Anthocyanins in Leaves: Distribution, Phylogeny and Development

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ABSTRACT

Red pigments, products of different metabolic pathways, occur in terrestrial plants. The flavonoid pathway contributes the greatest diversity, culminating in the prevalence of anthocyanins in the angiosperms. Anthocyanins are produced in flowers and fruits, and also in vegetative organs, but have been poorly researched in the latter. Anthocyanins are commonly produced in:

1. rapidly expanding leaves of tropical plants;
2. senescing leaves of temperate plants;
3. undersurfaces of floating leaves of aquatic plants;
4. abaxial surfaces of leaves of understory plants; and
5. leaves subjected to various environmental stresses.

The distribution of anthocyanins in leaves, both in presence and in tissue distribution, is influenced by both phylogeny and development. Few species produce anthocyanins in leaf tissues derived from both dermal and ground embryonic tissue. These influences will be important in resolving the ecological roles of anthocyanins in leaves.

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I. INTRODUCTION

An important context for understanding the functions of anthocyanins in plants is knowledge of their distributions, among organs and in tissue within organs. Certain functions require specific tissue distributions, and tissue distributions may be influenced by evolutionary history and developmental constraints. This article addresses these and other factors influencing anthocyanin distribution. First, it is important to review the history of this research and also to acknowledge that red coloration may be due to the production of other pigments.

A. HISTORICAL BACKGROUND

With the invention of the microscope it was not difficult to observe the distribution of pigments in specific cell layers of plant organs. Anthocyanins are sequestered in vacuoles, comprising the bulk of the volumes of most plant cells. Without any chemical knowledge, this color was viewed as caused by a single class of pigments, forming the 'colored cell sap' in plant organs. Marquart (1835) called the pigment 'anthocyanin'. Observers found anthocyanins in discrete layers within leaves. Morren (1858; Plate 1) observed the pigments in epidermal layers of red cabbage and a native lily. Hassack (1886) used his observations of pigment distributions in leaves to argue for the photoprotective function of these pigments. Later, Stahl (1896; Plate 2) observed pigmentation in both mesophyll and epidermal tissues of leaves of tropical rainforest understory plants. Such descriptions of variations in tissue distributions of anthocyanins in leaves led to some comprehensive surveys. Parkin (1918) reported on the results of a survey of 400 species of plants. Many of these species produced pigments within the mesophyll, but the report omitted distributions in specific cell layers and did not list the species surveyed. An extensive survey by Gertz (1906; described by Wheldale, 1916) showed that anthocyanins were more commonly produced in certain families and suggested there might be

some systematic relationships between the presence of anthocyanins among different families.

B. RED PIGMENTATION IN TERRESTRIAL PLANTS

Although the large majority of red-violet colors observed in plants are due to anthocyanins, many exceptions occur, and other pigments have evolved (Fig. 1). Most of these pigments are produced by the flavonoid pathway, but other metabolic pathways produce red pigments, including the terpenoid pathway. A variety of structural modifications shift absorbances toward longer wavelengths in xanthophylls (Moss and Weedon, 1976); some absorb at wavelengths above 500 nm and produce red colors, such as lycopene and rhodoxanthin (Fig. 1A). All pigments vary in occurrence among major plant groups, but the anthocyanins are by far the most important in the angiosperms.

Some bryophytes produce red shoots, particularly in response to low temperatures; these are particularly common in New Zealand forests. Sphagnorubin (Fig. 1E) is produced in *Sphagnum* and a few other bryophytes, and is a flavonoid aglycone perhaps derived from 3-deoxy anthocyanidin (Fig. 1D), bound to cell walls and distinct from anthocyanins (Markham, 1988; Mues, 2000). Similar red flavonoid aglycones, riccionidins A and B (Fig. 1H), have been observed in the cell walls of the liverwort *Ricciocarpos natans* and in three other genera. Such a compound may have been observed by Post and Vesk (1992) in the cold and high irradiance responses of an Antarctic liverwort, *Cephaloziella exiliflora*. Some bryophytes may redden in response to low temperatures by producing xanthophylls. Post (1990) observed the induction of violaxanthin in the photoprotection of an Antarctic moss, *Ceratodon purpureus*. In general, anthocyanins may be unusual in the bryophytes (Markham, 1988; Geiger *et al.*, 1997; Mues, 2000) but may be common in certain areas (as New Zealand, Kevin Gould, personal communication) and certainly deserve more study among these plants.

Some pteridophytes produce red leaves, particularly during expansion. *Selaginella erythropus*, popular in cultivation, produces red undersurfaces from the accumulation of an unknown pigment. Although anthocyanins have been reported from a variety of ferns (Soeder, 1985), these may not be true anthocyanins, but 3-deoxy anthocyanidins (Fig. 1D). These are flavonoid pigments, but are derived from flavanones rather than the flavones and 3-hydroxy flavanones as anthocyanins are (Stafford, 1994). Thus, they are biosynthetically slightly different than anthocyanins. Such pigments have been observed in a variety of ferns (Harborne, 1966; Markham, 1988).

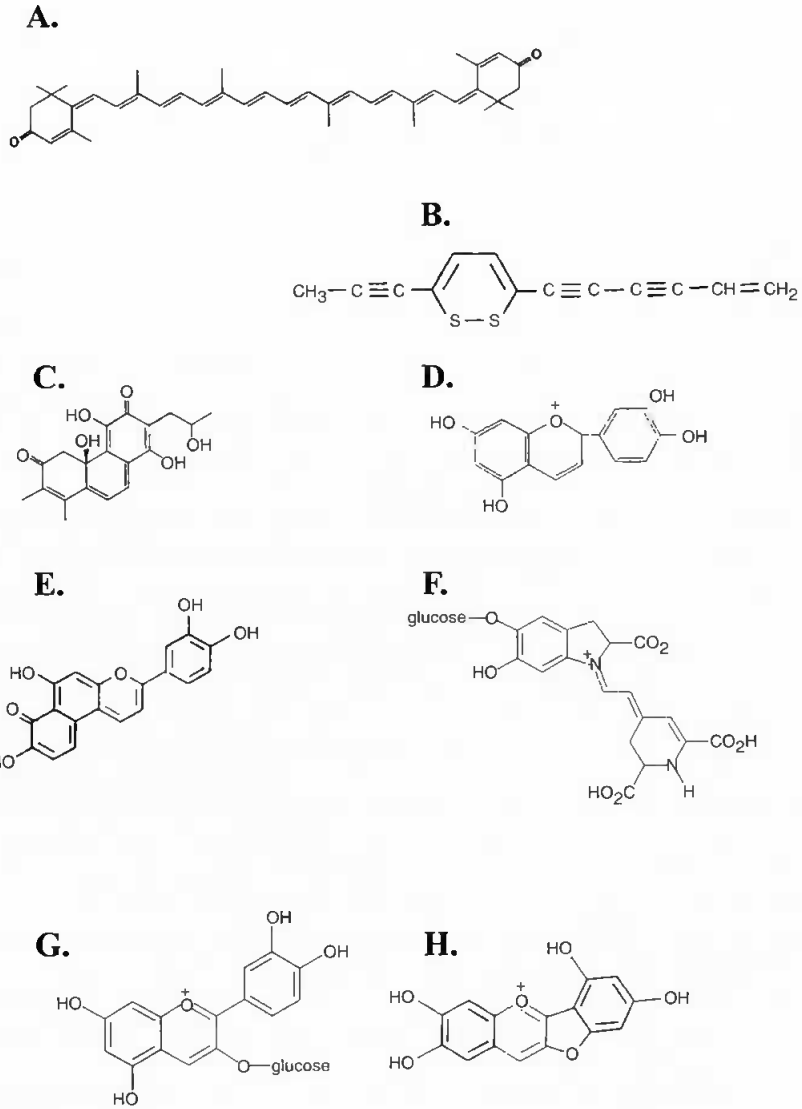

Fig. 1. Red pigment molecules that have been elucidated from various terrestrial plants. (A) Rhodoxanthin, a xanthophyll (Goodwin, 1976); (B) Thiarubrin-A, a polyacetylene from several taxa in the Asteraceae (Rodriguez *et al.*, 1985); (C) Coleone-E, a triterpenoid from *Coleus* sp. (Menthaceae; Thompson, 1976)); (D) 3-deoxy anthocyanidin, common in ferns and in a few angiosperms (Harborne, 1966); (E) Sphagnorubi sporadically isolated in members of the Bryophyta (Markham, 1988); (F) Betanin, nitrogenous pigment found exclusively in members of the Centrospermae, the core Caryophyllales (Stafford, 1994); (G) Cyanidin-3-glucoside, the most commonly isolated anthocyanin from leaves of angiosperms (Harborne, 1976); and (H) Riccionidin-A, seen in the cell walls of a few liverworts (Mues, 2000).

Gymnosperms produce red or brown-red pigmentation, during development or when induced under low temperatures. Goodwin (1976) reported the production of rhodoxanthin (Fig. 1A) in winter needles of *Cryptomeria*. In a survey of tropical taxa that included five gymnosperms, Lee and Collins (2001) observed reddish-brown expanding leaves in three species, all of these due to the accumulation of pigments in plastids. These were probably xanthophylls and certainly not flavonoids. Yet, some gymnosperm species do produce anthocyanins, particularly in response to cold. In *Pinus banksiana* seedlings, low temperatures induced the production of anthocyanin (cyanidin-3-glucoside; Fig. 1G). This helped increase tolerance to photoinhibition (Krol *et al.*, 1995). Niemann (1988) reported a few examples of foliar anthocyanin production, primarily in the odocarpaceae.

Anthocyanins occur in angiosperms in considerable diversity (Gianassi, 1988), but the principal type present in most leaves, early in development and during senescence, is cyanidin-3-glucoside (Fig. 1G; Harborne, 1976). Closer inspection using contemporary analytical techniques is beginning to reveal a greater diversity, particularly as different glycones and modifications of the cyanidin structure. Although red-violet coloration in flowering plants is generally due to anthocyanin accumulation, there are notable exceptions.

The most significant is the production of the nitrogenous betacyanin pigments in the Centrospermae, or core Caryophyllales. Species in this group of families produce flavonoids, but not anthocyanins, and betacyanins (as betanin; Fig. 1F) are responsible for reddish colors in flowers and vegetative organs (Stafford, 1994). There are other exceptions. In the Gesneriaceae reddish colors may be due to 3-deoxy anthocyanidin (Fig. 1D); reported in ferns as previously mentioned (Harborne, 1966). Reddish colors may also be produced by xanthophylls, as rhodoxanthin (Fig. 1A) in *Potamogeton* (Goodwin, 1976). Ida *et al.* (1995) observed the reddening of *Buxus sempervirens* leaves to be due to an unusual red carotenoid, anhydroeschscholtzxanthin. Xanthophyll pigments were responsible for reddish senescing leaves in *Colubrina elliptica* (Lee and Collins, 2001). Unusual pigments may cause the reddening of vegetative tissues in some plants. Thiarubrin-A, a polyacetylene compound (Fig. 1B) in *Aspilia* and other genera in the Asteraceae, produces the red color in an oil that is primarily in roots but also in lower branches and leaves (Rodriguez *et al.*, 1985). Thompson (1976) mentioned other unusual molecules present as red pigments in plants. Coleone (Fig. 1C) is a triterpenoid pigment produced in the leaves of *Coleus*. Fuerstione is a similar molecule present in leaf glands of *Fuerstia Africana*, also in the Menthaceae.

II. ANTHOCYANINS IN ANGIOSPERMS

Anthocyanins are produced in virtually all vegetative organs in flowering plants. They are produced in exposed roots, as the adventitious roots of *Ficus benghalensis* (unpublished observation). They are frequently produced in young shoots, including buds. They are produced in leaves, often in petioles as well as leaf blades. In leaves anthocyanins are present in:

1. mesophyll and abaxial epidermis of forest understory plants;
2. abaxial epidermis of floating aquatic plants as *Nymphaea*;
3. during leaf senescence;
4. during leaf expansion; and
5. tissues produced as a response to stress, as from nutrient deficiency, extreme temperatures, high irradiance and disease (Chalker-Scott, 1999 and this volume).

With the exception of the Centrospermae, anthocyanins are thought to be constitutively produced in all angiosperms. However, in a survey of 399 tropical species Lee and Collins (2001) observed 79 (19.8%) with no visible evidence of anthocyanin production.

Anthocyanin distribution within leaf tissues and among taxonomic groups is relevant to understanding their function in leaves. If these pigments have a physiological function in leaves, then we would expect their presence and distributions to vary with the environmental conditions that would be mitigated by this function, and for them to be absent in conditions without any selective advantage for pigmentation. Anthocyanins may not only differ in their presence but also their distributions in leaf tissues. The general assumption has been that anthocyanins are produced in epidermal layers of leaves, perhaps providing defense against damage by UV radiation. Although the documentation from earlier research is poor, such early surveys showed that anthocyanins were produced in the mesophyll of many plants (Wheldale, 1916; Parkin, 1918). Recent results by Gould and Quinn (1999) and Lee and Collins (2001) reveal considerable variation in the tissue distribution among different species.

A. ENVIRONMENTAL FACTORS

Although we lack good quantitative comparative data, anthocyanin production in leaves appears to be associated with certain environmental factors. Within species, anthocyanin production, both in development and senescence, may be enhanced by exposure to high irradiance. Anthocyanin production also appears to be enhanced by low temperatures (perhaps in association with high irradiance). This may occur

during leaf senescence, or annually in evergreen species whose leaves last several years. Leaf undersurface coloration is more frequent in species of forest understory in both temperate and tropical regions. These are environments of extreme shade, with brief exposures to the high irradiances of sunflecks. Finally, a variety of stresses during the leaf life span may induce the synthesis of anthocyanins. Abilities of plants to produce anthocyanins may be the result of the natural selection of traits for the production of these molecules, or the plasticity to produce them in response to environmental stimuli.

In addition to ecology (or habitat partitioning), two other factors may influence anthocyanin distribution:

1. phylogenetic influences, or inertia; and
2. developmental constraints.

These constraints on anthocyanin production may obscure the ecological relationships.

B. PHYLOGENY

There is a growing literature on the analysis of potential phylogenetic influences on character distribution in organisms (Felsenstein, 1985; Harvey and Pagel, 1991; and Miles and Dunham, 1993). The critical element in such analyses is a robust phylogeny. The molecular phylogenies routinely produced in plant systematics provide the frameworks for such analyses, and the potential benefits are beginning to be appreciated (Lord *et al.*, 1995; Ackerly and Donoghue, 1998; Ackerly and Reich, 1999).

Here I discuss some implications for such a large-scale analysis, very recently published (Lee and Collins, 2001). In this analysis of 399 woody tropical taxa, characters of anthocyanin tissue distribution during leaf expansion and senescence were mapped on a large-scale three-gene molecular phylogeny of the angiosperms (Soltis *et al.*, 1999). We determined the presence of red-violet pigmentation in vacuoles of leaf cell layers or features: adaxial epidermis, hypodermis (if present), palisade mesophyll, spongy mesophyll, bundle sheath cells only, abaxial epidermis, and trichomes. Additional structure within the family level was provided from other research, and the phylogeny was pruned to include families represented by the 399 species.

As an illustration of character distribution in one portion of the phylogeny, the Malvaceae is illustrated (Fig. 2). This large family is an amalgamation of four traditional families of primarily tropical woody plants: Malvaceae, Sterculiaceae, Bombaceae and Tiliaceae (APG, 1998). Additional structure in this tree was provided by recent molecular

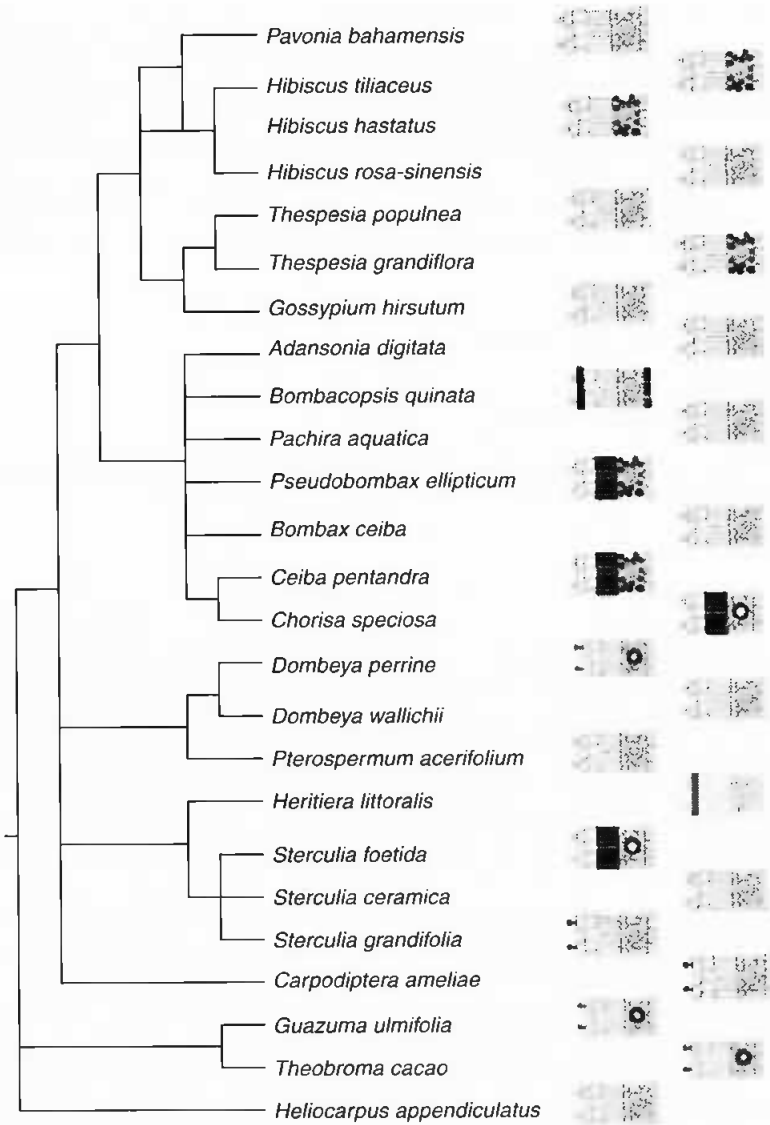

Fig. 2. The distribution of anthocyanins in leaf tissues of expanding leaves of species within the Malvaceae (APG, 1998) surveyed by Lee and Collins (2001). The cartoons depict the locations of anthocyanins in vacuoles of cells of the adaxial epidermis, hypodermis, palisade parenchyma, spongy mesophyll, bundle sheath cells, abaxial epidermis, trichomes.

research (Alverson *et al.*, 1998; Whitlock *et al.*, 1999; David Baum, personal communication), and the distribution of taxa among the families explains the establishment of a larger Malvaceae. In this figure the

presence of anthocyanins in different cell layers is illustrated for individual species. Fourteen of 25 species produced anthocyanins in leaf tissues in development, and these were produced in six different tissue combinations. Five of these species were unusual among the larger sample in producing anthocyanins in bundle sheath cells; there was only one other example (*Strongylodon macrobotrys*, Fabaceae) in the entire sample. This tissue combination was found in species of three of the four clades in this tree; only the taxa formerly in the Malvaceae *sensu stricto* had no such character.

These results visually suggest considerable variation in anthocyanin distribution within a single large family, particularly in presence and absence of production as well as in tissue distribution. However, the presence of anthocyanins in bundle sheath cells was almost unique to this family.

Distribution of these characters for leaf expansion within the large data set can also be compared between families by selecting only those with five or more species in the sample, and then presenting the mean for the family (Fig. 3). Here the tissue distributions are summarized for the germ layers from which they are derived, either dermal or ground tissue (or in a few cases, both), for 32 families. The clades represent the major lineages in the angiosperms. We see large differences among different families. For instance, almost all members of the Myrtaceae produced anthocyanins (mostly in the palisade mesophyll), and all members of the Combretaceae and Lythraceae. Thus the Myrtales has members with pronounced production of anthocyanins during development. On the other hand, few members of the Asteraceae, Araliaceae, Verbenaceae and Solanaceae produced anthocyanins in development or senescence. Thus the percentage production was lower in the Asteridae, particularly the Euasteridae II (APG, 1998). However, The Bignoniaceae had a fairly high percentage of members producing anthocyanins, particularly in dermal tissue.

The distribution of anthocyanins during senescence was quite different than during development. In the phylogeny of the Malvaceae (Fig. 2) only three species of the 25 species produced anthocyanins during senescence: *Carpodiptera ameliae*, *Gossypium hirsutum* and *Dombeya perrine*. Only seven families in the larger scale phylogeny (Fig. 3) produced 40% or more species with anthocyanins during leaf senescence: Euphorbiaceae, Malpighiaceae, Polygonaceae, Bignoniaceae, Flacourtiaceae, Combretaceae and Lythraceae. Only in the last three families did red leaf senescence appear in the large majority of the species sampled. Most families in this largely tropical survey did not develop any anthocyanins during leaf senescence.

In this sample of tropical taxa the percentage of taxa producing anthocyanins during senescence was much lower than during development, 13.5% versus 44.9%. This contrasts strongly with a second sample of 90 woody species from mixed deciduous forests in Central Massachusetts

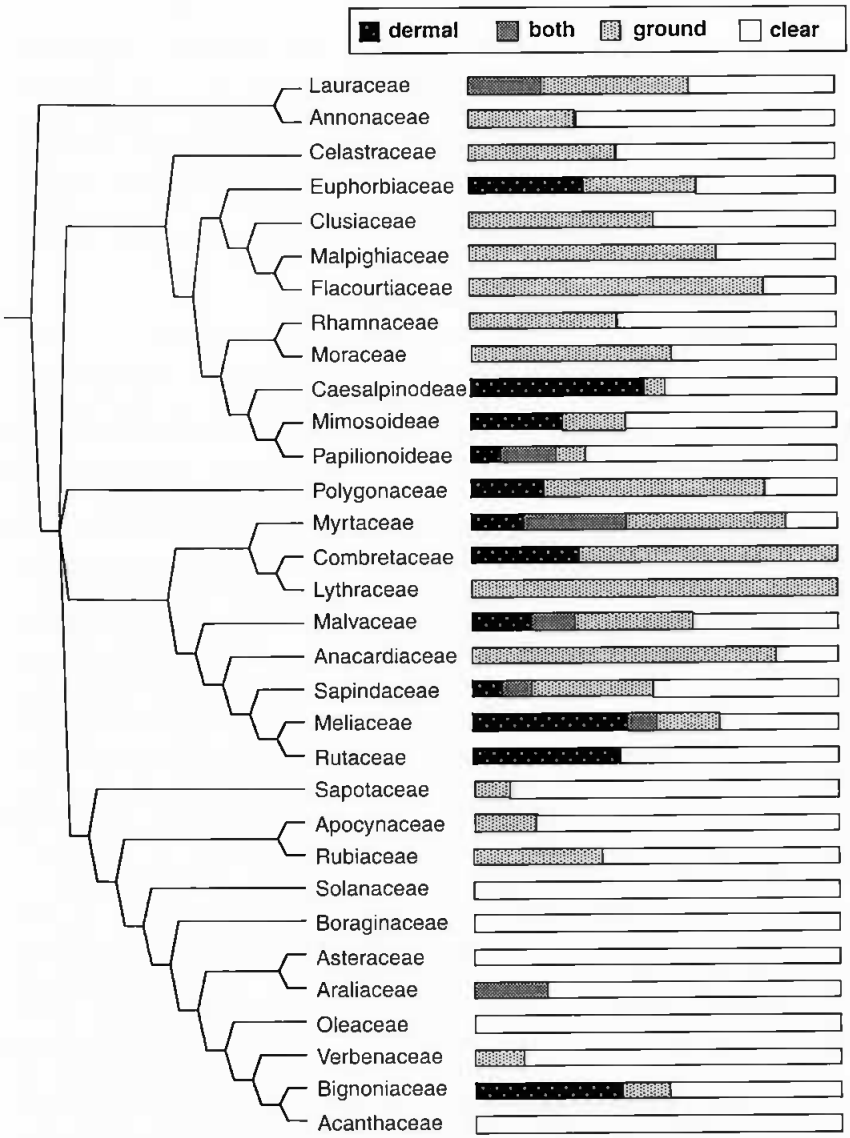

Fig. 3. Means of tissue presences in developing leaves of various angiosperm families for which there was a minimal sample of five species, in the survey of Lee and Collins (2001). Bar graphs indicate the frequency of presence of anthocyanins in dermal tissues (adaxial and abaxial epidermis and trichomes), ground tissues (hypodermis, palisa parenchyma, bundle sheath and spongy mesophyll cells), both, or absent from all tissues.

(Lee *et al.*, 2002). The large majority (62, or 70%) of these species produced anthocyanins during leaf senescence. All but two of them produced

anthocyanins in palisade mesophyll cells, and 11 species in both epidermis and palisade mesophyll cells. Certain families were particularly well-represented with anthocyanic taxa: Caprifoliaceae, Cornaceae and Rosaceae. Other families were poorly represented, as the Betulaceae (Lee *et al.*, 2002).

Mapping tissue distributions of anthocyanins on a phylogeny certainly indicates that the distributions are not random. The real strength of this approach is that it is amenable to hypothesis testing by statistical analysis. Are these distributions influenced by phylogeny, suggestive of phylogenetic 'inertia' (Maddison and Slatkin, 1991)? This question was tested on the phylogeny of 399 species in the following way (Lee and Collins, 2001). We generated both random character distributions and random resolutions of the polytomies on the study tree. We then compared the numbers of steps for characters of the resolutions of the study tree with e number of steps with the randomly shuffled character states. We also calculated retention indices; when RI = 1, phylogenetic inertia is maximized. We found that the number of steps differed significantly in four characters during development: epidermis (RI = 0.14), palisade parenchyma (RI = 0.25), spongy mesophyll (RI = 0.19) and lower epidermis (RI = 0.16). Steps differed during senescence only for palisade parenchyma (RI = 0.24). These differences were weak, yet highly significant, and suggest that these five characters were influenced by evolution. The relative ease of loss of expression due to mutation probably partially obscures this inertia. Thus, in this sample of tropical plants phylogeny played some role in the distribution of anthocyanins in leaf tissues.

C. DEVELOPMENT

A variety of factors independent of phylogeny may constrain the distributions of anthocyanins in leaf tissues. Consider the number of tissue layers or structures in which anthocyanins can be produced: adaxial epidermis, hypodermis (if present), palisade parenchyma, spongy mesophyll, bundle sheath cells, adaxial epidermis, and trichomes (if present). The first and last two layers/structures are dermal in embryological origin, and the rest originate from ground tissue (Westhoff, 1998; Sinha, 1999). Anthocyanins could theoretically be produced in these different tissues singly or in multiple combinations, for a total 127 combinations (126 if the absence of anthocyanins is subtracted), or 2^7.

Lee and Collins (2001) found that a small fraction of this total curred in their sample, both during leaf expansion and senescence (Table I). During expansions a mere nine tissue combinations accounted for 88.4% of all leaves producing anthocyanins. During senescence only seven tissue combinations accounted for 96.3% of all leaves. Ground tissue (particularly palisade parenchyma) was the prevalent site of

TABLE I

Distribution of anthocyanins in tissues of developing and senescent leaves in a survey of 399 tropical woody plants (Lee and Collins, 2001). These combinations are the most frequent among those observed in the survey.

Tissue type[a]									
UEP	HYP	PAL	SPM	VBU	LEP	TRC	Tissue origin	% in development	% in senescence
–	–	*[b]	*	–	–	–	ground	26.3[c]	18.5
–	–	*	–	–	–	–	ground	19.0	50.0
–	–	–	*	–	–	–	ground	10.6	0.0
–	–	*	–	*	–	–	ground	3.4	0.0
–	–	–	–	*	–	–	ground	3.4	0.0
*	–	–	–	–	*	–	dermal	14.5	1.9
*	–	–	–	–	–	–	dermal	9.5	7.4
*	*	*	–	–	–	–	both	0.0	7.4
*	–	*	–	–	–	–	both	0.6	7.4
*	*	–	–	–	–	–	both	1.1	3.7
Total								88.4 (9)	96.3 (7)

[a] UEP = adaxial epidermis; HYP = hypodermis, if present; PAL = palisade parenchyma; SPM = spongy mesophyll; VBU = parenchyma adjacent to vascular bundles; LEP = abaxial epidermis; TRC = trichomes or scales.

[b] * indicates presence in cell vacuoles in this tissue layer.

[c] Values are in % of taxa with anthocyanins, and numbers in parentheses are the numbers of tissue combinations accounting for the percentages.

anthocyanin accumulation during expansion (62.7%) and senescence (68.5%).

Dermal tissue accumulated anthocyanins in much lower percentages both during expansion (24.0%) and senescence (9.3%). The most striking result is the low percentages of leaves producing anthocyanins on tissues of both germ lines, only 6.7% out of the species producing anthocyanins in development, 18.5% in senescence.

There are seven possible tissue combinations of dermal origin ($2^3 - 1$) and 15 combinations of ground origin ($2^4 - 1$). We observed six of those combinations of dermal origin, and 13 of those combinations of ground origin. Surprisingly, there are 105 possible combinations of the tissues of both origins, and we observed only nine combinations. A conservative test of the significance of this deviation was performed by comparing the observed character combinations with those of 100 random simulations. Anthocyanin expression in both dermal and ground tissues was less in our data than in the simulations ($P<0.01$).

Thus, there are developmental constraints on the production of anthocyanins in leaves, against certain tissue combinations. Most of the loci controlling anthocyanin expression in plant tissues affect dermal tissue (Dooner *et al.*, 1991; Mol *et al.*, 1996), and those concerning ground (mesophyll) tissue are fewer and less studied (Kubo *et al.*, 1999). In maize, virtually any tissue combination in any organ can be reproduced experimentally (Virginia Walbot, personal communication). Yet, relatively few tissue combinations, and therefore more limited systems of genetic control, are commonly encountered in nature, revealed in this survey.

D. ADDITIONAL INFLUENCES

Other factors may affect anthocyanin expression in leaves. Production of anthocyanins in other plant parts, particularly flowers and fruits, may be correlated with anthocyanins in leaves. Since these organs develop from leaf-like segments, such correlations would not be surprising (Fineblum and Rausher, 1997). Lee and Collins (2001) found that 38% of the 127 species without anthocyanins in other plant parts produced anthocyanins in the leaf blade in expansion and/or senescence. In the majority of species producing anthocyanins in other organs, 53% produced anthocyanins in leaves.

The expression of anthocyanins in leaves during development may be ancestral to expression during senescence (or *visa versa*), and the patterns were similar or identical in those 35 species. In three heteroblastic vines (*Ficus pumila*, *Marcgravia rectiflora* and *Macfadyena unguis-cati*) the patterns of anthocyanin production were identical at all stages. These

are further minor constraints on anthocyanin expression in leaves. In other heteroblastic species, patterns of anthocyanin distribution may differ among leaves of heteroblastic stages. Patterns differ in leaf stages of *Hedera helix* (see Hackett in this volume), as they also do in different stages of the New Zealand tree, *Pseudopanax crassifolius* (Gould, 1993).

III. SUMMARY

Leaves of flowering plants are generally distinguished by the frequent production of anthocyanins at different stages in the leaf life span. Such production is rare in bryophytes, pteridophytes and seed plants, although other red pigments may be produced. In flowering plants, anthocyanin distribution in leaves is clearly influenced by:

1. ecological pressures;
2. phylogeny; and
3. developmental constraints.

The influence of development and phylogeny complicate the analysis of the relationship between ecology and chemistry. There are even larger more difficult questions. Within families and similar functional types, as trees of a genus growing in tropical deciduous forests (many of the plants analyzed by Lee and Collins, 2001), some plants produce anthocyanins and others do not. If anthocyanins confer some physiological/selective advantage, how do the species lacking anthocyanins compensate? Certain families do not produce anthocyanins in leaves. If there is an advantage, how do they compensate? Finally, a series of families in the Caryophyllales produce betacyanins and not anthocyanins. Some of the species in the Nyctaginaceae and other families produce betacyanins during leaf expansion, perhaps analogous to anthocyanin production in other families.

Do these molecules, chemically quite distinct but with similar absorbance and antioxidant activity, perform a similar physiological function?

Although we have not moved very far to attack these problems, what is now encouraging is that we have arrived at a level of understanding in different disciplines to begin working cooperatively to solve them. This volume illustrates the future directions of that research.

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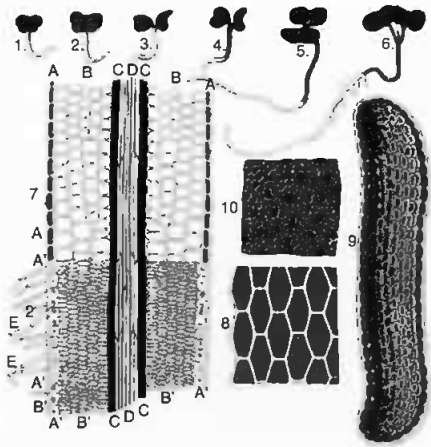

Plate 1. The distribution of anthocyanins in the cell sap of various organs of young seedlings of red cabbage, *Brassica oleracea*, from microscope observations by Édouard Morren (1858).

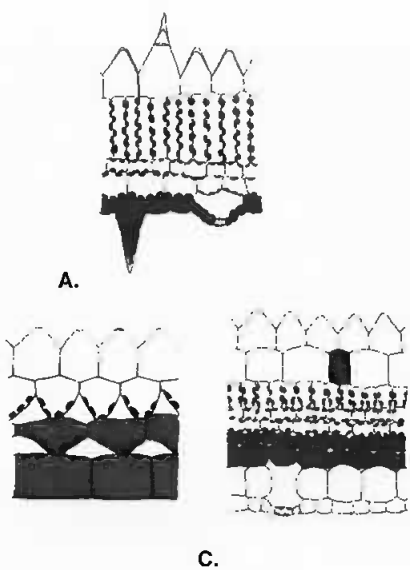

Plate 2. Distribution of anthocyanins in leaf tissues of tropical rainforest understory plants observed in Java (Stahl, 1896). A. *Eranthemum cooperi* (Acanthaceae), lower epidermis. B. *Piper porphyraceum* (Piperaceae), 'spongy' mesophyll. C. *Begonia falcifolia* (Begoniaceae), lower epidermis and single layer of spongy mesophyll.