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RESEARCH PAPER

# Why some stems are red: cauline anthocyanins shield photosystem II against high light stress

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## Abstract

**Red-stemmed plants are extremely common, yet the functions of cauline anthocyanins are largely unknown. The possibility that photoabatement by anthocyanins in the periderm reduces the propensity for photoinhibition in cortical chlorenchyma was tested for *Cornus stolonifera*. Anthocyanins were induced in green stems exposed to full sunlight. PSII quantum yields ($\Phi_{PSII}$) and photochemical quenching coefficients were depressed less in red than in green stems, both under a light ramp and after prolonged exposures to saturating white light. These differences were primarily attributable to the attenuation of *PAR*, especially green/yellow light, by anthocyanins. However, the red internodes also had less chlorophyll and higher carotenoid:chlorophyll ratios than the green, and when the anthocyanic periderm was removed, small differences in the $\Phi_{PSII}$ of the underlying chlorenchyma were retained. Thus, light screening by cauline anthocyanins is important, but is only part of a set of protective acclimations to high irradiance. Hourly measurements of $\Phi_{PSII}$ on established trees under natural daylight indicated a possible advantage of red versus green stems under sub-saturating diffuse, but not direct sunlight. To judge the wider applicability of the hypothesis, responses to high light were compared for red and green stems across five further unrelated species. There was a strong, linear, interspecific correlation between photoprotective advantage and anthocyanin concentration differences among red and green internodes. The photoprotective effect appears to be a widespread phenomenon.**

**Key words:** Anthocyanin function, chlorophyll *a* fluorescence, *Cornus stolonifera*, photoinhibition, photoprotection, red stems.

## Introduction

In many vascular plants, stems are pigmented red even though their leaves may be green. Stems may be entirely red, or, more commonly, coloured red only at the basal or at the apical regions of the shoot (Wheldale, 1916). In some species, the red pigment is restricted to the internodes or to the nodes only; in others it is localized around wounds, glands, or lenticels throughout the stem (Fig. 1A). Variation is evident in heteroblastic plants, such as *Hedera helix*, which produce red stems only in their juvenile stages (Hackett, 2002). Red colouration may also develop in otherwise green stems in response to abiotic stressors such as drought (Chalker-Scott, 1999), low temperatures (Shichijo *et al.*, 1993; Tignor *et al.*, 1997), ultraviolet radiation (Yatsuhashi *et al.*, 1982), or high ratios of red:far-red light (Alokam *et al.*, 2002). Anthocyanins are the pigments responsible for red colouration in the stems of most herbaceous species, although some members of the Caryophyllales produce betalains, and in woody stems, proanthocyanidins, lignins, and several minor pigments may be involved (Davies, 2004). These pigments are most commonly located in epidermal and/or sub-epidermal tissues of the stem (Wheldale, 1916; Nozzolillo and McNeill, 1985).

Despite the abundance of red-stemmed plant species, there is a surprising dearth of information on the possible functions of cauline anthocyanins. To our knowledge, only one study has explicitly addressed this issue: the

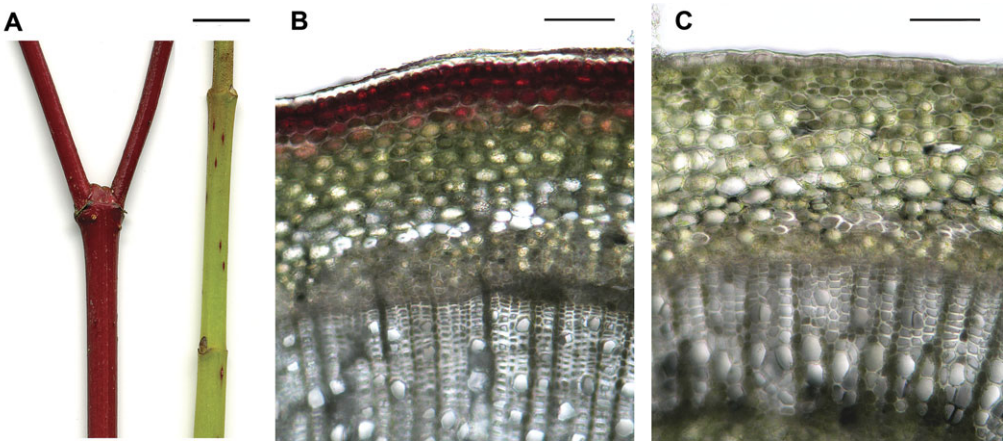


**Fig. 1.** *Cornus stolonifera* stems. (A) Surface view of red and green internodes. Bar, 5 mm. (B, C) Light micrographs of transverse sections through portions of (B) red and (C) green internodes. Bars, 100 μm.

anthocyanins in stems (and petioles) of *Ambrosia chamissonis* were convincingly demonstrated to absorb quanta that would otherwise degrade thiarubrine-A, a potent but photolabile defence compound (Page and Towers, 2002). However, for the majority of plant species, which do not hold thiarubrines, the functional significance of anthocyanic stems has never been investigated.

Red leaves, in contrast, have attracted heated scientific debate in recent years. Among the leading functional hypotheses for the presence of anthocyanins in leaves is that of photoprotection of chloroplasts; under saturated light, anthocyanins potentially mitigate photoinhibitory and photo-oxidative damage by absorbing a proportion of the photons surplus to the requirements of the light reactions of photosynthesis. This hypothesis does not appear to apply equally to all red-leafed plants; exceptions have been reported (Esteban *et al.*, 2008; Zeliou *et al.*, 2009), and alternative functional hypotheses, such as a role in herbivore defence, have been proposed (Archetti, 2009). However, empirical support for the photoprotective hypothesis is strong for many species (reviewed by Steyn *et al.*, 2002; Gould *et al.*, 2002*a*; Close and Beadle, 2003; Gould, 2004; Gould and Lister, 2005; Hatier and Gould, 2008). Particularly compelling evidence for photoprotection was presented in a comparison of the chlorophyll *a* fluorescence kinetics for anthocyanic and acyanic senescing leaves of *Cornus stolonifera* (Feild *et al.*, 2001). Exposure to saturating light resulted in a 60% reduction in the photochemical quantum yield of photosystem II (PSII) for red *C. stolonifera* leaves, but an almost 100% reduction in acyanic leaves. When returned to darkness, the red leaves recovered to their maximum value rapidly, yet the acyanic leaves did not attain their pre-treatment rates even after 6 h. Similar differences were noted between green and anthocyanic leaves in the evergreen herb, *Galax urceolata* (Hughes *et al.*, 2005; Hughes and Smith, 2007). Thus, foliar anthocyanins have the potential both to moderate the severity of chronic photoinhibition and to expedite recovery.

It is possible that photoprotection of chloroplasts could equally explain the presence of anthocyanins in some stems. Cortical chlorenchyma in stems are structurally similar to the palisade and/or spongy mesophyll in leaves (Bossard and Rejmanek, 1992; Yiotis *et al.*, 2006), and the photosynthesis of stems can contribute significantly to the carbon gain of plants (Nilsen, 1995). Moreover, both woody and herbaceous stems have been shown to be susceptible to photoinhibitory reductions in carbon assimilation at certain times of the year (Manetas, 2004; Berveiller *et al.*, 2007; Whittmann and Pfanz, 2007; Yiotis *et al.*, 2008).

This paper tests the hypothesis that light-shielding by anthocyanins in stems is associated with improved quantum efficiencies of PSII under saturating light. Using *Cornus stolonifera* as the focal species, the induction of anthocyanins and their effects on light transmittance through the periderm are described, and the chlorophyll fluorescence of red and green stems is compared after exposure to strong light at two temperatures. Finally, the applicability of the photoprotective hypothesis is assessed across five unrelated species which show variation in stem colour.

## Materials and methods

### *Plant material*

Fresh stem cuttings from six species that bore both red and green stems were harvested with permission from the Dunedin Botanic Garden, New Zealand, or the University of Otago Botany Department garden in mid-summer 2006. The species were: *Cornus stolonifera* Michx. (Cornaceae); *Leucothoe fontanesiana* (Steudel) Sleumer (Ericaceae); *Lobelia erinus* L. (Campanulaceae); *Salvia gesneriiflora* Lindl. and Paxton (Lamiaceae); *Solidago gigantea* Aiton (Asteraceae); and *Tanacetum parthenium* (L.) Schultz-Bip. (Asteraceae). Stems for which internodes were the most intensely pigmented red, or else were entirely green, were preferentially selected from at least five healthy, vegetative plants of similar ages per colour type per species. The *C. stolonifera* population was clonal, and most of the individuals from the Botanic Garden were probably genetically identical ramets. The severed ends of the stems were held in water for the duration of the photoinhibition experiments. For the longer-term light treatments, 10 *C. stolonifera* saplings of similar age growing in potting compost were purchased from a commercial nursery.

### Photoinhibitory treatments

The excised red and green stems were dark-adapted overnight for 12–15 h, and then values of $F_o$ (minimum chlorophyll fluorescence) and $F_v/F_m$ (ratio of variable to maximum fluorescence) at 22 °C were measured midway along the third-youngest fully expanded internode using a PAM-2000 (Heinz Walz GmbH, Effeltrich, Germany) chlorophyll fluorometer with red (630 nm) pulse-modulated measuring light.

These same portions of stems were then subjected to 1200 μmol $m^{-2}$ $s^{-1}$ collimated white light for (i) 0.5 h at 22 °C; (ii) 3 h at 22 °C; or (iii) 3 h at 4 °C. The light source was a 115 V halogen bulb with a colour temperature of 3350 K, delivered through a Novaflex fibre optic illuminator. Spectral output from the lamp was measured using an LI-1800 spectroradiometer (Li-Cor Biosciences, Lincoln, NE) fitted with a cosine receptor. Immediately following the light treatment, the light-adapted ratio of variable to maximum chlorophyll fluorescence ($F_v'/F_m'$) was measured using the PAM-2000. This experiment was repeated with fresh stem cuttings, but with their chlorophyll fluorescence monitored using a Walz Imaging PAM, which supplied blue (470 nm) pulse-modulated measuring light and white actinic light.

### Pigment location and quantification

Portions of internode that had been exposed to the photoinhibitory light fluxes were sectioned transversely, and the histological locations of anthocyanins noted in a Zeiss Axiostar Plus compound microscope. Pigment concentrations in these portions were estimated spectrophotometrically following the methods of Gould *et al.* (2000). Anthocyanins were extracted in 1.5 ml acidified methanol (MeOH:$H_2O$:3M HCl, 16:3:1 by vol.) at 4 °C for 24 h, clarified by centrifugation, and their maximum absorbance in the 500–600 nm waveband determined using an Ultrospec 2000 spectrophotometer. Anthocyanin concentrations were expressed as $A_{\lambda max}$ $g^{-1}$ FW, where $\lambda_{max}$ was normally 529 nm. Chlorophylls and carotenoids were extracted in 1.5 ml of 80% (v/v) acetone at 4 °C for 24 h in the dark, and concentrations determined from absorbances measured at 470, 647, and 663 nm using the equations of Lichtenthaler (1987).

### Chlorophyll fluorescence imaging

Five pairs of green and red stem cuttings from *C. stolonifera,* and one green, healthy leaf from each of the same stems, were dark-adapted at 22 °C for 12–18 h. A red and a green internode from two stems of similar developmental age were simultaneously attached to the stage of a Walz Imaging PAM, and subjected to an irradiance ramp comprising 21 steps, each lasting 3 min. *PAR* supplied by the fluorometer increased in intervals from 1 μmol to 1211 μmol $m^{-2}$ $s^{-1}$, then decreased to 136 μmol $m^{-2}$ $s^{-1}$. Values of $F_m'$ (maximum fluorescence in the light) and $F_t$ (steady-state fluorescence) were averaged from an approximately rectangular area of interest on each stem, and estimates of PSII photochemical efficiency ($\Phi_{PSII}$), photochemical quenching (*qP*), and non-photochemical quenching (*NPQ*) were calculated for each irradiance step using the equations of Genty *et al.* (1989) and Maxwell and Johnson (2000). Colour-indexed images of the stems showing chlorophyll fluorescence parameters were captured using the Imaging PAM software. Detached leaves from *C. stolonifera* were subjected to the same protocol.

### Anthocyanin induction

For each pot-grown *C. stolonifera* sapling, two green internodes of similar length, diameter, and developmental stage were randomly assigned to a sun or shade treatment. Their initial colour was measured from specular reflectance spectra using a reflectance probe attached to a USB2000 Ocean Optics (Dunedin, FL, USA) spectrometer. Stem redness was quantified by the ratio of reflectance in the red (600–699 nm) to green (500–599 nm) wavebands (Gamon and Surfus, 1999). A pilot study had shown this to be superior to other indices of redness for describing stem colour.

The intact stems were dark-adapted at 22 °C for 24 h, and then $F_o$ and $F_v/F_m$ were measured for each focal internode using the Walz PAM-2000. They were irradiated with 1200 μmol $m^{-2}$ $s^{-1}$ white light for 30 min, and then $F_v'/F_m'$ recorded.

The saplings were planted 1.5 m apart in a sunny, north-facing plot on the University of Otago grounds in February 2007. Stems were staked horizontally to the soil such that regions of interest on internodes assigned to the shade treatment faced the ground, and those assigned to the sun treatment faced upward to receive the maximum amount of solar radiation. The plot was watered twice weekly. Stems were harvested after 28 d, and internode colour, dark-adapted $F_o$, $F_v/F_m$, and $F_v'/F_m'$ post photoinhibition treatment were re-measured.

### Light transmittance through periderm

Small portions of the youngest fully-expanded internode were removed from nine red and nine green *C. stolonifera* stems. Strips of the periderm (*c.* 1×2 mm, and 0.1 mm deep) were excised from each portion under a dissecting microscope. These were arranged, inner periderm tissue uppermost, on a glass microscope slide in an Olympus AX70 compound microscope, an eyepiece of which delivered light from a 12 V/100 W Philips 7724 quartz halogen lamp to an Ocean Optics (Dunedin, FL) USB4000 spectrometer via a fibre optic probe. A custom-built attachment to the ocular tube ensured that the tip of the probe was perfectly centred in the optical field at the plane of focus, and prevented the entry and exit of stray light. The specimens were sub-illuminated with white light from the microscope, and transmittance, the proportion of incident light transmitted through the periderm, was measured at 0.2 nm intervals from 400–700 nm using SpectraSuite spectroscopy operating software.

### Periderm removal

To examine for intrinsic differences in the chlorophyll fluorescence properties of chlorenchyma in the cortex of red and green stems, the periderm and four outermost cortical layers were surgically removed from a small portion of the trunk on five red and five green potted *C. stolonifera*. Subjacent cortical cells lacking in anthocyanins and exposed by this procedure were subjected to an irradiance ramp, and photochemical yields measured using the Walz PAM 2000.

### Light-screening experiment

To simulate possible light-screening effects of red pigmentation on the chlorophyll fluorescence properties of green stems, one layer of polycarbonate film was wrapped around a small portion of green internode on five potted *C. stolonifera* plants. The Supergel Rosco (Sydenham, UK) no. 36 'medium pink' filter absorbed *c.* 40% green light, with a peak absorptance at 533 nm. Transmittance and chromaticity coordinates of the film are available at: www.rosco.com/uk/filters/supergel.asp#colors. Dark-adapted $F_v/F_m$ was measured at contiguous regions of covered and uncovered internode; these were then irradiated with 1200 μmol $m^{-2}$ $s^{-1}$ white light for 90 min at 22 °C, and the decline in $\Phi_{PSII}$ quantified.

### Diurnal changes in $\Phi_{PSII}$ in situ

To examine for possible photoprotective roles of anthocyanic stems *in situ*, advantage was taken of four established *C. stolonifera* trees growing in a north–south row at an exposed corner of a Wellington garden. The trees originated from the same nursery stock as the potted plants, with red and green-stemmed phenotypes alternating in the row. On 6 September 2009 (early Spring), a cloudless day with a light southerly breeze, measurements of $\Phi_{PSII}$ were recorded

at hourly intervals from 8 am until 5 pm on both the east- and west-facing aspects of three vertical stems per tree using a Walz PAM 2500 chlorophyll fluorometer. Stem temperatures were recorded with a Ni-NiCr thermocouple fitted with the Walz leaf clip holder 2030-B, and irradiance normal to the stem was measured using a Li-Cor (Lincoln, NE, USA) 250A light meter fitted with a cosine-corrected quantum sensor.

## Results

### Cornus stolonifera *stem pigments*

The population of *C. stolonifera* in the Dunedin Botanic Garden varied in stem colour from entirely red to entirely green (Fig. 1A). Individual stems often displayed a radial gradient in pigmentation, with the more shaded parts of the internode green, and the remainder predominantly red. The red pigment, shown previously to be an anthocyanin (Feild *et al.*, 2001; Bjorøy *et al.*, 2007), was located in the red stems in the periderm (where present) and/or up to four outermost layers of the stem cortex (Fig. 1B). In green stems, anthocyanins were either absent from cortical tissue (Fig. 1C), or else were evident at low concentration in the outermost subepidermal layer only. Portions of periderm surrounding lenticels and lesions were generally anthocyanic on both types of stem. Aside from vacuolar pigmentation, there were no obvious structural differences between the red and green stems. All expanded leaves were entirely green until the onset of senescence.

Cortical tissues in the red stems held substantially lower concentrations of the chlorophylls, both *a* and *b*, than did those in the green stems (Table 1). Total carotenoids (carotenes plus xanthophylls) were similar in concentration among both stem types and, consequently, there was a small but statistically significant greater ratio of carotenoids to total chlorophylls in the red stems. Anthocyanin concentrations were on average 3.6-fold greater in the red than in the green stems.

### *Photoinhibitory responses of* C. stolonifera *stem cuttings*

The maximum photochemical efficiencies of PSII, as estimated from chlorophyll fluorescence ($F_v/F_m$) using the Walz PAM 2000, were comparable for dark-adapted green (0.75±0.01) and red (0.74±0.01) *C. stolonifera* stems. When subjected to white light at 1200 μmol $m^{-2}$ $s^{-1}$, $\Phi_{PSII}$ declined markedly (Fig. 2A, B). After 30 min light at 22 °C, the reduction from initial $F_v/F_m$ values was statistically similar for green and red stems (ANOVA; $P$=0.6). By contrast, after 3 h light at 22 °C, $\Phi_{PSII}$ had declined approximately 33% more in the green than in the red stems, a statistically significant difference ($P$=0.03). Because the potential for photoinhibition is greatest under strong light at low temperatures, the responses of green and red stems were also compared after 3 h light at 4 °C. Under those conditions, $\Phi_{PSII}$ declined even further, and again the red stems were less affected than the green ($P$=0.01). There was a significant negative correlation between anthocyanin content and the magnitude of response to photoinhibitory conditions after 3 h at 22 °C ($r$= –0.65; $P$=0.04) and at 4 °C ($r$= –0.89; $P$=0.007). This correlation was not evident after only 30 min at 22 °C ($r$=0.58; $P$=0.08).

Similar trends were observed when further portions of red and green stems exposed to high light were examined under a Walz Imaging PAM (Fig. 3). This result demonstrated that the differences in chlorophyll fluorescence between red and green stems were unaffected by the quality of measuring light issued by the fluorometer, since the PAM-2000 supplied modulated pulses of red light, and the Imaging PAM blue light. Small differences in $\Phi_{PSII}$ were observable

**Table 1.** Concentrations of chlorophyll (Chl) *a* and *b*, carotenoids (Car), and anthocyanins (Ant) in the cortical tissues of green and red stem portions of *Cornus stolonifera*

Means of $n$=14 ±SE. Statistical differences between columns indicated by: *$P$ <0.01; **$P$ <0.0001.

| Pigment | Green | Red |
|---|---|---|
| Chl *a* (μg $g^{-1}$ FW) | 161±8.0 | 129±6.7* |
| Chl *b* (μg $g^{-1}$ FW) | 65±3.6 | 49±2.7* |
| Chl *a*:*b* | 2.5±0.03 | 2.6±0.02* |
| Car (μg $g^{-1}$ FW) | 53±2.4 | 49±2.8 |
| Car:Chl (a+b) | 0.24±0.005 | 0.27±0.004** |
| Ant ($A_{529nm}$ $g^{-1}$ FW) | 4.4±0.6 | 16.0±1.2** |

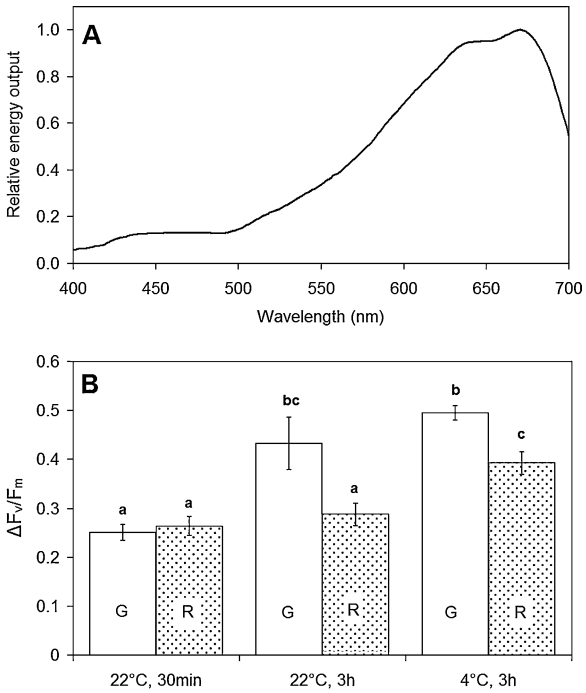


**Fig. 2.** (A) Normalized spectral output (W $m^{-2}$ $nm^{-1}$) from the lamp used for photoinhibition experiments. (B) Decline in $F_v/F_m$ for chlorophyll fluorescence of green (G) and red (R) *Cornus stolonifera* internodes following exposure to 1200 μmol $m^{-2}$ $s^{-1}$ white light for 30 min ($n$=9) or 3 h ($n$=5) at 22 °C or 4 °C. Means ±SE. Different letters above bars indicate statistical significance (ANOVA; $P$ <0.05).

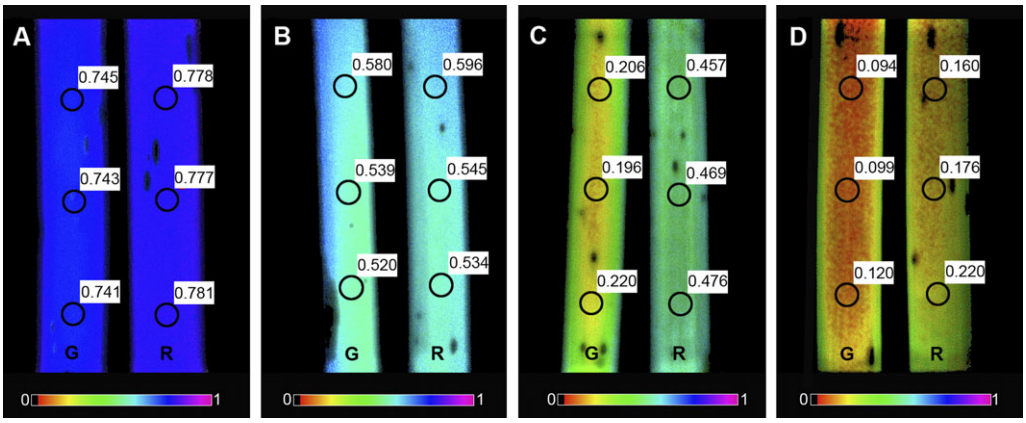


**Fig. 3.** Images of green (G) and red (R) stems of *Cornus stolonifera* colour-indexed for chlorophyll fluorescence. (A) Dark-adapted $F_v/F_m$ at 22 °C. (B-D) $F_v'/F_m'$ after 1200 µmol $m^{-2}$ $s^{-1}$ white light for (B) 30 min at 22 °C, (C) 3 h at 22 °C, (D) 3 h at 4 °C. Numbers show averages for circled areas of interest.

along the lengths of each internode, but these did not vary consistently among stems. Ignoring edge effects caused by the stem curvature, the Imaging PAM proved to be a useful, reproducible method to compare simultaneously the fluorescence kinetics of red and green stems under identical conditions.

### *Light response curves for chlorophyll fluorescence*

Relationships between incident light level and chlorophyll fluorescence kinetics differed markedly between the red and green stems of *C. stolonifera* (Fig. 4). $\Phi_{PSII}$ and photochemical quenching coefficients (*qP*) were both statistically greater in the red than in the green internodes when irradiated with between 80 and 750 µmol $m^{-2}$ $s^{-1}$ white light (ANOVA; $P$ <0.01), but were comparable in magnitude for the two stem types outside this irradiance range. Red stems were similar to the green *C. stolonifera* leaves in their light response curves for $\Phi_{PSII}$ and *qP* with irradiances between 100 and 600 µmol $m^{-2}$ $s^{-1}$ ($P$ >0.2). Non-photochemical quenching (*NPQ*), in contrast, was greater in the green than in the red stems with irradiances up to about 400 µmol $m^{-2}$ $s^{-1}$ ($P$ <0.01), although the trend reversed under stronger light. *NPQ* values for the leaves closely matched those of the green stems ($P$ >0.1 at all irradiance levels), but exceeded those of the red stems under light up to 400 µmol $m^{-2}$ $s^{-1}$ ($P$ <0.05).

### *Light attenuation through the periderm*

The periderm in *C. stolonifera* internodes substantially reduced the amount of light transmitted to subjacent cortical chlorenchyma (Fig. 5A). Of the *PAR* (400–700 nm) incident normal to the youngest, fully-expanded internode, 32±2% was transmitted through the periderm in green stems, and only 15±3% transmitted in red stems. Those numbers permitted the photochemical yield data in Fig. 4 to be re-expressed as a function of the calculated irradiance received at the cortex, rather than of the measured total light incident on the stem surface. The light curves thus obtained (Fig. 5B) were very similar for cortical cells in red

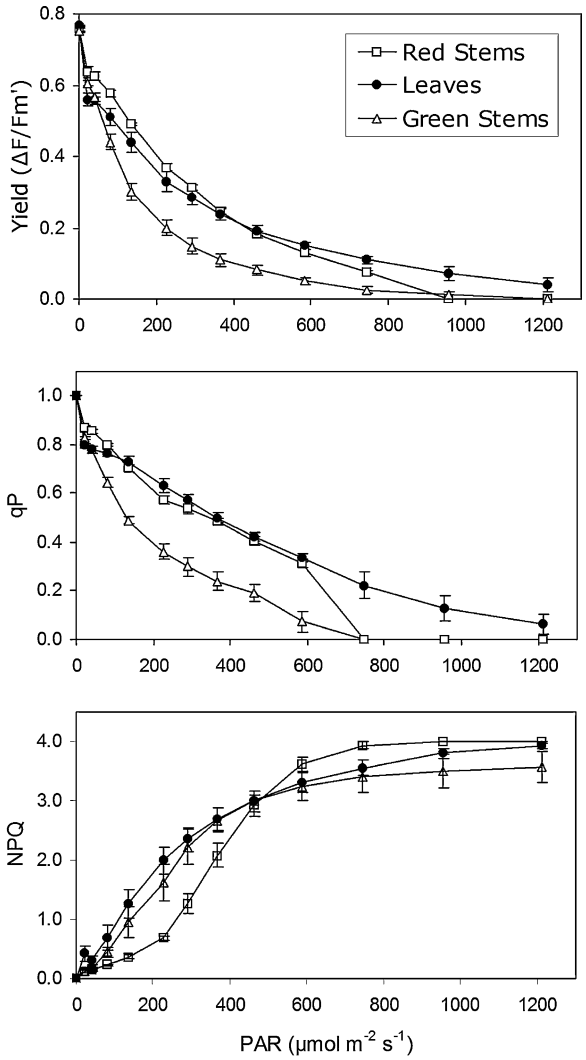


**Fig. 4.** Light response curves for photochemical quantum yield ($\Delta F/F_m'$), photochemical quenching (*qP*), and non-photochemical quenching (*NPQ*) of photosystem II at 22 °C for green and red stems and green leaves of *Cornus stolonifera*. Means ±SE, $n$=5.

and green stems at irradiances lower than 50 μmol $m^{-2}$ $s^{-1}$, although there was an apparent small disadvantage of the red stems under stronger light. However, the data in Fig. 5B possibly overestimate the effects of light attenuation on quantum yields of red stems, since anthocyanin in the periderm altered the quality as well as the quantity of transmitted light (Fig. 5A). Green stems transmitted comparable proportions of red light (600–700 nm), but significantly more blue (400–500 nm) and green/yellow light (500–600 nm) to the cortex than did red stems (ANOVA; $P_{red\ light}$=0.07, $P_{blue}$=0.017, $P_{green/yellow}$ <0.0001). The light incident on the cortex of green stems typically comprised 14±1% blue, 40±1% green/yellow, and 46±1% red. The corresponding composition of light inside red stems was on average 10 ±3% blue, 9±2% green/yellow, and 81±5% red, but these proportions were more variable than in the green stems; internodes with a thicker and more intensely red-pigmented periderm transmitted proportionately less blue and green/yellow light. Relative to that in green stems, the spectral composition at the cortex in red stems was significantly enriched in red light (Kruskal–Wallis non-parametric ANOVA; $P$=0.0003), depleted in green/yellow ($P$=0.0003), and comparable in blue ($P$=0.2).

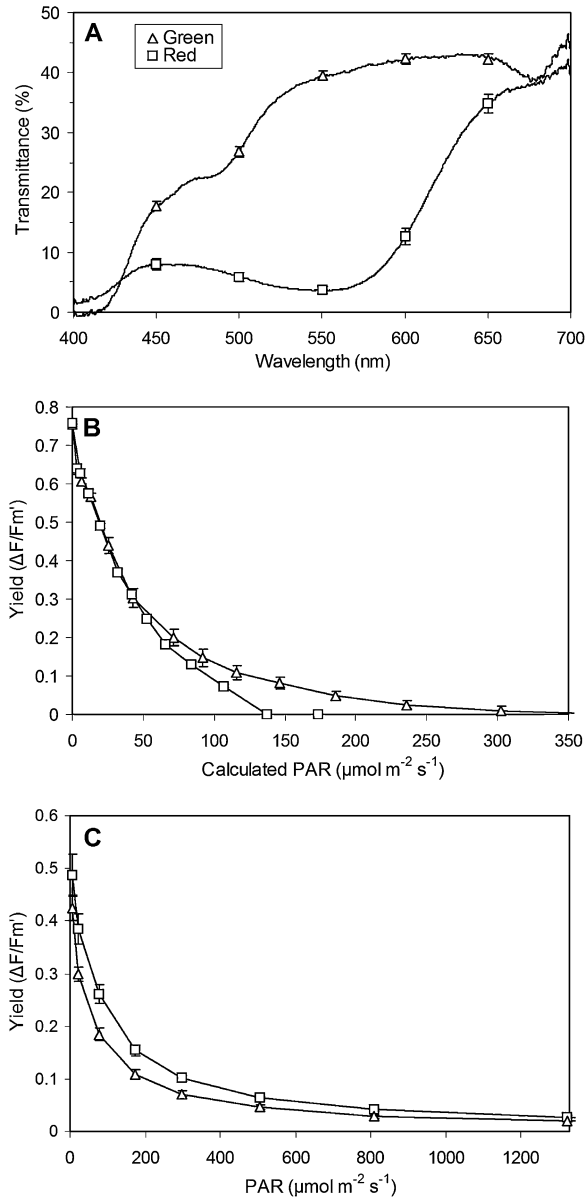


**Fig. 5.** (A) Spectral transmittance through the periderm of green and red stems of *Cornus stolonifera*. Symbols identify the lines; actual data points are spaced 0.2 nm apart. Means ±SE, $n$=9. (B) Light response curves for photochemical quantum yield ($\Delta F/F_m'$) as a function of calculated *PAR* incident on cortical chlorenchyma in red and green stems. Data from Fig. 4 were redrawn after accounting for light attenuation through the periderm. Means ±SE, $n$=5. Error bars for red stems are smaller than symbols. (C) Light response curves for photochemical quantum yield in cortical chlorenchyma following removal of the periderm. Means ±SE, $n$=5.

### *Periderm removal*

When the periderm and all anthocyanic tissues were surgically removed, the underlying cortical chlorenchyma in green and red stems showed only small differences in chlorophyll fluorescence profiles (Fig. 5C). $\Phi_{PSII}$ values were greater for the cortex of red than of green stems ($P$ <0.05) under irradiances between 21 μmol and 1300 μmol $m^{-2}$ $s^{-1}$, but were not statistically distinct outside this range. However, the differences were invariably smaller than those for the intact stems (Fig. 4). The maximum difference in $\Phi_{PSII}$ between the red and green stem cortex (0.09 units) was approximately 2-fold smaller than the corresponding difference for intact stems.

### *Light-screening experiment*

A polycarbonate filter, the optical properties of which approximated those of anthocyanin pigments, greatly reduced the photoinhibitory response of green stems to white light. When green internodes were irradiated for 90 min with 1200 μmol $m^{-2}$ $s^{-1}$ white light at 22 °C, the quantum efficiencies of PSII declined by 76±3% ($n$=5). By contrast, in contiguous regions of internodes that had been ensheathed with a single layer of polycarbonate, $\Phi_{PSII}$ declined by 60 ±2%. The difference was statistically significant (paired $t$ test; $P$ <0.002).

### *Anthocyanin induction and photoprotection*

When upright, predominantly green stems of *C. stolonifera* were staked horizontally in a garden plot, their uppermost portions, which were exposed to full sunlight at the height of summer, rapidly developed anthocyanin pigments, whereas the shaded, lower surfaces remained green. Upon harvest 28 d after treatment, the sun-exposed regions of the stems were 'very dark red' (Munsell notation: 2.5R 1/4); their ratios of red:green reflectance were, on average, 10-fold greater than those of the shaded regions (Fig. 6A). This difference was attributable to a reduction in the reflectance of green wavebands, rather than to any increase in reflectance in the red. Associated with this increase in anthocyanin pigmentation was an improved performance under

photoinhibitory light. When exposed to 1200 μmol $m^{-2}$ $s^{-1}$ white light for 30 min at 22 °C, $\Phi_{PSII}$ declined significantly less in the redder regions of the stems than in the green regions (Fig. 6B).

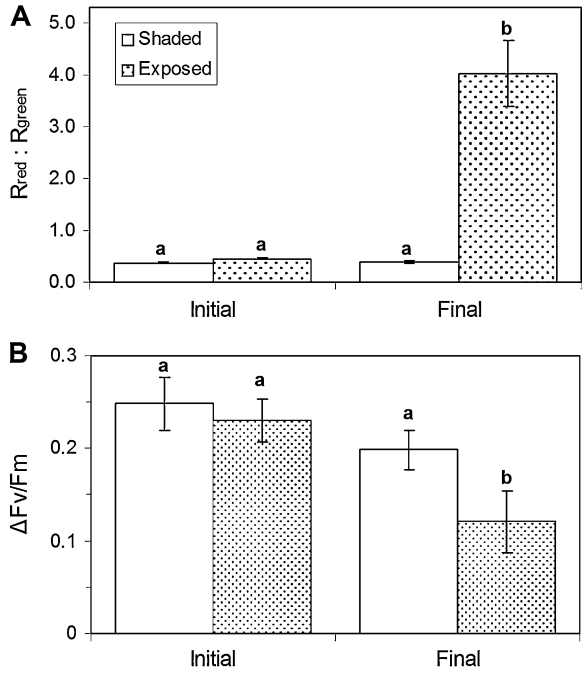


**Fig. 6.** Reflectance and chlorophyll fluorescence of sun-exposed and shaded regions of *Cornus stolonifera* stems before and after staking horizontally. (A) Ratio of reflectance of red (600–699 nm) to green (500–599 nm) wavebands. (B) Decline in $F_v/F_m$ after 1200 μmol $m^{-2}$ $s^{-1}$ white light for 30 min at 22 °C. Means ±SE, $n$=9.

### *Diurnal changes in* $\Phi_{PSII}$ in situ

Hourly measurements of $\Phi_{PSII}$ were recorded for east- and west-facing aspects of the vertical stems borne on established *C. stolonifera* saplings (Fig. 7). There was an apparent advantage of red over green stemmed trees under sub-saturating diffuse sunlight, but not under direct sunlight; $\Phi_{PSII}$ values were up to 27% higher in the red than in the green for east-facing cortical tissues during the early afternoon, and up to 42% higher for west-facing tissues in the late morning. At other times of the day, $\Phi_{PSII}$ values did not vary consistently between phenotypes. Stem temperature fluctuations were clearly unaffected by the presence of anthocyanins. Each data point in Fig. 7 represents the mean value of six measurements; these, however, were derived from only two trees per phenotype (the maximum available), and the data do not, therefore, lend themselves to rigorous statistical analysis.

### *Photoprotection across unrelated plant species*

To test the wider applicability of the photoprotective hypothesis for red stems, internodes from five further unrelated plant species were exposed to photoinhibitory fluxes and their decline in PSII photochemical efficiencies compared using the PAM-2000. Anthocyanin content varied 22-fold among those internodes, but did not correlate to concentrations of chlorophylls *a* and *b*, or total carotenoids (Table 2). For each species, the stems showed a greater decline from their original $F_v/F_m$ values when exposed to 1200 μmol $m^{-2}$ $s^{-1}$ white light for 3 h at 4 °C than for 30 min at 22 °C. The decline after 3 h light at 4 °C

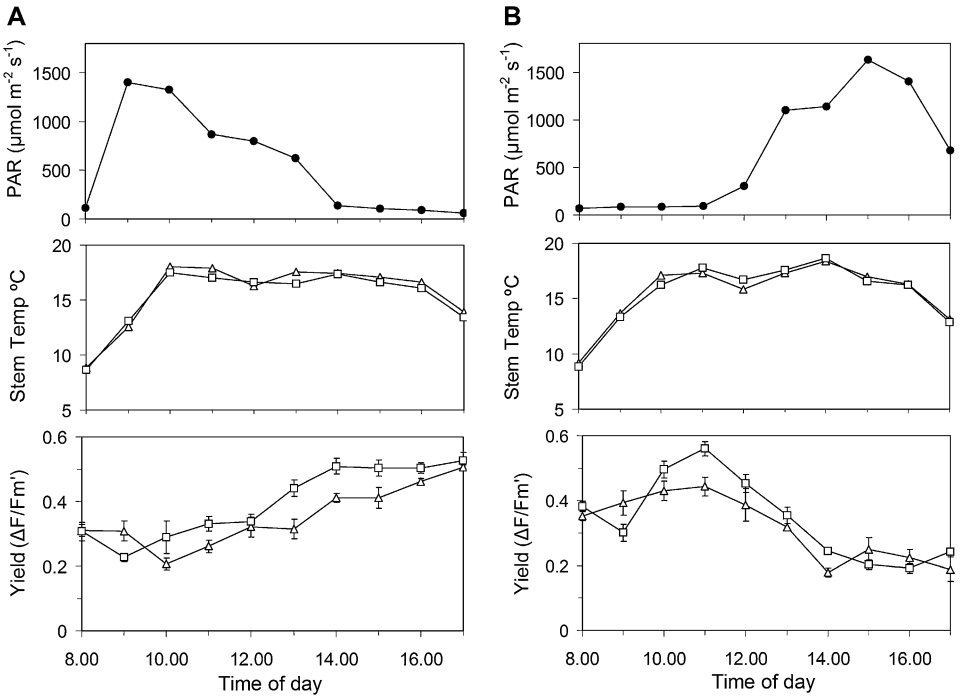


**Fig. 7.** Diurnal changes in incident *PAR*, stem temperature, and photochemical quantum yield for east-facing (A) and west-facing (B) cortical tissues of vertical internodes in red (open squares) and green (open triangles) stemmed *Cornus stolonifera* grown under natural conditions. Means ±SE, $n$=6 measurements for 2 trees per phenotype.

**Table 2.** Proportionate decline in $F_v/F_m$ for chlorophyll fluorescence of green and red internodes of six species following exposure to 1200 μmol $m^{-2}$ $s^{-1}$ white light for 30 min at 22 °C or for 3 h at 4 °C, and concentrations of anthocyanins (Ant), chlorophylls (Chl), and total carotenoids (Car); means ±SE, $n$=5.

| Species | Colour | $\Delta F_v/F_m$ (%) | | Ant | Chl *a* | Chl *b* | Car |
|---|---|---|---|---|---|---|---|
| | | 22 °C | 4 °C | ($A_{\lambda max}$ $g^{-1}$ FW) | (μg $g^{-1}$ FW) | (μg $g^{-1}$ FW) | (μg $g^{-1}$ FW) |
| *Leucothoe fontanesiana* | Green | 23±3 | 56±5 | 3.4±0.5 | 111±11 | 41±5 | 47±4 |
| | Red | 15±1 | 40±3 | 17.4±2.3 | 102±6 | 36±3 | 46±2 |
| *Lobelia erinus* | Green | 28±4 | 39±4 | 4.0±1.0 | 182±27 | 67±9 | 55±8 |
| | Red | 22±3 | 29±4 | 22.3±5.9 | 280±44 | 97±14 | 90±14 |
| *Tanacetum parthenium* | Green | 18±2 | 37±4 | 1.7±0.2 | 81±4 | 32±3 | 17±1 |
| | Red | 19±3 | 31±7 | 8.8±1.3 | 75±9 | 33±8 | 13±2 |
| *Solidago gigantea* | Green | 30±2 | 50±3 | 2.1±0.2 | 91±16 | 36±6 | 28±5 |
| | Red | 28±3 | 46±4 | 8.1±0.9 | 94±11 | 31±2 | 31±4 |
| *Salvia gesneriiflora* | Green | 33±4 | 58±6 | 1.0±0.1 | 45±8 | 18±3 | 23±3 |
| | Red | 31±3 | 57±1 | 4.9±1.0 | 41±8 | 18±3 | 26±5 |

was consistently greater in the green than in the red internodes for each species, and $\Delta F_v/F_m$ negatively correlated to total chlorophyll content ($r$= –0.8; $P$ <0.01).

The photoprotective advantage of anthocyanic stems was computed as the difference between green and red internodes in their proportionate declines in $F_v/F_m$ following a 3 h at 4 °C photoinhibitory treatment. When regressed against the anthocyanin differential (i.e. differences in anthocyanin content between red and green internodes), a robust positive, linear relationship ($R^2$=0.98; $P$ <0.001) was evident across five of the six species (Fig. 8); the redder the stems, the greater was the photoprotective advantage as compared with green stems. The outlier was an ornamental cultivar of *Lobelia erinus*, for which the photoprotective advantage was disproportionately lower in relation to its anthocyanin content as compared with the other species. *Lobelia* was also unusual in that (i) its red stems were the richest in anthocyanins, (ii) chlorophylls were much more concentrated in the red than in the green stems, and (iii) both green and red stems were richer in chlorophylls than were those of the other species (Table 2).

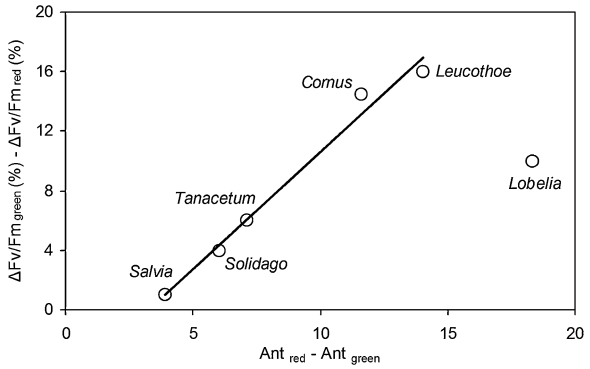


**Fig. 8.** Photoprotective advantage of red versus green pigmented stems as a function of anthocyanin concentration differential across six species. Differences in proportionate decline in $F_v/F_m$ between green and red internodes following 1200 μmol $m^{-2}$ $s^{-1}$ white light for 3 h at 4 °C regressed against differences in anthocyanin content. Regression line excludes data point for *Lobelia*; $y$=1.58$x$–5.13, $R^2$=0.98. Means, $n$=5.

## Discussion

Four sets of data presented here argue strongly in favour of a photoprotective role of anthocyanins in stems. First, anthocyanin levels greatly increased in internodes that had been staked horizontally and exposed to full sunlight at the height of summer (Fig. 6A). Second, anthocyanins in the periderm dramatically reduced the amount of *PAR* transmitted to cortical chlorenchyma (Fig. 5A). Third, for both upright and horizontal stems, $\Phi_{PSII}$ was depressed significantly less in red than in green internode portions following a protracted exposure to saturating white light at 4 °C or 22 °C (Figs 2, 6B). Finally, and most compellingly, the photoprotective advantage of red versus green stems was directly proportional to their difference in anthocyanin content across five of six species examined (Fig. 8).

Manetas (2004) has argued that, in comparison with leaves, woody stems may have reduced capacities both to utilize the absorbed quanta in photosynthesis, and to dissipate the excess light energy as heat. This is because, in the absence of stomata, high $CO_2$ concentrations ranging from 1% to 26% (Pfanz *et al.*, 2002) can accumulate in the cortical chlorenchyma; this, in turn, causes acidification of the chloroplast stroma, which inhibits the activities of some Calvin cycle enzymes and may interfere with the xanthophyll cycle for non-photochemical energy dissipation. Consistent with the hypothesis, $\Phi_{PSII}$ and $qP$ values for green *C. stolonifera* stems were lower than those for green leaves under non-saturating irradiance (Fig. 4). By contrast, those parameters were remarkably similar between red stems and green leaves. Red stems are evidently better equipped to deal with surplus incident photons, elevating their photochemical performance to that of green leaves.

That differences in photoprotection were measurable between red and green regions of the same stem (Fig. 6B)

indicates a response independent of genotypic variability. Nevertheless, the two regions had been manipulated such that they had developed under substantially different light environments, and their photosynthetic properties could have resulted from a variety of acclimatory features quite separate from possible effects of anthocyanins. Indeed, red *C. stolonifera* stems had the lower concentrations of total chlorophyll, and the larger ratios of carotenoids to chlorophylls (Table 1), which are characteristic of sun-acclimated plants. Moreover, when the periderm and other anthocyanic tissues were removed, the underlying chlorenchyma in red stems continued to show a small advantage in $\Phi_{PSII}$ relative to chlorenchyma in the green (Fig. 5C). However, much larger differences in $\Phi_{PSII}$ between red and green stems were observed when anthocyanins were present (Fig. 4). It is also noteworthy that *NPQ* values were invariably lower for red stems than for green stems (or green leaves) under non-saturating light (Fig. 4). Thus, anthocyanins in stems are important, but they are only a part of a complete set of biochemical and physiological acclimations to high irradiance, which in red stems includes the up-regulation of all photoprotection mechanisms. Collectively, these would reduce excitation pressure on the chloroplasts, so that Calvin cycle enzyme activity, although partly inhibited from stromal acidification, can adequately utilize electrons for carbon reduction.

Interestingly, among the species tested, the non-senescing leaves were usually acyanic (green) even though their supporting stem was partly or entirely anthocyanic. Indeed, in general, red stems are far more common than are red leaves (Wheldale, 1916). Most leaves, of course, have abundant stomata as well as a panoply of mechanisms for eliminating supernumerary quanta (Niyogi, 2000), and are unlikely, therefore, to encounter the problems associated with stromal acidification to the same degree as that in stems. This also suggests that there must be substantive differences in the internal biochemical environment (and perhaps anatomical structure) between stems that have the capacity to turn red, and those that do not, for the latter must have alternative means by which to withstand high light stress. Comparative studies between obligate green and facultative red stems would be fruitful for elucidating these differences.

The close correspondence in light curves for photochemical yield in red and green stems after accommodating differences in transmittance through the periderm (Fig. 5B) indicates that anthocyanins probably assist photoprotection indirectly by abating incident quantum fluxes, rather than by any direct mechanism. An alternative hypothesis, that anthocyanins shield thylakoid membranes from oxidative assault through the scavenging of reactive oxygen intermediates (Neill and Gould, 2003), seems improbable given that the red pigments are, for the most part, located some distance away from cortical chloroplasts (Fig. 1B), and most species of reactive oxygen do not diffuse freely across cell membranes or cell walls. A third possibility, that anthocyanins optimize photosynthesis by elevating stem temperatures, was not supported by *in situ* temperature measurements (Fig. 7). By contrast, the simple procedure of ensheathing a green stem in a red polycarbonate film, the optics of which approximated those of anthocyanins, reduced photoinhibition by 16%. The film reduced the flux of green light incident on the stem, but did not alter the transmission of red light. This is an important result because the photoprotective hypothesis (as it relates to red autumn leaves) has previously been criticized on the grounds that anthocyanins absorb those wavebands that are least used by the chlorophylls, and therefore would be ineffective at countering photoinhibition (Manetas, 2006; Archetti, 2009). Anthocyanins strongly absorb green and yellow light, with *in vivo* absorption maxima ranging between 537 nm and 542 nm (Merzlyak *et al.*, 2008), and like red leaves (Gould *et al.*, 2002*b*), the presence of anthocyanins in stems substantially reduced the amount of green light transmitted to subjacent chlorenchyma (Fig. 5A). Contrary to the argument used by opponents of the photoprotective hypothesis, green light has repeatedly been shown to drive photosynthesis, particularly in the lower mesophyll layers (Sun *et al.*, 1998; Evans and Vogelmann, 2003); indeed, a recent analysis found green light to be *more* effective than red light in driving photosynthesis when leaves are irradiated with strong white light (Terashima *et al.*, 2009). Thus, the absorption of green quanta by peridermal anthocyanins clearly has the potential to modulate such photosynthetic responses.

Differences in their photoprotective capacities appear to translate into a photosynthetic advantage of red over green *C. stolonifera* stems *in situ* (Fig. 7). The demonstration of an advantage of anthocyanins under natural conditions is important; the spectral output from lamps commonly used in photoinhibition experiments (e.g. Fig. 2A) can differ appreciably from that of sunlight, potentially leading to an underestimation of the natural effects of anthocyanins on green light-screening. That advantages of anthocyanins were most evident for west-facing stems only in the morning, and for east-facing stems only in the afternoon, suggests that anthocyanins assist the photoprotection of PSII more in sub-saturating diffuse light than in direct light. Differential effects of diffuse and direct light on photosynthetic yields have recently been reported for a variety of sun-acclimated leaves (Brodersen *et al.*, 2008), and it is not inconceivable, therefore, that photoabatement by anthocyanins might be more useful to stems under diffuse light. This interesting possibility warrants further investigation using larger sample sizes.

The induction of anthocyanin biosynthesis usually, though not always (Alston, 1958; Lewis *et al.*, 1998), requires exposure to light (Mancinelli, 1985). Brief exposures to visible and/or ultraviolet radiation are sufficient to induce anthocyanins in many species, while prolonged exposures can further enhance anthocyanin production (Steyn *et al.*, 2002). The horizontally-staked *C. stolonifera* stems were planted in fertile soil and irrigated regularly, and it seems probable, therefore, that the dark red colour exclusive to their uppermost surfaces was a response to elevated radiation levels rather than to other abiotic

stressors, although warmer internode temperatures may also have been involved. Horizontal stems would receive a greater proportion of the incident sunlight for most of the day than would erect ones, which explains why staking the stems resulted in a more intense pigmentation. This potentially also explains why the naturally prostrate stems of many other species bear anthocyanins on their uppermost surfaces yet are green underneath, and why stems that are colonized by twining vines are often red except for the narrow portions covered by the vine (KS Gould, personal observation). Thus, there appears to be a regulated feedback mechanism whereby the perception of high light upregulates anthocyanin biosynthesis in stems; this, in turn, protects the cortical chloroplasts from the adverse effects of prolonged exposures to high light.

The strong correlation between photoprotective advantage and anthocyanin content across five unrelated plant species (Fig. 8) indicates that the light-screening hypothesis for red-pigmented stems has widespread applicability. The mix of plants studied included annuals, perennials, shrubs, and herbs, yet irrespective of differences in their internode thickness, anatomy, or ratios of chlorophylls to carotenoids (Table 2), the photoprotective benefit of red over green stems varied as a linear function of the anthocyanin concentration differential. *Lobelia erinus* proved to be the exception in our study; although quantum efficiencies were depressed less in the red than in the green stems when subjected to saturating light, the measured benefit was smaller than might be predicted based on its anthocyanin content. The *Lobelia* used was an ornamental cultivar bred specifically for a dark purple/blue flower, and its exceptionally high levels of anthocyanins (and chlorophylls) in the stems (Table 2) are the likely outcome of artificial selection by plant breeders rather than of physiological requirement. Nonetheless, the inclusion of *Lobelia* stems in our study serves to indicate that the photoprotective hypothesis is not universally applicable. Indeed, photoprotection is unlikely to explain the more localized patterns of anthocyanin distribution, such as at the bases of internodes, and around lenticels and herbivore-induced wounds. There remains much to be learned about anthocyanin function in stems.

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