Research review

Anthocyanins in vegetative tissues: a proposed unified function in photoprotection

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Summary

The function of anthocyanins in green, vegetative tissues has always been a contentious issue. Here we evaluate their proposed photoprotective function since recent findings have shown that anthocyanins reduce photoinhibition and photobleaching of chlorophyll under light stress conditions. Anthocyanins generally accumulate in peripheral tissues exposed to high irradiance, although there are some exceptions (e.g. accumulation in abaxial leaf tissues and in obligatory shade plants) and accumulation is usually transient. Anthocyanin accumulation requires light and generally coincides with periods of high excitation pressure and increased potential for photo-oxidative damage due to an imbalance between light capture, CO_2 assimilation and carbohydrate utilization (e.g. greening of developing tissues, senescence and adverse environmental conditions). Light attenuation by anthocyanin may help to re-establish this balance and so reduce the risk of photo-oxidative damage. Although it has been suggested that anthocyanins may act as antioxidants, the association between anthocyanins and oxidative stress appears to relate to the ability of anthocyanins to reduce excitation pressure and, hence, the potential for oxidative damage. The various aspects of anthocyanin induction and pigmentation presented here are compatible with, and support, the proposed general role of anthocyanins as photoprotective light screens in vegetative tissues.

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Introduction

The visual function of anthocyanins in reproductive organs as an aid to pollination and seed dispersal is generally accepted (Harborne, 1965). However, ascribing a function to the transient accumulation of anthocyanins in green, vegetative tissues has proven elusive. This may be due to the diversity of inducers and the various patterns of red pigmentation in vegetative tissues. Recently, Smillie & Hetherington (1999) demonstrated that, by acting as visible light screens,

anthocyanins may protect photosynthetic tissues against photoinhibition. Subsequently, they proposed that anthocyanins have a general function in photoprotection of vegetative tissues that are predisposed to photoinhibition.

Our objective with this review is to evaluate the merit of the proposed general photoprotective function for anthocyanins in vegetative tissues. Our intention is to determine if the photoprotective function is congruent with the histological, developmental and environmental aspects of anthocyanin induction and variation in pigmentation. We are also interested

in evidence of any underlying physiological connection between the various inducers of red pigmentation. Initially we needed to establish whether other data exist to support this proposed role of anthocyanins in photoprotection.

Anthocyanins as Photoprotective Pigments

Photoinhibition and photoprotection

The harvesting of sunlight by green tissues is inherently hazardous. Energy capture occurs at a much faster rate than electron transport and dissipation, hence over-excitation of the photosynthetic apparatus is a constant threat. Over-excitation manifests itself as a repression of photosynthesis, phenomenon called photoinhibition (Long *et al.*, 1994). Chronic photoinhibition can significantly reduce productivity and may have a negative effect on survival (Ball *et al.*, 1991). Photoinhibitory conditions may lead to the formation of reactive oxygen species, which in turn cause photodynamic bleaching and perturbation of cellular metabolism (Foyer *et al.*, 1994).

Plants employ multiple mechanisms to balance energy capture with energy consumption and dissipation, thereby preventing oxidative damage (Demmig-Adams & Adams III, 1992; Niyogi, 1999). These include tolerance mechanisms that regulate energy distribution and dissipation, repair mechanisms, and avoidance mechanisms that decrease the absorbance of light by green tissues. Avoidance mechanisms include alteration of whole-leaf light absorption by paraheliotropic leaf orientation and leaf folding, enhanced reflectance through pubescence, salt deposition, epicuticular wax layers, and more permanent morphological adaptations, for example smaller leaf size, thicker leaves and compact growth habit. Internal measures to reduce light absorption include chloroplast movements and the accumulation of screening compounds. It is as visible light screens that some nonphotosynthetic pigments, for example anthocyanins, betalains and rhodoxanthin may exert their function by reducing light levels incident on chlorophyllous tissues (Weger *et al.*, 1993; Smillie & Hetherington, 1999).

Reduction of light levels by anthocyanins

Anthocyanins significantly modify both the quantity and quality of light incident on chloroplasts (Krol *et al.*, 1995; Ntefidou & Manetas, 1996). The red anthocyanins present in vegetative tissues preferentially absorb green and ultraviolet (UV) light and show lower absorbance of blue light, while little red light is absorbed (McClure, 1975). Absorbance of blue-green light by anthocyanins reduces light available to chlorophyll (Pietrini & Massacci, 1998; Smillie & Hetherington, 1999) in proportion to the anthocyanin concentration (Neill & Gould, 1999). This presents a mechanism to modulate light absorption in accordance with environmental and developmental requirements (Pietrini & Massacci, 1998). A low level of absorbance, or complete lack of it in the blue and red spectra, possibly allows accumulation of pigments to high levels without interference with photoreceptors, for example phytochrome and cryptochrome (McClure, 1975). The absorbance maximum of anthocyanin in the green spectrum of visible light is probably related to the deeper penetration of this colour light into green tissues and its greater contribution to total solar energy levels compared with other wavebands (Merzlyak & Chivkunova, 2000). This may be the basis for the apparent evolutionary convergence for red nonphotosynthetic pigments.

Evolutionary convergence for red pigmentation

Anthocyanins in vegetative tissues are mostly red cyanidin glycosides that are generally simpler in structure than those found in reproductive organs (Harborne, 1965), where blue colour and UV-patterning are important for guiding or directing pollinators (Harborne, 1965; Harborne & Grayer, 1994). Although anthocyanins are characteristic of higher plants (Harborne, 1965), the ability to impart red colour to plants is not restricted to anthocyanins. Families within the order Centrospermae, including taxa like prickly pear (*Opuntia* sp.) and paper flower (*Bougainvillea* sp.), display transient red coloration in vegetative tissues. However, in nine of the 11 families comprising the order, red colour is imparted by nitrogenous betalains, unrelated to anthocyanins, though colourless flavonoid precursors of anthocyanin are still present (Mabry, 1980). Certain plants accumulate red carotenoids (e.g. rhodoxanthin) in patterns and under inductive conditions typically associated with anthocyanins, such as acclimation to low temperature (Diaz *et al.*, 1990; Weger *et al.*, 1993).

The evolutionary convergence for the ability to accumulate red pigments in vegetative tissues suggests that this provides an adaptive advantage (Stafford, 1994). The selectivity for either red anthocyanins or betalains in different plant species suggests that these pigments fulfil a similar function. Since this function is unrelated to the origin and chemical characteristics of the pigments, the purpose of anthocyanin accumulation in vegetative tissues may lie in its ability to absorb visible light as a red pigment.

Ability of anthocyanins to afford photoprotection

The difficulty in obtaining a contrast between tissues containing or lacking anthocyanin, but not differing in any other respect, has hindered the study of anthocyanin function. Circumstantial evidence for the ability of anthocyanins to provide photoprotection has been obtained from studies of crosses made between yellow chlorophyll-deficient and red anthocyanin-containing hazelnut varieties for horticultural purposes (Mehlenbacher & Thompson, 1991). Chlorophyll-deficient

seedlings lacking anthocyanin died under field conditions while chlorophyll-deficient progeny containing anthocyanins survived.

Evidence for the participation of anthocyanins in photo-protection was obtained from studies on jack pine seedlings subjected to variable excitation pressures (Krol *et al.*, 1995). Seedlings acclimated at 5°C accumulated anthocyanins in needles exposed to direct light (250 µmol m^{-2} s^{-1} over the waveband 250–750 nm). Needles from the same seedlings shaded from direct light did not accumulate anthocyanin and were more susceptible to photoinhibition at moderate irradi-ance (600 µmol m^{-2} s^{-1}). Control seedlings kept at 20°C also did not accumulate anthocyanin and, upon exposure to high irradiance (1200 µmol m^{-2} s^{-1}), were twice as susceptible to photoinhibition than seedlings acclimated at 5°C. However, shaded needles of acclimated seedlings were more tolerant of photoinhibition than exposed needles of control seedlings, indicating that factors other than anthocyanin accumulation also participated in the acquisition of hardiness. Krol *et al.* (1995) attributed the increased tolerance of acclimated jack pine seedlings to photoinhibition to a combination of light attenuation by anthocyanin in the epidermis and an increased photosynthetic capacity that facilitates increased utilisation of absorbed light energy. Shading of conifer seedlings exposed to low temperatures and high irradiance had previously been found to reduce photoinhibition (Strand & Lundmark, 1987). Anthocyanin light screens may fulfil a similar role.

Smillie & Hetherington (1999) circumvented the prob-lems associated with studies of anthocyanin function by using white, red or blue-green light to subject pods of red and green *Bauhinia variegata* phenotypes to photoinhibitory condi-tions. Red light of high irradiance, which is not absorbed by anthocyanin, induced a similar degree of photoinhibition in pods of both colours. The increased ability of red pods to tolerate high intensities of blue-green and white light compared with green pods was attributed to the presence of anthocyanin. This was first conclusive evidence supporting a photoprotective function for anthocyanins that was not obviously confounded by other photoprotective measures.

Since then Feild *et al.* (2001) has used the same method to demonstrate that anthocyanins reduced photodamage in red compared with yellow senescing leaves of red-osier dogwood. Further evidence for anthocyanin-mediated photoprotection was provided by a study using apple peel tissue (Merzlyak & Chivkunova, 2000). Peel tissue ranging in colour from green to red, was subjected to severe light stress (4600 µmol m^{-2} s^{-1} photosynthetic photon fluence rate (PPFR)). The presence of anthocyanin reduced the susceptibility of chlorophyll to photobleaching, ostensibly by absorption of green-orange light.

However, Burger & Edwards (1996) found no difference in photoinhibition between leaves of red and green *Coleus* varieties exposed to severe photoinhibitory treatment (2 h at 1800 µmol m^{-2} s^{-1} PPFR). On the other hand, screening of

moderate irradiance by anthocyanin reduced the light use efficiency of photosynthesis, indicating that anthocyanin did, in fact, attenuate light. Krol *et al.* (1995) also found no difference in photoinhibition between control and acclimated seedlings at high irradiance (1200 µmol m^{-2} s^{-1} over the waveband 250–750 nm), even though anthocyanin was found to provide photoprotection at moderate irradiance. The failure to observe differences in photoinhibition at high irradiance leads us to believe that photoinhibition reaches a maximum at subsaturating irradiance and is not a good indi-cator of additional photostress at super-saturating irradiance.

The extent to which anthocyanins reduce light capture by chlorophyll depends on the histological distribution of the pigment, that is whether it is located in single or multiple layers in the epidermis, mesophyll or both.

Anatomical Aspects of Anthocyanin Function

Localization of anthocyanins in vegetative tissues

The distribution of anthocyanins within organs and tissues is genetically determined by tissue specific expression of regulatory genes. These genes control expression of structural genes in response to environmental and developmental cues (Mol *et al.*, 1996). Anthocyanin synthesis is a cell-autonomous response, meaning that colour development is controlled at the level of the individual cell (Nick *et al.*, 1993; Lancaster *et al.*, 1994). This allows local accumulation of anthocyanin resulting in a specific light screen, in contrast to other whole-leaf light avoidance measures. Cells without anthocyanin are found dispersed throughout red anthocyanin-rich apple peel (Lancaster *et al.*, 1994). Heterogeneity in cell response to stimuli allows the gradual increase in pigmentation at whole-organ-level with increasing intensity of stimulation (Nick *et al.*, 1993).

As can be expected of light screens, anthocyanins generally accumulate in peripheral tissues exposed to direct light, such as the upper epidermis (McClure, 1975; Chalker-Scott, 1999). They also accumulate throughout the leaf in mesophyll tissue (McClure, 1975) and even in trichomes (Ntefidou & Manetas, 1996). In leaves of *Quintinia serrata*, varying sizes and frequencies of red areas occurred on the lamina as a result of anthocyanin accumulation in mesophyll cells, both epidermal layers and/or vascular parenchyma at the midrib (Gould *et al.*, 2000). Generally, however, these red areas were more prevalent in leaves experiencing high light conditions.

Red pigmentation in the abaxial surfaces of expanding mango and cacao leaves (Lee *et al.*, 1987), mustard cotyledons (Drumm-Herrel & Mohr, 1985) and unfolding leaves of various fern species (unpublished observations) is, seemingly, incompatible with a photoprotective function. However, unfolding leaves and cotyledons are often orientated in such a way that, for a short period, abaxial surfaces are exposed to high irradiance while adaxial surfaces are shaded from direct

light (Drumm-Herrel & Mohr, 1985, unpublished observations). Abaxial leaf surfaces are also more light sensitive than adaxial surfaces (Sun *et al.*, 1996).

Purpling in response to phosphorous (P) and nitrogen (N) deficiency develops first in abaxial leaf surfaces before spreading to the whole leaf (Cobbina & Miller, 1987; Awad *et al.*, 1990). This may be attributed to differential stress sensitivity of different leaf tissues (Kingston-Smith & Foyer, 2000). Vital organs or tissues may be preserved in favour of more expendable components (Baysdorfer *et al.*, 1988). Hence, the lower leaf surface may either be more sensitive to nutrient stress, or P and N partitioning may favour the palisade mesophyll within deficient leaves.

Anthocyanins in shade leaves

The presence of a permanently pigmented or coloured layer immediately below the palisade mesophyll is characteristic of many plants growing in light-limiting environments (Lee *et al.*, 1979; Lee & Graham, 1986). Selectivity for red pigmentation does not apply because the coloured layer in some forest understory plants may be an iridescent blue (Lee & Graham, 1986). Gould *et al.* (1995) found higher levels of photoinhibition in green-leaved compared with red-leaved individuals of the same two shade species under photoinhibitory conditions. They proposed that the anthocyanin layer protects these shade leaves from photoinhibition. However, the red-leaved individuals also displayed greater photosynthetic capacities, confounding the measured effect. Although shade plants are extremely vulnerable to high light stress because of the large capacity for energy capture required in their ecological niche, the localization of anthocyanins within their leaves does not favour a photoprotective function. Any pigmentation pattern reducing light capture in extreme shade environments may, in fact, be a disadvantage. Attenuation of light by chlorophyll in upper leaf layers should impart considerable light protection to the lower layers (Sun *et al.*, 1996).

Lee *et al.* (1979) suggested that anthocyanins aid light capture in shade leaves through backscattering. This theory was not validated in a subsequent study (Lee & Graham, 1986). The very low light levels encountered in shade environments and the strong gradient of blue and red light attenuation (Vogelmann, 1993) ensures that mostly green light will reach the anthocyanin layer and be absorbed. For backscattering to occur, penetration and reflection of red light would be required.

Developmental Aspects of Anthocyanin Function

Developmental patterns

Anthocyanin accumulation is associated with seasonal changes in growth conditions (Nozzolillo *et al.*, 1990; Krol

et al., 1995) and with greening and etiolation, for example early seedling growth, leaf expansion and senescence (Drumm-Herrel & Mohr, 1985; Krause *et al.*, 1995; Hoch *et al.*, 2001). Anthocyanins accumulate when either environmental or developmental changes render plants more sensitive to the environment. The ability to induce anthocyanin accumulation may sometimes be limited to the juvenile phase (Murray *et al.*, 1994), or is lost with increasing age and reduced sensitivity to environmental stress, as in many conifers (Nozzolillo *et al.*, 1990; Richter & Hoddinott, 1997). Developmental patterns of anthocyanin accumulation may also differ according to the developmental strategy of different plant species. For instance, *Craterostigma wilmsii*, a resurrection plant species that maintains high chlorophyll levels during dehydration, resumes photosynthesis and degrades anthocyanin as soon as water becomes available (Sherwin & Farrant, 1998). By contrast *Xerophyta viscosa*, which degrades chlorophyll during dehydration, maintains anthocyanin during rehydration and re-assembly of the photosynthetic apparatus.

Young, expanding leaves, as well as senescing leaves, are more susceptible to photoinhibition and photobleaching of photosynthetic pigments than mature, presenescent leaves (Kar *et al.*, 1993; Krause *et al.*, 1995; Hoch *et al.*, 2001). The increased sensitivity is mainly due to a lower ability to utilize absorbed light energy (Krause *et al.*, 1995; Bukhov, 1997). Also, environmental conditions in temperate regions may be more limiting during early leaf development than later when leaves are mature (Fryer *et al.*, 1998). An inability to export carbohydrate may impose a further 'feedback' limitation on photosynthesis in developing leaves (Barker *et al.*, 1997). Anthocyanin accumulation may also precede the accumulation of other photoprotective pigments, such as xanthophylls (Gamon & Surfus, 1999). Physical light barriers, such as wax layers, that will later protect mature leaves are absent in developing leaves (Barker *et al.*, 1997). This function may be accomplished by dense trichome layers which, together with anthocyanins, strongly attenuate light (Ntefidou & Manetas, 1996; Choinski & Wise, 1999). Recent molecular studies suggest that anthocyanin synthesis and trichome development are mutually regulated, at least in *Arabidopsis* (Payne *et al.*, 2000).

Hoch *et al.* (2001) proposed that anthocyanins reduce the potential for photo-oxidative damage to senescing leaf cells. Evidence was presented in a subsequent study (Feild *et al.*, 2001). While red senescing leaves were able to recover from a photoinhibitory treatment, yellow senescing leaves suffered photodamage. Red light caused a similar degree of photoinhibition in both red and yellow senescing leaves, but anthocyanins reduced photoinhibition in red leaves irradiated with blue-green light. The photoprotection afforded by anthocyanins is thought to increase the efficiency of nutrient retrieval from senescing leaves (Feild *et al.*, 2001; Hoch *et al.*, 2001).

Transient and permanent pigmentation

A feature of the developmentally-regulated accumulation of anthocyanin is its transient nature (Harborne, 1965). Seedlings and expanding leaves typically attain maximum pigmentation a few days after germination or sprouting, whereafter anthocyanins disappear rapidly, and apparently deliberately (Kubasek *et al.*, 1992). In developing leaves the disappearance of anthocyanins seems to coincide with the transition from sink to source (Choinski & Wise, 1999). Chawla *et al.* (1999) found that the constitutive expression of anthocyanin regulatory genes in transgenic plants may be deleterious or lethal at certain developmental stages, probably by interfering with normal metabolism. Apparently, anthocyanin accumulation is normally suppressed at developmentally sensitive stages. Evidence of anthocyanin suppression through desensitisation of certain structural genes and/or negative regulation by other interrelated biosynthetic pathways has been reported (Nick *et al.*, 1993; Bowler *et al.*, 1994).

The prolonged presence of anthocyanin is usually restricted to tissues that do not have carbon assimilation as primary function, for example petioles, veins, stems and lower layers of shade leaves (Harborne, 1965; Lee *et al.*, 1979), or to inactive growth stages, for example dormancy (Sherwin & Farrant, 1998; Leng *et al.*, 2000). Taking into account that light is often limiting, especially on a whole plant level, permanent light screens are undesirable, except maybe in arid, high light habitats (Björkman & Demmig-Adams, 1995). Reduced photosynthesis due to reduced light capture in constitutively red plants may offset any potential benefit with regard to photoprotection (Burger & Edwards, 1996).

Anthocyanins are usually more permanent in horticultural plants because many constitutively red or variegated leaves or fruit of garden and crop plants have been selected for aesthetic reasons (Harborne, 1965). While mutations in genes of anthocyanin biosynthesis do not usually affect plant growth and development (Holton & Cornish, 1995), increased pigmentation is not necessarily an advantage. Although anthocyanin reduced photoinhibition in fruit of purple mango cultivars, these fruit were more susceptible to sunburn than fruit of green-fruited cultivars, presumably a result of higher heat-absorbing capacity of the darker peel (Schroeder, 1965; Hetherington, 1997). Red pear cultivars (selected bud mutations of green cultivars) are reported to be more difficult to grow, less vigorous and less productive than their parents. Martin *et al.* (1997) found that the mean maximum net photosynthetic rate and Rubisco activity in green, mature leaves of three red-fruited sports was 30–40% lower compared with their respective green-fruited parents. Photosynthesis in two of the red sports appeared to be saturated at lower light levels.

Accumulation and maintenance of anthocyanins carries an energy cost, may reduce light capture and ultimately carbon assimilation (Drumm-Herrel & Mohr, 1985; Burger & Edwards, 1996). Therefore, the transient accumulation of anthocyanin probably forms part of a short-term defence strategy to limit damage during developmental or environmental changes. Acclimation to new conditions entails the replacement of anthocyanins by more long-term physical, photosynthetic or metabolic adjustments that re-establish homeostasis between the plant and the environment as illustrated by the following three examples. First, postharvest synthesis of colourless flavonoids and anthocyanins in apples was reduced in proportion to previous light exposure (Lancaster *et al.*, 2000). Second, nutrient starved *Eucalyptus* seedlings contained high anthocyanin levels and were severely photoinhibited at planting, but photosynthetic efficiency recovered during winter while the anthocyanin content of such plants, unlike cold-stressed nutrient sufficient seedlings, did not increase (Close *et al.*, 2000). Third, exposure of lodgepole pine seedlings to conditions favouring acclimation (short daylengths and moderate temperatures) reduced subsequent anthocyanin synthesis in response to low temperatures (Camm *et al.*, 1993).

The metabolic cost of a transient presence of anthocyanin should be considerably lower than the cost attributable to damage incurred during rapidly changing environmental conditions or associated with other protection measures, for example permanent light screens and the down-regulation of the photosynthetic and assimilatory apparatus.

Environmental Aspects of Anthocyanin Function

Light

Effect on anthocyanin accumulation Consistent with a function in photoprotection, light exposure is a prerequisite for significant anthocyanin synthesis in vegetative tissues in response to both environmental (Franceschi & Grimes, 1991; Krol *et al.*, 1995) and developmental factors (Mancinelli, 1983). Depending on the species and developmental stage, red, blue or UV light may effect synthesis through mediation by phytochrome, cryptochrome or the putative UV-receptor (Mancinelli, 1983; Mol *et al.*, 1996 for review on signal perception and transduction). Generally, induction of anthocyanin synthesis requires high light intensities, and anthocyanin levels in plants and in individual leaves vary in relation to light exposure levels (Mancinelli, 1983; Krol *et al.*, 1995). Endogenous signals, developmental stage, environmental factors and previous light exposure modify the effect of light on anthocyanin synthesis (Mancinelli, 1983).

Physiological studies Recently, molecular tools have broadened our understanding of the light regulation of anthocyanin synthesis during photomorphogenesis. The underlying molecular basis for the high light requirement for anthocyanin synthesis and the synchronization of anthocyanin accumulation with other photomorphogenic processes, such as greening, has been established in etiolated tomato seedlings (Bowler *et al.*, 1994).

Anthocyanin, PSI and PSII synthesis are regulated through the phytochrome–mediated activation of their respective signal transduction pathways (Bowler *et al.*, 1994). Negative reciprocity between the pathways ensures synthesis of anthocyanins and suppression of greening during early seedling growth when seedlings are most susceptible to light-induced stress (Drumm-Herrel & Mohr, 1985). Anthocyanin synthesis is suppressed as chlorophyll starts to accumulate and emphasis shifts to carbon assimilation. The signalling pathway leading to anthocyanin synthesis is less sensitive to the signalling compound shared by the pathways (Bowler *et al.*, 1994). The result is that stronger signals are required to trigger the anthocyanin pathway, which is the basis for the high light requirement for anthocyanin synthesis. Anthocyanin synthesis requires an investment of carbohydrate reserves before seedlings become self-sufficient (Drumm-Herrel & Mohr, 1985). The high light requirement and the strict regulation of synthesis ensure that anthocyanin will only accumulate to the concentrations required and only at specific times and locations (Drumm-Herrel & Mohr, 1985).

Recently, Iida *et al.* (2000) described a gene apparently involved in acclimation to visible light stress. This gene was rapidly induced in proportion to intensity and duration of irradiation stress. Over-expression of the gene resulted in constitutive high-light tolerance, anthocyanin accumulation and adaptive phenotypic changes, such as thicker leaves, usually associated with acclimation to high light, suggesting that anthocyanin accumulation is part of the general plant response to light stress.

Anthocyanins and UV-B protection The UV-inducibility of anthocyanins and the ability of anthocyanins to absorb UV-B radiation have led to suggestions that these pigments protect plants from UV-B. High concentrations of anthocyanin can provide protection against UV-B radiation in cells and tissues where it is the major UV-absorbing compound (Takahashi *et al.*, 1991; Stapleton & Walbot, 1994; Burger & Edwards, 1996). However, a general UV-protective function for anthocyanins through the attenuation of UV-B radiation is unlikely.

Like the colourless flavonoids, the perfect UV-B screen should be permanent, ubiquitous in peripheral cell layers where most attenuation of UV-B occurs (DeLucia *et al.*, 1992) and should accumulate to high levels without any negative effect on photosynthetic yield (Teramura, 1983). However, anthocyanin accumulation is mostly transient, not confined to the epidermis (Gould *et al.*, 2000) and may reduce photosynthesis (Burger & Edwards, 1996). Furthermore, anthocyanins have a lower UV absorbance than colourless flavonoids and simpler phenolics (Caldwell *et al.*, 1983; Teramura, 1983; Landry *et al.*, 1995) and, when present, often contribute little to total UV-B absorbance (Lee *et al.*, 1987; Woodall & Stewart, 1998). Increased UV-B radiation has been found to reduce anthocyanin levels, in

some instances while UV-B absorbance increases due to accumulation of phenols and flavonoids (Moorthy & Kathiresan, 1997).

But why does anthocyanin accumulate in response to UV-B radiation if it does not have a general function in attenuation of UV-B radiation? UV-B radiation induces the down-regulation of photosynthesis primarily by damaging PSII (Teramura & Sullivan, 1994) and reducing the content and activity of Rubisco and other Calvin-cycle enzymes (Jordan *et al.*, 1992; Allen *et al.*, 1997), thereby increasing susceptibility of plants to photoinhibition. It is conceivable that anthocyanins protect the photosynthetic apparatus against photodamage by reducing visible light under conditions when UV-radiation inhibits photosynthesis. However, high visible light levels alleviate many of the detrimental effects of UV-B radiation (Teramura, 1980; Caldwell *et al.*, 1994).

Experimental conditions in studies of plant responses to UV radiation were often unrealistic in the past and bore no resemblance to field conditions (Björn, 1996; Allen *et al.*, 1998). UV-B levels much higher than would occur naturally have often been combined with low visible light levels, exacerbating the effect of UV-B (Teramura, 1980). Realistic levels of UV-B irradiance together with corresponding levels of white light do not appear to have a significant influence on photosynthesis in many species (Allen *et al.*, 1998). Rather, UV-B induction of anthocyanin synthesis, down-regulation of photosynthesis, altered growth habit and changes in leaf morphology seem to form part of an adaptive rather than injurious general photomorphogenic response to UV-B (Björn, 1996). Under natural conditions, anthocyanin induction by UV-B may perform a photoprotective role similar to that which has been proposed for visible light induction of anthocyanin via phytochrome (Drumm-Herrel & Mohr, 1985; Bowler *et al.*, 1994).

Temperature

Suboptimal temperatures, experienced either as sudden, short-term cold spells or long-term seasonal reductions in temperature, induce anthocyanin synthesis (Nozzolillo *et al.*, 1990; Christie *et al.*, 1994; Leng *et al.*, 2000), while high temperatures reduce synthesis and are associated with net pigment loss (Oren-Shamir & Levi-Nissim, 1997; Haselgrove *et al.*, 2000). Anthocyanin accumulation often coincides with acclimation or deacclimation of overwintering tissues and, although it appears to be a general response to cold stress (Christie *et al.*, 1994), does not seem to be involved in the acquisition of hardiness (Steponkus & Lanphear, 1969; Leyva *et al.*, 1995). Evidence suggests that anthocyanin synthesis and hardening are linked at a regulatory or biochemical level (McKown *et al.*, 1996). Environmental cues other than temperature that participate in the acquisition of hardiness, for example photoperiod, may also induce anthocyanin accumulation (Howe *et al.*, 1995). Interestingly, four of seven

Arabidopsis mutations showing reduced freezing tolerance, displayed reduced pigmentation (McKown *et al.*, 1996).

Maximal pigmentation usually requires low night temperatures (10°C) followed by mild day temperatures (25°C). Low temperatures enhance transcription of anthocyanin regulatory and structural genes, but post-transcriptional events leading to anthocyanin synthesis require higher temperatures (Christie *et al.*, 1994). Temperatures that effectively induce synthesis vary between species, cultivars and tissues as well as developmental stage and growth conditions. For example, mature apple fruit will colour at night temperatures below 15°C (Curry, 1997) while maximum pigmentation in dormant apple shoots occurs at −20°C (Leng *et al.*, 2000). A heat treatment of short duration (3 h at 30°C) was found to reduce the effect of preceding inductive low temperatures on anthocyanin accumulation (Reay, 1999), suggesting intervention of high temperatures at molecular level in preventing anthocyanin accumulation. Light and high temperatures have been found to increase anthocyanin and betacyanin degradation in solution, preserves and whole fruit (Attoe & Von Elbe, 1981; Marais *et al.*, 2001). Increased heat load and a reduction in carbon gain due to the presence of anthocyanin at high temperatures (Schroeder, 1965; Burger & Edwards, 1996), may be reasons for the negative relationship between temperature and anthocyanin.

Anthocyanins disappear with the resumption of growth or with increasing temperature, although it may persist with the continuation of cold conditions (Nozzolillo *et al.*, 1990; Oren-Shamir & Levi-Nissim, 1997). Conversely, anthocyanin synthesis may coincide with resumption of photosynthetic activity and increasing temperatures in cold environments. Starr & Oberbauer (2002) found that anthocyanin levels in three arctic evergreens increased as light intensity increased with melting of the snow cover. Environmental and growth conditions that predispose the photosynthetic apparatus to photoinhibition and photooxidation may increase the extent of anthocyanin accumulation in response to low temperature (Nozzolillo *et al.*, 1990; Close *et al.*, 2000). For example, newly planted *Eucalyptus* seedlings displayed photoinhibition and anthocyanin accumulation during winter, while established saplings did not (Close *et al.*, 2000).

While light capture and O_2 evolution are temperature insensitive, enzymatic assimilatory reactions decrease with decreasing temperature (Huner *et al.*, 1998). Consequently, light levels required to saturate photosynthesis decrease while the probability of photoinhibition at a constant light level increases with decreasing temperature (Hetherington & Smillie, 1989; Falk *et al.*, 1990). Low temperatures may have an even greater effect on carbohydrate metabolism by limiting assimilate utilization and decreasing sink strength (Azcón-Bieto, 1983; Paul *et al.*, 1992). Either shading or protecting conifer seedlings against frost reduced photoinhibition (Strand & Lundmark, 1987). Decreasing the source : sink ratio through shading may also relieve the low temperature-

imposed sink-limitation on photosynthesis (Paul *et al.*, 1992). Similarly, attenuation of light by anthocyanins probably provides protection against photoinhibition in tissues exposed to a combination of low temperatures and light (Krol *et al.*, 1995; Starr & Oberbauer, 2002).

Nutrient deficiency

Anthocyanin accumulation is a distinctive symptom of P deficiency in many plants, though N deficiency may also induce purpling (Cobbina & Miller, 1987; Nozzolillo *et al.*, 1990; Close *et al.*, 2000). Highest anthocyanin yield in suspension cultures was obtained when N and/or P concentrations were low (Dedaldechamp *et al.*, 1995). Addition of N to cell suspension cultures reduced anthocyanin accumulation (Pirie & Mullins, 1976; Sakamoto *et al.*, 1994).

An *Arabidopsis* mutant deficient in the ability to maintain adequate internal P levels displayed at least a 100-fold greater anthocyanin content than the normal phenotype (Zakhleniuk *et al.*, 2001). Similarly, *Arabidopsis* mutants with diminished expression of two RNase genes usually induced by P starvation and thought to sequester P, displayed increased anthocyanin levels in P adequate and deficient growth medium (Bariola *et al.*, 1999). Anthocyanin synthesis in flooded (Andersen *et al.*, 1984) or cold soils (Cobbina & Miller, 1987) may be due to reduced P uptake experienced under these conditions (Engels *et al.*, 1992; Topa & Cheeseman, 1992). Salinity stress, reported to result in anthocyanin accumulation, induces P deficiency in leaves of tomato and increases the P requirement of young leaves (Awad *et al.*, 1990).

P and N deficiency results in growth reduction, carbohydrate accumulation, sugar-repression of photosynthesis, and increased susceptibility to photostress (Lauer *et al.*, 1989; Paul & Driscoll, 1997; Verhoeven *et al.*, 1997; Nielsen *et al.*, 1998). Very low P levels may eventually limit photosynthesis due to the insufficient regeneration of ribulose bisphosphate (Rao & Terry, 1995). Interestingly, low temperatures may predispose leaves to phosphate limitation by suppressing photorespiration and therefore cycling of orthophosphate (P_i) (Leegood & Furbank, 1986) and by causing loss of the synchronization between activity of enzymes such as sucrose phosphate synthase and diurnal assimilatory activity of photosynthesis (Jones *et al.*, 1998). Cold tolerance in some, mainly herbaceous, plants is achieved through greater availability of P_i. This is brought about by a change in carbon sinks from exporting fixed carbon to support new growth to increased flux of fixed carbon to storage (Huner *et al.*, 1993). The reduced sensitivity to photoinhibition displayed by hardened rye leaves could partially be reproduced by feeding P to nonhardened leaves (Hurry *et al.*, 1993).

Gaume *et al.* (2001) attributed increased tolerance to P deficiency in maize to the accumulation of anthocyanins. Shading of N-deficient leaves prevented carbohydrate accumulation and the subsequent repression of photosynthesis

(Paul & Driscoll, 1997) providing an indication of how anthocyanin light screens possibly protect nutrient deficient plants against light stress.

Wounding and pathogen attack

Plants often accumulate anthocyanin in response to wounding (Bopp, 1959) and pathogen infection (Hammerschmidt & Nicholson, 1977a; Hipskind *et al.*, 1996). Fungal elicitors enhance anthocyanin accumulation in cell cultures (Rajendran *et al.*, 1994; Fang *et al.*, 1999) although, in other cases, fungal inoculation or elicitors were found to reduce anthocyanin accumulation (Gläßgen *et al.*, 1998; Lo & Nicholson, 1998). This probably relates to regulation of the phenyl-propanoid pathway ensuring allocation of resources from less essential metabolic activities to those of immediate concern for survival (Gläßgen *et al.*, 1998; Lo & Nicholson, 1998).

Environmental conditions may modify anthocyanin accumulation in response to pathogens and wounding. Sweetcorn hybrids infected with barley yellow dwarf virus accumulated anthocyanin during a cool, but not during a warm season (Itnyre *et al.*, 1999). Anthocyanin accumulation in response to methyl jasmonate or jasmonic acid (JA) was greater in cooled than in uncooled tulip bulbs (Saniewski *et al.*, 1998). Sucrose has a synergistic effect on JA-induced gene expression in the light while P partially inhibits the JA effect (Berger *et al.*, 1995). Pests and pathogens damaging and reducing root function may give rise to reddening probably by inducing P deficiency (Cobbina & Miller, 1987).

Exogenous JA application, acting at transcriptional level, induces anthocyanin accumulation in various tissues (Franceschi & Grimes, 1991; Tamari *et al.*, 1995; Saniewski *et al.*, 1998). The effect of JA could be reproduced by wounding (Tamari *et al.*, 1995). In at least some host–pathogen interactions, anthocyanin induction may proceed via the jasmonate-wounding pathway (Feys *et al.*, 1994). Coronatine, a supposed jasmonic acid mimic produced by some *Pseudomonas syringae* pathovars, and jasmonic acid both induced anthocyanin synthesis in *Arabidopsis* seedlings, while coronatine insensitive mutants did not accumulate anthocyanin and were resistant to the pathogen.

Increased anthocyanin accumulation is often indicative of resistance or hypersensitivity responses while anthocyanin accumulation is repressed in susceptible host-parasite combinations (Hammerschmidt & Nicholson, 1977b; Heim *et al.*, 1983; Hipskind *et al.*, 1996). Anthocyanin does not seem to play a direct role in the pathogen–host interaction, but accumulates in healthy uninfected epidermal cells surrounding restricted lesions only after fungal growth is repressed (Heim *et al.*, 1983; Hipskind *et al.*, 1996). Rather, anthocyanin synthesis may be related to the accumulation of carbohydrates, reduced photochemical quenching and local demise of the photosynthetic apparatus that are, typical responses to pathogen infection (Balachandran *et al.*, 1997).

Water status

Although drought is said to increase pigmentation (Balakumar *et al.*, 1993; Yang *et al.*, 2000), no evidence of drought-induced anthocyanin synthesis could be found. Combinations of high UV-B radiation and water stress increased pigmentation in cowpea (Balakumar *et al.*, 1993) and cucumber (Yang *et al.*, 2000) seedlings, but not relative to UV alone. On its own, water stress had no significant effect on pigmentation. Additional, nonphotosynthetic pigmentation could presumably increase the heat-load of tissues (Schroeder, 1965; Hetherington, 1997) and high leaf temperature has been found to aggravate photoinhibition in water-stressed plants (Ludlow & Björkman, 1984). Many environmental stresses, including water stress, may induce leaf senescence (Gan & Amasino, 1997) and so bring about anthocyanin pigmentation.

The red carotenoid, rhodoxanthin, accumulates in *Aloe vera* in response to high light and drought stress and is thought to provide protection against the resultant photo-oxidative stress (Diaz *et al.*, 1990). Also, water stress predisposes leaves to photo-oxidative damage, which can be reduced or prevented through light avoidance mechanisms (see section above entitled 'Photoinhibition and photoprotection') (Ludlow & Björkman, 1984; Smirnoff, 1993).

Some resurrection plants accumulate anthocyanins in exposed surfaces in response to severe dehydration (Farrant, 2000). The anthocyanins are thought to reduce light stress and provide protection against oxidation. Anthocyanin accumulation in these plants should, however, be seen as part of a distinct developmental strategy analogous to the development of dormancy in response to low temperature, and not as a response to drought stress.

Biochemical Commonality Between Inducers of Anthocyanin Synthesis

According to Foyer *et al.* (1997), plant response to changing environmental conditions involves changes in the expression of two sets of genes, those involved in antioxidative defence and those involved in carbohydrate metabolism. Photoinhibitory conditions and excess excitation increase the levels of reactive oxygen species, which may result in oxidative damage to cells (see section above entitled 'Photoinhibition and photoprotection'). Changes in carbohydrate metabolism comprise partitioning of resources for employment of defence mechanisms (Foyer *et al.*, 1997) and are required for the adjustment of source activity to reduced sink strength, a general effect of various stresses (Sheen, 1994).

Antioxidative defence

Tissues may experience increased oxidative stress at sensitive developmental stages, for example early leaf development

(Fryer *et al.*, 1998). Many environmental stresses including those associated with anthocyanin synthesis, for example low temperature (Prasad *et al.*, 1994), UV radiation (Landry *et al.*, 1995), wounding and pathogen infection (Grantz *et al.*, 1995; Lamb & Dixon, 1997), also increase the levels of oxidants and induce the expression of genes involved with protection against oxidative stress. Oxidative stresses, for example ozone (Foot *et al.*, 1996) and salt (NaCl and $CaCl_2$) stress (Kennedy & Filippis, 1999; Donahue *et al.*, 2000), were found to induce anthocyanin accumulation. Anthocyanins probably provide protection against oxidative metabolites produced during the expression of disease resistance (Hipskind *et al.*, 1996), dehydration of resurrection plants (Sherwin & Farrant, 1998) and P deficiency (Gaume *et al.*, 2001). Chromoplast-specific carotenoids accumulate in green tissues in response to oxidative stress where they effectively quench free radicals (Bouvier *et al.*, 1998). These carotenoids include rhodoxanthin, which is thought to play a photo-protective role similar to that of anthocyanin (Diaz *et al.*, 1990; Weger *et al.*, 1993).

Flavonoids, including anthocyanins, are potent antioxidants (Yamasaki *et al.*, 1997; Yamasaki, 1997), but are spatially separated from sites of oxidant generation in the chloroplast and mitochondria. Despite rigorous quenching in these organelles, H_2O_2 may leak to the vacuole during severe stress. Yamasaki (1997) suggested that the H_2O_2 is quenched by anthocyanin and other phenolics. However, the equal effectiveness of other colourless flavonoids and phenolics as antioxidants suggests that the putative photooxidative protection afforded by anthocyanins should be unrelated to their ability to quench oxidants. Rather, it is conceivable that anthocyanins protect plants against photooxidation through the attenuation of visible light and consequent reduction of excitation pressure (Smillie & Hetherington, 1999; Merzlyak & Chivkunova, 2000; Feild *et al.*, 2001).

Sink–source regulation

Carbohydrate accumulation, locally or at a whole plant level, is a common response to all the main environmental inducers of anthocyanin synthesis, for example low temperature (Strand *et al.*, 1997), nutrient deficiency (Paul & Stitt, 1993), wounding and pathogen infection (Balachandran *et al.*, 1997), flooding (Topa & Cheeseman, 1992) and oxidative stress (Foot *et al.*, 1996). Exogenous sucrose and hexose sugars strongly induce anthocyanin synthesis in suspension cultures, detached leaves and leaf disks (Murray *et al.*, 1994; Decendit & Mérillon, 1996; Larronde *et al.*, 1998). Anthocyanin synthesis in response to treatments that increase carbohydrate levels, such as girdling (Jeannette *et al.*, 2000), CO_2 enrichment (Tripp *et al.*, 1990; Stitt, 1991), sink removal (Hussey, 1963) and treatment with sulfonylurea herbicides (Hall & Devine, 1993; Nemat Alla & Younis, 1995) may be related to this. So may the reduction in pigmentation in

response to treatments that reduce carbohydrate levels such as source removal (Hussey, 1963) and phenylurea herbicides (Downs *et al.*, 1965). Expression of chalcone synthase (CHS), a key enzyme in anthocyanin synthesis, has been found to be induced by sugar (Tsukaya *et al.*, 1991; Takeuchi *et al.*, 1994).

Apart from anthocyanin biosynthesis, sugar regulation of gene expression may also affect processes as diverse as photosynthesis, carbohydrate metabolism, oxidative stress defence and senescence (Sheen, 1994; Ehness *et al.*, 1997). Generally, sugar-mediated regulation of gene expression is thought to assist plants in balancing carbohydrate supply with demand in response to environmental change and the transition from heterotrophic to autotrophic growth (Sheen, 1994). The jasmonic acid-mediated accumulation of vegetative storage proteins, induction of anthocyanin synthesis and repression of the assembly of the photosynthetic apparatus in sink cells that have a low capacity to export or store carbon is thought to have a similar function by creating a carbon and nitrogen sink, releasing phosphate from sugar-phosphate pools and reducing light levels incident on chloroplasts (Sadka *et al.*, 1994; Creelman & Mullet, 1997; and also refer to section above entitled 'Nutrient deficiency').

P exercises a direct effect on anthocyanin synthesis by inhibiting sucrose-stimulated expression of CHS (Sadka *et al.*, 1994). Depletion of P induces expression of CHS even in the absence of sucrose (Sadka *et al.*, 1994). This is probably related to the role P plays in the regulation of carbohydrate metabolism and the balancing of source capacity with sink demand (Stitt, 1991; Marschner, 1995).

Anthocyanin accumulation and reduced expression of Calvin-cycle enzymes in response to sink limitation probably represents a mechanism to down-regulate photosynthesis in order to restore the source to sink balance, and to prevent photoinhibition and subsequent photooxidative damage (Creelman & Mullet, 1997; Jeannette *et al.*, 2000).

Conclusion

Light may become toxic to green tissues under environmental stress as well as at certain stages during normal development. In this review a picture has emerged of anthocyanins as effective and flexible light screens allowing the sensitive modulation of light absorption, and so reducing photoinhibition in photosynthetic tissues. Currently, there is proof of the photoprotective role of anthocyanins in senescing leaves, but evidence also supports a photoprotective function in de-etiolating tissues and in plants experiencing environmental stress. Light attenuation may be especially beneficial under conditions that impose a sink limitation on plants and may help to re-establish a balance between light capture, CO_2 assimilation and carbohydrate utilization. Reduced light capture may also decrease the potential for photo-oxidative damage in cells experiencing high excitation pressure.

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