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Nitrogen metabolism and remobilization during senescence

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Abstract

Senescence is a highly organized and well-regulated process. As much as 75% of total cellular nitrogen may be located in mesophyll chloroplasts of C_3-plants. Proteolysis of chloroplast proteins begins in an early phase of senescence and the liberated amino acids can be exported to growing parts of the plant (e.g. maturing fruits). Rubisco and other stromal enzymes can be degraded in isolated chloroplasts, implying the involvement of plastidial peptide hydrolases. Whether or not ATP is required and if stromal proteins are modified (e.g. by reactive oxygen species) prior to their degradation are questions still under debate. Several proteins, in particular cysteine proteases, have been demonstrated to be specifically expressed during senescence. Their contribution to the general degradation of chloroplast proteins is unclear. The accumulation in intact cells of peptide fragments and inhibitor studies suggest that multiple degradation pathways may exist for stromal proteins and that vacuolar endopeptidases might also be involved under certain conditions. The breakdown of chlorophyll-binding proteins associated with the thylakoid membrane is less well investigated. The degradation of these proteins requires the simultaneous catabolism of chlorophylls. The breakdown of chlorophylls has been elucidated during the last decade. Interestingly, nitrogen present in chlorophyll is not exported from senescencing leaves, but remains within the cells in the form of linear tetrapyrrolic catabolites that accumulate in the vacuole. The degradation pathways for chlorophylls and chloroplast proteins are partially interconnected.

Key words: Chlorophyll catabolism, chloroplast, compartmentation, membrane integrity, protein degradation, vacuole.

Introduction

Senescence represents the final stage of leaf development and is characterized by the transition from nutrient assimilation to nutrient remobilization (Feller and Fischer, 1994; Masclaux *et al*., 2000). The catabolic processes are well organized on the whole plant level (Noodén, 1988), on the organ level (Feller *et al*., 1977), on the cellular level (Zeiger and Schwartz, 1982), and on the subcellular level (Matile, 1992). In mesophyll cells, chloroplasts are dismantled in an early phase of senescence, while mitochondria remain functional. Up to 75% of the nitrogen present in mesophyll cells is located in the chloroplasts (Peoples and Dalling, 1988). Stromal enzymes, mainly Rubisco, represent the major fraction of chloroplast nitrogen. The rate of senescence and the remobilization of leaf nitrogen are related to the nitrogen nutrition status of the plant and on source/sink relations (Crafts-Brandner *et al*., 1996, 1998; Ono *et al*., 1999; Masclaux *et al*., 2000).

Chlorophyll and protein catabolism inside the chloroplasts

Several lines of evidence support the conclusion that at least the initial steps of chlorophyll and chloroplast protein degradation can occur in the intact organelles. On the other hand, some steps are or might be located outside the plastids (Fig. 1). It must also be considered that alternative catabolic pathways may exist and that they may be compartmentalized in a manner not yet discovered.

Degradation of stromal enzymes

Regardless of the fate of thylakoid constituents, stromal enzymes are degraded early during senescence leading to the decline of photosynthetic capacity. Enzymes involved in carbon and nitrogen assimilation are lost and the amino acids derived from their catabolism may

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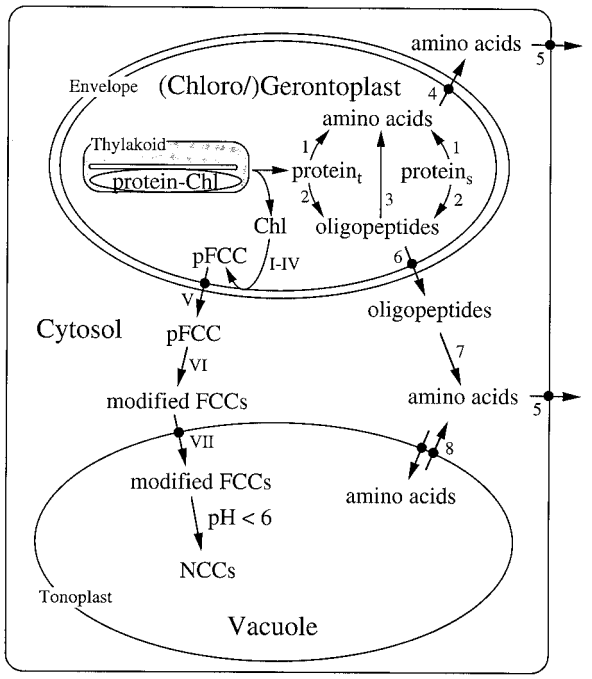

I, chlorophyllase
II, Mg dechelatase
III, Pheide *a* oxygenase
IV, RCC reductase
V, Chl catabolite translocator
VI, FCC-modifying enzymes
VII, ABC transporter

1, exopeptidases
2, endopeptidases
3, exopeptidases
4, amino acid transporter (envelope)
5, amino acid exporter (plasma membrane)
6, oligopeptide transporter
7, cytosolic exopeptidases
8, amino acid transporters (tonoplast)

Fig. 1. Pathways for the degradation of Chl (roman numbers) and of chloroplast proteins (arabic numbers). Protein$_t$, thylakoid proteins; protein$_s$, stromal proteins.

be exported via the phloem with or without prior modification (e.g. production of amides from other amino acids). Glutamine synthetase, the key enzyme in the assimilation of ammonia, is present in the chloroplasts and in the cytosol. The plastidial form is lost in cereal leaves in an early phase of senescence, while the cytosolic form remains active longer (Streit and Feller, 1983). Glutamine synthetase is rather susceptible to proteolysis in isolated chloroplasts and it is degraded far more rapidly than Rubisco or other enzymes involved in carbon assimilation (Mitsuhashi and Feller, 1992; Thoenen and Feller, 1998). The cytosolic form of glutamine synthetase was shown to be localized in the vascular bundles of rice leaves (Sakurai *et al.*, 1996). Based on immunological data, a shift in ammonia assimilation from the chloroplast to the cytosol of mesophyll cells of tobacco was suggested recently (Brugière *et al.*, 2000).

It has been reported that stromal enzymes are degraded in isolated pea chloroplasts (Mitsuhashi and Feller, 1992; Thoenen and Feller, 1998). In these studies, the chloroplasts were isolated, incubated under defined conditions and re-isolated from each sample to ensure that only chloroplasts remaining intact throughout the incubation period were analysed. It may be important from a technical point of view to consider that EDTA and DTT (common ingredients in isolation and incubation buffers) can severely interfere with proteolysis in chloroplasts (Roulin and Feller, 1998*a*, *b*). DTT inhibits violaxanthin de-epoxidase and ascorbate peroxidase, two enzymes involved in the detoxification of reactive oxygen species in chloroplasts (Neubauer, 1993; Stieger and Feller, 1997). EDTA on the other hand most likely interferes with a proteolytic enzyme depending on divalent cations (Roulin and Feller, 1998*b*). In contrast to the quite rapid degradation of stromal enzymes such as Rubisco, glutamine synthetase and glutamate synthase observed in intact chloroplasts isolated from mature pea leaves, only a very slow catabolism of the same enzymes was observed after chloroplast lysis (Roulin and Feller, 1997).

The degradation of the predominant stromal protein in C_3-plants, Rubisco, has been the subject of several studies (Mitsuhashi *et al.*, 1992; Desimone *et al.*, 1996; Ishida *et al.*, 1997, 1998, 1999). In intact chloroplasts, degradation of Rubisco causes the accumulation of different fragment patterns depending on the specific experimental conditions (e.g. pH, energy status, solute concentrations, radicals) during incubation (Mitsuhashi *et al.*, 1992; Hildbrand *et al.*, 1994; Herrmann and Feller, 1998). It has often been suggested that Rubisco degradation may be initiated by reactive oxygen species (Desimone *et al.*, 1996, 1998; Ishida *et al.*, 1998; Roulin and Feller, 1998*a*; Stieger and Feller, 1997). A direct (non-enzymic) cleavage of the large subunit of Rubisco into a 37 kDa and a 16 kDa fragment induced by reactive oxygen has been reported (Ishida *et al.*, 1997).

The complete hydrolysis of proteins to free amino acids depends on the action of endo- and exopeptidases (Brouquisse *et al.*, 2001). Endopeptidases are essential for the first cleavage(s) of peptide bonds in a protein and, therefore, also for the initiation of the catabolism of this protein. Several types of endopeptidases have been detected in plastids. Evidence for the involvement of a metalloendopeptidase in stroma protein degradation has been presented by several groups (Bushnell *et al.*, 1993; Roulin and Feller, 1998*b*). Aminopeptidases are also present in the plastids and may also contribute to the complete degradation of stromal proteins, especially by degrading the peptides generated by endopeptidase activity. On the other hand, carboxypeptidases (vacuolar enzymes) were not detected in intact plastids (Feller and Fischer, 1994, and references therein). Together these data suggest that metalloendopeptidase and aminopeptidases are key enzymes for the degradation of stromal proteins.

The presence of the Clp system in plastids has been reported by several groups during the past few years.

The Clp protease with the plastome-encoded proteolytic subunit ClpP and the nuclear-encoded ATPase subunit ClpC was identified in several species at the transcript level and at the protein level (Shanklin *et al*., 1995; Crafts-Brandner *et al*., 1996, 1998; Weaver *et al*., 1999). It appears likely that this proteolytic system plays a role in chloroplast development or housekeeping (Shikanai *et al*., 2001). The Clp protease may also be important for the degradation of polypeptides in the cytochrome b_6f complex (Majeran *et al*., 2000). Hence this proteolytic system is likely to be important mainly before the onset of senescence and the involvement of Clp in the catabolism of mature proteins during senescence must be questioned. To this end there is no good evidence available for a key regulatory role of Clp in the remobilization of stromal proteins from senescing leaves.

Protein catabolism in plastids may not only be controlled by the levels of proteolytic activities, but also by the susceptibility of the substrate proteins. Besides catalytic and regulatory properties of an enzyme, its intrinsic susceptibility to proteolytic attack must be considered as a physiologically highly relevant aspect. This type of post-translational control is often overlooked. Plastidial isoforms of glutamine synthetase (Streit and Feller, 1983) and of glutamate pyruvate transaminase (Thomas and Feller, 1993) are far more susceptible to proteolysis than the cytosolic forms. The results indicate that the stroma contains some rather fragile enzymes. Furthermore, stromal enzymes can be protected from degradation by interaction with ligands (Thoenen and Feller, 1998; Khan *et al*., 1999). For example, in intact pea chloroplasts, plastidial glutamine synthetase is protected from degradation by the inhibitor methionine sulphoximine which is a substrate analogue that binds tightly to the glutamate binding site (Thoenen and Feller, 1998). Rubisco on the other hand is protected against breakdown by trypsin or soluble chloroplast proteases by the natural inhibitor 2′-carboxy-D-arabinitol 1-phosphate (Khan *et al*., 1999). Chloroplast enzymes are involved in protein turnover throughout leaf development. It is not known if the proteolytic activities involved in protein turnover are also involved in the net degradation of chloroplast proteins during senescence. It is possible that such catabolic enzymes may function in the initial (reversible) phase of senescence and that other proteolytic systems become more relevant as senescence progresses.

Breakdown of thylakoid-bound proteins

The morphological analysis of senescing chloroplasts reveals dramatic changes of the thylakoid membrane system. The contact between grana stacks is loosened and, finally, the membranes disappear in conjunction with an increase in the number and size of plastoglobuli. Concomitant with the disappearance of the membrane system is the degradation of membrane constituents such as lipids, proteins and chlorophyll (Chl) (Matile, 1992). Most important in terms of nitrogen content are the thylakoid-bound apoproteins of chlorophyll, mainly LHCP II. Hence, in addition to Rubisco, degradation of LHCP II is a major contributor to the overall loss of protein during foliar senescence (Matile, 1992).

Unfortunately, thylakoid protein turnover or degradation during senescence has not been studied in great detail. Exceptions are LHCP II and the D1 protein of photosystem II. D1 requires Chl for stable integration into the thylakoid membrane (Mullet *et al*., 1990) and has an extremely rapid turnover rate in the light (Mattoo *et al*., 1984). It has been shown that this rapid turnover rate of D1 proceeds into late senescence (Roberts *et al*., 1987) and is accompanied by the turnover of Chl (Raskin *et al*., 1995).

LHCP II has been investigated during the course of leaf development and senescence in several species (Hidema *et al*., 1992). Studies using stay-green genotypes of *Festuca pratensis* and *Phaseolus vulgaris* demonstrated that LHCP II degradation requires the simultaneous loss of Chl. In wild types, LHCP II and Rubisco subunits were degraded at similar rates during senescence. By contrast, the mutants which retained most of their Chl into late senescence also retained LHCP II, whereas Rubisco subunits were catabolized as in the wild types (Hilditch *et al*., 1989; Bachmann *et al*., 1994).

There are no data available concerning the enzymes responsible for the digestion of thylakoid-bound proteins. The question can be raised whether there are specific proteases that exclusively hydrolyse the apoproteins of Chl. However, it seems more likely that these undefined proteases have a broader substrate specificity. Thus, whether a thylakoid or stromal protein is prone to digestion may be regulated by the accessibility of the particular protein rather than by changes of the proteolytic machinery. There is striking evidence that Chl-binding proteins like LHCP II are stabilized through the insertion of Chl and proteolysis is prevented as long as the pigment–protein complexes are retained (White and Green, 1987). A net loss of apoproteins during senescence only occurs after the removal and degradation of Chl. In stay-greens where Chl catabolism is blocked, LHCP II remains stabilized and proteolytic cleavage is restricted to a small N-terminal region of the protein that protrudes into the stroma (Thomas and Donnison, 2000). Based on these data, it seems obvious that catabolism of Chl is a prerequisite for the proteolysis of its apoproteins. Disassembly of pigment–protein complexes causes the release of potentially hazardous Chl that can cause photo-oxidative damage. Thus, degradation of this photosynthetic pigment is necessary for the remobilization of nitrogen located in the Chl-apoproteins.

Chlorophyll catabolism in senescing chloroplasts

The catabolism of Chl during leaf senescence is accompanied by the loss of green colour and is one of the most spectacular phenomena in nature. The breakdown pathway has been nearly completely elucidated during the last decade, starting in 1991 with the first description of a Chl catabolite from barley (Kräutler *et al*., 1991). More recently, non-fluorescent Chl catabolites (NCCs) have been found in a number of different species (Mühlecker and Kräutler, 1996; Curty and Engel, 1996) and are believed to represent the final breakdown products of Chl that are deposited in the central vacuole. Chemically they represent tetrapyrroles derived from Chl by oxygenolytic opening of the porphyrin macrocycle. Hence, the four moles of nitrogen associated with each mole of Chl are not recycled during senescence, but are lost when senescent leaves shed.

Catabolism of Chl is catalysed by at least five enzymic reactions commonly found in all species that have been investigated. The first four reactions take place in the senescing chloroplasts (gerontoplasts) (Fig. 1) starting with the removal of the phytol chain by chlorophyllase producing chlorophyllide (Chlide). Chlorophyllase, first described by Stoll (Willstätter and Stoll, 1913), is localized in the inner envelope membrane (Matile *et al*., 1997) and exhibits a rather unusual latency. Thus *in vitro*, activity can only be measured in the presence of high concentrations of detergents or solvents (Trebitsh *et al*., 1993). The spatial separation of Chl and chlorophyllase invokes the necessity of an as yet unknown factor which establishes the physical contact between substrate and enzyme and which could represent a regulator for the initiation of Chl catabolism. Chlorophyllase genes have been recently identified from *Arabidopsis*, *Citrus* and *Chenopodium* (Jakob-Wilk *et al*., 1999; Tsuchiya *et al*., 1999) with surprising results: all deduced proteins are soluble and some of them may be extraplastidially located. This fact raises the question whether other subcellular compartments may be involved in the early catabolism of Chl (see below).

Mg-free forms of Chl have been identified in several plant species during senescence (Ziegler *et al*., 1988). Using senescent rape cotyledons, considerable quantities of pheophorbide (Pheide) *a* accumulated, when the subsequent cleavage of the porphyrin macrocycle was inhibited (see below). By contrast, during normal yellowing, Pheide *a* did not accumulate (Langmeier *et al*., 1993). Thus, the removal of the central atom, Mg, of Chlide by Mg dechelatase to yield Pheide was postulated to represent the second enzyme in the Chl catabolic pathway. This activity has not yet been investigated in much detail, but it appears to be associated with a heat stable substance of low molecular weight (Shioi *et al*., 1996).

The third reaction in the Chl catabolic pathway is most important for leaf yellowing because it is responsible for the loss of green colour. Actually, two enzymes are necessary for the formation of the first identifiable cleavage product of Pheide *a*, a primary fluorescent Chl catabolite (pFCC). Firstly, Pheide *a* oxygenase (PaO) opens the porphyrin macrocycle by the introduction of oxygen. This yields a red Chl catabolite (RCC) intermediate that is released from PaO after a site-specific reduction catalysed by RCC reductase (RCCR) (Rodoni *et al*., 1997*a*). RCCR activity has been detected in roots, and is measurable in the stroma at all stages of leaf development (Rodoni *et al*., 1997*a*; Wüthrich *et al*., 2000). By contrast, PaO is bound to the inner envelope membrane (Matile and Schellenberg, 1996) and its activity is only detectable during senescence (Hörtensteiner *et al*., 1995). Despite their different locations, it is evident that PaO and RCCR interact closely during catalysis, and metabolically channel RCC. Both partial reactions use reduced ferredoxin (Fd) as a source for electrons; and reduced Fd is regenerated either by the pentose phosphate cycle or by photosystem I.

Metal chelation and reconstitution experiments have shown that PaO is a non-haem iron containing monooxygenase (Hörtensteiner *et al*., 1998). It is specific for Pheide *a* and, *in vitro*, Pheide *b* acts as a competitive inhibitor. This remarkable specificity is reflected by the NCCs which exclusively are derived from Chl *a*, suggesting that Chl *b* is converted to Chl *a* prior to entering the Chl catabolic pathway. Indeed, a Chl *b*-reducing system has recently been described in barley and cucumber, which could be important for Chl *b* to *a* conversion during senescence (Ito *et al*., 1996; Scheumann *et al*., 1999).

RCCR is a novel reductase that does not require a prosthetic group for the transfer of electrons from Fd to RCC (S Hörtensteiner, B Kräutler, unpublished data). The properties of RCCR are remarkable as it acts in a stereospecific manner when assayed together with PaO *in vitro*. Depending on the source of RCCR, only one of two possible epimers of pFCC occurs (Hörtensteiner *et al*., 2000; Mühlecker *et al*., 2000). This stereospecificity is lost when assays are performed with RCCR on its own using artificial RCC as substrate: under this condition, both epimers are formed simultaneously. RCCR has been partially purified from barley and was subsequently cloned from *Arabidopsis* (Rodoni *et al*., 1997*b*; Wüthrich *et al*., 2000). The identification of accelerated cell death (*acd*) mutants in *Arabidopsis* (Greenberg *et al*., 1994) has led to the isolation of *acd2* that is defective in the RCCR gene (Mach *et al*., 2001). The accumulation of phototoxic Chl degradation products such as RCC has been suggested to cause the hypersensitive phenotype observed in *acd2*, but there are several indications that RCCR may have other as yet undefined functions during plant

development. Thus, the *acd2* phenotype occurs even at presenescent stages of development, i.e. before PaO activity could cause the accumulation of RCC and, in addition, analysis of RCCR expression revealed the presence of the enzyme not only in senescent leaves, but also in non-green tissues such as roots (Wüthrich *et al*., 2000).

Interactions between chloroplasts and other subcellular compartments

Although the first steps in the degradation of chlorophylls and of proteins are catalysed by enzymes present inside the chloroplasts, interactions with other subcellular compartments are also relevant in this context. Catabolites generated inside the chloroplasts may be further metabolized after the release across the chloroplast envelope.

Release of catabolites across the chloroplast envelope and further degradation in other compartments

When isolated gerontoplasts of barley or oilseed rape were examined for the production of Chl catabolites, FCCs were synthesized *in organello* when energy was supplied in the form of ATP or glucose-6-phosphate (Matile *et al*., 1992; Ginsburg *et al*., 1994). In addition, in the presence of ATP, export of FCCs was observed. The export could not be activated by UTP and required hydrolysis of ATP, indicating that an ATP-dependent transport system at the envelope was responsible for the release of Chl catabolites from senescent chloroplasts (Matile *et al*., 1992). The nature of the respective transport protein has not been identified.

After export from gerontoplasts and extraplastidial modification (see below), Chl catabolites are eventually imported into the vacuole by a tonoplast-bound transport system (Fig. 1). The nature of this carrier has been identified as a primary active ATPase (Hinder *et al*., 1996) of the type of multidrug resistance-associated proteins that are members of the class of ATP-binding cassette transporters (Tommasini *et al*., 1998).

From the chemical structures of pFCC (Mühlecker *et al*., 1997) and the final NCCs, two additional reactions commonly occurring in the Chl catabolic pathway were postulated. First, an ethyl side-chain (C8) of pFCC is hydroxylated, most probably by an as yet undefined P450-dependent hydroxylase. Whether this enzyme is located at the endoplasmatic reticulum, similar to other P450 systems, is unclear. The finding that modified FCCs, in addition to pFCC, are formed within and exported from gerontoplasts (Matile *et al*., 1992; Ginsburg *et al*., 1994) suggests that the hydroxylase could also be located inside the chloroplast.

The second modification concerns the conversion of FCCs into NCCs. This tautomerization is a non-enzymic reaction, most probably catalysed by the acidic pH of the vacuolar sap (S Hörtensteiner, unpublished data).

In addition to these common reactions, there are species-specific modifications of Chl catabolites that could be postulated based on the chemical structures of the respective NCCs. For example, in oilseed rape, a total of three different NCCs accumulate which differ at the C8-ethyl side-chain. Whereas it is hydroxylated in *Bn*-NCC-3 (for a nomenclature of Chl catabolites see Ginsburg and Matile, 1993), the OH-group is conjugated with malonic acid in *Bn*-NCC-1 and with glucose in *Bn*-NCC-2 (Mühlecker and Kräutler, 1996). The formation of *Bn*-NCC-1 that is catalysed by a malonyltransferase which uses malonyl-coenzyme A as the co-substrate has been reported (Hörtensteiner, 1998).

There are a number of reports describing the oxidative bleaching of Chl *in vitro* (Lüthy *et al*., 1986). However, in all of these reports the respective degradation products identified are not natural derivatives of Chl. The positive correlation of Chl-bleaching activities with senescence (Johnson-Flanagan and Spencer, 1996) does not imply their *in vivo* contribution to Chl breakdown. An indication that catabolism of Chl is extraplastidial was supported by the finding of a mass exodus of Chl-containing plastoglobules from senescent soybean chloroplasts (Guiamèt *et al*., 1999). The combination of extraplastidial-located chlorophyllase (Tsuchiya *et al*., 1999) and unspecified vacuolar oxidases has been postulated as an alternative Chl-catabolic pathway (Takamiya *et al*., 2000). Whether this pathway is important *in vivo* remains to be shown. It is not likely that a second complete extraplastidial pathway involving the PaO/RCCR system exists, as RCCR has been demonstrated to be located in the stroma and encoded by a single copy gene in barley and *Arabidopsis* (Wüthrich *et al*., 2000).

Proteolytic activities are also present in the cytosol, in the vacuole, and in the peroxisomes and may function in the degradation of peptides released from chloroplasts (Distefano *et al*., 1999; Brouquisse *et al*., 2001). There is no direct proof for such a release of peptides from chloroplasts (Fig. 1), but peptide translocators have been identified in plants (Jamai *et al*., 1994). Aminopeptidases and oligopeptidases in the cytosol may cleave oligopeptides into free amino acids. The proteasome and other endoproteolytic enzymes are located in the cytosol and may also be involved in the catabolism of larger fragments (Brouquisse *et al*., 2001). In particular, the 20S proteasome may contribute to the degradation of denatured proteins in an ATP-independent manner.

High cysteine endopeptidase and carboxypeptidase levels are present in the vacuole. Increased transcript levels (Buchanan-Wollaston, 1997; Ueda *et al*., 2000) and increased activities (Feller *et al*., 1977) of cysteine

endopeptidases have been observed during senescence. Their function may be restricted to degradation processes occurring after cell death (see below).

Import of nuclear encoded catabolic enzymes into the intact chloroplasts

There are different lines of evidence for the involvement of extraplastidial components in the intraplastidial degradation of Chl to FCCs during senescence (Fig. 2A). For example, isolated mature chloroplasts are not capable of degrading Chl to FCCs as long as they have not started to senesce. In gerontoplasts, however, FCCs are produced *in organello* when energy is supplied either from the photosynthetic machinery, by external ATP or glucose-6-phosphate (Matile *et al*., 1992).

It has been shown that *de novo* cytoplasmic protein synthesis is required for the induction of Chl catabolism and treatment of excised leaves with inhibitors of nuclear protein biosynthesis inhibits normal yellowing (Ginsburg *et al*., 1994). With the exception of PaO, all known components of the Chl catabolic pathway are constitutive. Again, cycloheximide-treated senescent barley leaves exhibit significantly reduced PaO activity, indicating that PaO is a nuclear encoded protein that is specifically expressed during senescence (Rodoni *et al*., 1998). In the stay-green mutant Bf 993 of *Festuca pratensis*, Chlides and Pheide *a* accumulate due to the absence of PaO activity (Vicentini *et al*., 1995). The accumulation of these dephytylated pigments does not occur when senescent leaves of this mutant are treated with protein biosynthesis inhibitors (Thomas *et al*., 1989). This suggests that activity of a nuclear-encoded enzyme is required prior to the interaction of chlorophyllase with Chl.

Chlorophyllase and RCCR are nuclear-encoded enzymes which contain transit peptides directing the respective proteins into the chloroplasts (Jakob-Wilk *et al*., 1999; Wüthrich *et al*., 2000). In addition, ATP-dependent import into isolated chloroplasts and processing of the precursor protein has been demonstrated for RCCR (Wüthrich *et al*., 2000). All in all, it is clear that Chl breakdown is dependent on the import of several extraplastidial components.

Degradation of chloroplast vesicles or entire chloroplasts after import into the vacuole?

Evidence for an autophagic nature of vacuoles has largely been documented by electron micrographic studies. These studies show cytoplasmic structures located within vacuoles, and the results were interpreted as evidence for the engulfment of cytosolic material by the tonoplast and subsequent invagination and budding off into the vacuole (reviewed in Matile, 1975). There are justified doubts concerning this concept of autophagic vacuoles, i.e. possible artefacts due to improper tissue fixation. In addition, serial sections clearly demonstrating the invagination process are largely missing (Matile, 1997).

Evidence for vacuolar autophagy of senescing chloroplasts is not convincing. No obvious transfer of plastid material (e.g. in the form of vesicles) or of entire chloroplasts into the vacuole (Fig. 2B) has been demonstrated by electron microscopy (Matile, 1992, 1997). In addition, in tobacco it has been shown that gerontoplasts remain fully intact during leaf senescence and are able to redifferentiate into chloroplasts during a cytokinin-induced regreening of decapitated plants (Zavaleta-Mancera *et al*., 1999). A recent exception is a report that describes a mass

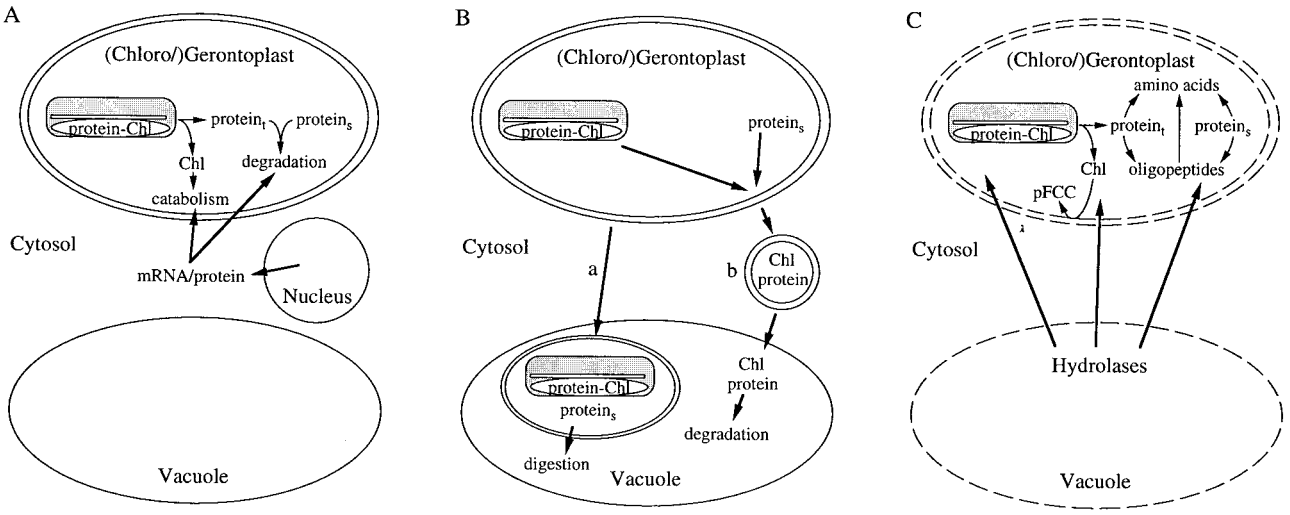

Fig. 2. Extraplastidial contributions to the degradation of chloroplast constituents. (A) Involvement of nuclear-encoded enzymes in the degradation of proteins and of Chl inside the chloroplast. (B) Hypothetical model for the catabolism of chloroplast-derived components in the vacuole after a transfer of entire chloroplasts (a) or of chloroplast vesicles (b). (C) Degradation of chloroplast constituents by extraplastidial enzymes after loss of membrane intactness. Protein$_t$, thylakoid-bound proteins; protein$_s$, stromal proteins.

exodus of Chl-containing plastoglobules from senescent chloroplasts (Guiamét *et al.*, 1999). Together with the identification of chlorophyllase genes which imply an extraplastidic (vacuolar?) location (Tsuchiya *et al.*, 1999) this was interpreted in terms of an alternative Chl degradation pathway located outside the plastid (Takamiya *et al.*, 2000). Whether such a system could also participate in the metabolism of chloroplast proteins, for example, by delivering proteins to cytosolic or vacuolar proteases, remains to be demonstrated.

Autophagy of entire chloroplasts during leaf senescence would cause a continuous decrease in the number of plastids per mesophyll cell. Yet, assessments of chloroplast numbers in mesophyll protoplasts or cross-sections of senescent leaves have demonstrated that the number of plastids remains constant until late in senescence, whereas plastid markers such as Rubisco or Chl decrease (Martinoia *et al.*, 1983). These observations strongly suggest that Chl and chloroplast proteins disappear through degradation within the intact plastids without a significant loss of gerontoplast numbers.

Degradation after loss of membrane intactness

Vacuoles have a lysosomal character as documented by the presence of a large number of different hydrolases such as proteases, nucleases, phosphatases, and glucosidases. Most of these enzymes have an acidic pH optimum and are rather unspecific enzymes (Boller and Wiemken, 1986). The potential of vacuolar hydrolases for the digestion of cellular constituents is demonstrated by the rapid digestion of proteins and nucleic acids after artificial disruption of the subcellular organization. Thus, *in vivo*, if cellular components are exposed to vacuolar hydrolases, the process must be tightly regulated. As outlined above, evidence for import into and subsequent digestion of cytoplasmic or plastidial constituents inside the vacuole during senescence is not shown. Despite this, vacuolar hydrolases, and proteases in particular, have been shown to be expressed during senescence (Buchanan-Wollaston, 1997). Their function has not been analysed biochemically, but most probably it is associated with digestion processes occurring after the rupture of the tonoplast at the end of senescence, i.e. during autolysis (Fig. 2C).

This process, also called apoptosis or programmed cell death (PCD), is genetically regulated and has been described for many developmental processes such as formation of tracheary elements in *Zinnia elegans* (Fukuda, 1996). As for senescence, new hydrolases, among them a cysteine protease, are highly expressed shortly before autolysis is initiated. It is probable that during PCD the vacuolar hydrolases are responsible for the digestion of all remaining subcellular constituents. This would include components of the mitochondria that, during senescence, remain structurally and functionally intact (Matile, 1992), but could also include the complete degradation of undigested gerontoplast constituents (Matile, 1997).

Cysteine endopeptidases are present in the vacuole, but a role in protein degradation in intact chloroplasts has not been demonstrated unambiguously so far. However, vacuolar enzymes may act on stromal proteins after a loss of membrane intactness. The following observations may support this idea. Under low light and low CO_2, a 50 kDa fragment of the large subunit of Rubisco (LS) accumulated in wheat leaf segments (Fig. 3), while this fragment was absent in samples of intact plants under N-deprivation or in segments exposed to high light (data not shown). The 50 kDa fragment was shown to be generated by the removal of the N-terminus from intact LS, since it was detected by antibodies raised against the denatured LS, but not by antibodies raised against the N-terminus of LS (Thoenen, 2000). By contrast, isolated chloroplasts accumulate a series of fragments in the range of 30–50 kDa. These fragments still contain the original N-terminus, indicating that they were produced by other catabolic steps than the 50 kDa fragment mentioned here. In addition, the 50 kDa fragment was formed while the subunit was still integrated in the holoenzyme complex and the cleavage was inhibited by E-64, a cysteine protease inhibitor (Thoenen, 2000). The Rubisco fragment

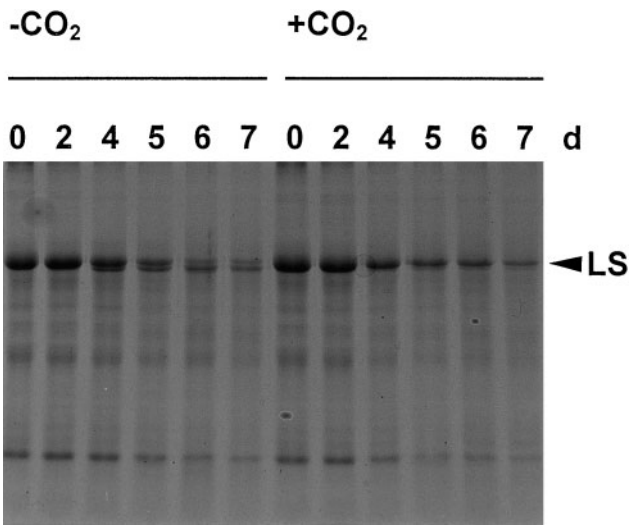

Fig. 3. Accumulation of a 50 kDa fragment of the large subunit of Rubisco (LS) in CO_2-free air. Wheat leaf segments floating on nutrient solution (Hildbrand *et al.*, 1994) were incubated in Erlenmeyer flasks in permanent light (photosynthetic active radiation: 50 µmol m^{-2} s^{-1}). The flasks were either bubbled through with CO_2-free air ($-CO_2$) or with ambient air ($+CO_2$). Samples were taken after 0, 2, 4, 5, 6, and 7 d and analysed by SDS-PAGE followed by staining with Coomassie Brilliant Blue R250. Each lane was loaded with 5 µl extract containing three 5 cm segments per ml^{-1}. The additional band below LS was cross-reactive with antibodies against LS (data not shown) and was produced during incubation in CO_2-free air, but not in ambient air.

was also formed during incubation of wheat leaf extracts. However, it was not detected in wheat leaf segments with high carbohydrate levels or in isolated pea chloroplasts (Roulin and Feller, 1998*b*).

The involvement of vacuolar enzymes in a progressive amino-terminal degradation of the large subunit of Rubisco in French bean leaves has been proposed earlier (Yoshida and Minamikawa, 1996). However, conditions under which vacuolar hydrolases may contact stromal proteins are not clear, although the release of vacuolar enzymes into other compartments after membrane rupture represents a likely possibility (especially during the final phase of senescence). An extracellular metalloproteinase may be important for the degradation of proteins in the extracellular matrix and may also contribute to the degradation of proteins from other compartments after the loss of membrane intactness (Delorme *et al*., 2000).

Conclusions

The initial steps in the degradation of chlorophylls and of chloroplast proteins can occur in the intact organelles. Chloroplasts isolated from mature pea leaves are able to degrade stromal proteins including the predominant enzyme Rubisco. On the other hand, degradation of chlorophylls and of major membrane proteins depends on the synthesis and import of at least one nuclear-encoded catabolic enzyme, PaO, at the onset of senescence. The fact that chloroplast constituents can be degraded in the intact organelles does not necessarily mean that they are under all circumstances degraded while the chloroplast envelope is still intact. It appears possible that different environmental situations (e.g. energy supply) affect not only the velocity, but also the compartmentation of the catabolism of chloroplast proteins and chlorophylls. The initial (reversible) phase of senescence may differ considerably from the final (irreversible) phase. Vacuolar and other extraplastidial enzymes may contact chloroplast constituents after membrane rupture, whilst before senescence or during early senescence these catabolic enzymes are separated from stroma and thylakoid components by membranes. Based on current information, the control of membrane intactness has emerged as a key issue of senescence regulation.

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