophyll fluorescence was used to assess relative photooxidative stress of leaves in this study. Before measurement, plants were dark-adapted for 30 min using Handy-PEA leaf clips. A Handy-PEA 1000 fluorescence analyzer (Hansatech Institute, Cambridge, UK) emitting a 2-s 3 mmol m^{-2} s^{-1} saturating pulse was then used to measure the ratio of variable fluorescence to maximum fluorescence (F_v/F_m), representing maximum efficiency of photosystem II (PSII) (Maxwell & Johnson, 2000).

High-stress recovery

The fluorescence-based responses of red and green leaves with equal starting F_v/F_m values were monitored during and after exposure to a high light-stress period to test the hypothesis

that anthocyanins confer photoprotection under high light stress. For each test, five leaves were removed from either a single red or a single green clone (located within 50 m of each other) between 09 : 00 and 10 : 00 h during February 2004. The petiole of each leaf was cut under water, and remained submerged throughout the experiment. As F_v/F_m values of red leaves in the field are naturally much lower than those of green leaves (reflecting adaptation to a higher light environment), red and green leaves were placed in separate transient environments for 4 d before experimentation, in order to approximately equalize slow-recovery nonphotochemical quenching, and thus F_v/F_m, before experimentation. This was done so that any divergence in fluorescence between the groups during experimental high light exposure could be more readily attributed to anthocyanin-based differences rather than merely differences in starting sustained xanthophyll pigment molecule ratios and/or differences in activated D1/D2 protein/PSII cores. The green leaf transient period consisted of 4 d within an outdoor protected enclosure, where leaves were exposed to 10 h 175 (±25) μmol m^{-2} s^{-1} at field temperatures (−10 to 15°C). Light was supplemented during this time by a 1000-W metal halide lamp equipped with a UV filter, a water bath, and shade cloths to obtain the desired PPFDs. Red leaves were simultaneously placed indoors and exposed to similar PPFDs at a constant 18°C. Once values of F_v/F_m were no longer significantly different between the two groups, both sets of leaves were placed in the dark within the outdoor protected enclosure overnight. At 07 : 00 h, starting F_v/F_m was measured for each group, and the high-stress treatment was subsequently applied. This treatment consisted of 10 h high light (1150 ± 150 μmol m^{-2} s^{-1} from the metal halide bulb) combined with cold temperatures (circulating outside air, which ranged from 0 to 10°C during the day, and −15 to 0°C during the night). A glass water bath placed between the light source and the leaves absorbed heat emitted by the bulb during this period. After 3 d in the high-stress environment, plants were moved into a low-stress environment [18°C constant temperature, 10 h 175 (±25) μmol m^{-2} s^{-1} provided by a 65-W incandescent flood bulb] until F_v/F_m recovered to starting values. F_v/F_m was measured at 17 : 00 h on each day of the high-stress and recovery treatments. This experiment was conducted on both abaxial and adaxial surfaces of separate sets of pretreated leaves, as well as on adaxial surfaces of leaves that had been removed from the field 1 h before the experiment (with no pretreatment).

This experiment was also repeated on adaxial surfaces of leaves using light filtered through either red or green (750 and 550 nm peak transmittances, respectively) glass filters (Schott Glass, Grünenplan, Germany), to assess more specifically the effects of anthocyanin's absorptive properties on photoprotection. Before experimentation, leaves were harvested and pretreated as described above. PPFDs under both filters ranged from 400 to 800 μmol m^{-2} s^{-1}. Because of the wide variation of PPFDs beneath the filters, red and green leaves were arranged and rotated daily so that leaves within both groups experienced the entire range of PPFDs.

Antioxidant capacity

Low molecular-weight antioxidant activities were evaluated using the stable radical α,α-diphenyl-β-picrylhydrazyl (DPPH) protocol described by Neill *et al.* (2002). Summer leaves were collected in July 2003, winter leaves in January and February 2004. All leaves were collected from Long Arm Mountain between 16 : 00 and 17 : 00 h. Leaves were immediately placed in liquid nitrogen, then freeze-dried before analysis. Freeze-dried tissue (25 mg) was extracted in 5 ml extraction buffer, and the assay was run using a 180 μM DPPH solution instead of 18 μM. Molarity of DPPH was altered because of the increase in tissue : extractant used in this study relative to Neill *et al.* (2002). Anthocyanin content was measured directly using an aliquot of the extracted solution. LMWA activity was quantified using IC_{50} values, which represent the concentration of leaf extract (μg d. wt ml^{-1}) needed to neutralize the DPPH by 50%.

Vein severing

A scapel was used to sever major veins <1 cm from the center main vein on one side of five intact high-light green leaves in early autumn, to inhibit carbohydrate export and observe the inducibility of anthocyanin synthesis in response to increases in leaf carbohydrate concentration. After 10 d, anthocyanins and TNCs were quantified using the protocols described previously.

Statistical analyses

Regression analyses were used to correlate light intensity with F_v/F_m values, anthocyanin, chlorophyll and carbohydrate concentrations, as well as to correlate anthocyanin with carbohydrate concentration and antioxidant activity. Trends in fluorescence were analyzed using repeated-measures ANOVAs. A two-factor ANOVA was used to compare pigment and IC_{50} values, and pigment and carbohydrate ratios between sun and shade plants within and between seasons. Two-sample *t*-tests were used to compare total energy absorbed between 400 and 700 nm; energy absorbed at blue/green wavelengths (500–600 nm) for red and green leaves; and mean IC_{50} values of leaf groups. Anthocyanin and TNC levels of severed and unsevered sides of leaves were compared using paired *t*-tests. Significance for all tests was assumed if $P < 0.05$.

Results

Effects of light intensity on anthocyanin, chlorophyll and TNC levels

Of the three naturally occurring *Galax* plots sampled in this study, leaves that exhibited reddening were found in

environments exposed to substantially more light than leaves that remained green. Total daily PPFDs incident upon red leaves on a clear winter day averaged *c.* 135 (±9) mmol m^{-2}, whereas average total PPFDs of green leaves averaged only 12 (±1.5) mmol m^{-2}, a *c.* 10-fold difference. Additionally, maximum PPFDs incurred by red leaves often surpassed 1000 µmol m^{-2} s^{-1}, while PPFDs of green plots seldom exceeded 400 µmol m^{-2} s^{-1}. A winter diel PPFD pattern for a typical plot containing both red and green leaves, with corresponding F_v/F_m values of sample leaves, is illustrated in Fig. 1.

High-light leaves that had been sequentially shaded with neutral density cloth before reddening exhibited an anthocyanin gradient that increased linearly with light intensity [$P < 0.0001$, $r^2 = 0.76$ for potted plants (Fig. 2a); $r^2 = 0.84$ for field plants (Fig. 2b)]. Leaves from the 0% shade treatment exhibited an average 23-fold higher anthocyanin content than leaves in the 80% shade treatment. In both experiments, leaves in the 80% shade treatment were the only leaves that exhibited no visible reddening. *Galax* leaves exhibiting various shades of reddening are depicted in Fig. 3(a).

At the tissue level, anthocyanins were observed to occur in the vacuoles (Fig. 3b) of peripheral mesophyll cells closest to the epidermis (Fig. 3c). Pigmentation was observed to occur on both abaxial and adaxial surfaces, depending on the orientation of the leaf relative to the light source. For those leaves oriented with adaxial surfaces facing the light source, with abaxial surfaces covered by litter or another leaf, the adaxial surface alone exhibited pigmentation. For those leaves oriented upside down, only the abaxial surface exhibited pigmentation. If the light was incident upon the adaxial surface, and the leaf's abaxial surface was uncovered by litter or other leaves, both adaxial and abaxial surfaces were frequently observed to exhibit pigmentation (Fig. 3c).

Levels of chlorophyll *a* and *b*, and the ratio of chlorophyll *a* : *b*, did not differ significantly between sun and shade plants during the summer. However, during the winter the ratio of chlorophyll *a* : *b* was significantly lower in sun plants compared with shade ($P = 0.005$), and was found to decrease significantly with light intensity [$P < 0.005$, $r^2 = 0.36$ for potted plants (Fig. 2c); $r^2 = 0.42$ for field plants (Fig. 2d)]. This decline appeared to be caused by decreases in chlorophyll *a*, as sun leaves exhibited an average of 10% less chlorophyll *a* than shade leaves, but only 2% less chlorophyll *b* (Table 1).

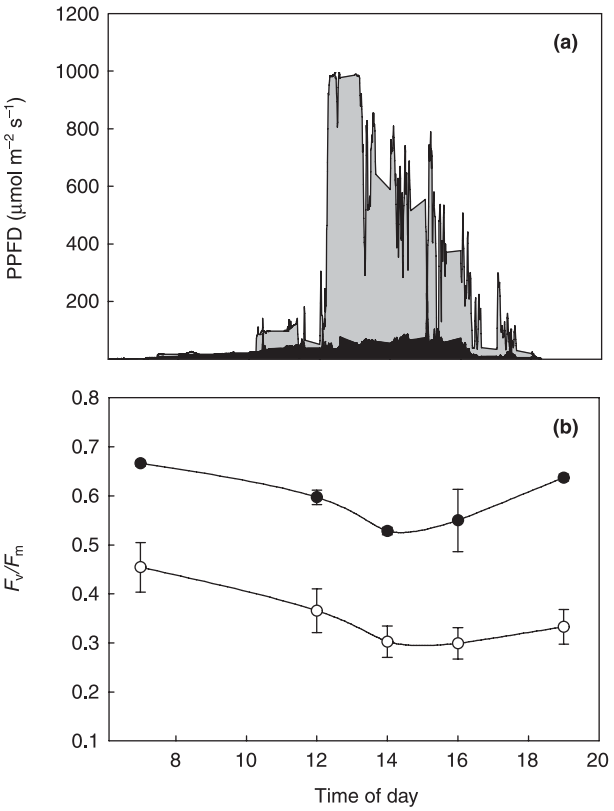

Fig. 1 Typical diel pattern for (a) PPFD; (b) ratio of variable to maximum fluorescence (maximum PSII efficiency, F_v/F_m) of red (light shading or open symbol) and green (dark shading or closed symbol) *Galax urceolata* leaves in the field (taken 3 March 2004).

Table 1 Levels of biochemicals for summer and winter high- and low-light leaves of *Galax urceolata* in the field

Leaves	Anthocyanin content (A_{530})	Chl *a* (µg cm^{-2})	Chl *b* (µg cm^{-2})	Total Chl (µg cm^{-2})	Chl *a* : *b*	Starch (mg g^{-1})	Soluble sugars (mg g^{-1})	TNC (mg g^{-1})
Summer sun	0.0201^b	34.4^a	12.9^a	47.3^a	2.70^a	25.3^a	45.7^b	71.0^a
	(0.0010)	(2.1)	(1.2)	(3.2)	(0.11)	(3.1)	(1.2)	(3.6)
Summer shade	0.0229^b	36.4^a	15.6^a	52.0^a	2.47^a	0.00^c	32.7^b	32.7^b
	(0.0012)	(2.4)	(2.1)	(4.3)	(0.19)	(0.0)	(1.2)	(1.2)
Winter sun	1.36^a	24.8^b	11.1^b	35.9^b	2.24^b	0.300^b	74.6^a	74.9^a
	(0.17)	(1.2)	(0.52)	(1.7)	(0.019)	(0.30)	(0.8)	(1.0)
Winter shade	0.0500^b	27.6^b	11.3^b	38.9^b	2.45^a	0.100^c	71.8^a	71.9^a
	(0.010)	(0.69)	(0.46)	(1.1)	(0.046)	(0.10)	(3.8)	(3.8)

Winter sun leaves are red, all others are green. Values are means from five samples (± SE). Means within columns not followed by the same letter are different at $P < 0.06$. TNC, total nonstructural carbohydrates. Means within a column followed by different letters are different at $P < 0.06$.

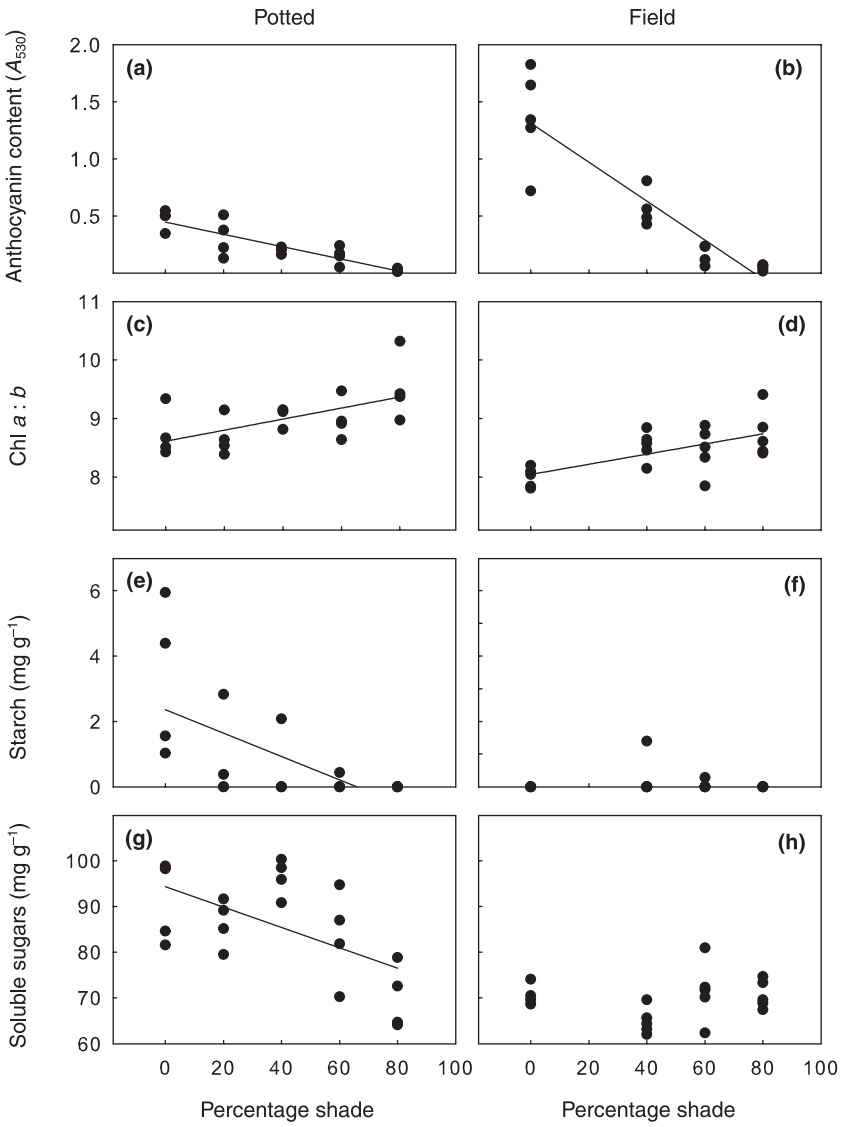

Fig. 2 Percentage shade effects on leaf chemistry for *Galax urceolata* plants in pots and plants in the field: (a,b) anthocyanin content; (c,d) chlorophyll *a* : *b* ratio; (e,f) starch content; and (g,h) soluble sugars. Coefficients of regression for relationships with $P < 0.05$: (a) $r^2 = 0.76$; (b) $r^2 = 0.84$; (c) $r^2 = 0.36$; (d) $r^2 = 0.42$; (e) $r^2 = 0.39$; (g) $r^2 = 0.33$.

The effects of shade on TNCs yielded less consistent results between field and potted plant groups during the winter. Shade had no effect on starch or soluble sugar content in field plants ($P = 0.95$ and 0.65, respectively, Fig. 2f,h), but a very significant effect in potted plants ($P < 0.01$, $r^2 = 0.386$; $P < 0.01$, $r^2 = 0.329$, respectively, Fig. 2e,g), with starches and soluble sugars both increasing with light intensity. Potted plants also generally exhibited higher levels of carbohydrates than field plants at most shade levels.

UV light was not necessary to induce anthocyanin synthesis, as green leaves were able to synthesize anthocyanins in both the presence and absence of UV (data not shown). This was observed in plants in the natural habitat (under UV screens); plants that had been potted (under UV screens); and green leaves that had been cut at the petiole and placed in water (exposed to light through a UV filter).

Seasonal variation in pigments and nonstructural carbohydrates

Chlorophyll *a* and *b* levels were both significantly lower in winter leaves compared with summer leaves ($P = 0.002$ and $P = 0.058$, respectively). Seasonal comparison of TNCs showed that starch was significantly higher in summer sun leaves than winter sun leaves ($P < 0.0001$), although starch levels did not differ significantly between summer and winter shade leaves (both groups averaged close to 0 mg g^{-1}). Both sun and shade winter leaves exhibited significantly higher soluble sugar content than summer leaves, with a nearly twofold increase ($P < 0.0001$). Total nonstructural carbohydrates did not differ significantly between summer and winter sun plants, although TNCs were twofold higher in shade winter leaves compared with shade summer leaves ($P < 0.0001$)

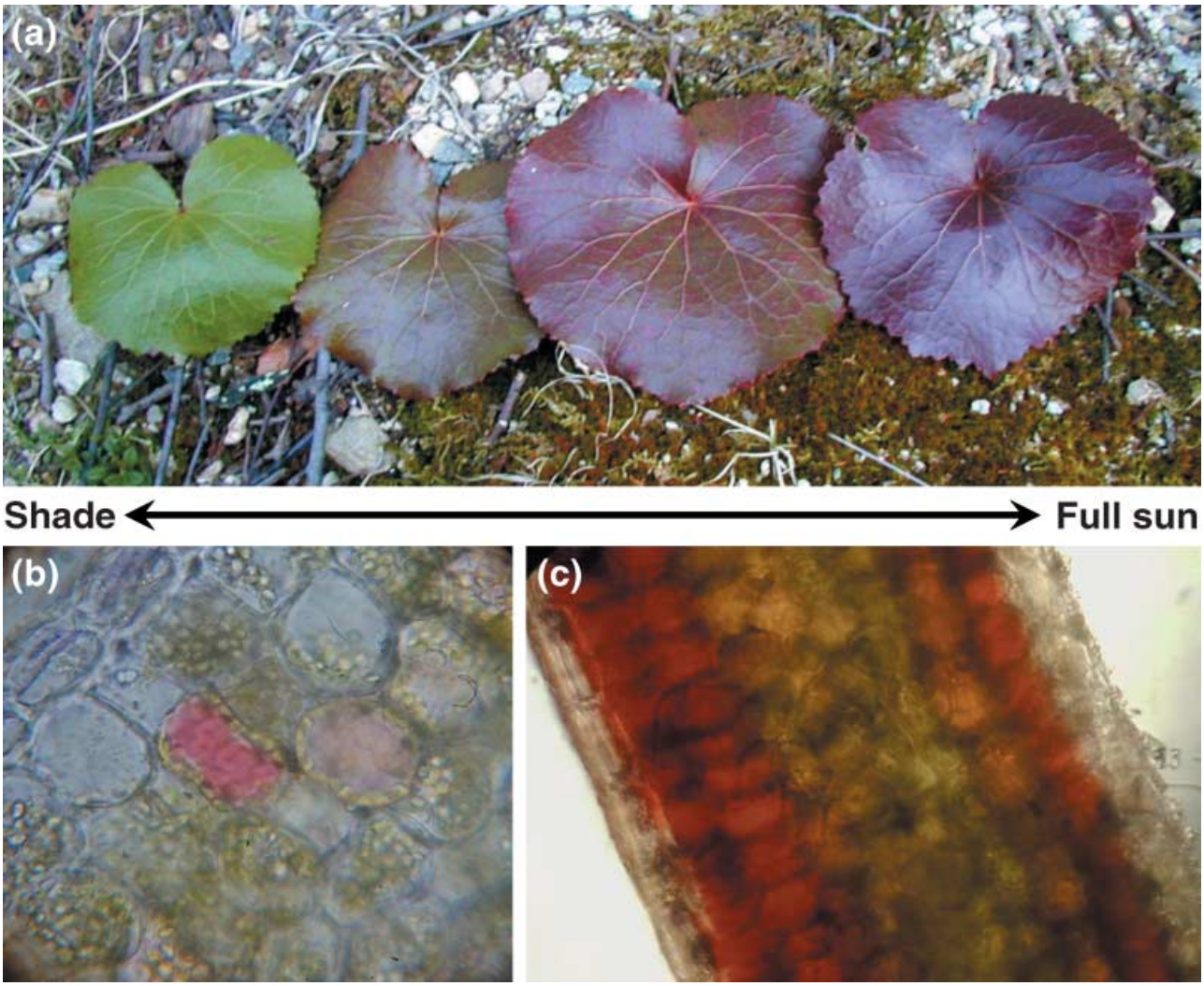

Fig. 3 (a) *Galax urceolata* leaves grown in varying levels of shade; (b) localization of anthocyanins in vacuoles (×430); (c) anthocyanins in mesophyll tissues on both adaxial (left-facing) and abaxial (right-facing) leaf surfaces (×100).

because of the increased soluble sugar content. Anthocyanins were never observed visually in summer plants, and were present only at low concentrations in shade winter plants, although they were observed consistently in winter sun plants.

Chlorophyll fluorescence profiles of red and green leaves

Red leaves exhibited significantly and consistently lower dark-adapted maximum PSII efficiency values (F_v/F_m) than green leaves in the field ($P < 0.0001$). This difference corresponded with the level of irradiance incident upon the leaves, as F_v/F_m decreased with light intensity ($r^2 = 0.87$, Fig. 4).

Leaf optics

Red leaves absorbed significantly more light energy compared with green leaves, most notably in the green (500–600 nm) wavelengths (Fig. 5). When percentage absorbance was converted to energy equivalents, red leaves were found to absorb 11% more light energy in green wavelengths than green leaves, a difference which was highly significant ($P < 0.0001$).

High-stress recovery

When adaxial surfaces of red and green leaves of similar starting F_v/F_m values were exposed to a high white light-stress treatment, mean green leaf F_v/F_m was observed to decline by 86%, while F_v/F_m of red leaves declined by only 55%. Subsequently, red leaves recovered to near-starting F_v/F_m values after 1 d, while green leaves required 5 d (Fig. 6a). In leaves exposed to the high-stress treatment immediately following removal from the field (without a pretreatment period to equalize F_v/F_m), very similar trends were observed. Green leaves exhibited declines in F_v/F_m of *c.* 78%, while red leaves exhibited declines

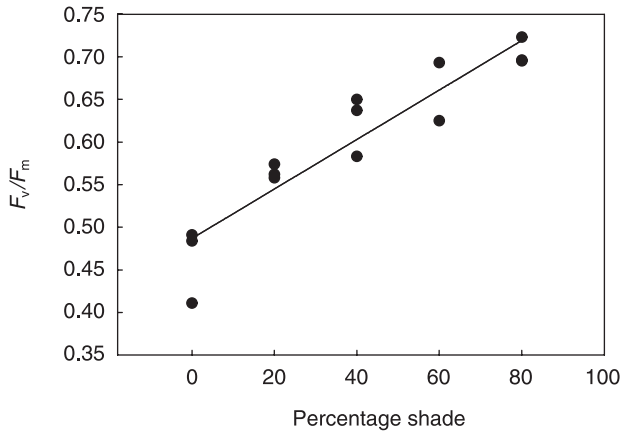

Fig. 4 Ratio of variable to maximum fluorescence (maximum PSII efficiency, F_v/F_m) as a function of light intensity, $r^2 = 0.87$. Data derived from sequentially shaded field *Galax urceolata* plants on 15 December (minimum temperature −4°C, maximum temperature 0°C). Points represent three randomly selected leaves from each shade treatment.

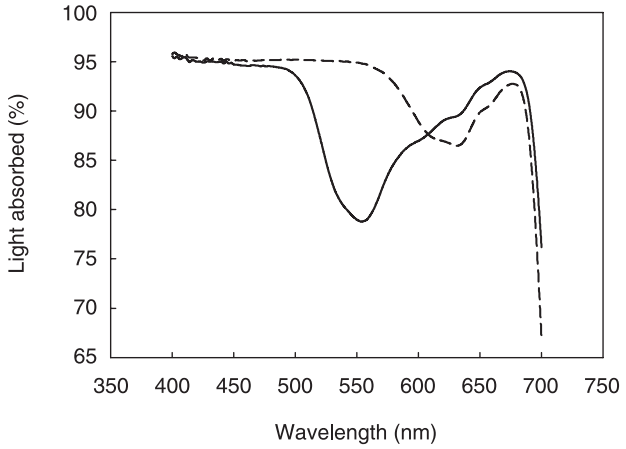

Fig. 5 Absorbance spectra for red (dashed line) and green (solid line) *Galax urceolata* leaves. Lines represent means of five leaves.

of only 52% (Fig. 6b). Additionally, red leaves recovered to starting F_v/F_m after 1 d, while green leaves required 4 d.

This experiment was repeated (with pretreatment) on the green abaxial surfaces of red and green leaves to compare F_v/F_m recovery rates without interference by anthocyanins (Fig. 7). Decline and recovery of the green undersides of both red and green leaves were nearly identical, with a decrease in F_v/F_m of *c.* 88% during the high-stress period. Recovery rates of both groups of leaves were similar to those exhibited by the green adaxial surfaces (Fig. 6a).

In response to a green light-stress treatment (Fig. 8a), red leaves exhibited significantly less decline in maximum PSII efficiency compared with green leaves, as red leaf F_v/F_m declined by 19% in response to the high-light treatment, while green leaf F_v/F_m declined by 72%. Red leaves required 2 d to recover to starting F_v/F_m values, while green leaves required 3 d. On exposure to red light stress (Fig. 8b), however, declines in maximum PSII efficiency were nearly identical between groups, with both red and green leaf groups exhibiting a 63% decline in F_v/F_m; all leaves in this treatment recovered to near starting F_v/F_m values after 1 d of recovery.

Antioxidants

LMWA activities of winter leaves were significantly higher than summer leaves ($P < 0.0001$), with both sun and shade

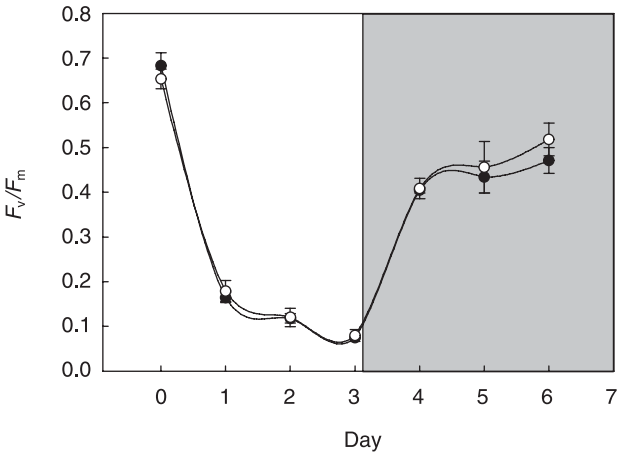

Fig. 7 Recovery of maximum PSII efficiency (F_v/F_m) for pretreated red (open symbols) and green (closed symbols) *Galax urceolata* leaves. Light was shone on acyanic abaxial surfaces only. White area, high-stress treatment; gray area, recovery period. Points depicted are means ± SE of five replicates.

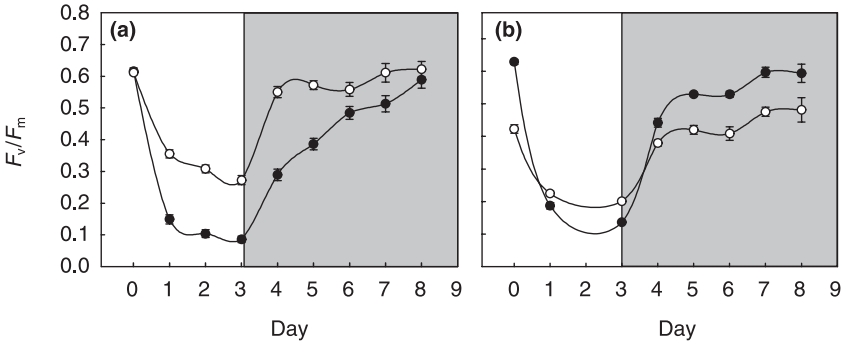

Fig. 6 Recovery of maximum PSII efficiency (F_v/F_m) for (a) pretreated; (b) nonpretreated red and green *Galax urceolata* leaves. Light was shone on adaxial surfaces only. Red leaves, open symbols; green leaves, closed symbols; white area, high-stress treatment; gray area, recovery period. Points depicted are means ± SE of five replicates.

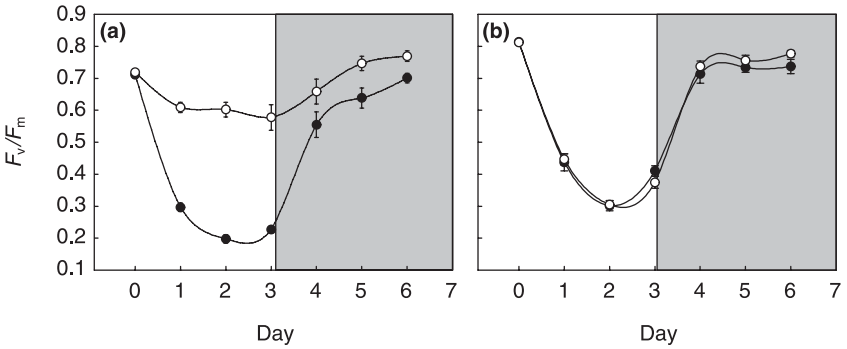

Fig. 8 Recovery of maximum PSII efficiency (F_v/F_m) for pretreated red (open symbols) and green (closed symbols) *Galax urceolata* leaves to high-stress period consisting of (a) green light; (b) red light. Light was shone on adaxial surfaces only. White area, high-stress period, gray area, recovery period. Points depicted are means ± SE of five replicates.

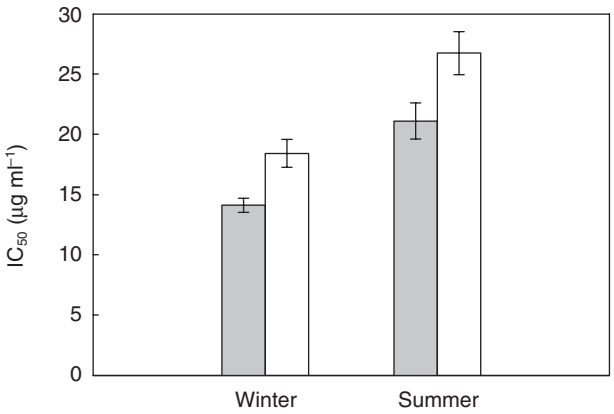

Fig. 9 Mean IC_{50} by light environment and season. Bars represent means ± SE for five to 14 replicates. Gray columns, sun; white columns, shade.

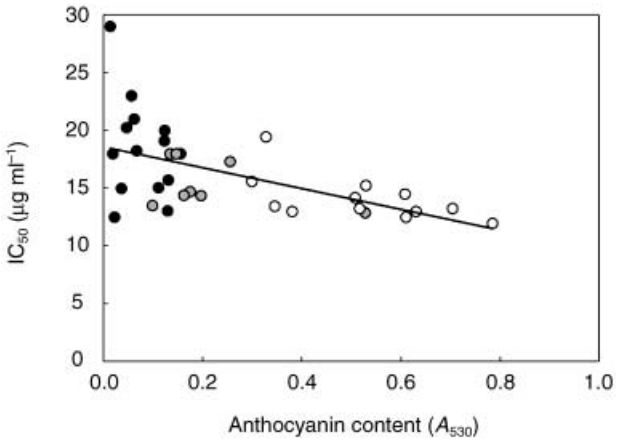

Fig. 10 IC_{50} as a function of anthocyanin content (A_{530}); $r^2 = 0.32$. Black circles, green leaves; gray circles, intermediate leaves; white circles, red leaves.

groups averaging 1.5× lower IC_{50} values during the winter (Fig. 9). Sun leaves exhibited antioxidant activities higher than shade leaves in both seasons, although these differences were significant only during the winter ($P = 0.003$); during the summer the difference was only marginally significant ($P = 0.066$). Anthocyanin content was positively correlated with antioxidant activity ($P = 0.0005$), with leaves of the highest anthocyanin concentrations tending to exhibit the highest antioxidant activities; the r^2 value for this relationship was 0.32 (Fig. 10).

Vein severing

Severing of major veins resulted in significant increases in starch ($P = 0.0003$), soluble sugars ($P = 0.0002$), and hence TNCs ($P = 0.0002$) relative to the half of the leaf where veins had not been severed (Fig. 11a). Furthermore, the halves of leaves with severed veins produced significantly and visibly more anthocyanins ($P = 0.0002$) than unsevered halves (Fig. 11b). However, no consistent linear relationships between anthocyanins and starches, sugars or TNCs were seen in this experiment.

Discussion

The fundamental assumption that anthocyanins occur in leaves most susceptible to light stress was illustrated clearly in this study. As light intensity increased under cold conditions, susceptibility of *Galax* plants to photooxidative damage increased proportionally, as illustrated by decreases in F_v/F_m observed in plants grown across a light gradient (Fig. 4). This decline in F_v/F_m in response to increasing irradiance has been documented in several species, and has been attributed to the upregulation of PSII-specific photoprotective mechanisms implemented as a response to increased photostress. These photoprotective responses include increases in xanthophyll pool size, increased conversion of violaxanthin to zeaxanthin, and selective degradation and/or sustained-phosphorylation of D1/D2 protein and whole PSII cores (Adams *et al.*, 1994; Verhoeven *et al.*, 1996; Adams *et al.*, 2001; Ebbert *et al.*, 2001). The observation that anthocyanin synthesis in winter leaves is proportional to light intensity (Fig. 2a,b) and occurs concomitantly with declines in maximum PSII efficiency (Fig. 4), therefore strengthens the prospect that anthocyanins are produced in response to increasing light stress, and thus may serve some type of photoprotective function.

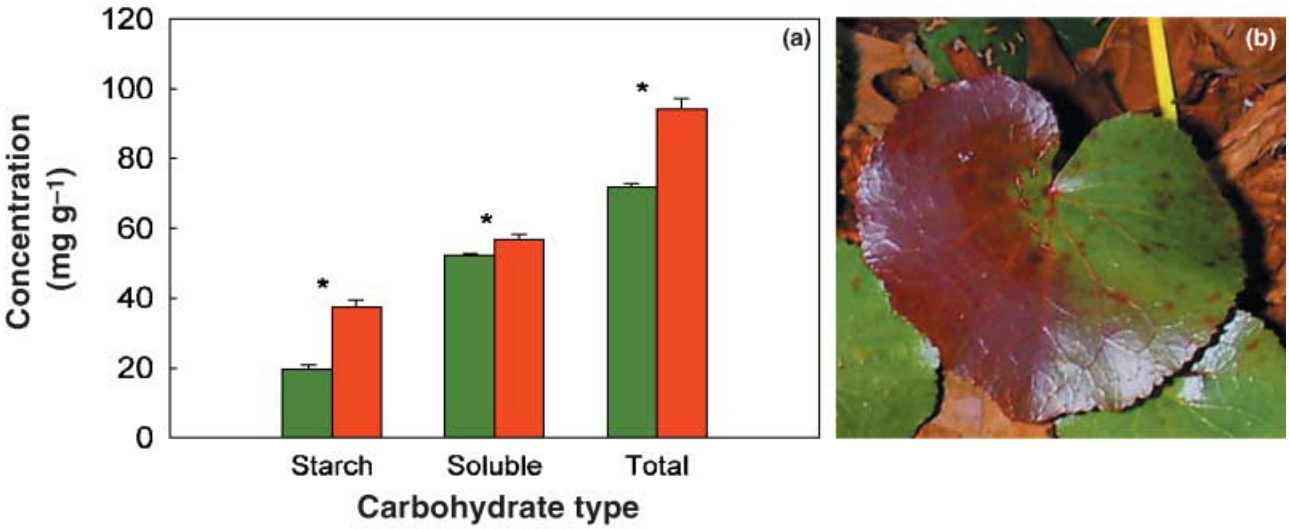

Fig. 11 (a) Effects of vein severing on starch, soluble sugar and total nonstructural carbohydrate concentrations. Bars represent means ± SE of five replicates; significant differences denoted by asterisks. Green bars, unsevered; red bars, severed. (b) Effects of vein severing on anthocyanin synthesis. In the *Galax urceolata* leaf pictured, veins on the left side of the leaf were severed (<1 cm from center main vein) while veins on the right side were not (16 October 2003).

The first hypothesis described in this study proposed that anthocyanins protect underlying mesophyll cells in high-light leaves by absorbing blue-green light. In contrast to the heat-dissipating process of the xanthophyll pigments, which are located in the antennae complex and accept energy directly from excited chlorophyll molecules, anthocyanins are exclusively vacuolar (Fig. 3b) and therefore intercept and dissipate light energy as yet unabsorbed by other pigments. The light attenuation hypothesis predicts that the presence of anthocyanins should cause cyanic leaves to exhibit less photooxidative stress than acyanic leaves when exposed to equally high levels of irradiance perpendicularly to the cyanic surface, assuming starting levels of other photoprotective mechanisms to be roughly equal. However, if the light is shone on any acyanic regions of red leaves, such as the green abaxial surface, those tissues should exhibit declines in F_v/F_m comparable with green leaves. Both these predictions gained experimental support in this study. As shown in Fig. 6(a), leaves with red and green adaxial surfaces and equal starting F_v/F_m values exhibited drastically different fluorescence responses when exposed to high PPFDs, as the decline in F_v/F_m of green leaves was 29% greater than that observed in red leaves. Furthermore, green leaves required four more days than red leaves to recover to starting maximum PSII efficiency, suggesting that red leaves had experienced a lesser degree of photooxidative stress than green leaves during the high-light period. When the light was shone on the acyanic abaxial surfaces, however, maximum PSII efficiency of red and green leaves both declined by 88% (Fig. 7) and exhibited recovery rates similar to those of adaxial green surfaces.

In order to attribute the decreased high light-stress susceptibility of red leaves more specifically to anthocyanin, adaxial surfaces of red and green leaves were again exposed to high levels of irradiance, but at wavelengths that were either strongly or poorly absorbed by anthocyanins. On exposure to green light (Fig. 8a), which anthocyanins absorb strongly, green leaves exhibited a significantly higher degree of photooxidative stress than red leaves; however, when leaves were exposed to red light (Fig. 8b), which anthocyanins absorb poorly, red and green leaves exhibited equal declines in F_v/F_m. These findings indicate that anthocyanin-containing leaves are less susceptible than acyanic leaves to photooxidative damage induced by green wavelengths, but that they are equally susceptible to damage induced by red wavelengths. As anthocyanin is presumably the only plant pigment present in *Galax* which absorbs strongly in green wavelengths and weakly in red, the hypothesis that anthocyanins function to protect high-light leaves from excess irradiance gains strong support.

The absorbance of blue-green light by anthocyanins may also be responsible for the changes in chlorophyll ratios observed in cyanic leaves during the winter. As shown in Fig. 2(c,d), chlorophyll *a* : *b* ratios were observed to decrease significantly as light intensity (and anthocyanin concentration) increased. This trend is unusual, given that pigment analyses of evergreens in previous studies have shown increases in chlorophyll *a* : *b* in response to increasing irradiance, rather than decreases, presumably corresponding to higher ratios of reaction centers to light-harvesting complexes, indicative of a physiological shift away from light capture and towards carbon fixation (Cui *et al.*, 1991; Grace & Logan, 1996; Demmig-Adams, 1998). It is interesting to note, however, that chlorophyll ratios of other plant species that produce anthocyanins also exhibit lower chlorophyll *a* : *b* ratios in red leaves compared with green, even when there is no difference in the light

environment in which they are grown. Such species include the tropical understory plants *Begonia pavonina* and *Triolena hirsuta*, and the evergreen shrub *Mahonia repens* (Gould *et al.*, 1995; Grace & Logan, 1996). The propensity for red leaves consistently to exhibit lower chlorophyll *a* : *b* ratios than green conspecifics suggests either that anthocyanin concentration is somehow related to this change, or that chlorophyll spectrophotometric assays are affected by the presence of anthocyanin. Unpublished data of Sims & Gamon (1999) support the latter explanation, as their study suggests that failure to account for anthocyanin concentrations in spectrophotometric assays could indeed affect chlorophyll *a* : *b* ratios, resulting in patterns observed here and in the studies described previously. Further studies should be conducted to elucidate this relationship.

Results from leaf antioxidant analyses further support our assumption that high-light winter leaves are prone to greater photooxidative stress than summer leaves and leaves that are shaded during winter. This is illustrated in Fig. 9, where winter sun (red) leaves are shown to exhibit the highest mean antioxidant activity of all leaf groups, reflecting a need for increased protection from photooxidation (Grace & Logan, 1996). Our study also showed a significant positive correlation between anthocyanin content and antioxidant activity (Fig. 10); however, this correlation does not necessarily suggest that the anthocyanins are the proximate cause for this elevation of LMWA activity, although it is possible that they may be involved. It is apparent from data in Fig. 10 that many green leaves exhibited antioxidant activities equal to or greater than those of some very red leaves. The latter results indicate that increases in antioxidant potential are not necessarily contingent on anthocyanin synthesis, and therefore anthocyanins are probably not synthesized solely for their adaptive value as antioxidants in *Galax*. The likelihood that anthocyanins are active contributors to the antioxidant pool in *Galax* is reduced further by the fact that anthocyanins are sequestered in the vacuoles (Fig. 3b), rather than the chloroplasts or cytosol, and are thus spatially separated from the parts of the cell in which radical oxygen species are most abundant. Subsequent assays that test the relative contributions of individual antioxidants in the LMWA pool would help to elucidate these results.

The above findings regarding high LMWA activity in green leaves may initially appear counterintuitive, given observations that antioxidants levels are typically inversely proportional to shade levels in cold-acclimated, broad-leaved evergreen species (Grace & Logan, 1996). However, they could be explained by the fact that shade leaves used in our study were prone to periodic exposure by potentially damaging sunflecks. Although winter green leaves are shaded throughout most of the day, sunflecks exceeding 500 μmol $m^{-2}s^{-1}$ were observed to occur in some green plots (Hughes, 2004), including those sampled in this study. A well known cost of keeping photosystems primed for energy capture in the deep shade is an increased vulnerability to photooxidative damage caused by sunflecks when they occur (Demmig-Adams & Adams, 1992; Watling *et al.*, 1997). It is conceivable, then, that a shade plant which experiences frequent sunflecks may maintain a steady pool of antioxidants as a protective measure throughout the day, as the intensity of sunflecks may result in high-light stress (and hence increased ROI production), but their brevity would not allow plants effectively to engage PSII-related photoprotective mechanisms. The fact that the leaves used in this study were sampled from their natural environment, where sunflecks readily occur, should be taken into account when comparing these results with those of studies where plants were grown in homogeneous light environments, such as Grace & Logan's (1996) study on broad-leaved evergreens.

Analyses of leaf carbohydrates yielded results that suggest anthocyanins are not produced to alleviate source : sink imbalances in high-light leaves during the winter. The major assumptions of the carbon overflow hypothesis were: (1) that high light (red) leaves fix a greater amount of carbon than green leaves; and (2) that carbon sinks are limited during the winter because of cessation of growth, inhibition of translocation caused by soil freezing and/or reduced metabolic rates. These two factors in combination would then result in an overflow of carbon into an alternative sink (the phenylpropanoid pathway), leading to the production of anthocyanins. Regarding the first assumption, our results do suggest that high-light leaves fix more carbon than their shaded counterparts. This was supported by observations showing nonstructural carbohydrates in potted plants increased across a light gradient (Fig. 2e,g), and high-light (red) leaves exhibited higher rates of photosynthesis (up to three times higher) on warm winter days than shaded plants in the field (data obtained with a Li-Cor 6200, not shown). One might expect, then, that if sinks were limited in field plants, and anthocyanins were indeed functioning to alleviate source : sink imbalances, plants grown under increasing PPFDs would exhibit TNC concentrations that leveled off once anthocyanins began to form, while anthocyanin concentrations would continue to increase; however this trend was not observed (Fig. 2). Furthermore, one would expect reddening to be most intense in potted plants, which had substantially shorter rhizomes and experienced lower soil temperatures than field plants (thus exacerbating any source : sink imbalances). While leaves of potted plants did accumulate much higher levels of carbohydrates than field plants (up to a sixfold difference), they actually exhibited lower anthocyanin levels, with the reddest leaves possessing only 25% of the anthocyanin content observed in some field leaves, even those with lower TNC concentrations (Fig. 2). In this case, it seems that plants which could have benefited the most from an additional carbon sink actually contained some of the lowest levels of anthocyanin. For these reasons, the supposition that anthocyanin concentration increases in response to increases in carbohydrate content was not supported by this study.

Although red and green leaves in the field did not differ with regard to TNCs, it should not escape notice that all winter leaves exhibited a twofold increase in soluble sugar content relative to summer leaves (Table 1). Previous studies have shown that plants often upregulate soluble sugars as a mechanism to prevent tissue freezing during the winter (O'Neill, 1983; Sasaki *et al.*, 1996). This cryoprotectant function would certainly be advantageous for evergreen plants such as *Galax*, which are susceptible to temperatures that plunge well below freezing at night, and could therefore be a legitimate ultimate cause for their synthesis and retention. Although not tested in this study, it is also possible that anthocyanins function in this role as well. Because sun leaves experience a greater, and more rapid, plunge in leaf temperature from day to night than shade leaves, it seems logical that they could benefit from the depressed freezing point afforded by an increase in solutes such as anthocyanin (Chalker-Scott, 1999).

Results from the vein-severing experiment suggest that this increase in soluble sugar content may also serve an additional function – perhaps as a cue necessary to initiate anthocyanin synthesis in the presence of high light (Yamakawa *et al.*, 1983; Gollop *et al.*, 2002). As shown in Fig. 11(b), *Galax* could be induced to synthesize anthocyanins by severing veins of a leaf in warm, moderate-to-high light conditions. Under no other conditions were *Galax* leaves observed to synthesize anthocyanins in this study without the presence of cold temperatures. Figure 11(a) shows that starch and sugar concentration significantly increased in the side of the leaf where veins had been severed, and anthocyanins were also produced only on that side of the leaf, despite roughly equal PPFDs on both cut and uncut sides. As described previously, several studies have observed anthocyanin synthesis to be initiated in response to increases in sugar-feeding and girdling (Jeanette *et al.*, 2000; Onslow, 1925), and the necessity of light for anthocyanin synthesis has been very well documented (for review see Chalker-Scott, 1999). In addition, studies by Yamakawa *et al.* (1983) and Gollop *et al.* (2002) have demonstrated that high sucrose, in combination with light, induces anthocyanin synthesis in *Vitis*. Perhaps the increase in soluble sugars that occurs during the winter in *Galax* serves a dual function of acting as a cryoprotectant as well as a biochemical cue for initiating anthocyanin synthesis in the presence of light. This explanation seems to be consistent with the data, as it would explain why high-light summer leaves fail to produce anthocyanins (as most sugars are stored as starch, Table 1), why leaves with severed veins are able to turn red in warm temperatures (Fig. 11), and why shaded winter leaves fail to produce anthocyanins (Table 1).

In summary, the results of this study lend strong support to a light-attenuating function of anthocyanins in high-light winter leaves of *G. urceolata.* However, further studies should be conducted to evaluate more clearly the relative contribution of anthocyanins to the antioxidant pool, and the carbohydrate dynamics involved in anthocyanin synthesis.

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References

Adams WW III, Demmig-Adams B, Verhoeven AS, Barker DH. 1994. 'Photoinhibition' during winter stress: involvement of sustained xanthophyll cycle-dependent energy dissipation. *Australian Journal of Plant Physiology* **22**: 261–276.

Adams WW III, Demmig-Adams B, Rosenstiel TN, Ebbert V, Brightwell AK, Barker DH, Carter CR. 2001. Photosynthesis, xanthophylls, and D1 phosphorylation under winter stress. In: *PS2001 Proceedings 12th International Congress on Photosynthesis*. Melbourne, Australia: CSIRO Publishing.

Baker NR. 1994. Chilling stress and photosynthesis. In: Foyer CH, Mullineaux PM, eds. *Causes of Photooxidative Stress and Amelioration of Defense Systems in Plants.* Boca Raton, FL, USA: CRC Press, 105–126.

Chalker-Scott L. 1999. Environmental significance of anthocyanins in plant responses. *Photochemistry and Photobiology* **70**: 1–9.

Cui M, Vogelmann TC, Smith WK. 1991. Chlorophyll and light gradients in sun and shade leaves of *Spinacia oleracea*. *Plant, Cell & Environment* **14**: 493–500.

Demmig-Adams B. 1998. Survey of thermal energy dissipation and pigment composition in sun and shade leaves. *Plant Cell Physiology* **39**: 474–482.

Demmig-Adams B, Adams WW III. 1992. Photoprotection and other responses of plants to high light stress. *Annual Review of Plant Physiology and Plant Molecular Biology* **43**: 599–626.

Demmig-Adams B, Adams WW III. 1996. The role of xanthophylls cycle carotenoids in the protection of photosynthesis. *Trends in Plant Science* **1**: 21–26.

Ebbert V, Demmig-Adams B, Adams WW III, Mueh KE, Staehelin LA. 2001. Correlation between persistent forms of zeaxanthin-dependent energy dissipation and thylakoid protein phosphorylation. *Photosynthesis Research* **67**: 63–78.

Feild TS, Lee DW, Holbrook NM. 2001. Why leaves turn red in autumn. The role of anthocyanins in senescing leaves of red-osier dogwood. *Plant Physiology* **127**: 566–574.

Gollop R, Even S, Colova-Tsolova V, Perl A. 2002. Expression of the grape dihydroflavonol reductase gene and analysis of its promoter region. *Journal of Experimental Botany* **53**: 1397–1409.

Gould K, Kuhn D, Lee D, Oberbauer S. 1995. Why leaves are sometimes red. *Nature* **378**: 242–243.

Gould KS, Markham KR, Smith RH, Goris JJ. 2000. Functional role of anthocyanins in the leaves of *Quitinia serrata* A. Cunn. *Journal of Experimental Botany* **51**: 1107–1115.

Gould KS, Vogelmann TC, Han T, Clearwater MJ. 2002. Profiles of photosynthesis within red and green leaves of *Quintinia serrata*. *Physiologia Plantarum* **116**: 127–133.

Grace SC, Logan BA. 1996. Acclimation of foliar antioxidant systems to growth irradiance in three broad-leaved evergreen species. *Plant Physiology* **112**: 1631–1640.

Grace SC, Logan BA, Adams WW III. 1998. Seasonal differences in foliar content of chlorogenic acid, a phenylpropanoid antioxidant, in *Mahonia repens*. *Plant, Cell & Environment* **21**: 513–521.

Hoch WA, Zeldin EL, McCown BH. 2001. Physiological significance of anthocyanins during autumnal leaf senescence. *Tree Physiology* **21**: 1–8.

Hughes NM. 2004. Functional role of anthocyanins in high light winter leaves of the evergreen herb, *Galax urceolata*. MSc thesis, Boone, NC, USA: Appalachian State University.

Jeanette E, Reyss A, Gregory N, Gantet P, Prioul J-L. 2000. Carbohydrate metabolism in a heat-girdled maize source leaf. *Plant, Cell & Environment* **23**: 61–69.

Kaku S, Iwaya-Inoue M, Toki K. 1992. Anthocyanin influence on water proton NMR relaxation times and water contents in leaves of evergreen woody plants during the winter. *Plant and Cell Physiology* **33**: 131–137.

Landry LG, Chapple CCS, Last RL. 1995. *Arabidopsis* mutants lacking phenolic sunscreens exhibit enhanced ultraviolet-B injury and oxidative damage. *Plant Physiology* **109**: 1159–1166.

Lee DW, Gould KS. 2002. Why leaves turn red. *American Scientist* **90**: 524–531.

Logan BA, Grace SC, Adams WW III, Demmig-Adams B. 1998. Seasonal differences in xanthophyll cycle characteristics and antioxidants in *Mahonia repens* growing in different light environments. *Oecologia* **116**: 9–17.

Maxwell K, Johnson GN. 2000. Chlorophyll fluorescence – a practical guide. *Journal of Experimental Botany* **51**: 659–668.

McCarron JK. 1995. Ecophysiological adaptations of *Galax aphylla* to the understory of southern Appalachian forests. MSc thesis, Boone, NC, USA: Appalachian State University.

Merzlyak MN, Chivkunova OB. 2000. Light-stress-induced pigment changes and evidence for anthocyanin photoprotection in apples. *Journal of Photochemistry and Photobiology B* **55**: 155–163.

Mittler R. 2002. Oxidative stress, antioxidants and stress tolerance. *Trends in Plant Science* **7**: 405–410.

Murray JR, Hackett WP. 1991. Dihydroflavonol reductase activity in relation to differential anthocyanin accumulation in juvenile and mature phase in *Hedera helix* L. *Plant Physiology* **97**: 343–351.

Neill SO, Gould KS. 2003. Anthocyanins in leaves: light attenuators or antioxidants?. *Functional Plant Biology* **30**: 865–873.

Neill SO, Gould KS, Kilmartin PA, Mitchell KA, Markham KR. 2002. Antioxidant activities of red versus green leaves in *Elatostema rugosum*. *Plant, Cell & Environment* **25**: 539–547.

O'Neill SD. 1983. Osmotic adjustment and the development of freezing resistance in *Fragaria virginiana*. *Plant Physiology* **72**: 938–944.

Onslow MW. 1925. Physiological conditions and factors influencing the formation of anthocyanins. *The Anthocyanin Pigments of Plants.* London, UK: Cambridge University Press, 93–101.

Ort D. 2001. When there is too much light. *Plant Physiology* **125**: 29–32.

Parker J. 1962. Relationship among cold hardiness, water-soluble protein, anthocyanins, and free sugars in *Hedera helix* L. *Plant Physiology* **37**: 809–813.

Pietrini F, Iannelli MA, Massacci A. 2002. Anthocyanin accumulation in the illuminated surface of maize leaves enhances protection from photo-inhibitory risks at low temperature, without further limitation to photosynthesis. *Plant, Cell & Environment* **25**: 1251–1259.

Porra RJ. 2002. The chequered history of the development and use of simultaneous equations for the accurate determination of chlorophylls *a* and *b*. *Photosynthesis Research* **73**: 149–156.

Sasaki H, Ichimura K, Oda M. 1996. Changes in sugar content during cold acclimation and deacclimation of cabbage seedlings. *Annals of Botany* **78**: 365–369.

Sims DA, Gamon JA. 1999. Estimating chlorophyll, carotenoid and anthocyanin concentration using hyperspectral reflectance. *Annual Meeting of the Ecological Society of America, August, 1999, Spokane, WA, USA.* Lawrence, KS, USA: Allen Press.

Smillie RM, Hetherington SE. 1999. Photoabatement by anthocyanin shields photosynthetic systems from light stress. *Photosynthetica* **36**: 451–463.

Steyn WJ, Wand SJE, Holcroft DM, Jacobs G. 2002. Anthocyanins in vegetative tissues: a proposed unified function in photoprotection. *New Phytologist* **155**: 349–361.

Tsukaya H, Ohshima T, Naito S, Chino M, Komeda Y. 1991. Sugar-dependent expression of the *CHS-A* gene for chalcone synthase from petunia in transgenic *Arabidopsis*. *Plant Physiology* **97**: 1414–1421.

Verhoeven AS, Adams WW III, Demmig-Adams B. 1996. Close relationship between the state of the xanthophyll cycle pigments and photosystem II efficiency during recovery from winter stress. *Physiologia Plantarum* **96**: 567–576.

Watling JR, Robinson SA, Woodrow IE, Osmond CB. 1997. Responses of rainforest understorey plants to excess light during sunflecks. *Australian Journal of Plant Physiology* **24**: 17–25.

Yamakawa T, Kato S, Ishida K, Kodama T, Monoda Y. 1983. Production of anthocyanin by *Vitis* cells in suspension culture. *Agricultural and Biological Chemistry* **47**: 2185–2191.