Anthocyanins

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Editors

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Biosynthesis, Functions, and Applications

Springer

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ISBN: 978-0-387-77334-6 e-ISBN: 978-0-387-77335-3
DOI: 10.1007/978-0-387-77335-3

Library of Congress Control Number: 2008933450

Printed on acid-free paper

springer.com

Preface

Amongst the range of naturally occurring pigments, anthocyanins are arguably the best understood and most studied group. Research into their occurrence, inheritance and industrial use encompasses hundreds of years of human history, and many volumes are dedicated to describing the prevalence, type and biosynthesis of anthocyanins. Only recently have studies begun to explain the reasons for the accumulation of these red pigments in various tissues of plants. Indeed it has only been within the last 20 years, coinciding with the ability to genetically manipulate plants, that we have begun to tease out the multitude of roles that these compounds play within plants. Alongside these fundamental advances in understanding the functional attributes of anthocyanins *in-planta* we are now beginning to realise the potential of anthocyanins as compounds of industrial importance, both as pigments in their own right and also as pharmaceuticals.

With this backdrop, the 4th International Workshop on Anthocyanins was convened in Rotorua, New Zealand in January 2006. The programme was designed to bring together a wide range of researchers across an array of disciplines to highlight the increasing importance of these pigments as a research area but also as chemicals of wider importance to human activity. The chapters in this book represent a collection of recent and highly relevant reviews prepared by participants in this symposium who are internationally recognised experts in their respective fields.

The book is divided into 10 chapters that address a wide range of topics including the proposed roles of anthocyanins *in vivo*, methods for manipulating the biosynthesis of anthocyanins to both produce new pigments as well as pigments that exhibit greater stability, use of plant and microbial cell cultures for large scale production of anthocyanins for industrial uses and the effectiveness of anthocyanins as pharmaceutical compounds.

Chapter 1 reviews the current thinking about the role of anthocyanins in leaves, stems roots and other vegetative organs. The authors point out that current roles for anthocyanins such as participation in photoprotective, UV-B protective, and protection from oxidative stresses do not adequately explain the range of spatial and temporal distributions of anthocyanins observed in plants. They hypothesise that anthocyanins possess a more indirect role in signalling and developmental regulation in response to oxidative stress.

The discussion in Chapter 1 is expanded on in Chapter 2, whose authors proceed to examine the potential roles of anthocyanins in plant/animal interactions. In particular this chapter reviews current thought and recent experimental evidence on plants' use of anthocyanins as visual clues that provide information to animals about palatability of plant structures and the potential role of anthocyanins in camouflage, undermining insect crypsis and in mimicry of defensive structures.

The molecular basis for spatial and temporal regulation of anthocyanin biosynthesis forms the focus of Chapter 3. This review concentrates on the recent advances in our understanding of the biosynthesis and molecular regulation of this pathway and how this information has been used in conjunction with recombinant DNA technologies to manipulate anthocyanin production in plants for both scientific and commercial applications.

Picking up again the theme of *in vivo* roles for anthocyanins, Chapter 4 reviews the role of anthocyanin pigmentation in fruits and adaptive advantages accumulation of these pigments confers to plants. In particular the author concentrates on the accumulation of anthocyanin pigments in fruits in response to environmental factors, seed disperser visual systems and fruit quality parameters. Accumulation of pigments contributing differing hues to fruit is discussed with respect to the interaction with animal dispersers and as a measure of fruit ripeness and quality.

Chapter 5 provides an in-depth review of the use of plant cell cultures for the industrial production of anthocyanins for use as high quality food pigments. A wide range of plant species are reviewed as to their ability to produce cell cultures capable of production of anthocyanins in cell culture, the types of cultures obtained and the pros and cons of using these cell types as production systems for anthocyanins. Methodology to increase the production of anthocyanins from these cultures, and limitations of these cultures for anthocyanin production are discussed alongside potential methods for overcoming production barriers that currently prevent large scale anthocyanin production from plant derived cell cultures.

Anthocyanin stability and the colour imparted to plant tissues by anthocyanin accumulation are in part due to the extent and nature of secondary modifications to the anthocyanin aglycone. Glycosylation, acylation and methylation are especially important in altering the chemical characteristics of anthocyanins both *in vivo* and *in vitro*. In Chapter 6 the authors review the current advances in our understanding of the biochemical pathways that lead to these chemical modifications and how this information may be utilized to modify and stabilize anthocyanins both in plants and for industrial uses such as those described in Chapter 5 and later in Chapter 9.

While production of anthocyanins in plant cell cultures is well documented, production of these compounds in microbial cell culture systems is a relatively new concept. With the increasing knowledge of the biosynthesis of anthocyanins in plant systems it has become feasible to engineer microbial species to contain a functional anthocyanin pathway. Chapter 7 reviews current advances in this area and is split into two sections. The first deals with advances in the metabolic engineering of bacterial and yeast species for anthocyanin production while the second section reviews the endogenous biotransformations carried out by host species and provides a excellent counterpoint to natural modification schemes found in plants that is reviewed in Chapter 6.

Continuing the theme of industrial application of flavonoids and anthocyanins developed in earlier chapters, Chapter 8 turns our attention to the utility of this ubiquitous chemical group in agricultural systems. In particular the roles of anthocyanins, related flavonoids and their derivatives in forage and forage legume species is discussed in the context of the value of these compounds in these crops from an animal health and nutrition perspective. Potential methods for manipulating the levels of important compound classes are reviewed from both a genetic modification and traditional breeding standpoint.

Returning to anthocyanins as food colorants, Chapter 9 develops themes outlined in Chapter 5 with a review of how anthocyanins are currently utilized in the food industry from sources thorough isolation and analytical methodologies employed. While industrial utilization of anthocyanins is still to be realized on a wide scale, this review covers the potential and perspectives for anthocyanins and their derivatives in food products.

Finally Chapter 10 concludes the book with a discussion of anthocyanins and other flavonoids as phytochemicals that promote human health. This very relevant topic is reviewed with a particular emphasis on the interaction of these compounds with other components of diet to protect and enhance human heath. The chapter describes how plant cell cultures and research models are being used to increase our understanding of the complex and multi-faceted roles that interacting phytochemicals play in the human body, specifically in the context of developing novel insights into the competing mechanisms of action, bioavailability and distribution *in situ*.

The editors, Associate Professor Kevin Gould, Dr. Kevin Davies and I hope that this book will both provide a valuable reference resource and provide inspiration for new researchers in this exciting and rapidly expanding field.

Dr. Chris Winefield
January 2008

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Anthocyanin Function in Vegetative Organs

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Abstract. Possible functions of anthocyanins in leaves, stems, roots and other vegetative organs have long attracted scientific debate. Key functional hypotheses include: (i) protection of chloroplasts from the adverse effects of excess light; (ii) attenuation of UV-B radiation; and (iii) antioxidant activity. However, recent data indicate that the degree to which each of these processes is affected by anthocyanins varies greatly across plant species. Indeed, none of the hypotheses adequately explains variation in spatial and temporal patterns of anthocyanin production. We suggest instead that anthocyanins may have a more indirect role, as modulators of reactive oxygen signalling cascades involved in plant growth and development, responses to stress, and gene expression.

1.1 Introduction

> “Yet it is difficult to find a hypothesis which would fit all cases of anthocyanin distribution without reduction to absurdity. The pigment is produced, of necessity, in tissues where the conditions are such that the chemical reactions leading to anthocyanin formation are bound to take place. For the time being we may safely say that it has not been satisfactorily determined in any one case whether its development is either an advantage or a disadvantage to the plant”.
>
> From Muriel Wheldale’s *The Anthocyanin Pigments of Plants*, 1916.

The possible physiological roles of anthocyanins in vegetative tissues have perplexed scientists for well over a century. Anthocyanins are to be found in the vacuoles of almost every cell type in the epidermal, ground, and vascular tissues of all vegetative organs. They occur in roots, both subterranean and aerial, and in hypocotyls, coleoptiles, stems, tubers, rhizomes, stolons, bulbs, corms, phylloclades, axillary buds, and leaves. There are red vegetative organs in plants from all terrestrial biomes, from the basal liverworts to the most advanced angiosperms. Plants also show tremendous diversity in anthocyanin expression. In leaves, for example,

K. Gould et al. (eds.), *Anthocyanins*, DOI: 10.1007/978-0-387-77335-3_1,

anthocyanins may colour the entire blade, or else be restricted to the margins, stripes, patches, or seemingly random spots on the upper, lower or both lamina surfaces. In some leaves, only the petiole and major veins are pigmented red, in others it is the interveinal lamina tissue, or the stipules, or domatia that are anthocyanic. Some leaves turn red shortly before they abscise, others are red only while they are growing, yet others remain red throughout their lives. In many species, anthocyanins are produced only when the plant is unhealthy or has been exposed to environmental stress, but there are some that develop the red pigments even under optimal growth environments. Given this enormous variation in location, timing, and inducibility of anthocyanins in vegetative tissues, it is not surprising that a unified explanation for the presence of these pigments has thus far eluded scientific investigation. Muriel Wheldale's statement that a unified explanation would require "reduction to absurdity" remains as valid today as it was when she wrote it in 1916.

Although the selective pressure that has driven the evolution of anthocyanins in such disparate vegetative structures remains far from obvious, plant physiologists have nevertheless made significant progress over the past decade in elaborating the *consequences* of cellular anthocyanins on plant function. Reflecting the resurgence of scientific interest in anthocyanin (and betalain) function in vegetative organs, several reviews have been written on this topic in recent years (Chalker-Scott 1999, 2002; Hoch et al. 2001; Gould et al. 2002b; Lee and Gould 2002a, 2002b; Steyn et al. 2002; Close and Beadle 2003; Gould 2004; Gould and Lister 2005; Stintzing and Carle 2005; Manetas 2006). Those reviews provide a comprehensive summary of contemporary knowledge, particularly in relation to leaf physiology, on which most research has been done. It is not our intention to duplicate that information in this chapter, although for completeness we do briefly summarise the three leading hypotheses for anthocyanin function in leaves. Rather, with the use of selected examples, we hope to demonstrate the extraordinary versatility in anthocyanin function. Thus, any two species might benefit from anthocyanins in very different ways and to different degrees, even though the chemical nature and histological location of the pigment are identical. Finally, in acknowledgement of the recent paradigm shift in relation to the role of reactive oxygen species (ROS) in plants (see Foyer and Noctor 2005), we develop an argument for a novel function of anthocyanins in leaves – that of a modulator of signal transduction cascades in physiological responses to stress.

1.2 Anthocyanins and Stress Responses

Foliar anthocyanins most commonly occur as vacuolar solutions in epidermal and/or mesophyll cells, although in certain bryophytes these red pigments bind to the epidermal cell wall (Post 1990; Gould and Quinn 1999; Gould et al. 2000; Lee and Collins 2001; Post and Vesk 1992; Kunz et al. 1994; Kunz and Becker 1995; Hooijmaijers and Gould 2007). Irrespective of their cellular location, however, anthocyanin biosynthesis in many leaves is generally upregulated in response to one or more environmental stressors. These include: strong light, UV-B radiation, temperature extremes, drought, ozone, nitrogen and phosphorus deficiencies, bacterial and fungal infections, wounding, herbivory, herbicides, and various pollutants (McClure 1975; Chalker-Scott 1999). Because of their association with such biotic and abiotic stressors, anthocyanins are usually considered to be a stress symptom and/or part of a mechanism to mitigate the effects of stress. Much of the physiological work undertaken in recent years has attempted to unravel the phytoprotective functions of anthocyanins that would enhance tolerance to these stress factors.

1.3 Photoprotection

Photoprotective roles of foliar anthocyanin have probably received more attention in recent years than any other functional hypothesis. It had long been suggested that anthocyanins might shield photosynthetic cells from adverse effects of strong light (see Wheldale 1916), yet the first experimental confirmation of this was not achieved until the 1990s, following the advent of the field-portable pulse amplitude modulated (PAM) chlorophyll fluorometer which permitted non-invasive comparisons of the quantum efficiencies of photosynthesis in red versus green leaves (Gould et al. 1995; Krol et al. 1995). Although photosynthesis is driven by light, quanta in excess of the requirements of the light reactions can adversely affect the photosynthetic system components (antenna pigments, reaction centres, accessory proteins, and electron transport carriers), and can lead to secondary destructive and repair processes in thylakoid membranes (Adir et al. 2003). Photoinhibition, the term given to the decline in quantum yield of photosynthesis attributable to excessive illumination, can be quantified directly using PAM chlorophyll fluorometers (Genty et al. 1989; Krause and Weis 1991). One of the most useful parameters for this is the ratio of variable to maximum chlorophyll fluorescence (Fv/Fm) for dark-adapted leaves, which correlates to the maximum quantum yield of photosystem II (Maxwell and Johnson 2000). Fv/Fm values are typically around 0.83 for pre-dawn, healthy plants, but they can be considerably lower in plants under stress. Measurements of Fv/Fm values for red and green leaves before and after exposing them to photoinhibitory light fluxes provide a convenient method to compare their relative tolerances to light stress.

Anthocyanic leaves typically absorb more light in the green and yellow wavebands than do acyanic leaves (Neill and Gould 1999; Gitelson et al. 2001). The fate of these absorbed quanta is unknown, but it is very clear that their energy is not transferred to the chloroplasts. Indeed, the chlorenchyma of red leaves may receive considerably less green light than do those of structurally comparable green leaves (Gould et al. 2002c), and red leaves may develop the morphological and physiological attributes of shade leaves (Manetas et al. 2003). This light-filtering effect of anthocyanins has been shown many times both to reduce the severity of photoinhibition and to expedite photosynthetic recovery in red as compared to green leaves (see reviews by Steyn et al. 2002; Gould and Lister 2005). In point of fact, sufficient experimental evidence of a photoprotective function of anthocyanins has accrued to justify its elevation from hypothesis to theory.

Anthocyanins confer measurable photoprotection when present both in senescing foliage of deciduous trees (Feild et al. 2001; Hoch et al. 2003) and in the mature, overwintering foliage of evergreen plants (Hughes et al. 2005). Young, developing leaves can also benefit significantly from these pigments (Cai et al. 2005). Indeed, nascent chloroplasts in immature leaves are particularly vulnerable to the effects of light stress (Pettigrew and Vaughn 1998; Choinski et al. 2003). Strong support for a photoprotective role of anthocyanins in developing leaves was provided recently by Hughes et al. (2007), who followed the timing of anthocyanin production and degradation across three unrelated species: *Acer rubrum*, *Cercis canadensis*, and *Liquidambar styraciflua*. In all three species, anthocyanins were produced early in leaf development, and persisted until leaf tissues had fully differentiated. The subsequent decline in anthocyanin levels occurred only after leaves had synthesised approximately 50% of the total chlorophylls and carotenoids, and had attained close to their maximum photosynthetic assimilation rates. The authors suggested that the strong coupling between the timing of anthocyanin reassimilation and those of leaf developmental processes indicates that anthocyanins serve to protect tissues until other photoprotective mechanisms mature.

In view of the immutable property of the coloured anthocyanins to absorb light that might otherwise strike chloroplasts, it is perhaps surprising that the degree to which anthocyanins contribute to the photoprotection of leaves seems to vary substantially from species to species. In *Galax urceolata*, for example, *Fv/Fm* values for green leaves decreased 36% more than did those for red leaves following exposure to photoinhibitory conditions (Hughes and Smith 2007). Differences of a similar magnitude were noted between yellow and red senescent leaves of *Cornus stolonifera* (Feild et al. 2001), and between green, flushing leaves of *Litsea dilleniifolia* and the red flushing leaves of *Litsea pierrei* and *Anthocephalus chinensis* (Cai et al. 2005). However, much larger differences (ca. 75%) have been reported for the green adult and red juvenile leaves of *Rosa* sp., and in *Ricinus communis* the decline in *Fv/Fm* for green leaves was almost double that of red leaves (Manetas et al. 2002). In contrast, photosynthetic efficiencies of young red leaves of *Quercus coccifera* were only marginally greater than those of young green leaves under photoinhibitory light flux (Karageorgou and Manetas 2006), and red-leafed species of *Prunus* actually performed worse than green-leafed species under saturating light (Kyparissis et al. 2007).

The reasons for these large interspecific differences in photoprotection by anthocyanin are unknown. It is uncertain whether they reflect true physiological differences, or else are the result of disparities in the experimental conditions under which measurements were taken. Karageorgou and Manetas (2006) suggested that the photoprotective capacity of foliar anthocyanins might vary simply as a function of leaf thickness. They argued that because green light contributes to photosynthesis only in the lowermost tissues of the leaf lamina (Sun et al. 1998; Nishio 2000), then those leaves which are relatively thick and whose mesophyll contain large amounts of chlorophyll would benefit most from the abatement of green light by anthocyanins. Photosynthesis of thinner leaves that contain low amounts of chlorophyll would be driven almost exclusively by red and blue light, and therefore their propensity for photoinhibition would not be affected greatly by the presence of anthocyanins. Accordingly, the immature red leaves of *Quercus coccifera*, which are less than 200 μm thick and hold 11 μg m^{-2} chlorophyll, show little evidence of photoprotection by anthocyanin (Karageorgou and Manetas 2006). In contrast, the mature leaves of that species, which are twice as thick and hold four times as much chlorophyll, show a sizeable benefit from anthocyanins (Manetas et al. 2003). The "leaf-thickness hypothesis" warrants further testing, although the recorded benefits of anthocyanins to the photosynthesis of thin, immature leaves in certain other species (Hughes et al. 2007) would suggest that the hypothesis is not universally applicable.

At least some of the variation among reports of photoprotection by foliar anthocyanins is likely to be attributable to differences in the experimental protocol. Photoinhibition is often intensified when, in addition to excess photon flux, plants experience other types of abiotic stressor (Long et al. 1994). By limiting the rates of CO_2 fixation, environmental factors such as chilling and freezing temperatures, high temperatures, and nitrogen deficiency have been shown to exacerbate the photoinhibitory responses to strong light. It seems possible, therefore, that the photoprotective capacity of anthocyanins would assume greater importance in plants that face combinations of such stressors. Evidence for this was presented recently in a comparison of green- and red-leafed genotypes of maize; the beneficial effects of anthocyanins were apparent only after the plants had experienced a combination of strong light (2000 μmol m^{-2} s^{-1}) and a 5ºC chilling treatment (Pietrini et al. 2002). Light quality is also important; reductions in *Fv/Fm* have been found to be greater for green than red leaves when irradiated with white or green light, yet they are similar in magnitude under red light (Hughes et al. 2005). Thus, the experimental conditions under which leaves are tested for photoinhibition can have a significant bearing on measurements of chlorophyll fluorescence.

The interpretation of chlorophyll fluorescence signals can itself be problematic for red leaves. In a recent report describing the common pitfalls of chlorophyll fluorescence analysis, Logan et al. (2007) explained that anthocyanins may absorb a proportion of the measuring light issued from the PAM fluorometer, and therefore reduce the intensity of the emitted chlorophyll fluorescence that is collected for detection. This can lead to low signal to noise ratios, and therefore compromise the accuracy of the data. Fluorescence output can be improved by increasing the intensity of the measuring light, yet this runs the risk of the measuring beam becoming actinic (i.e. driving photosynthesis), which would artifactually reduce *Fv/Fm* values. Some machines perform better than others for measuring chlorophyll fluorescence in red leaves; Pfündel et al. (2007) showed that because anthocyanins attenuate about half of the incident radiation at 470 nm, a fluorometer that issues pulses of blue measuring light can be inferior to one that emits red pulses. It is also noteworthy that the chlorophyll fluorescence signals can alter as a leaf ages, and can even vary from region to region across a leaf lamina (Šesták and Šiffel 1997). Thus, the comparison of young (red) and old (green) leaves, or else red and green parts of the same leaf blade, may yield differences in chlorophyll fluorescence that are unrelated, or only partially related to the presence of anthocyanins.

There are in addition to anthocyanins many other mechanisms by which plants can avoid or dissipate excess light energy. These include morphological features, such as hairs or a waxy cuticle that reflect and scatter incident radiation from the lamina surface, and physiological processes such as thermal dissipation by the xanthophyll cycle pigments and the triplet chlorophyll valve, and the transfer of excess electrons to alternative sinks (Niyogi 2000). The degree to which each of these mechanisms is utilised apparently varies from species to species, as well as with the intensity and duration of exposure to abiotic stress (Demmig-Adams and Adams III 2006). Accordingly, the requirement for supplementary photoprotection, such as that provided by anthocyanins, would also vary. Consistent with this, the young leaves of *Rosa* sp. and *Ricinus communis* contain only low levels of xanthophyll pigments, yet they are resistant to photoinhibitory damage possibly because of their high anthocyanin concentrations (Manetas et al. 2002). Similarly, the combined effects of pubescence and anthocyanins in certain cultivars of grapevine (*Vitis vinifera*) apparently compensate for their reduced xanthophyll contents relative to levels in green, glabrous cultivars (Liakopoulos et al. 2006). In their analysis of mutants of *Arabidopsis thaliana*, Havaux and Kloppstech (2001) concluded that the flavonoids might actually be *more* important than the xanthophylls in regard to long-term protection from photoinhibitory damage, although the anthocyanins were less effective as photoprotectants in that system than were the flavonols and dihydroflavonols. Interspecific differences in requirements for supplementary photoprotection probably best explain why reports of the capacity of foliar anthocyanins to protect leaves from photoinhibiton vary so greatly.

1.4 Protection Against Ultraviolet Radiation

In addition to their capacity to protect plant tissues from excess visible radiation, anthocyanins have also been implicated in the protection from ultraviolet (UV) radiation. UV radiation is often classified as UV-A (320–390 nm), UV-B (280–320 nm) and UV-C (<280 nm). Stratospheric ozone (O_3) absorbs most of the UV-C and part of the UV-B. UV-A radiation, however, is not filtered by stratospheric O_3. With an absorption maximum ($A_{\lambda\text{max}}$) at 260 nm, DNA is particularly vulnerable to the adverse effects of highly energetic UV rays (Hoque and Remus 1999).

To fortify themselves against the harmful effects of UV radiation, plants have developed multifarious mechanisms to diminish UV penetration into plant tissues, including the synthesis of UV-absorbing phenolic compounds (Ryan and Hunt 2005). The biosynthesis of anthocyanins and other flavonoids is known to be activated in many plant species by UV exposure (Takahashi et al. 1991; Mendez et al. 1999; Singh et al. 1999), although exceptions have been noted (Jordan et al. 1994; Buchholz et al. 1995; Solovchenko and Merzlyak 2003). Most anthocyanins, especially those that are acylated, can absorb biologically-active UV radiation (Markham 1982; Giusti et al. 1999), and it has been suggested that their function in vegetative organs may be to buffer tissues against UV damage by attenuating the excess energy (Takahashi et al. 1991; Li et al. 1993; Koostra 1994). Support for a protective role of anthocyanins was provided by Burger and Edwards (1996), who noted that following exposure to UV-B or UV-C radiation, the photosynthetic capacities of green-leafed varieties of *Coleus* were lower than those of red-leafed varieties.

However, UV filtering is unlikely to be the primary role of anthocyanins in leaves. Foliar anthocyanins tend not to be acylated, and are therefore less effective absorbers of UV radiation than are certain other flavonoids (Woodall and Stewart 1998). Moreover, to be an efficient screen, anthocyanins must intercept incident UV radiation before it reaches the chloroplasts (Caldwell et al. 1983). In the case of leaves, this means that the pigments should reside in the vacuoles and/or cell walls of epidermal or hypodermal tissues (Day et al. 1992; Ålenius et al. 1995; Gorton and Vogelmann 1996; Olsson et al. 1999). In some species, anthocyanins can indeed be found in these superficial foliar tissues. More commonly, however, anthocyanins occur in the vacuoles of the chlorenchyma cells themselves (Wheldale 1916; Gould and Quinn 1999; Gould et al. 2000; Lee and Collins 2001), a suboptimal location for UV filtering.

It was shown recently that the presence of anthocyanins might in the long term be detrimental rather than beneficial to plants that face high UV levels (Hada et al. 2003). The authors found that in purple-leafed rice, anthocyanins absorb a portion of the blue/UV-A radiation that would otherwise activate the DNA-repairing enzyme photolyase. Such inhibition of DNA repair would offset any short-term gain from UV absorption by anthocyanins.

1.5 Free Radical Scavenging

Environmental stressors such as saturating light flux or high levels of UV radiation can augment the production of free radicals in plant cells (Foyer et al. 1994; Gould 2003). It has been suggested that by absorbing a proportion of the incident quanta, and by scavenging the free radicals thus formed, foliar anthocyanins might serve to abate this oxidative insult.

A free radical is any chemical species capable of independent existence that contains one or more unpaired electron (Halliwell and Gutteridge 1999). The oxygen radicals have been most extensively studied in plants, although there is increasing awareness of the roles of nitrogen-centred radicals. The collective term "reactive oxygen species" (ROS) or "reactive oxygen intermediates" (ROI) is often used to include both the oxygen radicals and non-radical derivatives of oxygen which have similar chemical properties. These include the superoxide radical (O_2^-), hydroxyl radical (OH), peroxyl radical (ROO), and alkoxyl radical (RO^-), as well as the non-radical intermediates such as singlet oxygen ($^1O_2$), hydrogen peroxide (H_2O_2), and ozone (O_3).

In plant cells, chloroplasts and mitochondria are the principal sources of ROS, which are generated via the aerobic reactions involved in photosynthesis and respiration (Mittler 2002; Rhoads et al. 2006). ROS are also produced in the peroxisomes during photorespiration and fatty acid oxidation (Corpas et al. 2001). Enzymatic sources of ROS have been identified, including NADPH oxidase in the plasma membrane (Grant and Loake 2000), oxalate oxidase and amine oxidase in the apoplasm (Allan and Fluhr 1997; Dat et al. 2000), and peroxidases in the cell wall (Kawano 2003). Under optimal growth conditions the production of ROS from routine metabolic processes is low: 240 $\mu M\ s^{-1}\ O_2^-$, and a steady-state level of 0.5 $\mu M\ H_2O_2$ in chloroplasts (Polle 2001). However, environmental stressors can increase levels of ROS three-fold (Polle 2001).

A superabundance of ROS potentially causes cellular damage to phospholipid membranes, proteins, and nucleic acids, and this has traditionally been considered detrimental to plant functioning (Alscher et al. 1997). Guarding against oxidative damage, plants have evolved elaborate antioxidant defence mechanisms in the different intracellular compartments. These serve to control concentrations of ROS, to improve the plant's resistance to stressors, to repair damage to proteins, particularly those in photosystem II, and to re-activate key enzymes (Halliwell and Gutteridge 1999). An antioxidant may be defined as any substance which, when present at low concentrations compared with those of an oxidisable substrate, significantly delays or prevents oxidation of that substrate. The major antioxidants are enzymes, and include superoxide dismutase (SOD), catalase (CAT), various peroxidases such as ascorbate peroxidase (APX), and glutathione reductase (GR) (Polle 1997). There are in addition a number of low molecular weight antioxidants (LMWAs) in plant cells: ascorbate (vitamin C), tocopherols (vitamin E), glutathione, β-carotene, and phenolic compounds such as the flavonoids.

Certain flavonoids, including the more common anthocyanin pigments, have ROS-scavenging capacities up to four times greater than those of vitamin E and C analogues (Rice-Evans et al. 1997; Wang et al. 1997). Their potency stems from a high reactivity as proton and electron donors, from their ability to stabilize and delocalize unpaired electrons, and from their capacity to chelate transition metal ions (Rice-Evans et al. 1996; van Acker et al. 1996; Brown et al. 1998). Flavonoids have been shown *in vitro* to neutralise most of the biologically important ROS and nitrogen-centred radicals. Recently, compelling evidence was presented for the scavenging of ROS by flavonoids *in vivo*. Agati et al. (2007) infused leaves of *Phillyrea latifolia* with DanePy, a fluorochrome whose fluorescence is quenched exclusively by $^1O_2$. Microscopic examinations of cross-sections through those leaves revealed that the scavenging of $^1O_2$, which had been generated by subjecting the leaves to strong light, was largely attributable to flavonols and flavones specifically associated with chloroplasts in the mesophyll cells.

Could antioxidant activity explain the presence of anthocyanins in vegetative tissues? Two mechanisms by which anthocyanins might reduce the oxidative load in leaves have been proposed. First, by reducing the numbers of high-energy quanta incident on the photosynthetic cells, anthocyanins might prevent or moderate the light-driven reactions that generate ROS. This is an old concept. Indeed, Wheldale (1916) herself described an experiment in which a solution of chlorophyll, when illuminated behind a glass vessel containing a red solution, remained green for longer than when illuminated behind a colourless solution. Chlorophyll bleaching is a classic symptom of oxidative damage (Kato and Shimizu 1985). More recently, Neill and Gould (2003) showed that chloroplasts suspended in a buffered solution produced fewer O_2^- radicals, and were bleached less, when irradiated with monochromatic red light than with white light of comparable intensity. However, the benefits of anthocyanin as an optical shield have yet to be demonstrated *in situ*.

Second, anthocyanins might directly scavenge ROS. Anthocyanins are usually colourless or light blue at the pH of the cytoplasm, but they turn red after being transported into the vacuole. Both the colourless and the red tautomers of cyanidin glycosides have been demonstrated to scavenge O_2^- produced by a suspension of chloroplasts under light stress (Neill and Gould 2003). Clearly, cytosolic anthocyanins would be better located than would vacuolar anthocyanins for scavenging ROS produced by organelles such as chloroplasts, mitochondria, and peroxisomes. However, the question of the common occurrence of anthocyanins in both cytosol and vacuole is one that requires further attention. It remains unclear how anthocyanins are transported to the vacuole from their site of synthesis at the endoplasmic reticulum. If they move to the vacuole by diffusion, then they would transiently pass through the cytosol. Alternatively, there is growing evidence of a route from the endoplasmic reticulum directly into vesicles, which then migrate to the vacuole, completely bypassing the cytosol (Poustka et al. 2007). Irrespective of their intracellular location, however, anthocyanin-containing leaf cells have been observed under the microscope to remove H_2O_2 more swiftly than acyanic cells (Gould et al. 2002a). It is possible, therefore, that antioxidant activity may be one of the major functions of anthocyanins in vegetative tissues.

The available data indicate that anthocyanins contribute to the total antioxidant pool more in some species than in others. For example, in *Elatostema rugosum*, a sprawling understorey herb from New Zealand, extracts from red leaves had a significantly greater LMWA activity than did those from green leaves, which could be attributed primarily to the presence of anthocyanins (Neill et al. 2002b). In contrast, in the canopy plant *Quintinia serrata*, extracts from red and green leaves showed similar ranges in antioxidant potential (Neill et al. 2002c). Similarly, in the sugar maple (*Acer saccharum*), antioxidant activity correlated strongly with anthocyanin content in extracts from juvenile leaves, but the correlation was only weak in extracts from senescing leaves (van den Berg and Perkins 2007). Reasons for these differences are not known, though it has been suggested that the location of anthocyanic cells within the leaf tissues may be important. Kytridis and Manetas (2006) compared the effects of methyl viologen, a herbicide, on various species for which foliar anthocyanins were located in different cell types. Methyl viologen inhibits photosynthetic electron transport, generating ROS that lead to the destruction of chloroplast membranes. The authors claimed that red leaves for which anthocyanins were located in the mesophyll were more resistant to methyl viologen treatment than were those that held anthocyanin in the epidermis. Although antioxidant activities were not measured in that study, the data are consistent with the hypothesis that when anthocyanins are located in the mesophyll, they can contribute to the LMWA pool.

In addition to the anthocyanins, concentrations of other LMWAs, as well as certain enzymatic antioxidants, can also be higher in red than in green leaves. For example, the red-leafed morphs of *Elatostema rugosum* had higher levels of caffeic acid derivatives and greater SOD and CAT activities than had the green-leafed morphs (Neill et al. 2002b). Similarly, leaves of maize cultivars that had been exposed to toxic copper concentrations upregulated the production of anthocyanin as well as the activities of superoxide dismutase, ascorbate peroxidase, and glutathione reductase (Tanyolaç et al. 2007). Anthocyanins might well supplement the antioxidant potential in such plants, but they clearly do not substitute the major LMWA and enzymatic antioxidants.

1.6 Paradigm Shift

Recent genetic studies have indicated that the potential for ROS to cause unrestricted damage to plant cell components is realised far less commonly than had been previously thought. On the contrary, there is a growing body of empirical evidence to suggest that ROS may serve many useful functions in plants. Indeed, ROS appear to be actively produced by plant cells for their use as signalling molecules in processes as diverse as growth and development, stomatal closure, pathogen defence, programmed cell death, and abiotic stress responses. This evidence led Foyer and Noctor (2005) to state, “The moment has come to re-evaluate the concept of oxidative stress.” They proposed that the processes by which ROS are generated and scavenged might better be described as “oxidative signalling”, and should be regarded as “an important and critical function associated with the mechanisms by which plant cells sense the environment and make appropriate adjustments to gene expression, metabolism and physiology.”

The arguments in favour of a signalling role for ROS have been expertly summarised in several reviews (Dat et al. 2000; Mittler 2002; Vranová et al. 2002; Mahalingam and Fedoroff 2003; Apel and Hirt 2004; Laloi et al. 2004; Mittler et al. 2004; Foyer and Noctor 2005; Pitzschke et al. 2006). Among the more compelling lines of evidence for ROS signalling is the work by Wagner et al. (2004) on the *flu* mutant of *Arabidopsis thaliana*. The *flu* mutant, when transferred from darkness to the light, generates singlet oxygen in the plastids, ultimately leading to chlorophyll bleaching and death of seedlings, or to the arrest of growth in more mature plants. However, when a single gene, *EXECUTER1*, is inactivated in this species (as in the *flu/executer1* double mutant), both seedlings and mature plants grow normally despite the continued production of $^1O_2$. The authors concluded that in wild type plants, $^1O_2$ does not damage cellular components directly, but rather, activates a genetic switch that initiates a signalling cascade leading to programmed cell suicide.

ROS-induced programmed cell death may be useful for plants facing pathogenic attack since it potentially limits the spread of disease from the point of infection. However, cell death would probably not be beneficial under conditions of abiotic stress such as those imposed by strong light and elevated UV-B. Mittler (2002) argued that the steady state levels of ROS may be used by plants as a gauge of intracellular stress. When levels of ROS rise in response to abiotic stress, plants face the challenge of removing excess ROS to avoid programmed cell death, yet retaining sufficient low levels of the different types of ROS for signalling purposes. This would require the finely-tuned modulation of ROS production and scavenging mechanisms. Specificity in response may be achievable by the coordinated production of LMWAs such as ascorbate and glutathione (Foyer and Noctor 2005). The flavonoids, too, seem likely to play a role in this.

1.7 Modulation of Signalling Cascades: A New Hypothesis

That ROS can be at once the products of plant stress as well as mediators in plant stress responses presents the possibility for a new functional hypothesis for the presence of anthocyanins in vegetative tissues. We propose that the anthocyanins, along with some other flavonoids, provide multifarious mechanisms for the modulation of signalling cascades that mitigate the effects of abiotic and biotic stressors. As explained below, this role is achievable in three interrelated ways: (i) by protecting antioxidant enzymes; (ii) by scavenging ROS directly; and (iii) by interactions with other molecules in the signal transduction pathways.

Many of the putative roles of anthocyanins in plant physiology could equally be achieved by antioxidant enzymes. For example, like the anthocyanins, the ROS-scavenging enzymes of the so-called "water-water cycle" (SOD and APX) in the chloroplasts result in a reduced propensity for photoinhibition and photo-oxidation (Asada 1999, 2000; Rizhsky et al. 2003). These enzymes scavenge O_2^- and H_2O_2 with extreme efficiency, and are undoubtedly key players in the modulation of ROS signalling cascades. Under certain conditions, however, these enzymes may be inactivated. Strong light combined with chilling stress, for example, reduces the efficiency of APX, leading to the accumulation of H_2O_2 to levels can inactivate APX, SOD, and CAT (Jahnke et al. 1991; Wise 1995; Casano et al. 1997; Streb et al. 1997; Asada 1999). For the water-water cycle to function properly, its enzymatic antioxidants need to be protected from free-radical attack.

It is perhaps no coincidence that the very conditions that can lead to the inactivation of such enzymes may also stimulate the formation of anthocyanins in plant tissues. Anthocyanins may prevent the inactivation of antioxidant enzymes by restricting the amount of light within a photosynthetic cell (thereby reducing the production of ROS). The strong antioxidant capacities of anthocyanins mean that they could also scavenge supernumerary ROS and therefore spare the antioxidant enzymes from inactivation. Thus, for a one-time investment in the production of anthocyanin pigments, plants might achieve the long-term protection of these pivotal components of plant stress responses. Indeed, the capacity of plants to maintain or enhance their antioxidant enzyme activities is regarded as a key feature in the acclimation of plant tissues to environmental stress (Bowler et al. 1992; Anderson et al. 1995; Pinhero et al. 1997; Scebba et al. 1999; Kuk et al. 2003).

It is possible that anthocyanins interact with stress signal transduction cascades more directly. This has been demonstrated already in human tumour cells; two anthocyanin aglycones, cyanidin and delphinidin, were found to inhibit tumour cell growth by shutting off downstream signalling cascades that would otherwise lead to the production of growth factors (Meiers et al. 2001). Interactions between phenolic compounds and ROS signalling have also been documented for plants. For example, the softening of plant cell walls, which is necessary for cell expansion, results partly from OH radical attack on cell wall polysaccharides (Fry 1998), and is terminated by the cross-linking of phenolic compounds (Rodríguez et al. 2002). Because anthocyanins can scavenge a variety of free radicals and oxidants such as H_2O_2 (Takahama 2004), they have the potential directly to influence the balance between ROS production and ROS scavenging in stress responses. H_2O_2 is considered a particularly important molecule in plant signalling because of its relative stability, as well as its ability to diffuse rapidly across membranes and between different cell compartments (Dröge 2002; Neill et al. 2002a). H_2O_2 is a known activator of MAP kinase cascades, and has been shown to regulate the expression of certain genes

(Bowler and Fluhr 2000). Despite its efficient scavenging by enzymes in the chloroplasts, mitochondria, and peroxisomes, H_2O_2 may leak into the cytosol and possibly the vacuole during periods of severe stress (Yamasaki et al. 1997). Vacuoles typically occupy more than 70% of the mature plant cell volume, and as a consequence of their size, vacuoles are one of the closest neighbours of all the major sources of organelle-derived ROS. For this reason, vacuolar LMWAs such as the anthocyanins are likely to have a crucial role, especially in preventing the symplastic movement of ROS from one cell to another (Mittler et al. 2004).

Finally, anthocyanins may interact with secondary messengers downstream of the ROS signalling pathway, or else be involved in the crosstalk with other response pathways. An intriguing possibility is the interaction between anthocyanins and sucrose. The anthocyanin biosynthetic pathway is strongly upregulated by sucrose in plants as diverse as radish (*Raphanus sativus*), English ivy (*Hedera helix*), and *Arabidopsis thaliana* (Murray and Hackett 1991; Hara et al. 2003; Solfanelli et al. 2006). Soluble sugars, especially sucrose, glucose, and fructose, are now known to play central roles in the control of plant development, stress responses, and gene expression (Gibson 2005). Sugar accumulation has been associated with improved tolerance to diverse stressors including drought, salinity, high light, cold, anoxia and herbicides (Roitsch 1999; Couée et al. 2006). It has been suggested that these roles relate to the regulation of the pro-oxidant and antioxidant balance in plant cells; sucrose is known to be involved in both ROS-producing and ROS-scavenging metabolic pathways (Couée et al. 2006). However, the mechanism by which sucrose, anthocyanins, and ROS might contribute to plant function remains to be established.

A signalling role for anthocyanins is attractive because it potentially also explains why anthocyanins often accumulate in organs that do not photosynthesise, or else for which photosynthetic carbon assimilation is not the primary function, such as stems petioles, and adventitious roots. It can also explain why anthocyanins are in some species preferentially produced at certain developmental stages, such as seed dormancy, leaf initiation or leaf senescence, or in certain seasons such as autumn or spring. Establishing possible relationships between the cellular redox balance and anthocyanin function presents the promise of an exciting, new line of investigation into this intriguing class of plant pigments.

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