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The Colors of Autumn Leaves as Symptoms of Cellular Recycling and Defenses Against Environmental Stresses

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The color changes that occur during foliar senescence are directly related to the regulation of nutrient mobilization and resorption from leaf cells, often under conditions of biotic and abiotic stress. Chlorophyll is degraded through a metabolic pathway that becomes specifically activated in senescence. Chlorophyll catabolic enzymes and genes have been identified and characterized and aspects of their regulation analyzed. Particular genetic interventions in the pathway lead to disruptions in protein mobilization and increased sensitivity to light-dependent cell damage and death. The chemistry and metabolism of carotenoid and anthocyanin pigments in senescing leaves are considered. Bright autumn colors observed in the foliage of some woody species have been hypothesized to act as a defense signal to potential insect herbivores. Critical consideration of the biochemical and physiological

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features of normal leaf senescence leads to the conclusion that accumulating or unmasking compounds with new colors are unlikely to represent a costly investment on the part of the tree. The influences of human evolutionary and social history on our own perception of autumn coloration are discussed. The possibility that insect herbivores may respond to volatiles emitted during leaf senescence, rather than to bright colors, is also presented. Finally, some new approaches to the analysis of protein recycling in senescence are briefly considered.

I. Introduction

This discussion considers the significance of the striking changes in pigmentation that occur when green plant tissues undergo senescence. The metabolic events underlying the highly visible symptoms of senescence are directly concerned with the functional and structural transdifferentiation (Thomas *et al.*, 2003) of cells, from units with a primary assimilation role into centers of nutrient mobilization and recovery. Plants, as sessile organisms, experience nonoptimal environments as a way of life. A period of remodeling, such as occurs in senescence, is a potentially vulnerable time for tissues, organs, and the whole plant, and this is reflected in physiological changes accompanying the developing nutrient-recycling function that serve to defend against the intrusion of abiotic and biotic challenges. Here again, pigments are diagnostic of defenses against stress. In this review, we focus on two specific aspects of recycling and stress resistance: recent developments in understanding the molecular and cellular control of chlorophyll degradation, and autumn colors as potential signals in biotic interactions between plants and animals.

II. The Role of Chlorophyll in Protein Recycling

A. The Biochemistry of Chlorophyll Degradation in Senescing Leaves

Protein mobilization in senescence is regulated by a network of processes (Dangl *et al.*, 2000; Hortensteiner and Feller, 2002; Thomas and Donnison, 2000), among which the induction of chlorophyll degradation is an early and, for plastid membrane polypeptides, essential event (Thomas *et al.*, 2002). Net loss of chlorophyll from green tissues during senescence and other terminal developmental events culminates in the accumulation of colorless products (nonfluorescent chlorophyll catabolites, or NCCs) (Miihlecker and Krautler, 1996). The enzymic pathway of NCC formation from chlorophyll (Hortensteiner, 2004) commences with chlorophyllase, which dephytylates

chlorophyll a. Magnesium (Mg) is removed from chlorophyllide a by a dechelatase activity. The tetrapyrrole macrocycle of the product of Mg removal is opened oxygenolytically by pheophorbide a oxygenase (PaO), producing a red bilin, RCC. A reductase immediately converts RCC into a colorless fluorescent product, FCC. Further enzymic and nonenzymic reactions metabolize FCC to NCCs in a species-specific manner (Hortensteiner and Feller, 2002; Thomas *et* al., 2001). Catabolites of chlorophyll b are not normally observed in senescing tissues, leading to the notion that there is interconversion between chlorophyll(ide) a and b and catabolism exclusively by the a-specific pathway. An enzymic activity capable of converting chlorophyllide b to a has been shown to become elevated during senescence (Scheumann *et* al., 1999). Terminal catabolites are sequestered in the cell vacuole. There is no evidence that the N of the chlorophyll ring is exported from the cell during senescence. Chlorophyll catabolism is summarized in Fig. 1.

B. Genes and Genetic Variation for Chlorophyll Degradation

Genes for most of the steps in the chlorophyll catabolism pathway have been cloned (Gray *et* al., 2002; Jakob-Wilk *et* al., 1999; Pruiinska *et* al., 2003; Tanaka *et* al., 2003; Tommasini *et* al., 1998; Tsuchiya *et al.*, 1999; Wiithrich *et* al., 2000). A number of mutations, genetic variants, and transgenics modifying chlorophyll catabolism have been described (Pruiinska *et* al., 2003; Thomas and Howarth, 2000; Thomas *et* al., 2001). In general, they fall into two main categories:

1. Stay-greens are genetic variants in which the yellowing of senescing leaves is delayed, or slowed, or both, relative to comparable normal genotypes. Stay-greens have been differentiated in turn into two kinds—functional and cosmetic (Thomas and Smart, 1993). In functional stay-greens, the link between enhanced stability of chlorophyll, retention of photosynthetic capacity, and delayed protein mobilization is maintained. In cosmetic stay-greens, yellowing is disabled but photosynthetic rate usually declines over a similar time-course to normally senescing yellowing genotypes, and there is partial stabilization of protein. Section II.C discusses this further.

2. Photosensitive genotypes. As described in more detail in Section II.D, variants with deficiencies in particular steps of tetrapyrrole metabolism display pathological symptoms that often mimic disease lesions and are consistent with the accumulation of photodynamic intermediates upstream of the metabolic blockage (Hortensteiner, 2004). Interestingly, in those cosmetic stay-greens in which the location of the metabolic

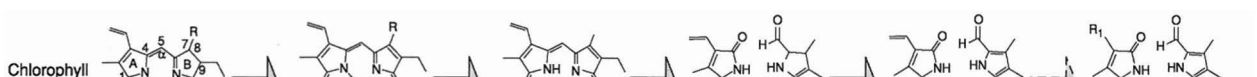

Figure 1 The chlorophyll degradation pathway. 1, Chlorophyllase; 2, magnesium dechelatase; 3, pheophorbide *a* oxygenase; 4, RCC reductase; 5, species-specific enzymic and nonenzymic conversion of FCC to nonfluorescent terminal catabolites.

deficiency has been studied, the evidence points to disruption of one or the other step in the chlorophyll catabolic pathway but no corresponding increase in photosensitivity (Bachmann *et al.*, 1994; Roca *et al.*, 2004). Conversely, and contrary to expectation, photosensitive chlorophyll catabolism variants seem not to behave as cosmetic stay-greens when induced to senesce in the absence of light (Tanaka *et al.*, 2003).

C. Chlorophyll as a Regulator of Protein Metabolism in Senescing Cells

As long as the nitrogen requirements of growing tissues and organs can be met by uptake from the soil and assimilation in roots and leaves, senescence does not usually make a major contribution to the plant's internal nitrogen cycle. However, if sink demand cannot be met by current assimilation—as may happen when development switches from the vegetative to reproductive phase, for example—nitrogen reserves become remobilized. First, low-molecular-weight sources, such as free amino acids and vacuolar nitrate, are drained from the system, then polymers begin to be catabolized. Chloroplasts are protein storage bodies as well as photosynthetic organelles. The onset of senescence marks the functional transition of plastids from assimilation to remobilization, of which chlorophyll catabolism is the visible symptom. Yellowing and protein nitrogen remobilization are generally quite well correlated (Thomas *et al.*, 2002). Genetic and environmental factors that interfere with chlorophyll degradation during senescence also modify protein degradation. For example, a mutant gene that confers cosmetic stay-greenness in *Festuca* and *Lolium* species has the effect of stabilizing chloroplast membrane proteins during senescence (Roca *et al.*, 2004; Thomas *et al.*, 2002). *On* the evidence of mutants and *in vitro* reconstitution experiments, pigment-binding proteins must be properly complexed with chlorophyll if they are to fold correctly, otherwise they are vulnerable to proteolytic attack (Thomas, 1997). Because pigment proteolipids have both a photosynthetic function and a role in thylakoid structure (Allen and Forsberg, 2001), stabilizing chlorophyll–protein complexes in senescence confers durability on chloroplast membranes and reduces the lability of membrane-associated components that are not themselves directly stabilized by chlorophyll, such as cytochrome **f** (Bachmann *et al.*, 1994; Davies *et al.*, 1990). Conversely, an analysis of the behavior of the light-harvesting chlorophyll complex of photosystem 2 during senescence of a stay-green *Festuca* mutant revealed that part of an otherwise deeply-buried thylakoid intrinsic protein that extends into the stroma may be subject to proteolysis, just like soluble-phase plastid proteins (Thomas and Howarth, 2000).

D. Senescence in Relation to Programmed Death of Green Plant Cells

In the present discussion, the term *senescence* is used in the specialized context of the controlled recovery of nutrients from green tissues and is associated with the transdifferentiation of cells and organelles from centers of primary photoassimilation into remobilizing storage structures. Senescence, as the name implies, usually occurs at the end of the life of the leaf and is often classified as an aspect of programmed cell death (Dangl *et al.*, 2000; van Doorn and Woltering, 2004). Nevertheless, senescence in the context of green cell transdifferentiation has features that distinguish it from other cell death processes in a fundamental way. Particularly significant is its reversibility. In many, perhaps most, species it is possible to induce regreening of senescent leaves by interfering with source-sink or hormonal status, or both. Zavaleta-Mancera *et al.* (1999a,b) showed that during regreening of tobacco leaves, gerontoplasts were redifferentiated into chloroplasts, senescence-enhanced genes and their products were turned off, and components of the plastid assembly machinery were reactivated. Thomas *et al.* (2003) argued that the reversibility of senescence, among other characteristics, classifies the process as a differentiation event and not an aspect of programmed cell death. In some ways, it is unfortunate that history has left us with the term *senescence* to describe a phenomenon that, mechanistically, is better understood in developmental rather than deteriorative terms. It may be significant that scientists in the field of animal aging and cell death have moved away from employing the word senescence in recent years because of its imprecise, ambiguous associations (Gordon Lithgow, personal communication). We suggest that we accept the inappropriate etymology and choose to define senescence in our own pragmatic way (in the words of Humpty Dumpty, "When I use a word ... it means just what I choose it to mean—neither more nor less.") (Carroll, 1872), thereby avoiding the distractions of semantics (van Doorn and Woltering, 2004). If senescence is accepted as being functionally distinct from, rather than a form of, programmed cell death, a fruitful area of study opens up concerned with the relationship between the two phenomena in plant development and survival. Hortensteiner (2004) has expressed this most dramatically in terms of the obligate requirement for correct expression of the senescence syndrome to avoid the pathological consequences of cell death. In other words, programmed senescence and (programmed) cell death are mutually antagonistic.

This has become increasingly clear from recent studies in which the molecular genetics of lesion formation and programmed cell death has converged with the identification and cloning of genes for the enzymes of the chlorophyll catabolism pathway. Mach *et al.* (2001) cloned the ACD2 (accelerated cell death 2) locus of *Arabidopsis* and found it to be identical with the gene encoding the chlorophyll catabolic enzyme RCC reductase

(Wiithrich *et al.*, 2000). The knockout has a phenotype that takes the form of light-dependent spreading lesions. Subsequently, the ACD1 (accelerated cell death 1) gene of *Arabidopsis*, a mutation of which also gives a photosensitive cell death phenotype, was shown to encode PaO, the enzyme that opens the chlorophyll macrocycle during senescence (Pružinská *et al.*, 2003; Tanaka *et al.*, 2003). Abnormalities of tetrapyrrole metabolism are well known to lead to pathological photosensitivity, and not just in plants (Hortensteiner, 2004). There remains the enigma of the contrasting senescence and light-response behavior of photosensitive mutants and cosmetic stay-greens as described earlier in Section II.B. It will be necessary to understand in much greater detail the mechanisms by which senescing leaves respond to light and other abiotic stresses, and the control mechanisms by which these stresses are resisted or avoided, before this paradox can be resolved.

III. Non-Green Pigments in Senescing Leaves

A. Revelation of Autumn Colors

Removal of chlorophyll is a defining feature of leaf senescence in all higher plant species. In contrast, the coloration remaining, after the chlorophyll has been catabolized and before tissue death, is much more variable, depending on both genetic background—differences can be found within as well as between species—and environmental factors, particularly stresses due to low temperature, high light, drought, and so on. Optical brighteners synthesized in senescing leaves can enhance the color contributed by other pigments; a striking example is the compound 6-hydroxykynurenic acid, which, by reinforcing carotenoid coloration, imparts the brilliant golden shade characteristic of senescent *Ginkgo biloba* leaves (Matile, 1994). However, the range of leaf colors from yellow through orange to red, pink, and purple is mainly due to two classes of compound: carotenoids and anthocyanins (Matile, 2000).

B. Carotenoids

Leaf carotenoids are highly hydrophobic, and most are yellow or orange in color. The most abundant carotenoids in the chloroplast of a green leaf are typically beta-carotene and alpha-carotene, and the xanthophylls (oxygenated carotenoids) violaxanthin, neoxanthin, antheraxanthin, zeaxanthin, and lutein. The proportions vary with species, leaf age, and environmental conditions (reviewed in Biswal, 1995). In green mesophyll cells in the light, carotenoids function as accessory pigments in the photosynthetic apparatus

and as protectants against photooxidative damage. During leaf senescence, disappearance of the chlorophyll reveals these previously masked compounds. Thus, in the many plant species that do not accumulate hydrophilic pigments such as anthocyanins, the senescing leaf appears yellow or orangish. Although red carotenoids do exist, they are more common in reproductive and dispersal structures, the best-known example being lycopene, which is synthesized in solanaceous fruits as they ripen. One unusual example of red carotenoids in tree leaves was observed by Hormaetxe *et al.* (2004), who found eschscholtzxanthin and derivatives in the foliage of box trees under photoinhibitory conditions encountered during winter acclimation. Like senescent chloroplasts (see Section II.D), and in contrast to fruit chromoplasts, the red plastids in these leaves are able to redifferentiate to green chloroplasts when environmental conditions change; unlike many of the phenylpropanoid compounds described in the next section, the red carotenoids are not terminal metabolites.

In general, remobilization of carotenoids during leaf senescence does not appear to be a consistently high priority for the plant. Being composed almost entirely of carbon and hydrogen (Fig. 2), they do not contain elements that are in short supply at this stage. Chlorophyll and its derivatives, the breakdown products of which are also not exported from the senescing leaf, present a potential hazard to leaf cells as photosynthesis declines and are therefore catabolized by the detoxification process described in Section II, but carotenoids represent no such threat. Rather, as antioxidants they may have a role to play in protecting against photooxidative damage during a vulnerable phase of the life of the leaf. For example, in leaves of the mastic tree *Pistacia lentiscus,* lutein and neaxanthin levels remained constant during the early stages of senescence (up to 20% chlorophyll loss) while beta-carotene levels increased by 9%; only once chlorophyll had largely disappeared did carotenoid levels decline (neoxanthin by approximately 20%; lutein and beta-carotene by approximately 35%) (Munne-Bosch and Penuelas, 2003). Taken in conjunction with similar behavior by other antioxidant compounds, including alpha-tocopherol and ascorbate, the authors inferred a photoprotective role for carotenoids during the chlorophyll catabolism phase of leaf senescence. Similarly, Merzlyak and Gitelson (1995) considered that the retention of carotenoids responsible for the intense yellow color of *Acer platanoides* leaves in autumn was required for protection against blue light irradiation. Whatever the extent of their participation in protection against light damage, it is certainly the case that in many plant species, ranging from beech trees (Garcia-Plazaola and Becerril, 2001) to temperate grasses (Biswal *et al.,* 1994), the disappearance of carotenoids is retarded relative to that of chlorophylls during leaf senescence (reviewed in Biswal, 1995). In green leaves, the carotenoids are almost exclusively localized in the thylakoid membranes, where they form part of the photosynthetic pigment–protein

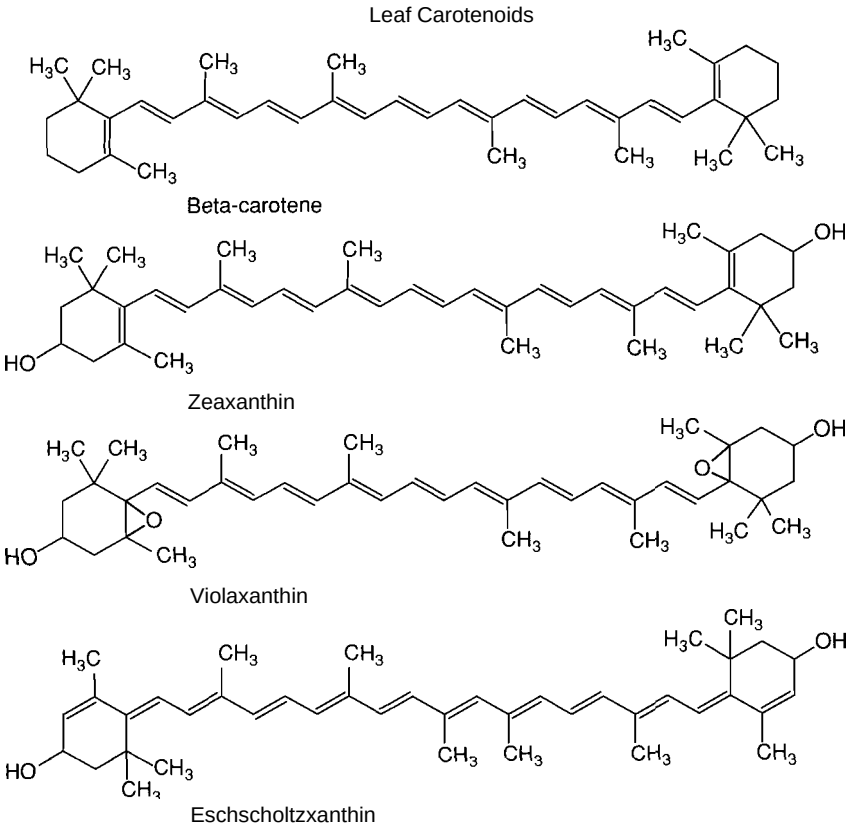

Figure 2 Some of the carotenoid pigments that contribute to autumn colors in senescing leaves.

complexes. During senescence, they relocate mainly to the lipid-rich spherical bodies known as plastoglobuli, which are a striking feature of the plastids in senescent tissues (Steinmuller and Tevini, 1985; Tevini and Steinmuller, 1985). In this respect, senescing leaf chloroplasts resemble the specialized chromoplasts of flower petals and fruit, in which plastoglobuli also often contain the carotenoid pigments.

In addition to the change in cellular compartmentation, the complement of carotenoid compounds also often alters during leaf senescence. Frequently, the proportion of esterified carotenoids increases at the expense of unesterified forms (Biswal ***et al.,*** 1994; Garcia-Plazaola and Becerril, 2001; Tevini and Steinmuller, 1985; Young ***et al.,*** 1991). Some or all of the xanthophylls zeaxanthin, violaxanthin, antheraxanthin, and lutein often become more abundant relative to other carotenoids (Afitlhile ***et al.,*** 1993; Garcia-Plazaola

et al., 2003). Both esterification and changes in relative proportions of these pigments can result in subtle alterations to the yellow-orange coloration of leaves during senescence.

Carotenoids, therefore, can undergo degradation, relocation, and chemical modification during leaf senescence, but there is little evidence for *de novo* synthesis of carotenoids in senescing leaves; genes encoding enzymes of the carotenoid biosynthesis pathway, such as geranyl-geranyl pyrophosphate synthase, phytoene synthase, and phytoene desaturase, are not reported among the wide range of genes whose expression is upregulated during leaf senescence (Andersson *et al.*, 2004; Buchanan-Wollaston *et al.*, 2003). It appears, therefore, that any contribution that carotenoids make to the colors of senescent leaves depends mainly on their preexistence in those leaves before the onset of senescence.

C. Anthocyanins and Other Flavonoids

The major classes of flavonoid polyphenols contributing to the color of flowers, leaves, and fruits are the anthocyanins, flavonols, chalcones, and aurones (Fig. 3). The anthocyanins are the most widespread and recognizable group of plant pigments after chlorophyll, because these water-soluble compounds are responsible for nearly all of the red, pink, mauve, violet, blue, and purple colors in the petals, leaves, stems, and fruits of plants. Anthocyanins are present in nature as heterosides whose aglycone (or anthocyanidin) is a derivative of the flavylium ion. This combination with sugars is important in the case of flower pigments in providing solubility and stability to light and may be important in leaf development as a way of keeping potentially toxic metabolites in an inactive form within the cell.

The three most common anthocyanidins are cyanidin (magenta), pelargonidin (orange-red, with one less hydroxyl group than cyanidin), and delphinidin (purple-blue, with one more hydroxyl than cyanidin). Ji *et al.* (1992) surveyed the leaf pigments of 119 taxa within the aceraceae and found that cyanidin and delphinidin glycosides accounted for most of the anthocyanins in the highly-colored autumnal foliage of these species. Cyanidin, pelagonidin, and delphinidin correspond to the three main flavonols (quercetin, kaempferol, and myricetin) in order of increasing B-ring hydroxylation. Three anthocyanidin methyl esters are also quite common, peonidin derived from cyanidin and petunidin and malvidin derived from delphinidin. Each of these anthocyanidins occurs in plants with various sugar attachments as a range of O-glycosides (anthocyanins) rather than as the aglycones (anthocyanidins). The main variation is in the type of sugar (glucose, galactose, rhamnose, xylose, or arabinose), the number of sugars (mono, di, or triglycosides) and the attachment of the sugar (usually at the 3 OH or the

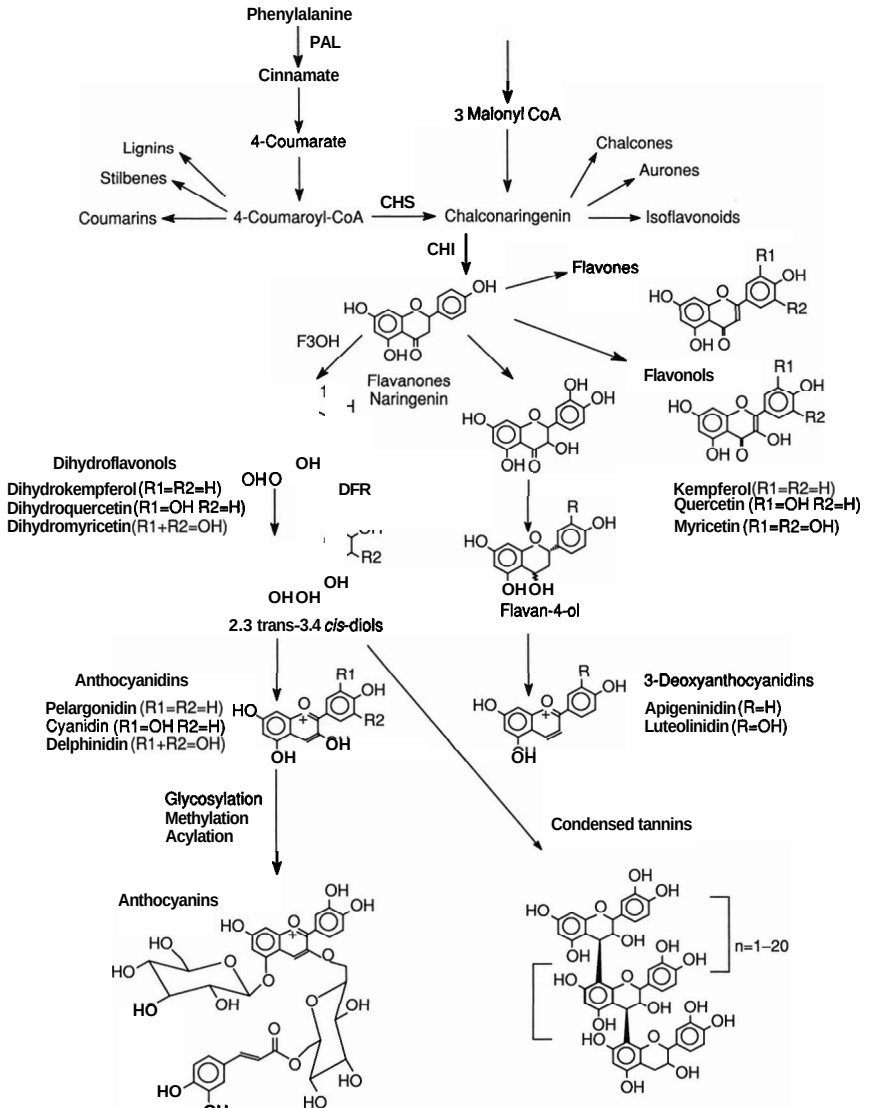

Figure 3 Generalized biosynthetic pathway of flavonoids leading to anthocyanin and related pigments in plants. Key enzymes: CHI, chalcone isomerase; CHS, chalcone synthase; DFR, dihydroflavonol reductase; F3OH, flavanone-3-hydroxylase; PAL, phenylalanine ammonia lyase.

3-and 5-OH). Anthocyanins with acyl attachments are also quite common in some genera such as the solanaceae. The anthocyanins are therefore all based on the single aromatic structure of cyanidin (see Fig. 3), and their structural diversity is derived by modification of hydroxyl groups either by methylation or by glycosylation, or by acylation with different organic acids. These compounds are nearly universal in vascular plants except for the centrospermae, where they are replaced by a chemically distinct group of pigments, the betacyanins, derived from the amino acid L-dopa, and typified by the major pigment of beetroot (Beta vulgaris).

In summary, anthocyanin biosynthesis proceeds via p-coumaroyl-CoA, derived from L-phenylalanine in general phenylpropanoid metabolism, which enters a condensation reaction with three molecules of malonyl-CoA to form a C15 tetrahydroxychalcone intermediate. In the subsequent flavonoid pathway (see Fig. 3), this cyclizes to the corresponding flavanone and is hydroxylated and then reduced to produce the flavan-3-4-*cis* diol precursors of anthocyanins and also of condensed tannins. The flavan-3-4-cis diols then undergo dehydration to form the flavylium cation, although the details of the latter steps of anthocyanin biosynthesis are still incomplete.

Important regulatory steps in the metabolic sequence leading to anthocyanidin synthesis occur at phenylalanine ammonia lyase. chalcone synthase, and dihydroflavonol reductase. In general, these activities, and transcription of the genes that encode them, are sustained or even increased with organ age, but there seems to be no clear obligate relationship with leaf senescence. For example, Romero-Puertas and Delledonne (2003) have described how nitrous oxide delays leaf senescence but also activates the expression of phenylalanine ammonia lyase and chalcone synthase genes as part of a disease resistance/cell death mechanism. On the other hand, Kannangara and Hansson (1998) observed an interaction between anthocyanin and chlorophyll metabolism in young Euphorbia leaves, where several enzymes of chlorophyll biosynthesis sharply decreased in abundance at the onset of red anthocyanin accumulation. It would be interesting to know if a comparable metabolic relationship exists to enhance chlorophyll catabolism in leaves that turn red during senescence. In considering the metabolic costs of biosynthesising anthocyanins de *novo*, it should be kept in mind that the fluxes through the phenylpropanoid pathway do not need to be particularly large to result in a significant observable change. For example, even in Acer *rubrum*, which had the most intensely colored leaves analyzed by Lee et *al.* (2003), anthocyanins accounted for less than 6 μg cm^{-2}; by contrast, chlorophyll in presenescent leaves of this species amounted to more than five times this value.

The final step in anthocyanin biosynthesis is glycosylation, which has the effect of stabilizing the molecule. Further modifications then include additional methylations, glycosylations, and acylations of hydroxyl groups to

produce the great range of anthocyanin colours present in the plant kingdom. One of the largest monomeric anthocyanins known is the heavenly blue complex from the petals of *Zpomoea tricolor* (morning glory) and *Commelina* (Hondo *et al.,* 1992). This has a molecular weight for the flavylium cation of 1759 (Goto *et al.*, 1987), comprises peonidin with six molecules of glucose and three molecules of caffeic acid, and serves to illustrate the complexity that can arise in naturally occurring anthocyanins.

Anthocyanins are mainly found in flower petals and in developing fruits, where they impart a broad spectrum of colors to these tissues. However, they may also accumulate in roots, leaves, bracts, seeds, and stems of both developing seedlings and mature plants. Anthocyanin accumulation is very sensitive to climatic conditions and is often associated with stress responses, particularly to low temperatures, and this is generally under tight genetic control, often mediated by MYB domain and basic helix-loop-helix transcription factors (Koes *et al.,* 1994; Weisshaar and Jenkins, 1998). Anthocyanins accumulate in the cell vacuole (Alfenito *et al.,* 1998), within which they are often located in spherical organelles known as *anthocyanoplasts* (Pecket and Small, 1980). There is a suggestive parallel here with aspects of chlorophyll metabolism in senescence, where conjugation and translocation of glycosides across the tonoplast is the ultimate metabolic fate, followed in some cases by nonenzymic chemical modifications within the acid milieu of the vacuolar sap (Thomas *et al.*, 2001). The tissue distribution of anthocyanins in the autumnal foliage of a range of woody species has been comprehensively surveyed by Lee *et al.* (2003). With a few exceptions, in which the pigment was partly or entirely confined to cells of the adaxial epidermis (e.g., *Acer spicatum, Euonymus atropurporeus,* some *Prunus* spp.), anthocyanin concentrates in palisade parenchyma cells. This observation, taken with the complete absence of pigmentation from lower layers of mesophyll and the abaxial epidermis, is consistent with a function in light interception.

Anthocyanins are compounds that readily alter their structures and, hence, color through the action of different agents. The stability of anthocyanins increases with the number of methoxyl groups on the B ring and decreases with decreasing hydroxylation, and in general they are less labile at acid pH. In aqueous solution, anthocyanins are found equilibrated in four basic structures (the flavylium cation, quinonoidal base, carbinol base, and chalcone base), and the proportions of these forms (and hence the color) are determined principally by pH, with the red flavylium ion predominating in acid solution (Strack and Wray, 1989). At higher pH the color changes to anthocyanic forms that may be colored (bluish in the case of the quinonoidal base and yellow to orange for the chalcone base) or colorless (in the case of the carbinol base), depending on whether the A and B rings are conjugated. Hence, the relative amounts of the structural forms that coexist in equilibrium is a function of pH and the extent of addition of functional groups to the

basic anthocyanin structure. In acid solution, the colors range from orange-red (pelargonidin) through magenta (cyanidin) to mauve (delphinidin). If the pH is raised to near pH 7.0, solutions become colorless due to the formation of the pseudobase, and above pH 7.0 the bluer anhydrobases are formed, whereas at very high pH irreversible changes occur following ionization of the phenolic hydroxyls. Age-related acidification of the vacuole may well account for intensification of the red color of preexisting blue or leuko molecular species.

Although glycosylation of anthocyanidins at C3 to produce anthocyanins results in a marked shift in color, the amount of anthocyanin present in the tissue, which can vary from 0.01% to 15.0% of dry weight, has a much more marked effect. For example, in normal blue cornflowers the anthocyanin concentration is 0.05%, whereas in the deep purple varieties it is 13–14% (Goodwin and Mercer, 1972). Blueness of flowers can also be due to copigmentation between an anthocyanin and a flavonol. An example of this is in maroon and mauve Primula species, where the anthocyanin is malvidin-3-glucoside in both cases, the difference in color being due to copigmentation with high concentrations of kempferol glucosides in the mauve variety. Spectral shifts due to copigmentation occur at pH values of 1–7, but these are not limited to polyphenolics and can also occur with purines and alkaloids, resulting in bathochromic shifts in the visible region of the adsorption spectra.

Anthocyanins can also form complexes with several divalent or trivalent metals such as copper (Cu), aluminum (AI), or iron (Fe), leading to changes in color and varying as a function of pH. For example, Al^{+++} ions bond with anthocyanins with ortho-dihydroxyl groups on the B ring, causing a bathochromic shift to give more blue coloration. This is best illustrated by comparing the blue color of cornflowers (Centaurea *cyanus*) with the red color of roses: the anthocyanin is cyanidin in both cases, but the blue color of cornflowers is due to a combined effect of metal chelation with iron and copigmentation with apigenin diglycoside to form procyanin, a blue crystalline iron complex. It is also well known that if the mineral balance of Hydrangea species is correct, aluminum is easily accumulated and the petals turn blue, otherwise they are red. Many mineral elements, including Fe, are extensively mobilized from senescing leaves (Himelblau and Amasino, 2001), making it unlikely that concentration of metal ions by normal physiological mechanisms plays a part in color intensification, although toxic accumulation may be significant under some circumstances.

The brown color in some petals (e.g., wallflowers) is due to a combination of the magenta anthocyanin in the vacuole with the yellow carotenoids in the chromoplast. The yellows and browns of autumn foliage occurring at the end of the senescence period are accounted for mainly by the presence of carotenoids (see Section III.B.), and by the formation of dark oxidation

products of polyphenols such as condensed or hydrolyzable tannins as subcellular compartmentation collapses. Loss of anthocyanin pigments during this period may also occur by intramolecular rearrangements, resulting in the formation of the colorless pseudo-bases. A recently discovered fate for anthocyanins is via reduction to colorless 2,3-*cis*-flavan-3-ols (e.g., epicatechin), mediated by a newly discovered enzyme anthocyanin reductase, which has been proposed to be involved in synthesis of condensed tannins (Xie et al., 2003).

The factors affecting the determination of the color of plant tissues as a result of anthocyanin accumulation are therefore a combination of the extent of glycosylation and acylation, the pH in the vacuole, and the presence of metal ions and copigments such as flavonols and flavones (Mol et *al.*, 1998). The perception of anthocyanin color in vivo can also be appreciably altered by cell structure. For example, Noda et al. (1994) showed that a transcription factor regulating the intensity of Antirrhinum flower color does so via control of cell shape. It is clear, therefore, that the progression of autumn colors shown by leaves of many trees may not be due solely to the loss of chlorophyll revealing the underlying colors of anthocyanins. Senescence-induced changes in vacuolar pH, increased or decreased levels of metal ions, the degradation of copigrnents such as flavonols and carotenoids, and the polymerization and oxidation of condensed and hydrolyzable tannins may well combine to produce the progression of color changes, from reds, oranges, yellows, and finally browns, independent of levels of de novo synthesis of anthocyanins. Evidence for de novo synthesis of anthocyanins in leaves during senescence is currently weak, and, in fact, in senescing leaves of the copper varieties of beech and hazel, senescence is preceded by the loss of anthocyanin so that, for a while, the foliage turns as green as in wild-type trees (Matile, 2000). Furthermore, although condensed tannins (or proanthocyanidins) give rise to anthocyanidins on acid hydrolysis, there is no evidence that this occurs in planta even during senescence. The wide range of potential modifications to which anthocyanins are subject cautions against assumptions that enhanced coloration in autumn must be the result solely of net synthesis of these compounds and therefore metabolically costly.

IV. Pigments and Stress Defenses in Senescing Leaves

A. Color Changes in Senescence as Signals

We have seen that the recycling function of leaf senescence is potentially vulnerable to disruption by light stress, and that pigment metabolism is normally organized and controlled in senescence to minimize photodamage. Moreover, the relationship between, on the one hand, pigment metabolism

and, on the other, the formation of lesions mimicking the symptoms of fungal and bacterial disease, emphasizes the importance of senescence processes in plant responses to biotic stress. Developing the theme of pigments and biotic interactions further, the case of herbivorous predation on plant tissues illustrates the ecological significance of cellular events in foliar senescence. Archetti (2000) and Hamilton and Brown (2001) have elaborated the autumn signaling hypothesis, which raises interesting questions about the origins and functions of plant pigments and the way the animal eye responds to them. The hypothesis proposes that the autumn coloration observed in many tree species acts as an honest (handicap) signal to potential insect predators about the tree's investment in defensedefensively committed and vigorous trees should produce the most intense coloration and, hence, the greatest deterrent to insects. It is suggested that the signaling mechanism in trees, and the insects' avoidance response, are features that have coevolved. Although some subsequent publications have lent support to the hypothesis (e.g., Hagen *et al.*, 2003), others have provided experimental evidence (Holopainen and Peltonen, 2002) or theoretical grounds (Wilkinson *et* al., 2002) to refute it. The majority of work in this area has so far been published by ecologists. Here we examine some aspects of the hypothesis and the results presented to date in the context of our knowledge about leaf senescence and plant physiology.

B. Is Autumn Color a Costly Signal?

A concept widely used in the autumn signaling theory is that autumn coloration is costly for the tree to produce (and, by implication, must therefore have some definite purpose). This idea probably arises by analogy with animal metabolism, in which any biosynthetic process has requirements for both energy and carbon that must be met from food intake or by breaking down some of the animal's own tissue to generate fuel and raw materials. However, plants are autotrophic, and, provided they are exposed to adequate light, air, and water, carbon and energy are not limiting factors as they are for heterotrophs. Indeed, it has been argued that terrestrial plants have evolved physically and physiologically to dump excess carbon captured through promiscuous assimilation (Thomas and Sadras, 2001). According to this proposition, the general overabundance of carbon and energy with which plants are cursed has resulted in proliferation of the huge range of carbon-rich secondary compounds that are unique to the plant kingdom (Hadacek, 2002; Pichersky and Gang, 2000) as a consequence of a kind of speculative metabolic doodling that occasionally pays off in terms of improved fitness. In any event, it is questionable to assume that activities requiring carbon and energy are necessarily costly to a plant in the same

sense that they would be for an animal or other heterotroph. This is particularly true of autumnal processes occurring in vegetation. Growth in plants is more sensitive to reduced temperature than is photosynthesis (Hjelm and Ogren, 2003); hence, as the temperature declines in autumn, the tree stops growing, further reducing the need for carbon and energy, which it can easily obtain again when required. The recycling function of autumnal senescence is part of a strategy to safeguard winter survival and resumption of growth in spring and culminates in the discarding of foliar skeletons consisting largely of carbon, oxygen, and hydrogen in various combinations. This throw-away residue may include two classes of pigment containing no elements of great reclamation value: carotenoids, which, as we have seen, can be unmasked, transformed, or relocated, but not in general synthesized *de novo* during senescence; and anthocyanins, which often *are* newly synthesized during the senescence process (Ishikura, 1972), although, as discussed, the argument that to do so is metabolically costly is at best questionable.

C. Possible Functions of Leaf Color

The question as to why trees produce bright colors in autumn, if they are not an honest signal about defense capability, has been reviewed by Wilkinson *et al.* (2002). Briefly, plant biologists have two main hypotheses to explain the synthesis of anthocyanins. They may have a role in defense against abiotic stress (Steyn *et al.,* 2002), protecting against potentially damaging forms of oxygen and chemical radicals. As photosynthesis declines during foliar senescence, light energy must be dissipated in alternative ways, some of which lead to the generation of reactive oxygen species (Feild *et al.,* 2001). Efficient recapture of nutrients exported from the senescing leaf requires protection against photooxidative damage. Hoch *et al.* (2003) demonstrated the capacity of anthocyanins to facilitate nutrient recovery during leaf senescence in three deciduous woody species; Lee *et al.* (2003) similarly inferred a correlation between anthocyanin production and efficiency of nitrogen resorption in a number of deciduous forest species. It is noteworthy that many plant species also synthesize anthocyanins in the leaf in response to stresses such as cold, drought, or very high light intensity, when again carbon assimilation and demand are unmatched and there may be an increased risk of free radical production (Hoch *et al.,* 2001). The coevolution theory suggests that vigorous and defensively committed trees can "afford" the loss of photosynthate resulting from early senescence, whereas less vigorous trees need to continue photosynthesizing for longer—but, as pointed out earlier, during the autumn period it is not carbon and energy that are at a premium, but nitrogen and other nutrients. A tree lacking vigor because of nutrient deficiency or abiotic stress cannot make productive use

of photosynthesis and is more, rather than less, likely to undergo early senescence. Interestingly, Schaberg *et al.* (2003) found that the extent and earliness of onset of red coloration in maple leaves was positively correlated with foliar nitrogen deficiency, an observation that contradicts the hypothesis that it is vigorous and healthy trees that initiate leaf senescence early but is entirely in accord with plant scientists' observations of many species under many conditions.

Other suggested functions of phenylpropanoid pigments in protecting against abiotic stresses include roles as antioxidants (Tsuda *et al.,* 1994) and osmolytes (Chalker-Scott, 2002). An additional role not often considered is suggested by the striking fact that intense pigmentation is a characteristic of deciduous species. It may be that colored secondary compounds benefit the plant by contributing to the allelopathic properties of leaf litter (Wardle *et al.*, 1998). Alternatively, the anthocyanins may simply represent a convenient dumping ground for excess carbon, in a form that is not metabolizable by, or attractive to, potential predators and pathogens. The fact that leaves are colored may be coincidental; our own evolutionary and social history has led human beings to attribute great significance to pigments in the wavelength range we can perceive, but a compound's color may not have any particular correlation with its function (there is, for example, no particular reason why the human gall bladder needs to be green!). The comparative cell and molecular biology of foliar senescence supports the view that the senescing leaf is the evolutionary progenitor of brightly colored floral and reproductive structures attractive to animal pollinators and dispersers (Matile *et al.,* 1999; Thomas *et al.,* 2003). It follows that the heightened physiological and psychological sensitivity of humans to the colors of autumn foliage may not have any direct biological meaning. It may, rather, be a secondary consequence of spectral tuning by fruit and leaf color during evolution of the trichromatic primate visual system (Surridge *et al.,* 2003). The connection between the colors of fruits and autumn leaves has been considered by Stiles (1982), who suggested that trees bearing colored fruit in fall may have evolved synchronization between fruit ripening and leaf coloration as an additional signal to seed-dispersing birds.

D. Does Dishonesty Pay?

Even if the red coloration is not costly to produce, could the signaling hypothesis still hold good? Subsequent authors (Lachmann *et al.,* 2001; Wilkinson *et al.*, 2002) have pointed out that it is not essential that an honest signal be costly provided that dishonesty is penalized. In the case of autumn colors, dishonesty would consist in a poorly defended tree producing bright colors as a misleading deterrent. However, as indicated earlier, it is precisely

stressed, and therefore generally poorly defended, trees that do produce more of the bright autumn colors. If the coloration were indeed a deterrent, far from being a costly misdirection, it could in fact act in the tree's favor. Therefore, neither is the "signal" costly to produce, nor does its dishonest production penalize the tree. However, an alternative explanation should also be considered. The signaling theory would still be valid if trees that are poor in nutrients in the autumn ***subsequently*** defend their (limited) resources more heavily in the following spring, compared to "richer" individuals that may simply outgrow their pests. In this scenario, the signal becomes an honest and unfakeable indicator of resources, since early bright leaves represent low resources. If, for a given plant species, low nonrenewable nutrient resource is associated with increased defenses in spring, then insects would be expected to make appropriate evasive action in autumn. Future experimental work on the relationships between resource status and leaf coloration in autumn, insect responses, and defensive commitment the following spring will be necessary to clarify this issue.

E. Insect Preference for Green Leaves

Moving from the reason why trees develop the coloration in the first place to a consideration of the reasons why insect predators avoid brightly colored leaves, the composition of these leaves in comparison with green foliage should be taken into account. By the time a leaf is orange or red, it will have broken down and exported a high proportion of its total protein. Photosynthesis will have ceased, remaining low-molecular-weight carbohydrates will have been removed, and, in general, it will have a much lower content of nutrients that an insect could digest than would a green leaf on the same tree. Anthocyanins that have accumulated will not be digestible by insects; they, or other, colorless, secondary products accumulating at the same time, may even be unpalatable and act as antifeedants. Furthermore, antinutritional factors such as inhibitors of digestive tract proteases are prominent among the genes and gene products upregulated in senescence and cell death (e.g., Huang ***et al.***, 2001). It is therefore quite feasible that insects initially land on green and red/yellow leaves in equal numbers but quickly vacate the latter after an initial sampling—to assess this possibility would require more detailed observation of insect behavior than has been presented in any of the studies to date. Alternatively, the predators may indeed be responding to the visual signal, but its significance is not "this is a vigorous tree able to withstand your attack" but simply "this is a leaf with poor nutritional quality, possibly unpalatable." Wilkinson ***et al.*** (2002) point out that because an individual tree may simultaneously bear green, yellow, and red leaves, the hypothesis that leaf color signals the overall vigor or defensive

capability of the tree is suspect. However, it could feasibly signal the nutritional value of the individual leaf; because studies to date (Hagen *et al.,* 2003; Holopainen and Peltonen, 2002) have measured insect colonization or insect damage relative to the mean proportion of brightly colored leaves on a tree, rather than on an individual leaf basis, the question remains open.

To complicate the issue further, Holopainen and Peltonen (2002) point out that birch aphids preferentially land on yellow rather than on green or red leaves, and propose that such leaves, which are actively exporting low-molecular-weight nitrogenous compounds such as amino acids during early senescence, are a rich source of accessible nutrient for the insects. A difficulty in interpreting the data on insect behavior as a whole is that most studies (e.g., Hagen *et al.,* 2003) did not look at red and yellow leaves separately; it may indeed be the case that some insect species are attracted to yellowing leaves more than green leaves, but to red leaves least of all. This possibility would resolve some of the inconsistencies in the story so far, including the fact pointed out by Wilkinson *et al.* (2002) that yellow is normally an attractive color to aphids. An interesting perspective on this issue is provided by the common observation that the proportion of infertile individuals in a plant population increases under stress. In species with strongly expressed monocarpic or reproduction-associated senescence patterns, barren plants may remain green while fertile individuals degrade their chlorophyll normally. Such barren plants have been reported to benefit the population by acting as decoys, reducing herbivory pressures on individuals destined to produce the next generation (Thomas and Sadras, 2001).

F. Visual and Olfactory Signals

The color changes observed in tree leaves in autumn can be so spectacular that it is easy for humans to overlook the possibility that the predators may be responding to different signals entirely, arising from other biochemical changes that the tree may be undergoing at the same time. It is possible that the insects are deterred not by color at all, but by the volatile substances emanating from plant leaves during senescence. Most land plants emit significant amounts of volatiles such as aldehydes and isoprenes during natural or wound-induced senescence (de Gouw *et al.,* 1999; Fall *et al.,* 1999), and it is known that insects can perceive and respond to quite low concentrations of these compounds (Ruther *et al.,* 2002). There is also evidence that the volatiles may act as defense agents by attracting insect parasitoids (Hoballah and Turlings, 2001). Peak emission of such volatiles would be expected to coincide with the phase when the leaf was yellow or red but still alive. It would be informative to measure volatile emissions during the color change period and correlate the results with levels of insect colonization.

V. Conclusions

In recent years, a fairly complete picture has emerged of the metabolic molecular and subcellular networks responsible for pigmentation changes during the growth and senescence of foliage. This has allowed the development of a model of leaf senescence in which pigmentation changes partially set the pace for proteolysis and nitrogen recycling at the same time as they play a critical role in sustaining cell viability under conditions of abiotic and biotic challenge. Although rapid progress has been made in understanding pigment metabolism in senescence, the mechanism of protein degradation and its control remains poorly understood. The interconversions and relocations of the amino acid products of proteolysis in leaf tissues are quite well established (Dangl *et al.*, 2000), but the step between the intact protein and its hydrolysis products continues to elude definitive analysis. Many senescence-associated and upregulated protease genes have been described (Bhalerao *et al.*, 2003; Buchanan-Wollaston, 1997; Buchanan-Wollaston *et al.,* 2003), but it is unclear how many, if any at all, are necessary for normal protein breakdown. Some new developments may help to introduce much-needed innovative ideas into the field of proteolysis and its control in senescence. Improved microscopy techniques are beginning to provide evidence for traffic between plastids (which contain most of the mobilizable protein in senescing cells) and vacuoles, which have long been considered to be the main sites of intracellular proteolysis (Chiba *et al.,* 2003; Guiamet *et al.,* 1999). Cascades of caspase proteases are characteristic of programmed cell death in animal systems, but plant genomes seem not to include orthologues of caspase genes; nevertheless, recent sequence searches and functional analyses have revealed families of so-called metacaspases in plants that may fulfil various signaling and proteolytic roles in terminal and pathological plant processes, including senescence (Watanabe and Lam, 2004). Another potentially fruitful development is the application of quantitative trait mapping to test the relative map positions of genetic loci for, on the one hand, nitrogen assimilation and reallocation traits in crop development and, on the other, protease enzyme activities and gene sequences (Andreas Fischer, unpublished results). Much work needs to be done before protein recycling in plant senescence could be said to be a well-understood process, but new tools and approaches are being applied and rapid progress can be expected in the near future.

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