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Review

Biochemistry of Indian summer: physiology of autumnal leaf coloration

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Abstract

The autumnal coloration of foliage in deciduous trees represents a most spectacular phytogerontological phenomenon. It is primarily due to the progressive loss of chlorophyll coinciding with the partial retention of carotenoids. Leaf senescence is a developmental process that is aimed at the recycling of nutrients to perennial parts of the tree for reuse upon the production of new foliage in spring. The remobilization of protein in senescing chloroplasts requires the dismantling of pigment-protein complexes and concomitant photodynamic inactivation of chlorophyll. Detoxification of chlorophyll is achieved by enzymic opening of the porphyrin macrocycle followed by modifications of the resulting linear tetrapyrrole and storage of colorless final catabolites in the vacuoles of degreening leaf cells. The polychromatic beauty of autumnal trees is due to species-specific variations of the degree of carotenoid retention, new synthesis of red anthocyanins and, upon cell death, the formation of dark oxidation products of phenolics.

Keywords: Leaf senescence; Chlorophyll catabolism; Carotenoid retention; Gerontoplasts; Nutrient recycling; Anthocyanin; Melanin

1. Introduction

1.1. Leaf senescence and nutrient recycling

Plant development is associated with senescence and death occurring at different times and at various levels of organization. Leaf senescence is merely the most conspicuous example of phytogerontological events.

Leaves have limited lifespans. In deciduous trees, leaves senesce and are eventually shed at the end of the vegetation period, but leaves of evergreen trees retain their

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photosynthetic function for several years. The other extreme of life spans is represented by short–lived leaves such as the cotyledons of certain species that begin to senesce almost immediately after expansion and greening.

In many herbs and in grasses leaves senesce in a progressive fashion, i.e. degreening in old leaves takes place concomitant with emergence and growth of new leaves. Such progressive yellowing of old leaves indicates quite strikingly the significance of senescence for the recycling of growth-limiting nutrient elements, particularly of nitrogen but also phosphorous, potassium, magnesium, and sulfur. Thus, leaves represent potential sources of nutrients that are required for development in other parts of the plant. In apple trees, Millard and Thomson (1989) have demonstrated that a substantial proportion of internally cycled nitrogen is reused for leaf growth in spring. The disappearance of green color in senescing leaves is merely a visible sign of extensive remobilization of cytoplasmic constituents and translocation of nutrients to growing organs, seeds, tubers or, in the case of deciduous trees, to storage tissues in branches, twigs, and winter buds.

In deciduous trees, leaves are eventually shed because the genetic program of senescence includes the weakening of cell-to-cell adhesion in a specialized tissue at the base of the petiole. Upon the completion of differentiation in these abscission layers the tensional strength has become so weak that the leaf is shed by its own weight. Wind may cause the removal of leaves before the process of nutrient recycling is completed and, hence, has a negative effect on nutrient economy (Oland, 1963; Killingbeck et al., 1990).

The recycling of nutrients identifies senescence as a vitally important developmental process that requires the full viability of cells. Indeed, leaves remain turgescent throughout the period of yellowing, indicating the intactness of membranes and maintenance of subcellular compartmentation. Senescence is a metabolically very active process. This is documented by high respiratory activity and also by surprisingly high rates of amino acid incorporation into protein. As this biosynthetic activity coincides with the remobilization of some 60% and more of total leaf protein, turnover of protein is extremely high. The synthesis of new proteins is due to the expression of a large number of genes, some of them having functions at other stages of development, some encoding proteins that play a role in senescence exclusively (Smart, 1994; Buchanan–Wollaston, 1997).

2. Gerontoplasts

It is common knowledge that chlorophyll is responsible for the harvesting of light and the mediation of quantum energy to the photosynthetic apparatus. In the photosynthetic organelles, chloroplasts, chlorophyll resides in the internal membrane system (thylakoids). A single mesophyll cell contains a hundred and more chloroplasts. For the understanding of leaf senescence and the nutrient recycling associated with it, it is important that the bulk of leaf protein is located in the chloroplasts. A major protein component is represented by the soluble ribulose bisphosphate carboxylase, the enzyme responsible for the incorporation of CO_2 into organic matter. About a third of total chloroplast protein consists of a group of hydrophobic proteins that form specific complexes with chlorophyll and carotenoids (Green, 1996). These complexes are components of photosystems and light harvesting antennas and, hence, represent the core pieces of the photosynthetic machinery.

When leaves begin to turn yellow, chloroplasts are gradually transformed into a distinct senescent form termed gerontoplast (Matile, 1992). Developing gerontoplasts represent the principal source of nutrients that are withdrawn from senescing leaves. The organelle

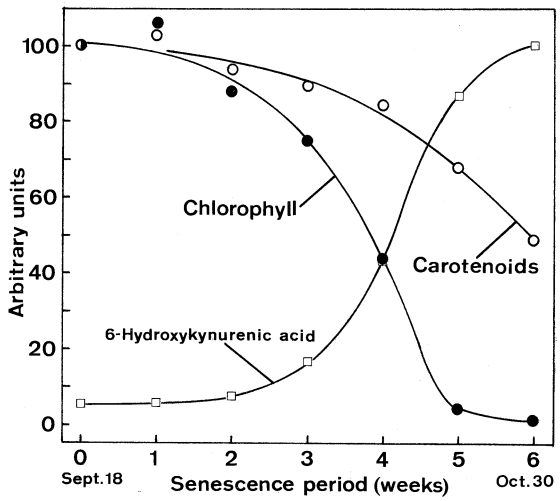


Fig. 1. Loss of chlorophyll, retention of carotenoids, and accumulation of 6-hydroxykynurenic acid during leaf senescence in *Ginkgo biloba*. Relative amounts per unit of fresh leaf weight. Data from Matile et al. (1992).

retains its membrane envelope whilst the thylakoids as well as the soluble proteins, ribosomes and other digestible constituents disappear. A fully developed gerontoplast consists of a still intact envelope wrapping up a number plastoglobules in which undigested lipophilic compounds such as carotenoids, carotenoid esters, phytol, and tocopherol are accumulated (Tevini and Steinmüller, 1985). Leaves turn yellow because, during disappearance of chlorophyll, carotenoids are partially retained. In the wide-spread ornamental tree, *Ginkgo biloba*, as much as half of total carotenoids originally present in the green leaf is retained by the time of shedding (Fig. 1). In other species of trees such as maples or linden, carotenoid retention is less pronounced but still high enough to cause a golden appearance of the autumnal foliage. Carotenoids are not newly synthesized in gerontoplasts. In this respect, gerontoplasts are different from chromoplasts, the yellow- or red-colored plastids that in fruits and flower petals develop from chloroplasts (Sitte et al., 1980).

Gerontoplast development is initiated by the extensive loss of the plastidic DNA (Sodmergen et al., 1991). This explains why the biochemical processes underlying the transformation of chloroplasts into gerontoplasts are exclusively under the control of the nuclear genome. Indeed, treatments of senescing leaves with inhibitors of cytoplasmic protein synthesis such as cycloheximide cause the arrest of chlorophyll loss.

3. Detoxification of chlorophyll

In the green leaves carotenoids have a vitally important function in the protection of the photosynthetic apparatus (Bartley and Scolnik, 1995). In the pigment-protein complexes they are closely associated with chlorophyll so that they can deactivate excited chlorophyll and thereby dissipate excessive energy. Chlorophyll is a potent photosensitizer and, hence, carotenoids prevent photodynamic damage of leaf cells caused by chlorophyll-mediated activation of oxygen. In the developing gerontoplasts the remobilization of chlorophyll-

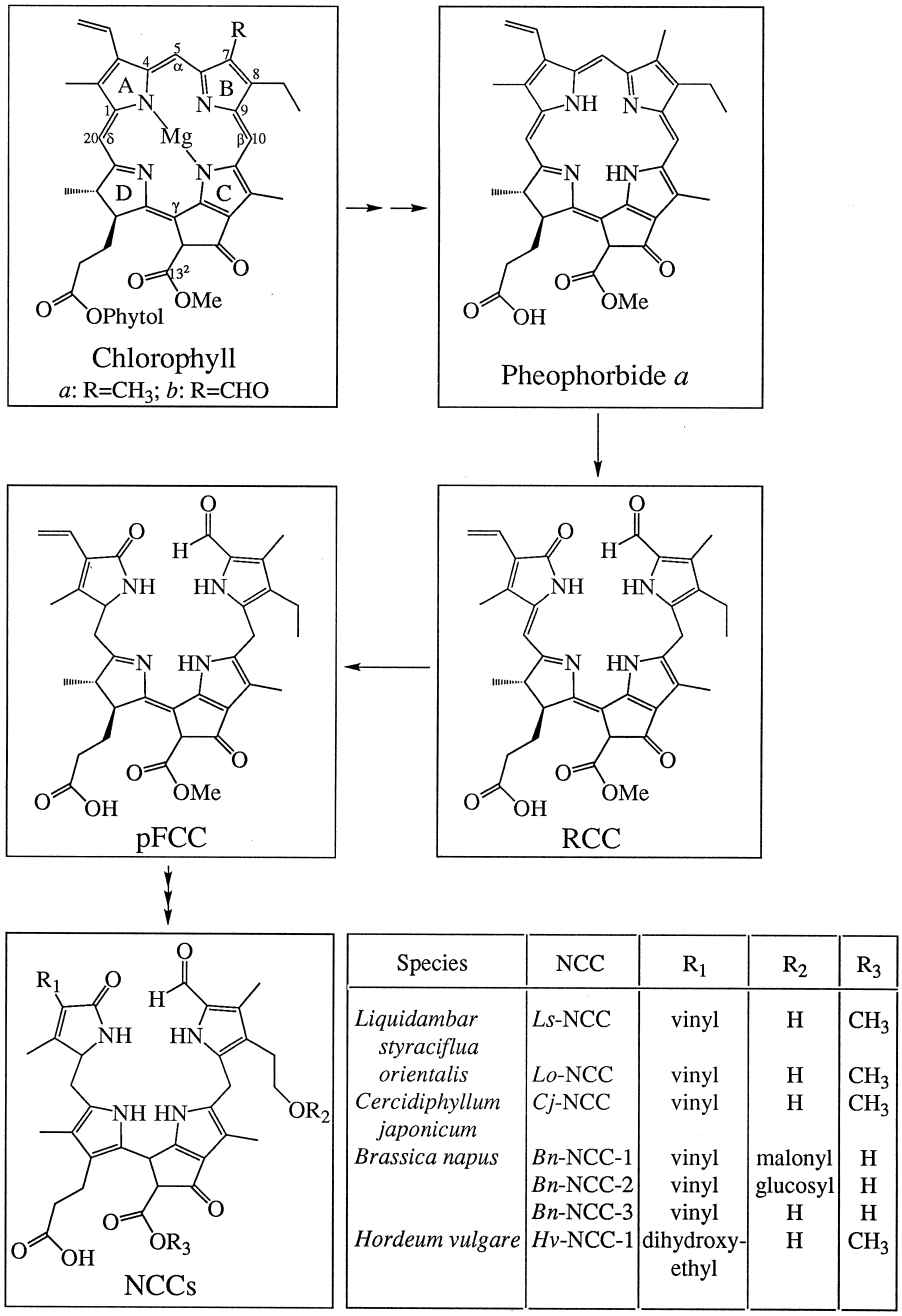
binding proteins requires the dismantling of pigment-protein complexes and, therefore, it is nothing but logical that chlorophyll must be photodynamically inactivated to prevent damage of senescing leaf cells. Although the porphyrin part of chlorophyll contains nitrogen, it is not utilized for nutrient recycling but converted into photodynamically harmless catabolites. Chlorophyll is not so much degraded than it is detoxified (Matile et al., 1999). The structures of some final products of chlorophyll catabolism that have recently been isolated from senescent leaves are shown in Fig. 2. They represent linear tetrapyrroles in which the conjugated system among the pyrrole units is completely abolished. These NCCs (nonfluorescent chlorophyll catabolites) are colorless and, therefore, probably the reason why, for a long time, they have escaped discovery.

4. Biochemistry of chlorophyll catabolism

The oxygenolytic opening of the porphyrin macrocycle represents the core piece of a rather complicated catabolic pathway that in the past few years has been elucidated (for recent reviews see Kräutler and Matile, 1999; Matile et al., 1999; Hörtensteiner, 1999). It is responsible for the loss of green color and for extensive photodynamic inactivation. The cleavage of the macrocycle yields a colorless blue-fluorescing product. The structure of this primary catabolite, pFCC (primary fluorescing chlorophyll catabolite) identifies it as a linear tetrapyrrole derived from chlorophyll *a* and pheophorbide *a*, respectively (Fig. 2; Mühlecker et al., 1997). The production of pFCC from pheophorbide *a* is catalyzed by two enzymes that, in a metabolically channeled reaction, cleave the macrocycle at the α-methine bridge and reduce the double bonds in the β- and δ-bridges. The reaction is initiated by the action of pheophorbide *a* oxygenase producing a red colored bilin (RCC) that is released from the oxygenase only upon interaction with RCC reductase catalyzing the saturation of the double bond in the δ-methine bridge (Hörtensteiner, 1999). It seems from the structure of pFCC that the fluorescence is due to the Schiff-base -N=C-C=C-N- of the still unsaturated γ-methine bridge and adjacent pyrroles C and D. It should be mentioned that the mechanism of action of pheophorbide *a* oxygenase has not yet been clarified in detail. Whereas RCC reductase, a soluble protein of the plastidic stroma, has recently been purified and the corresponding gene been cloned (Wüthrich et al., 2000), attempts at the purification of the membrane-bound oxygenase have so far been unsuccessful.

The cleavage of chlorophyll-porphyrin is strikingly reminiscent of heme conversion into biliverdin and reduction to bilirubin. Indeed, like heme oxygenase pheophorbide *a* oxygenase is a monooxygenase (Hörtensteiner et al., 1998); only the *O*-atom in C5 originates from dioxygen, the lactam-*O* in C4 is probably derived from water. But otherwise, there are conspicuous differences between chlorophyll- and heme-catabolism. Thus, the action of heme oxygenase is associated with the release of the methine bridge carbon as CO, whereas in the case of chlorophyll it is preserved as a formyl group. Other peculiarities of chlorophyll catabolism are represented by the requirements for reduced ferredoxin as electron donor (heme catabolism: NADPH) and by the tight coupling of oxygenolysis and reduction (Fig. 3). Still another difference concerns the central metal. ion: whereas in heme, Fe^{3+} is released upon ring opening, in the case of chlorophyll enzymic demetalation precedes macrocycle cleavage.

The insight into the mechanism of macrocycle cleavage has been gained largely through experimentation with chloroplasts from barley and oilseed rape. There are good reasons to believe, however, that the pheophorbide *a* oxygenase/RCC reductase system is



Species	NCC	R_1	R_2	R_3
Liquidambar styraciflua	*Ls*-NCC	vinyl	H	CH_3
orientalis	*Lo*-NCC	vinyl	H	CH_3
Cercidiphyllum japonicum	*Cj*-NCC	vinyl	H	CH_3
Brassica napus	*Bn*-NCC-1	vinyl	malonyl	H
	Bn-NCC-2	vinyl	glucosyl	H
	Bn-NCC-3	vinyl	H	H
Hordeum vulgare	*Hv*-NCC-1	dihydroxy-ethyl	H	CH_3

Fig. 2. Structures of intermediary and final chlorophyll catabolites arranged according to the catabolic pathway shown in Figs. 3 and 4. The list of final catabolites comprises NCCs isolated from yellowed leaves of two annuals (oilseed rape and barley) as well as from the ornamental trees, sweetgum (*Liquidambar*), and Katsura tree (*Cercidiphyllum*). For references see Matile et al. (1999).

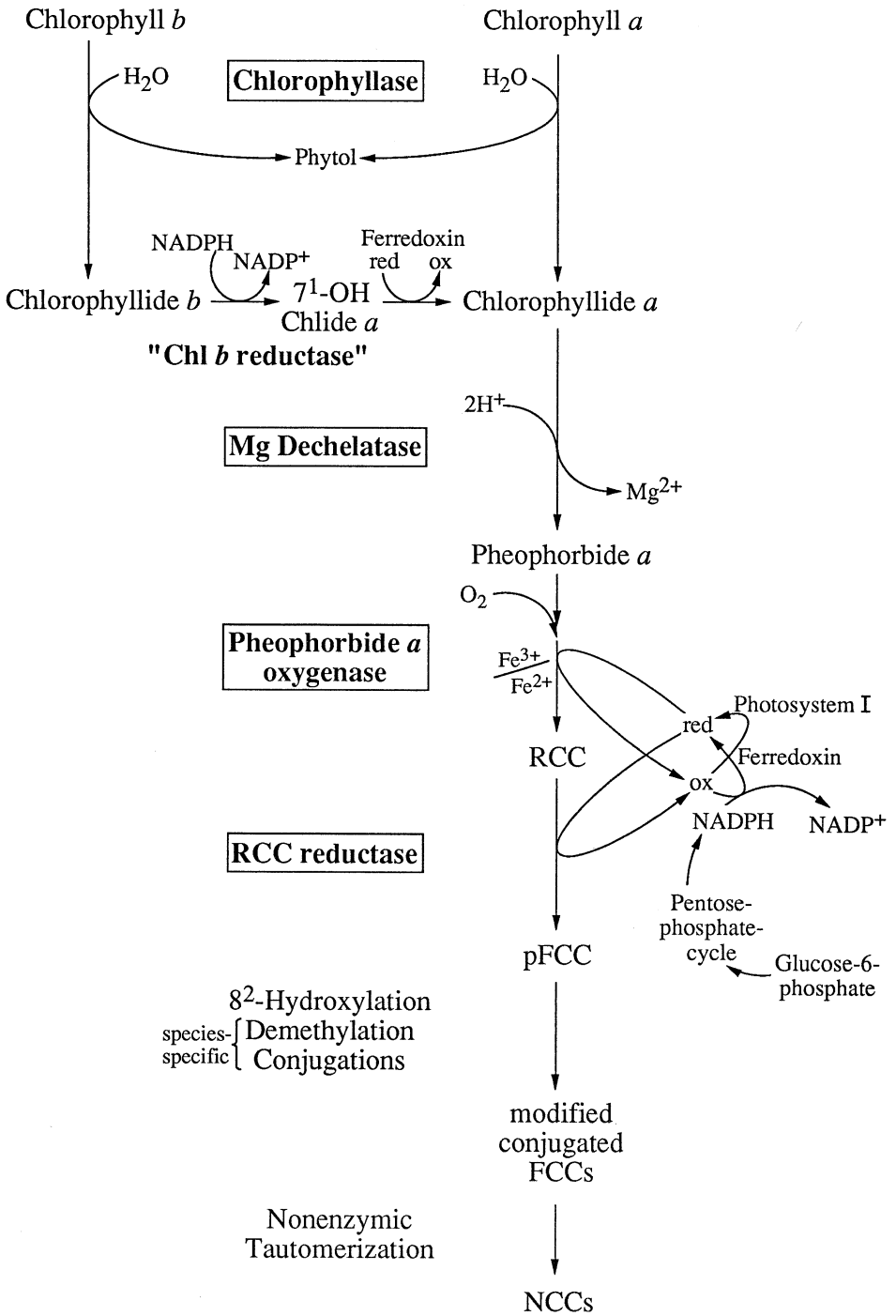


Fig. 3. The pathway of chlorophyll degradation in senescent leaves. Note that NCCs are derived from chlorophyll *a* because the ring-opening oxygenase has an absolute specificity for pheophorbide *a* as substrate. From Matile et al. (1999).

relevant for all vascular plants. Indeed, RCC reductase has been demonstrated to occur in a large number of species including primitive ferns, gymnosperms and angiosperms (Hörtensteiner et al., 2000). Detoxification of chlorophyll seems to be phylogenetically as ancient as chlorophyll biosynthesis and oxygenic photosynthesis. Whereas in green algae the process is restricted to oxygenolysis of the porphyrin and excretion of the resulting red bilin, RCC, into the surrounding water (Engel and Gossauer, 1996), in terrestrial plants the intracellular storage of catabolites requires a more extensive photodynamic inactivation of catabolites. The coupling of pheophorbide *a* oxygenase with RCC reductase seems to represent the decisive step toward intracellular storage of harmless NCCs.

It is worth mentioning that a non-yellowing mutant genotype of the grass, *Festuca pratensis*, has played a decisive role in the discovery of chlorophyll catabolites and subsequent elucidation of the catabolic pathway. Compounds that turned out to represent products of chlorophyll breakdown were observed to accumulate during senescence of wild type leaves but were not detectable in leaves of the mutant. Upon the discovery of pheophorbide *a* oxygenase and comparison of activities in senescing chloroplasts from wild type and mutant leaves, respectively, the high retention of chlorophyll in mutant leaves proved to be caused by largely lacking oxygenase activity (Vicentini et al., 1995). The same result has been obtained with genotypes of pea comparable to those employed by Gregor Mendel in his classical work on the inheritance of characters such as yellow and green seeds (Thomas et al., 1996). It had always been puzzling that the pea variety producing green seeds was the mutant, but now it is obvious that Mendel's green seed variety represents a stay-green mutant equivalent to the various non-yellowing cultivars of bean, soybean, tomato and other cultivated plants. With regard to protein breakdown in senescing leaves it is interesting that in stay-green genotypes the high retention of chlorophyll is always associated with an equally high retention of chlorophyll-binding proteins in the thylakoids. Hence, chlorophyll seems to protect the apoproteins from proteolytic degradation.

5. Pathway of chlorophyll degradation

It seems from the pathway depicted in Fig. 3 that chlorophyll catabolism is initiated by the hydrolysis of the ester bond linking the porphyrin moiety to its hydrophobic tail, phytol. The action of the corresponding enzyme, chlorophyllase, yields water-soluble chlorophyllide that is subsequently demetalized by Mg dechelatase to yield still green pheophorbide, the substrate of ring opening.

Chlorophyllase is a classical enzyme with a vast literature (partially reviewed in Matile et al., 1999). Since its discovery by Stoll (1912), the function has remained mysterious because the hydrolysis of chlorophyll in homogenized leaf tissue requires such unphysiological conditions as high concentrations of acetone or the presence of detergents (e.g. Amir–Shapira et al., 1986). Chlorophyllase is a hydrophobic protein of chloroplasts that gets into physical contact with the endogenous substrate only upon the solubilization of membranes. This structural latency has recently been explained in terms of differential locations of substrate in the thylakoids and enzyme in the chloroplast envelope (Matile et al., 1997). Chlorophyllase has been purified repeatedly from several sources and it seems to be the first chlorophyll-catabolizing enzyme whose gene has been cloned (E.E. Goldschmidt, personal communication).

Downstream macrocycle cleavage, pFCC is modified in various ways and eventually

converted to NCCs. The structures of NCCs (Fig. 2) suggest that hydroxylation in $C8^2$ is a common modification. Other modifications such as demethylation in $C18^2$ or conjugations of the $C8^2$ hydroxyl group occur in a species-specific fashion. A further common modification concerns a tautomerization resulting in the saturation of the γ-methine bridge and loss of fluorescence; it occurs non-enzymically under acidic conditions (Hörtensteiner, 1999).

It should be remembered that in the leaves there are two types of chlorophyll, *a* with a $C7^1$ methyl- and *b* with a $C7^1$ formyl group. For a while it was puzzling that all NCCs were identified as derivatives of chlorophyll *a*. The riddle was solved when, on the one hand, the oxygenase turned out to have an absolute specificity for pheophorbide *a* as substrate (Hörtensteiner et al., 1995) and, on the other hand, the reduction of chlorophyll *b* to *a* was discovered in senescent leaves (Folly and Engel, 1999; Scheumann et al., 1999).

6. Subcellular organization of chlorophyll catabolism

In the senescing leaf cells, the catabolic pathway extends over several subcellular and suborganellar compartments. Starting in the thylakoids and ending in the sap of central vacuoles it is not only associated with enzymic conversions but also with catalyzed transport across membranes (Fig. 4). A remarkable feature of this subcellular organization concerns the location of the initial steps within developing gerontoplasts. Contrary to expectation, chlorophyllase, oxygenase, and probably also Mg dechelatase are not located at the site of chlorophyll in the thylakoids but rather in the chlorophyll-free inner membrane of the gerontoplast envelope. This spatial separation of enzymes and substrate makes sense because chlorophyllase and dechelatase are constitutive enzymes. Only the oxygenase has so far been demonstrated to be present exclusively when chlorophyll is catabolized, the activity being positively correlated with rates of chlorophyll breakdown (Rodoni et al., 1998). However, such differential location of enzyme and substrate leaves us with the problem of how, during senescence, chlorophyll gets into contact with chlorophyllase. This riddle has not yet been solved but it is well established that the initiation of chlorophyll hydrolysis requires cytoplasmic protein synthesis (Thomas et al., 1989). It seems that the initiation of catabolism is due to a hypothetical carrier protein (marked X in Fig. 4) that is responsible for dismantling of pigment-protein complexes in the thylakoids and transport of chlorophyll molecules to the site of chlorophyllase in the envelope.

7. Polychromatic autumnal foliage

Beyond its significance for nutrient economy, leaf senescence has a widely appreciated aesthetical value, particularly with regard to deciduous trees. The polychromatic beauty of autumnal forests and parks is due to species-specific variations of a general theme: loss of green color associated with partial retention of yellow carotenoids. The variations comprise degrees of carotenoid retention and particularly various superpositions of yellow color with red colors of anthocyanins and brown colors of melanins (Fig. 5). During the short period of Indian summer, the foliage of deciduous trees runs through a spectacular

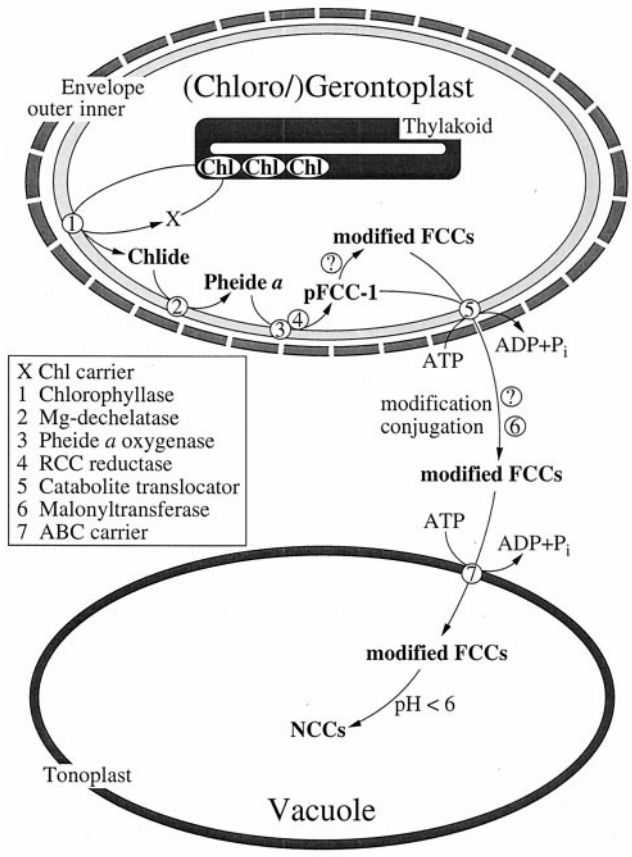


Fig. 4. Intracellular organization of chlorophyll catabolism in mesophyll cells of senescent leaves. The core piece of the pathway, pheophorbide (Pheide) *a* oxygenase, is located together with chlorophyllase and probably also Mg dechelatase, in the inner membrane of the gerontoplast envelope. The existence of a catabolite translocator (5) responsible for the export of FCCs into the cytosol has been demonstrated in isolated gerontoplasts. The final deposition of catabolites in the vacuole is catalyzed by a directly energized ABC (ATP-binding cassette) transporter. For further information and references see Hörtensteiner (1999).

play of colorations with species-specific patterns, gradations, shades, and tints of yellow, orange, red, purple, and brown.

Whereas the loss of chlorophyll and the accumulation of anthocyanins depend on the intactness of the metabolic machinery in the leaf cells, the browning is a consequence of cell death. In the living cells, enzymic oxidation of phenolics is prevented by differential location of substrates in the vacuoles and phenol oxidase in the plastids. At the end of the senescence period, the subcellular compartmentation collapses and the formation of dark melanins takes place as it does at cut surfaces of apple and potato. Phenolics represent a class of widespread secondary compounds. Phenol oxidase catalyses the hydroxylation of monophenols into diphenols and converts diphenols into quinons that polymerize to dark melanins. Most probably the decay of subcellular compartmentation and the browning reaction associated with it represents a case of programmed cell death (Yen and Yang, 1998). In some species such as beech, browning precedes leaf shedding whilst in others

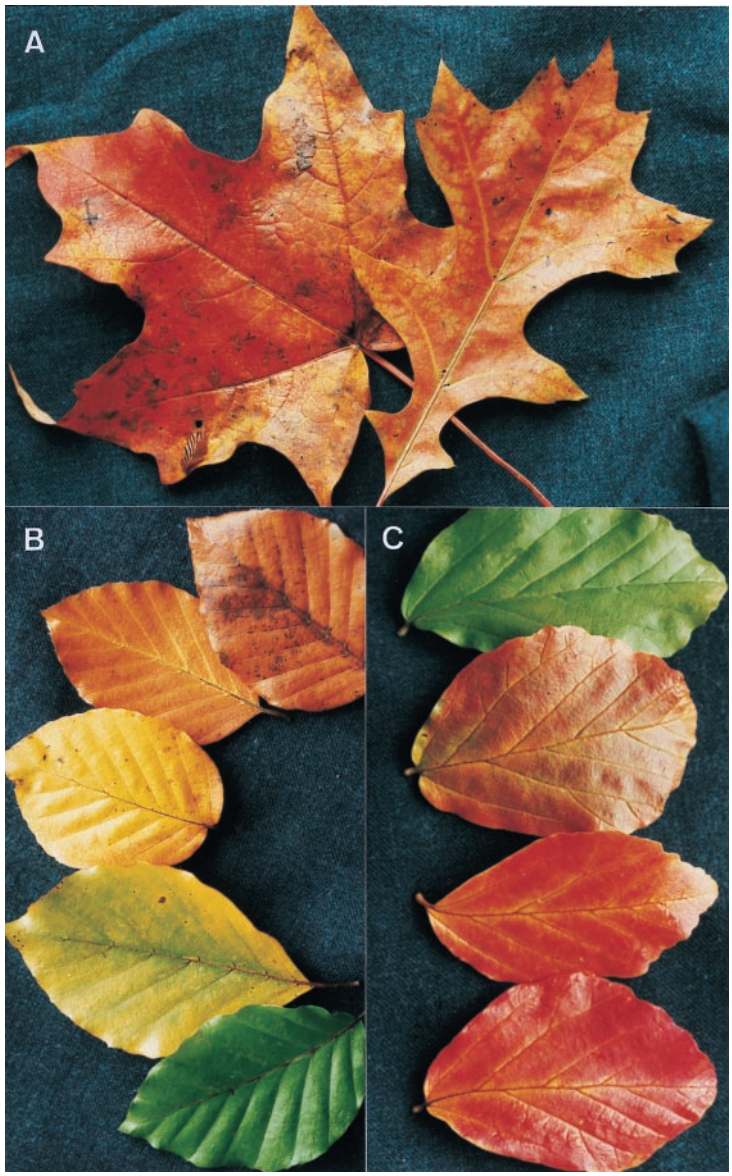


Fig. 5. a, Maple trees such as *Acer saccharum* (left) and oaks (*Quercus* spp., right) contribute mainly to the famous autumn coloration of forests during Indian summer in Northeast America). b, In beech (*Fagus sylvatica*) the yellowing of senescing leaves is superimposed by browning due to the oxidation of phenolics. Leaves are normally shed when browning is completed. c, The color changes during leaf senescence in the ornamental tree, Persian Ironwood (*Parrotia persica*), are due to superposition of red-colored newly synthesized anthocyanin with yellow of retained carotenoids.

such as maples or linden, shed leaves remain yellow and turgescent for many days before they eventually turn brown.

Whereas the degreening of foliage is a phylogenetically highly conserved part of the senescence program, the accumulation of anthocyanins in the vacuoles of leaf cells seems

to represent a kind of extravagancy without a vital function. Over the years, the extent of new synthesis varies considerably. Within a species, it also varies from one individual tree to another (Chang et al., 1989) and even from one leaf to another. Hence, in senescing leaves anthocyanins have no obvious function and this is strikingly demonstrated by the copper-varieties of beech and hazel in which senescence is preceded by the loss of anthocyanin so that, for a while, the foliage turns as green as in wild-type trees. And yet, senescence-associated formation of anthocyanins has a significant aesthetical value and its genetic variation has led to the selection of a large choice of ornamental trees. It is also responsible for the autumnal coloration of maples and oaks in North–East American forests (Fig. 5A) that, during Indian summer, attract thousands of tourists.

Anthocyanins represent glycosides of phenolic aglycons with a flavan C6-C3-C6 skeleton. They are free of nitrogen and hence, the synthesis during senescence does not interfere with the *N*-economy of the tree. The phenylpropane part of the molecule is derived from phenylalanine that is committed to the flavan pathway by the action of phenylalanine-ammonia lyase (PAL). It seems that genes of PAL as well as of other enzymes involved in anthocyanin biosynthesis are expressed under the control of senescence-associated promoters. Co-pigmentation with other phenolic glycosides and formation of complexes with metal. ions in the vacuoles may contribute to the variation of red tints in autumn foliage as it is known to be the case in flower petals (Harborne, 1993).

Close examination of shed leaves quite often reveals an uneven pattern of yellowing with scattered green islands (Fig. 6A-C). This phenomenon is due to infections by fungi that produce a plant hormone, cytokinin, a well-known inhibitor of senescence. Gall flies deposit eggs in leaf tissue of beech and cause the local cell proliferation by virtue of secreted cytokinin; during leaf senescence, cytokinin delays chlorophyll degradation and is responsible for the persistence of green and viable tissue surrounding the galls (Fig. 6C). Such phenomena indicate that senescence, like any other developmental process, is under endogenous hormonal control. External factors such as temperature and photoperiod play a role in the regulation as well but exact knowledge with regard to trees is very scarce. The reason for such lack of precise information is simply that experimentation with trees is a most difficult task.

Antisenescence effects of cytokinin have been demonstrated unambiguously in suitable experimental plant such as tobacco. This annual plant is characterized by sequential (progressive) leaf senescence. Levels of endogenous cytokinin decrease markedly concomitant with leaf yellowing, and the application of exogenous cytokinin causes retardation (Singh et al., 1992). The effect of cytokinin on age-dependent senescence of tobacco leaves has also been demonstrated in plants transformed with a bacterial gene for cytokinin synthesis (Gan and Amasino, 1995). Of the other classical plant growth regulators, the senescence-accelerating effects of ethylene and also of abscisic acid are well documented. Among the environmental factors, light is certainly the most important one. Depending on the experimental system both positive and negative effects as well as effects of photoperiod and light dosage have been reported. Whether the regulation of leaf senescence in trees can be inferred from data obtained through experimentation with herbs and grasses is uncertain.

Perhaps the most astonishing example of extravagant biochemistry of leaf senescence is represented by *Ginkgo biloba*, an ornamental tree originating from China. The magnificent golden-radiant appearance of its autumnal foliage is due to an unusually high retention of carotenoids in conjunction with an optical brightener that is produced in large quantities reaching the highest level when the leaves are eventually shed (Fig. 1; Figs. 6C

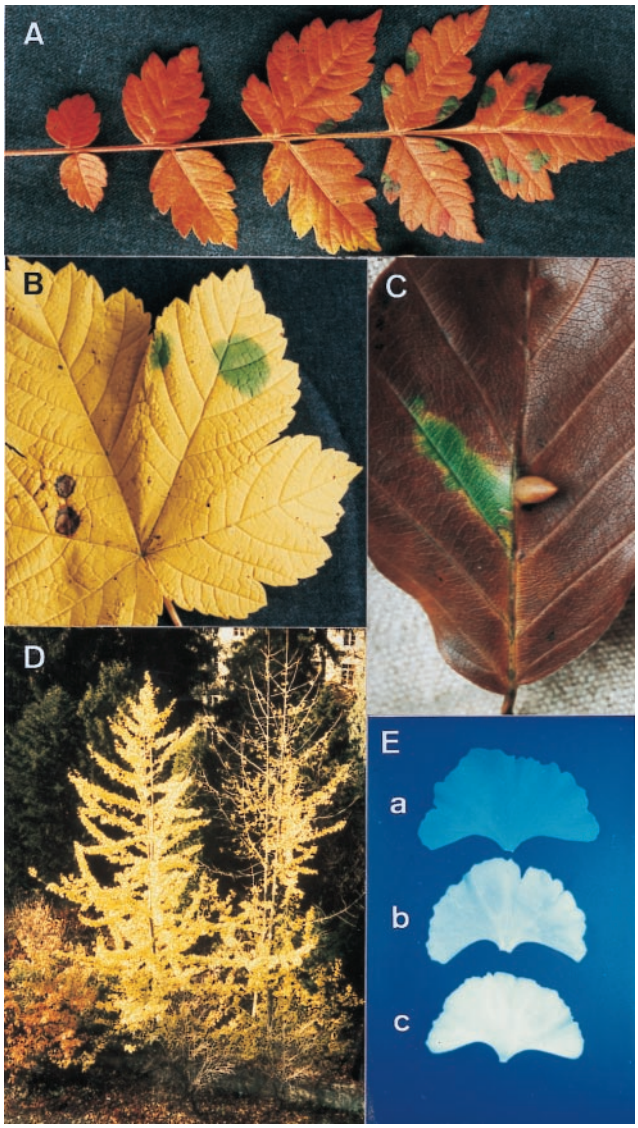


Fig. 6. a-c, Green islands in fully senescent leaves of Pride of India (*Koelreuteria paniculata*) (a), Sycamore (*Acer pseudoplatanus*) (b), and beech (*Fagus sylvatica*) (c). Local delays of degreening are caused by fungal infections (a, b) or by a gall (c). In both cases the phenomenon is due to the production of the antisenescence hormone, cytokinin. d, The brilliant golden appearance of autumn foliage in *Ginkgo biloba* is caused by the marked retention of carotenoids in conjunction with the accumulation of an optical brightener, 6-hydroxykynurenic acid (see also Fig.1). E, A mature green leaf (a) and fully senescent shed leaves (b, c) as viewed under ultraviolet radiation; note conspicuous fluorescence in the senescent leaves.

and D; Matile et al., 1992). This fluorescent compound has been identified as 6-hydroxykynurenic acid (Schennen and Hölzl, 1986), a derivative of tryptophane originally discovered in the urine of dog. The conversion of tryptophane into this secondary metabolite requires several enzymes. It seems that in senescing leaves of *Ginkgo*, corresponding genes are expressed in individual cells that develop into fluorescing idioblasts

(Matile, 1994). As in the case of anthocyanins, there is no obvious function associated with this premortal biosynthetic effort that does not seem to have a negative effect on the nitrogen economy of the tree. Such biochemical extravagancies associated with leaf senescence may have evolved in the absence of selection pressure. Whereas they seem to have no function in the trees, they contribute to the autumnal feast for the human eyes.

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