Chapter 18

Chloroplast Protein Degradation: Involvement of Senescence-Associated Vacuoles

Maria L. Costa, Dana E. Martínez, Facundo M. Gomez,
Cristian A. Carrión and Juan J. Guiamet*
Instituto de Fisiología Vegetal, Universidad Nacional de La Plata-CONICET, cc 327, 1900 La Plata, Argentina

Summary

Senescence, the last developmental phase in the life of a leaf, is characterized by massive degradation of chloroplast proteins and redistribution of the released amino acids and peptides to other parts of the plant. Chloroplast protein degradation plays an important role in the nitrogen economy of plants. Loss of chloroplast proteins is associated with cessation of protein synthesis and an increase in rates of protein degradation. For some photosynthesis-associated proteins, there is clear evidence for degradation within the plastid itself. For example, chloroplastic FtsH metalloproteases and DegP serine-proteases are involved in the breakdown of the D1 protein upon photoinhibition of photosystem II, and these same proteases might degrade D1 during senescence. The involvement of chloroplast proteases in the degradation of Rubisco, the most abundant leaf protein, is less clear. Senescence-associated vacuoles (SAVs) are a class of small, acidic, lytic vacuoles that occur in senescing leaf cells. They develop in chloroplast-containing cells (i.e., mesophyll and guard cells) and are characterized by high peptidase activity, particularly of cysteine-type proteases. SAVs seem

*Author for correspondence, e-mail: jguiamet@fcnym.unlp.edu.ar

B. Biswal, K. Krupinska and U.C. Biswal (eds.), Plastid Development in Leaves During Growth and Senescence, Advances in Photosynthesis and Respiration 36, pp. 417–433,
DOI 10.1007/978-94-007-5724-0_18,

to be different from "Rubisco Containing Bodies", and development of SAVs does not depend on the operation of the autophagic pathway. A role for SAVs in chloroplast protein degradation can be implied from the fact that stromal proteins of the chloroplast and PSI are re-located to SAVs during senescence. In vitro, cysteine-type proteases within SAVs degrade the chloroplast proteins contained in these vacuoles. SAVs may be part of an extra-plastidial degradation pathway for chloroplast stroma and PSI proteins.

I. Introduction

Chloroplasts are the main source of remobilizable N in leaves of most species, particularly in C_3 plants, having amounts of proteins, nucleic acids and other nitrogenous compounds which may represent up to 70% of leaf N (Mae 2004; Krupinska 2007). Chloroplast proteins are extensively degraded during senescence of leaves, and the peptides and amino acids released are exported to other parts of the plant (Mae 2004). Therefore, senescent leaves of deciduous trees and shrubs, or of annual plants, are usually shed with no more than traces of photosynthetic proteins (Guiamet et al. 2002; Mae 2004; Krupinska 2007). Chloroplast protein degradation during senescence can be viewed as a mechanism to recycle this vast resource of nutrients, which endows plants with a remarkable plasticity in terms of internal nutrient redistribution. Depending on environmental conditions and agronomic practices, in monocarpic crop plants N remobilization associated with senescence of leaves may contribute 20–80% of protein harvested in grains of, e.g., wheat, rice, maize and other crops (Mae 2004; Gregersen et al. 2008; Masclaux-Daubresse et al. 2008). In deciduous perennial trees, N resorption from senescing leaves is likewise important to store N in the bark over the winter to sustain early growth during the following season (May and Killingbeck 1992; Killingbeck 2004). Thus, degradation of chloroplast proteins during senescence plays an important role in the nutrient balance of plants, and in nutrient cycles in nature. However, this also causes a substantial decline in the photosynthetic capacity of leaves. At the same time crops are often in need for photosynthate to fill immature seeds/grains. Thus, in many crops it might be desirable to delay senescence-associated degradation of chloroplast proteins to extend the photosynthetic activity of the canopy (Thomas and Howarth 2000). Some evidence suggests that this has been already partially accomplished by plant breeding during the past decades (e.g., Ding et al. 2005; Giunta et al. 2008). In other cases, senescence acceleration might lead to increased nutrient redistribution and, thereby, increased mineral content of grains (Uauy et al. 2006). Whether the goal is to delay or to accelerate senescence, a better understanding of the mechanism(s) of chloroplast protein degradation might provide tools to manipulate senescence to increase crop yield or the nutritional quality of grains.

Early ultrastructural studies showed that chloroplasts are degraded ahead of other cellular compartments (Noodén 1988). In many cases chloroplast number per cell does not seem to decline during senescence, i.e., the components of each chloroplast are progressively broken down, whereas some studies have recorded a decrease in the number of plastids per cell (reviewed in Krupinska 2007). Mitochondria are lost at a later stage, and mitochondrial functions (e.g., respiration) are maintained until quite late during senescence (Keech et al. 2007). To a large extent, the early and mid stages of

Abbreviations: GFP – Green fluorescent protein; Lhcb – Apoproteins of the light-harvesting complex associated to photosystem II; PCD – Programed cell death; PSI – Photosystem I; PSII – Photosystem II; RCBs – Rubisco-containing bodies; Rubisco – Ribulose-1,5-bisphosphate carboxylase/oxygenase; *SAGs* – Senescence associated genes; SAVs – Senescence-associated vacuoles senescence seem to represent mostly a chloroplast dismantling process, leading to the appearance of "gerontoplasts" (Krupinska 2007). The dismantling of the photosynthetic apparatus is a massive process involving the degradation of the most abundant proteins on Earth (e.g., Rubisco and the light-harvesting chlorophyll-protein complexes of photosystem II).

II. Degradation of Chloroplast Proteins During Senescence

Typically, degradation of the stromal proteins involved in the carbon reduction cycle starts early, and the loss of these proteins (e.g., Rubisco, glyceraldehyde 3-phosphate dehydrogenase and ribulose 5-phosphate kinase) proceeds roughly in parallel, at similar rates (Krupinska 2007). Also non-photosynthetic, stromal proteins, such as those involved in nitrogen and sulfur assimilation are degraded early during senescence (e.g., Schmutz et al. 1983; Kamachi et al. 1992). Thylakoid membrane proteins and chlorophylls may persist a bit longer after the onset of stromal protein degradation. Among the thylakoid complexes involved in photosynthetic electron transport and ATP synthesis, there is evidence that the cytochrome b_6/f complex is lost ahead of photosystems I and II, and of ATP synthase, therefore the connection between PSII and PSI becomes the limiting step in the photochemical reactions of photosynthesis (Holloway et al. 1983; Humbeck et al. 1996; Guiamet et al. 2002). Although in many cases PSII and PSI are broken down with a similar time-course, there are also cases where PSI is lost before PSII (e.g., Miersch et al. 2000; Tang et al. 2005). Regarding each complex, degradation of PSII core proteins may be a coordinated event, since many studies have found a relatively constant F_v/F_m ratio, i.e., the maximum quantum yield of PSII, in the early phases of senescence (Miersch et al. 2000; Lu et al. 2001; Guiamet et al. 2002). However, F_v/F_m decreases during senescence of Arabidopsis and barley (Miersch et al. 2000; Woo et al. 2001). In contrast, breakdown of PSII antenna proteins may be uncoupled from degradation of PSII reaction centers, particularly under conditions of limiting irradiance where light-harvesting protein degradation is delayed (Hidema et al. 1991, 1992). There is even evidence that changes in irradiance differentially affect the degradation of the inner (CP29, Lhcb4) versus the peripheral (LHCII) antenna proteins of PSII in senescing leaves of barley (Humbeck and Krupinska 2003).

The differential timing in the degradation of the various complexes that make up the photosynthetic apparatus suggests that different, relatively specific proteases, proteolytic mechanisms or specific processes tagging proteins for degradation, might operate to achieve chloroplast protein degradation. This might afford a significant degree of control and adjustment to environmental conditions (e.g., irradiance). Particularly, balancing thylakoid versus stromal protein degradation might help to prevent over-reduction of the photosynthetic electron transport chain, which might otherwise lead to the excessive production of reactive oxygen species. For example, it may be crucial to maintain a sizable pool of oxidized Q_A (the primary electron acceptor of PSII) to lower the probabilities of free radical formation, and this may be achieved by balancing the rates of degradation of light harvesting complexes in relation to consumption of reducing power. In fact, photochemical quenching estimations suggest that the pool of reduced Q_A remains fairly constant during leaf senescence in some species (Lu et al. 2001; Guiamet et al. 2002). Chloroplast breakdown must be tightly regulated to prevent runaway oxidative stress and premature cell damage before nutrient redistribution is complete.

III. Senescence-Associated Proteases

Except possibly for the case of the D1 protein of PSII, which is actively translated even at late stages of senescence (Droillard et al. 1992), the loss of photosynthetic proteins is preceded by a decrease in their transcript levels (Lim et al. 2007) and shutdown of their synthesis (Mae 2004). However, the decisive event leading to decreased steady-state levels of photosynthetic proteins is an increase in the rates of protein degradation (Lamattina et al. 1985; Mae 2004). This is supported by correlative evidence showing an increase in proteolytic activity during senescence (e.g., Peoples and Dalling 1988; Feller 2004) as well as by the observation that inhibition of protease activity by pharmacological treatments with protease inhibitors can reduce chloroplast protein degradation in vivo (Thoenen et al. 2007).

Studies in Arabidopsis, Populus, wheat and barley have identified cysteine-, serine-, aspartic- and metalloproteases whose transcript levels increase during senescence of leaves (Buchanan-Wollaston et al. 2003; Andersson et al. 2004; Gregersen and Holm 2007; Parrott et al. 2010). Among these, the cysteine proteases appear to be the group of proteases most frequently associated with senescence. Also components of the autophagic machinery are up-regulated during leaf senescence (Thompson et al. 2005; Wada et al. 2009). The list of senescence-associated proteases includes proteins with a clear-cut transit peptide for chloroplast targeting as well as proteases synthesized by the endoplasmatic reticulum and targeted to their destinations by the secretory pathway. Apart from correlative evidence linking the expression (mRNA) of these proteases to the progression of senescence, increases in protein levels in parallel with mRNA have been shown for only a few proteases (e.g., SAG2 and SAG12 of Arabidopsis, Grbic 2003). There are cases of non-parallel changes in mRNA and its corresponding protein, for example the mRNA levels of ERD1, a regulatory subunit of the Clp proteolytic complex, increase during senescence of Arabidopsis leaves (Nakashima et al. 1997), whereas the ERD1 protein content declines (Weaver et al. 1999). Direct evidence implicating specific proteases with degradation of chloroplast proteins, e.g., through the use of knock out or knock down lines, is lacking in most cases, except for a few proteases which have been studied in more detail (see below).

A. Evidence for the Involvement of Chloroplast Proteases

The involvement of plastid-localized proteases in chloroplast protein degradation was put forward a few decades ago (reviewed in Gepstein 1988). More recently, genomic approaches revealed a large number of proteases with plastid targeting peptides (Adam and Clarke 2002; Sakamoto 2006), and transcriptomic analysis have shown that a number of these chloroplast proteases, particularly proteases of the Clp, DegP and FtsH families, are upregulated during senescence in various species (Bhalerao et al. 2003; Buchanan-Wollaston et al. 2003; Andersson et al. 2004; Guo et al. 2004; Gregersen and Holm 2007).

While some members of the ClpP family (i.e., nclpP3 and nclpP5) are up-regulated during senescence (Nakabayashi et al. 1999), the expression of other Clp proteases is constitutive, with no changes during leaf ontogeny (e.g., Shanklin et al. 1995). Clp proteases may play such an important role early during chloroplast biogenesis that knock out lines for the ClpP subunit are not viable (Zheng et al. 2006), which complicates the use of reverse genetic approaches to understand their role during senescence.

Proteases of the FtsH and DegP families seem to play a role in the degradation of the D1 protein of photosystem II. Members of the FtsH family are thylakoid-bound, ATP-dependent metalloproteases (Adam and Clarke 2002), and some of them seem to perform an important function in chloroplast biogenesis, as implied from the variegated phenotype of knock out mutants for FtsH2 and FtsH5 (Zaltsman et al. 2005). Interestingly, these alterations of chloroplast biogenesis are exacerbated in FtsH2/FtsH8 and FtsH5/FtsH1 double mutants, suggesting that these proteases act redundantly (Zaltsman et al. 2005). FtsH2 and FtsH5 seem to be involved in the degradation of the D1 protein during the PSII repair cycle following photoinhibition (Zaltsman et al. 2005; Kato et al. 2009). The DegP family encompasses a number of serine-type, plastid proteases (Adam and Clarke 2002). Chloroplastic Deg proteases may cooperate with FtsHs in D1 degradation during photoinhibition (Kapri-Pardes et al. 2007; Sun et al. 2007, 2010). Although senescence-associated degradation of D1 has not been studied in detail, the breakdown of D1 associated with the repair cycle of photoinhibition is active enough (Aro et al. 1993; Guiamet et al. 2002), that there does not seem to be a need for a separate, senescence-specific pathway.

In vitro studies with thylakoids isolated from knock-out lines for the thylakoid-associated FtsH6 metalloprotease linked this protease to the degradation of the Lhcb antenna complexes of photosystem II (Zelisko et al. 2005). Thylakoids isolated from senescing leaves degraded Lhcb3 in vitro, and this degradation was inhibited by phosphoramidon (an inhibitor of metalloproteases) and EDTA. Addition of Zn^{2+} to EDTA-treated thylakoids restored the proteolytic activity against Lhcb3. Degradation of Lhcb1 and Lhcb3 was not affected in knock-out lines for FtsH5 or FtsH11, but degradation of Lhcb3 was slowed down in thylakoids of an FtsH6 knock out line (Zelisko et al. 2005). This is consistent with previous observations showing that senescence-associated degradation of Lhcb3 in isolated thylakoids is carried out by a zinc-binding metalloprotease (Zelisko and Jackowski 2004). However, in vivo studies with an FtsH6 knock out line failed to confirm the involvement of this protease in Lhcb degradation during senescence or high-irradiance acclimation (Wagner et al. 2011).

There is no clear-cut evidence for senescence-associated breakdown of Rubisco within chloroplasts. In isolated chloroplasts, oxidative stress (e.g., high irradiance, high O_2 concentrations, addition of paraquat, etc.) leads to the formation of insoluble Rubisco aggregates, which are degraded (Desimone et al. 1996). Likewise, isolated chloroplasts can degrade Rubisco and other stromal proteins in the presence of light (Roulin and Feller 1998), even if irradiance is moderate to low, and, therefore, probably not relevant during oxidative stress. However, light, which accelerates Rubisco degradation in in vitro experiments, most often delays, rather than accelerates chloroplast protein degradation in vivo (Noodén and Schneider 2004). In darkness, isolated chloroplasts break down Rubisco at very low rates (Roulin and Feller 1998). In some cases, incubation of isolated chloroplasts yields Rubisco fragments, but degradation does not proceed beyond this initial cleavage (Kokubun et al. 2002). Autodigestion experiments with isolated chloroplasts do not provide clear and strong evidence for Rubisco breakdown within plastids of senescing leaves.

The involvement of the plastidic CND41 aspartic protease in Rubisco degradation was put forward based on phenotypic analysis of antisense lines (Kato et al. 2004, 2005). CND41 degrades partially denatured Rubisco in vitro (Kato et al. 2004), and the loss of Rubisco is retarded in antisense lines (Kato et al. 2005). However, CND41 is also a DNA-binding protein, and it might affect Rubisco degradation indirectly. For example, bioactive gibberellin levels are lower in CND41 antisense lines, therefore their growth is stunted (Nakano et al. 2003). Since leaf senescence during vegetative growth is controlled by correlative effects from the younger parts of the plant (i.e., the developing shoot apex, Noodén et al. 2004), delayed senescence and Rubisco degradation in CND41 antisense plants might be just an indirect effect of dwarfism in these plants (Martínez et al. 2008b). The direct involvement of CND41 in chloroplast protein degradation might be tested in plants where the dwarf phenotype is corrected through exogenous applications of gibberellin.

In brief, there is evidence for degradation of photosystem II proteins within the chloroplast itself, whereas the involvement of plastid proteases in the breakdown of Rubisco and other stromal proteins is not as conclusive. This is consistent with the observation that stay-green mutants of soybean, Festuca and rice specifically fail to degrade Lhcb proteins while breakdown of other chloroplast proteins proceeds normally (Thomas and Howarth 2000; Guiamet et al. 2002; Kusaba et al. 2007), which points to the operation of specific pathways for degradation of PSII light-harvesting proteins and Rubisco, respectively.

B. Chloroplast Protein Degradation in the Central Vacuole

Proteolysis within the central vacuole seems a logical alternative to degradation within plastids. Most of the acidic protease activity of a higher plant cell resides in the central vacuole (Matile 1997; De 2000), which is also the location of several senescence-associated cysteine-type proteases, e.g., SENU3 and SENU4 of tomato (Drake et al. 1996) and AtALEU/SAG2 of Arabidopsis (Hensel et al. 1993). The activity of four cysteine proteases localized to the central vacuole increased during senescence induced by incubation in darkness, water deficit, or during normal monocarpic senescence associated with reproduction in wheat (Martínez et al. 2007). Moreover, vacuolar lysates can degrade Rubisco in vitro (Yoshida and Minimikawa 1996; Srivalli et al. 2001). However, the spatial separation between chloroplast proteins and vacuolar proteases is maintained during senescence (Evans et al. 2010), which at first glance would rule out an important role of the vacuole in photosynthetic protein degradation. Few papers have, however, provided evidence for engulfment of entire plastids within the central vacuole (e.g., Minimikawa et al. 2001; Niwa et al. 2004; see also Krupinska and Mulisch, Chap. 14).

Chloroplast-derived vesicular structures have been implicated in Rubisco export out of chloroplasts as part of an extra-plastidial Rubisco degradation pathway (Chiba et al. 2003; Ishida et al. 2008; Wada et al. 2009; see also Wada and Ishida, Chap. 19). These Rubisco Containing Bodies (RCBs) are spherical vesicles (0.4–1.2 μm in diameter) bound by a double membrane. They contain Rubisco and glutamine synthetase II, but no thylakoid proteins (Chiba et al. 2003). The number of RBCs per cell increased by about threefold concomitantly with the start of Rubisco loss in the course of natural senescence of the first leaf of wheat (Chiba et al. 2003), suggesting that they are implicated in the degradation of the stromal proteins of the chloroplast. In Arabidopsis leaves expressing GFP fused to a chloroplast targeting peptide, GFP-containing vesicles are apparent in leaves treated with concanamycin A, an inhibitor of H^+-ATPases that causes an increase in vacuolar pH and, thereby, decreases vacuolar proteolytic activity (Ishida et al. 2008). Some GFP-vesicles (RCBs) were clearly located in the central vacuole, which points to a transport system between the chloroplast and the central vacuole mediated by RCBs (Ishida et al. 2008). Development of RCBs and their internalization into the central vacuole seemed to involve the autophagic pathway, since the number of RCBs decreased markedly in the cytoplasm and the central vacuole of concanamycin A-treated cells of autophagy mutants, i.e., knock out lines for *atg5* or *atg4a-4b* (Ishida et al. 2008; Wada et al. 2009; see also Ishida, Chap. 19). These observations point to the operation of an autophagic pathway in which RCBs carrying stromal proteins are internalized into the central vacuole, where chloroplast proteins may be broken down by vacuolar proteases. This predicts that inactivation of the autophagic machinery should slow down or inhibit chloroplast protein degradation. However, the loss of chloroplast proteins, including Rubisco, is significantly accelerated in autophagy mutants (Doelling et al. 2002; Thompson et al. 2005), which implies that an alternative, redundant pathway may operate to bring about extraplastidic chloroplast protein degradation in an autophagy-independent way.

IV. Senescence-Associated Vacuoles (SAVs)

A. Characterization of SAVs

Typically, mature mesophyll cells contain a single, large central vacuole which occupies most of the cell volume and performs a number of important functions, including osmoregulation, hydrolytic activities, sugar and amino acid storage, disposal of xenobiotics, etc. (De 2000). In special cases, mesophyll cells may have two types of vacuoles, as is the case for the salt-tolerant plant *Mesembryantemum crystallinum* where one vacuole sequesters NaCl as a mechanism to avoid Na^+ toxicity in the cytosol whereas a second vacuole accumulates malate at night during operation of CAM photosynthesis (Epimashko et al. 2004). Thus, there may be more than one type of vacuole in mesophyll cells.

During senescence of several dicots, e.g., Arabidopsis, soybeans and tobacco, "*S*enescence-*A*ssociated *V*acuoles" (SAVs) accumulate in chloroplast containing cells, i.e., mesophyll and guard cells (Otegui et al. 2005). SAVs are small (0.5–0.8 μm in diameter), and bound by a single membrane which contains at least one marker (i.e., vacuolar H^+ pyrophosphatase) in common with the central vacuole (Fig. 18.1). However, SAVs differ from the central vacuole in a number of features: SAVs are smaller and they lack

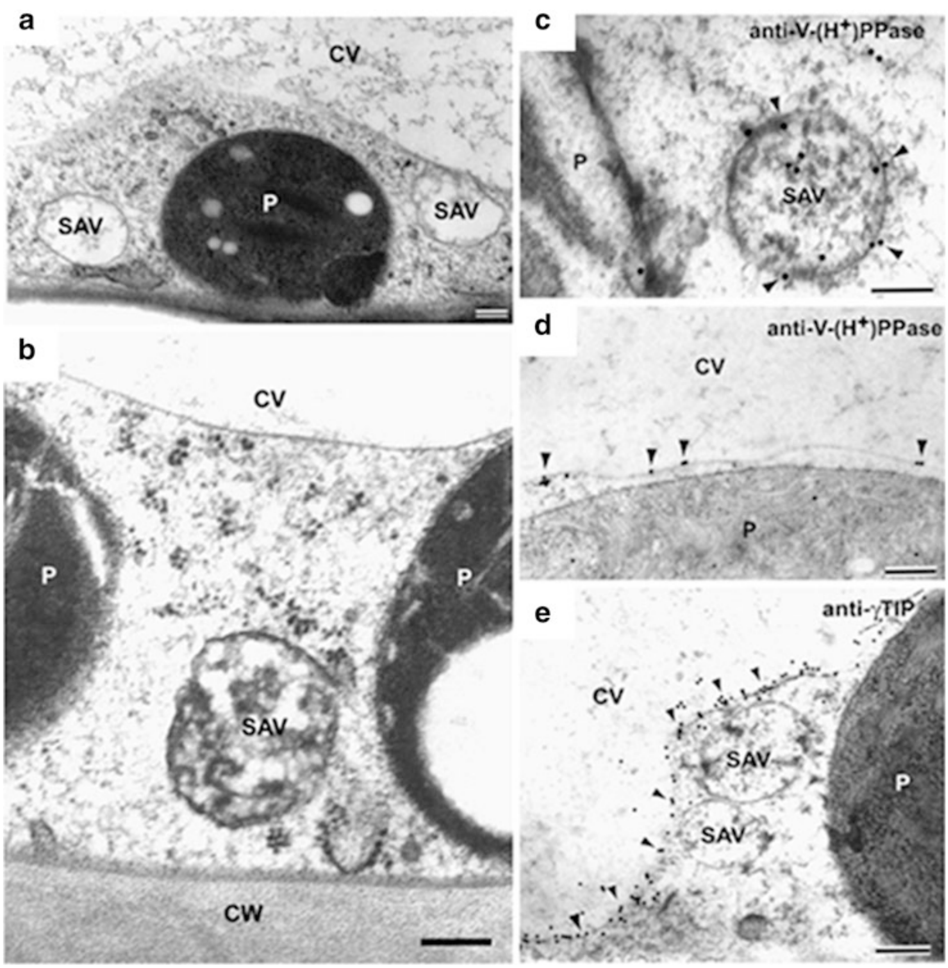

Fig. 18.1. Senescence-associated vacuoles (SAVs) of Arabidopsis and soybean observed by electron microscopy of high-pressure frozen/freeze-substituted samples. (**a**) SAVs develop in the peripheral cytoplasm surrounding the central vacuole of a senescing leaf cell of Arabidopsis. (**b**) SAV with dense contents in the peripheral cytoplasm of a senescing soybean cell. (**c**–**e**) Immunolocalization of vacuolar markers in senescing leaf cells of Arabidopsis. (**c**) and (**d**) Immunolocalization of the vacuolar H^+- pyrophosphatase, note labeling of the limiting membrane of SAVs (**c**) and the central vacuole tonoplast (**d**). (**e**) immunolabeling of γ-TIP, the tonoplast of the central vacuole is densely labeled, whereas the membrane of SAVs is not. *CV* central vacuole, *CW* cell wall, *P* plastid. *Scale bars* = 200 nm (**a** and **b**), 250 nm (**c**, **d**, and **e**). (Reprinted from Otegui et al. (2005).).

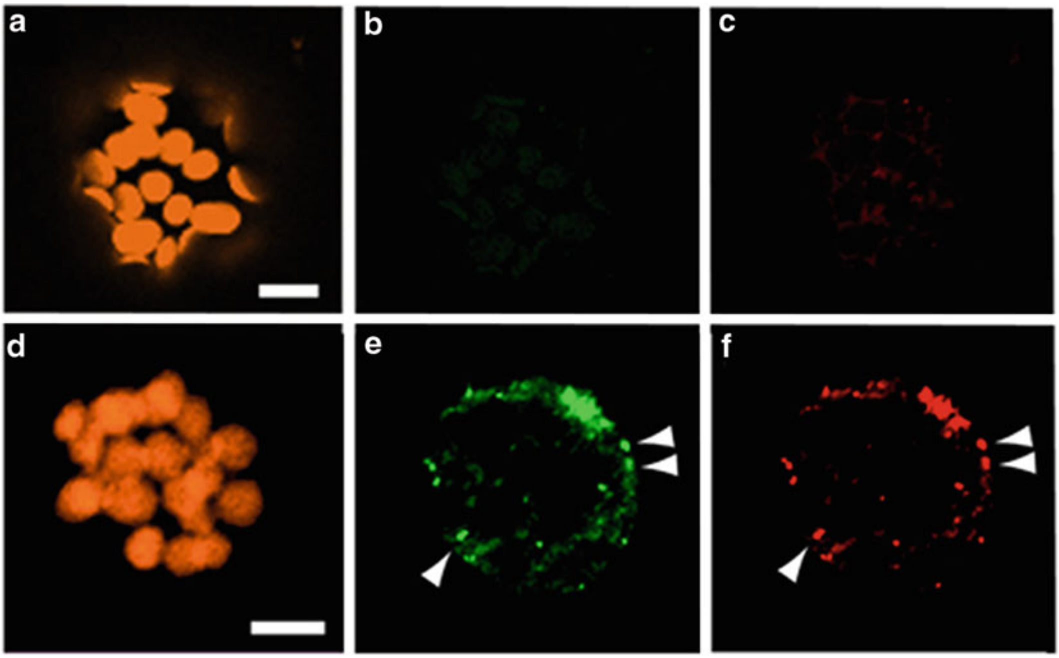

Fig. 18.2. Detection of cysteine-type protease activity in senescence-associated vacuoles. (**a** through **f**), protoplasts isolated from non-senescent (**a**, **b** and **c**) and senescent (**d**, **e** and **f**) leaves of Arabidopsis were incubated with Lysotracker Red (**c** and **f**) to label SAVs and the cysteine protease substrate R6502 (**b** and **e**). (**a** and **d**) show chlorophyll autofluorescence. Note the absence of Lysotracker-stained structures in non-senescent leaves, and the localization of R6502 within Lysotracker-stained SAVs in senescent leaves (*arrowheads* show a few representative SAVs). *Scale bar* = 10 µm. (Reprinted from Otegui et al. (2005).).

the vacuolar water channel γ-TIP (Otegui et al. 2005; Fig. 18.1). Besides, SAV are about 0.8 pH units more acidic than the central vacuole (Otegui et al. 2005), and this may be very significant in view of the fact that pH changes of this magnitude can exert a large influence on protein degradation within vacuoles (e.g., Hwang et al. 2003).

SAVs represent a novel class of lytic vacuoles, characterized by high peptidase activity. Confocal microscopy employing a fluorogenic peptidase substrate with affinity towards cysteine-type proteases showed that the highest peptidase activity of senescing mesophyll cells is located within SAVs (Otegui et al. 2005; Fig. 18.2). Although the interpretation of activity staining experiments in vivo may be complicated due to differences in surface-to-volume ratios of organelles with such contrasting sizes as SAVs and the central vacuole, subcellular fractionation studies confirmed these observations. Activity labeling with the biotinylated cysteine protease probe DCG-04 (Greenbaum et al. 2000; van der Hoorn et al. 2004) and detection in Western blots showed that labeled (and therefore active) cysteine proteases are mainly located in SAVs, compared to a crude leaf extract, or to chloroplasts isolated from senescing leaves (Fig. 18.3). Irrespective of the method employed for this analysis, it is quite clear that cysteine protease activity is enriched in SAVs, which is significant in view of the close association of cysteine proteases with the execution of the senescence program. SAG12, a strictly senescence-associated cysteine protease (Lohman et al. 1994), and a widely used marker for progression of senescence, also localized to SAVs (Otegui et al. 2005). Thus, we are tempted to conclude that SAVs represent the most active proteolytic compartment in senescing leaf cells.

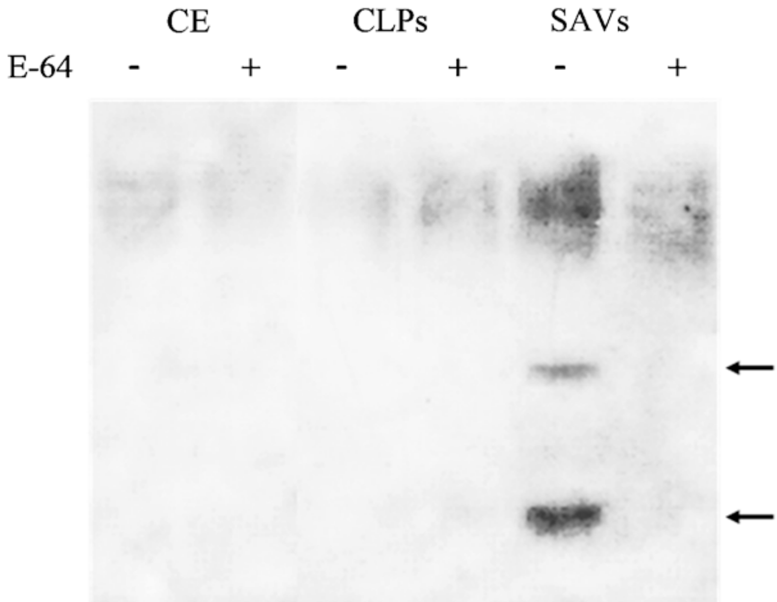

Fig. 18.3. Western blot analysis of cysteine-type proteases labeled with the biotinylated inhibitor DCG-04 and detected with a streptavidin-peroxidase conjugate. Equal amounts of protein (3 μg) from a crude extract (CE), purified chloroplasts (CLPs) and isolated SAVs were loaded in each lane. To control for non-specific binding of DCG-04 to proteins other than cysteine proteases, aliquots of the samples were incubated with the irreversible inhibitor E-64 prior to labeling with DCG-04 (Greenbaum et al. 2000; van der Hoorn et al. 2004). *Arrows* show two cysteine-type proteases of around 40 and 33 kDa apparent molecular mass which are detected only in SAVs; specific binding is confirmed by inhibition of labeling with E-64.

B. SAVs and Chloroplast Protein Degradation

The facts that SAVs develop in chloroplast containing cells during senescence, and that they have most of the cysteine protease activity of the cell suggest that SAVs might play a role in the breakdown of chloroplast proteins during senescence. The involvement of SAVs in breakdown of photosynthesis proteins would require re-location of chloroplast proteins to SAVs. To determine if a chloroplast protein may be re-located to SAVs during senescence, Martínez et al. (2008a) employed a transgenic tobacco line expressing GFP targeted to the plastid (Köhler et al. 1997). In mesophyll cells of mature, non-senescing leaves, GFP localized exclusively to chloroplasts (Martínez et al. 2008a). In contrast, in senescing leaves, a strong GFP signal was also detected in SAVs, indicating re-location of a chloroplast protein to SAVs (Fig. 18.4). Isolation and biochemical analysis of SAVs confirmed the presence of proteins from the chloroplast stroma (Rubisco and glutamine synthetase II, Martínez et al. 2008a), and photosystem I (Gomez et al. unpublished). Although chloroplasts can break down the large subunit of Rubisco (RbcL) to a 44 kDa fragment in vitro (Kokubun et al. 2002), normal sized RbcL was found in SAVs, which seems to rule out the possibility of an initial, partial proteolytic attack in plastids before transfer to SAVs (Martínez et al. 2008a). No PSII proteins were detected in SAVs, neither by immunological methods, modulated fluorescence measurements nor 77 K spectrofluorometry (Martínez et al. 2008a, Gomez et al. unpublished). These findings show that some chloroplast proteins are transported to SAVs, and hint at the operation of a novel sorting and trafficking pathway between chloroplasts and SAVs.

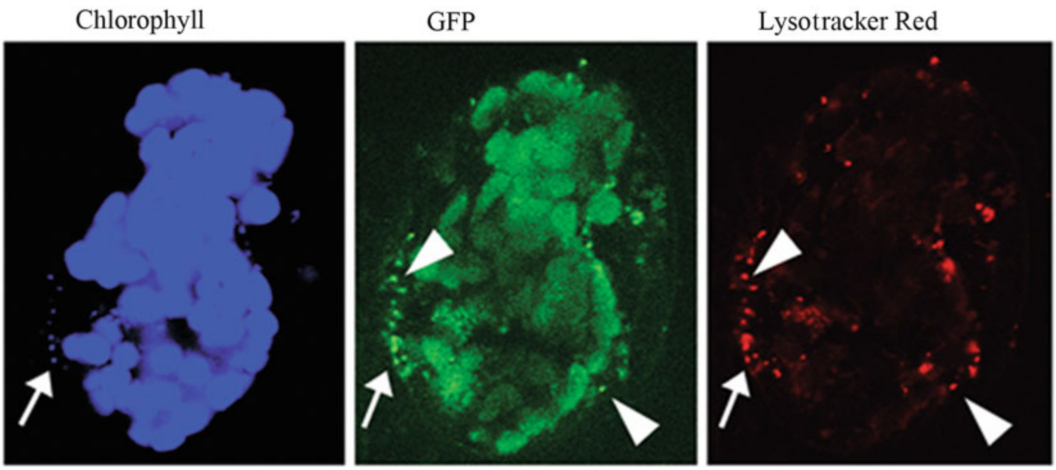

Fig. 18.4. Confocal section through a cell isolated from a senescing tobacco leaf expressing GFP targeted to the plastids and incubated with Lysotracker Red to detect SAVs. The punctuate pattern of GFP signal outside chloroplasts localizes to SAVs labeled with Lysotracker *Red* (*arrowheads* show a GFP-containing SAV). Less frequently, a fluorescence signal with the spectral properties of chlorophyll is also detected in SAVs (the *arrows* show SAVs with GFP and chlorophyll fluorescence). (Reprinted from Martínez et al. (2008a).).

Given their high peptidase activity, it is reasonable to presume that SAVs are not only a transport vesicle for chloroplast proteins. A role for SAVs in chloroplast protein breakdown seems likely, and this is supported by autodigestion experiments, where the chloroplast proteins contained in isolated SAVs were degraded by SAV-proteases (Martínez et al. 2008a; Gomez et al. unpublished). Though this supports the breakdown of chloroplast proteins in SAVs, *in planta* experiments (e.g., with knock out lines for SAV proteases) are required to confirm this issue.

C. Comparison with Other Lytic Compartments of Plant Cells

A number of small lytic compartments, different from the central vacuole, have been described in many tissues (for a review, see Müntz 2007). Of particular interest here are lytic compartments associated with senescence or cell death processes.

Aleurone cells of barley seeds develop small, acidic and proteolytically active secondary vacuoles after seed germination (Swanson et al. 1998). These secondary vacuoles contain cysteine protease activity, which might correspond, in part, to the papain-type protease aleurain. Gibberellic acid increases acidity of secondary vacuoles, whereas treatment with abscisic acid increases their lumenal pH (Swanson et al. 1998), which might affect the proteolytic activity of these vacuoles. Since gibberellins accelerate, while abscisic acid delays programmed cell death in aleurone cells, these observations seem to implicate secondary vacuoles in the execution of aleurone PCD.

Ricinosomes are small lytic vesicles in senescing endosperm cells of *Ricinus comunis* (Schmid et al. 1998, 1999; Gietl and Schmid 2001). Ricinosomes bud off the endoplasmic reticulum carrying the inactive 45 kDa precursor of the castor-bean cysteine endopeptidase Cys-EP, which carries a C-terminal KDEL motif for ER retention. The proCys-EP matures to the 35 kDa active form upon acidification of ricinosomes (Schmid et al. 2001). The number of ricinosomes per cell increases as nuclear DNA fragmentation (a marker for PCD) progresses in endosperm cells after germination (Schmid et al. 1999). At a late stage of PCD ricinosomes rupture releasing the active Cys-EP, which is then apparently involved in the final clearing of the dead cell body. Similar organelles are found in senescing petals of *Hemerocallis* sp. (Schmid et al. 1999) and sporophytic tissues of tomato anthers (Senatore et al. 2009). KDEL-tailed cysteine proteases have been found in a number of species, but they do not seem to be expressed in senescing leaves (Gietl and Schmid 2001). SAV proteins already identified as transported through the endomembrane system (i.e., the cysteine-protease SAG12 and V-H^+ pyrophosphatase) lack an ER-retention motif, which is an important difference between SAVs and ricinosomes. Moreover, ricinosomes seem to act at a very late stage in programmed cell death, whereas SAVs appear early (Otegui et al. 2005), when leaf senescence is still a reversible process (Zavaleta-Mancera et al. 1999).

D. SAVs and Rubisco Containing Bodies

There are a number of similarities between SAVs and RCBs which might suggest that these are the same or closely related structures, with a similar function. Both, SAVs and RCBs develop during senescence of leaves, and both contain Rubisco, glutamine synthetase, and chloroplast-targeted GFP (Chiba et al. 2003; Otegui et al. 2005; Martínez et al. 2008a). However, RCBs do not carry chlorophyll, and are limited by a double membrane (Chiba et al. 2003), whereas SAVs have a single limiting membrane (Otegui et al. 2005) and carry chlorophyll (Martínez et al. 2008a). SAVs and RCBs accumulate in senescing leaves, therefore both might be present simultaneously in the same cells during senescence. However, in tobacco leaves senescing under continuous darkness, there are SAVs containing an acidic interior and chloroplast-targeted GFP, but apparently no RCBs (i.e., vesicles containing chloroplast-targeted GFP but not an acidic lumen) (Martínez, Costa, Guiamet, unpublished observations), which seems to rule out the simultaneous occurrence of both organelles in the same cells. A thorough examination of the presence of SAVs and RCBs in the same tissues under diverse developmental and environmental scenarios, or in response to specific hormones is needed, but this will require the discovery of specific molecular markers for RCBs in conjunction with the use of SAG12 as a marker for SAVs.

The development of SAVs does not seem to depend on the autophagic machinery, as SAV development is apparently normal in an *atg7* knock-out line of Arabidopsis (Otegui et al. 2005), where autophagy is completely disrupted (Doelling et al. 2002). In contrast, formation of RCBs seems to be impaired in an autophagy mutant (Wada et al. 2009; Wada and Ishida, Chap. 19). Equally important, active proteolysis takes place inside SAVs but so far no proteolytic activity has been described for RCBs. In this regard, SAVs are clearly lytic compartments whereas RCBs might represent a “shuttle” for chloroplast proteins on their way to the central vacuole. Thus, the present evidence suggests that RCBs and SAVs are part of two different mechanisms for chloroplast protein degradation during senescence. The observation that chloroplast dismantling and breakdown of their components is accelerated, rather than delayed, in autophagy mutants (e.g., Doelling et al. 2002; Hanaoka et al. 2002; Thompson et al. 2005; Xiong et al. 2005; Wada et al. 2009), where formation of RCBs is impaired (Wada et al. 2009), reinforces the idea that an active chloroplast protein degradation machinery, probably involving SAVs, operates independently of autophagy and RCBs.

V. Conclusions and Prospects

The evidence collected so far suggests that SAVs are a lytic compartment with high peptidase activity, where stromal proteins and PSI proteins of the chloroplast are re-located during senescence, setting the stage for their degradation (Fig. 18.5). A number of important questions remain to be addressed, including the origin of SAVs, how stromal and PSI proteins are sorted out from other chloroplast components for transport to SAVs, the transport mechanism itself, and the final fate of SAVs. The origin of SAVs may involve

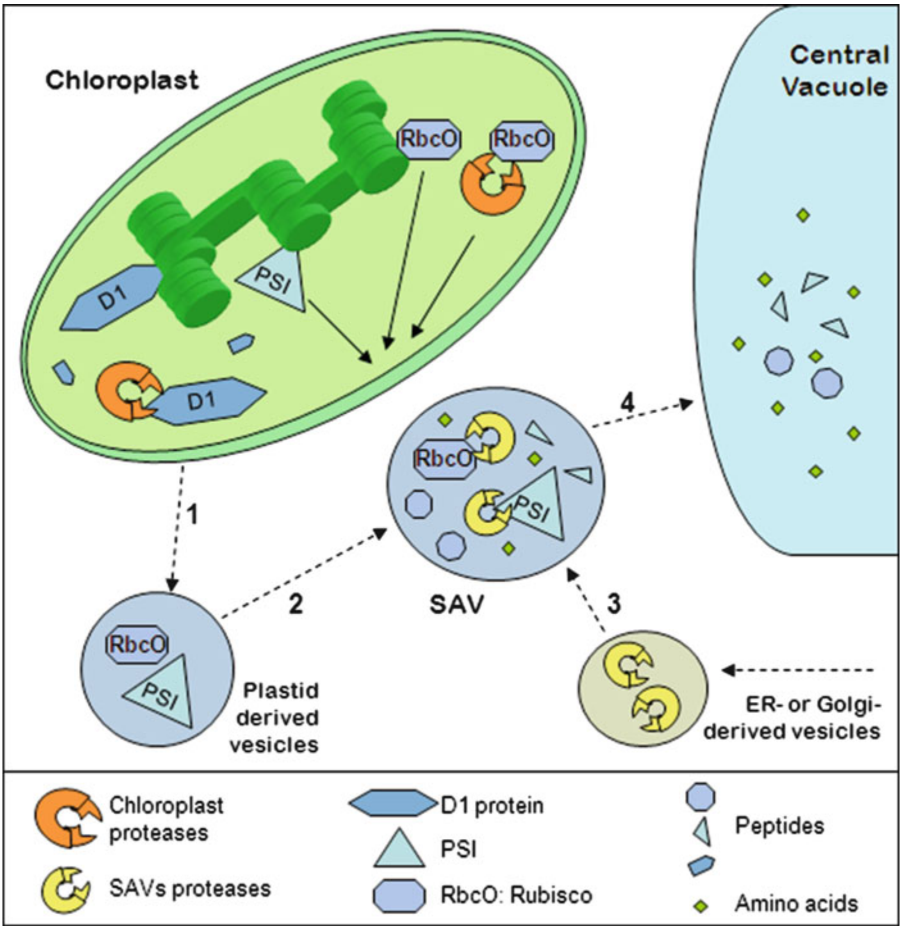

Fig. 18.5. A schematic view of the hypothetical involvement of senescence-associated vacuoles in the degradation of the most abundant chloroplast proteins during senescence of leaves. In this model, D1 (and possibly PSII and other chl-binding proteins) is broken down within the plastid by chloroplastic proteases (shown in *orange*). Rubisco (RbCO) and other stromal proteins are re-located from the plastid to SAVs (*1, 2*) and broken down there by non-chloroplastic proteases (shown in *yellow*). Probably whole PSI complexes are also re-located to SAVs for degradation. Fusion of SAVs with the central vacuole (4) might deliver amino acids and peptides (or even protein fragments which might be degraded to completion), and amino acids may be stored in the central vacuole until they are redistributed to other parts of the plant. The origin of SAVs possibly involving the fusion of plastid-derived (2) and ER- or Golgi-derived (3) vesicles, the mechanism sorting and transporting chloroplast proteins into SAVs and their final destination are areas that need further research. Modified from Martínez et al. (2008b).

events taking place in the chloroplast and in the secretory pathway, since some components of SAVs (e.g., SAG12) have a signal peptide for translocation into the endoplasmic reticulum (Grbic 2003), whereas other components of SAVs have a clear plastidic origin (e.g., chloroplast proteins). Plastids are fairly dynamic entities, with stromal protrusions (stromules) constantly extending out of, and retracting on the main plastid body (Gunning 2005; Hanson and Satterzadeh 2011). Stromule tips often enlarge, and cases where stromule tips were shed off have been recorded (Gunning 2005). Plastid protuberances, much shorter than stromules, are also common (Hanson and Satterzadeh 2011). Mass export of chloroplast components in the form of vesicles formed through scission of chloroplast protuberances or shedding of stromule tips might supply the chloroplast molecules found in SAVs. However, stromules are found more frequently in non-green plastids than in chloroplasts (Hanson and Satterzadeh 2011; see also Gray, Chap. 9), which seems to rule out the involvement of stromules in the biogenesis of SAVs. In any event, these observations highlight the dynamic nature of the chloroplast and its envelope, suggesting that similar changes might result in the formation of vesicles containing chloroplast components. Chloroplast-derived vesicles might later fuse with vesicles coming from the ER and Golgi apparatus carrying, e.g., proteases, hydrolytic enzymes, etc. (Fig. 18.5). We surmise that SAVs may eventually fuse with the tonoplast and discharge their contents in the central vacuole, which might be a site for transient storage of amino acids released from protein degradation. In this regard, it is interesting that the vacuolar concentration of amino acids increases during senescence (Matile 1997), possibly placing the central vacuole as the destination of SAV contents. A thorough analysis of the protein composition of SAVs employing mass spectrometry approaches combined with reverse genetic approaches may help to unravel some of these questions in the future.

Different mechanisms may be involved in chloroplast protein degradation (Hörtensteiner and Feller 2002), and these include (1) degradation by chloroplast-targeted, nuclear-encoded hydrolases, (2) export of chloroplast components to lytic vacuoles or (3) re-location of vacuolar hydrolases to the chloroplast. There is evidence for the operation of the first two mechanisms, whereas, as far as we know, there is not much evidence for re-direction of vacuolar proteases to chloroplasts. Collectively, the evidence may be accommodated into a scheme where PSII components are degraded within the plastid, while stromal and PSI proteins are degraded in SAVs, at least partially, and the resulting peptides and/or amino acids transported to the central vacuole (Fig. 18.5). This does not rule out other mechanisms, e.g., autophagy-mediated degradation of RCBs, which might operate independently of SAVs. Separate, independently regulated plastidic and extra-plastidic pathways for the degradation of different chloroplast proteins might endow senescing cells with a high degree of flexibility, for example to adjust light harvesting and carboxylation capacity to the environmental conditions (e.g., senescence under shade vs. sunlight), which may be quite important during the initial stages of senescence when residual photosynthesis may supply much of the energy consumed by the degradation and export machinery.

Acknowledgments

Work in the authors' laboratory has been supported by Agencia Nacional de Promoción Científica y Tecnológica (PICT 11885 and PICT 0784), Consejo Nacional de Investigaciones Científicas y Técnicas, and Universidad Nacional de La Plata (Argentina) and the DAAD (Deutscher Akademischer Austausch Dienst, Germany) – Ministerio de Ciencia, Tecnología e Innovación Productiva (Argentina) collaborative program. MLC and DEM are researchers, and CC and FMG hold fellowships, of Consejo Nacional de Investigaciones Científicas y Técnicas, Argentina. JJG is a researcher of Comisión de Investigaciones Científicas de la Provincia de Buenos Aires

References

Adam Z, Clarke AK (2002) Cutting edge of chloroplast proteolysis. Trends Plant Sci 7:451–456

Andersson A, Keskistalo J, Sjödin A et al (2004) A transcriptional timetable of autumn senescence. Genome Biol 5:R24

Aro E-M, Virgin I, Andersson B (1993) Photoinhibition of photosynthesis: inactivation, protein damage and turnover. Biochim Biophys Acta 1143:113–134

Bhalerao R, Keskitalo J, Sterky F, Erlandsson R, Björkbacka H, Jonsson Birve S, Karlsson J, Gardeström P, Gustafsson P, Lundeberg J, Jansson S (2003) Gene expression in autumn leaves. Plant Physiol 131:430–442

Buchanan-Wollaston V, Earl S, Harrison E, Mathas E, Navadpour S, Page T, Pink D (2003) The molecular analysis of senescence – a genomics approach. Plant Biotechnol J 1:3–22

Chiba A, Ishida H, Nishizawa NK, Makino A, Mae T (2003) Exclusion of ribulose-1,5-bisphosphate carboxylase/oxygenase from chloroplasts by specific bodies in naturally senescing leaves of wheat. Plant Cell Physiol 44:914–921

De DN (2000) Plant cell vacuoles. An introduction. CSIRO, Collingwood, 288pp

Desimone M, Henke A, Wagner E (1996) Oxidative stress induces partial degradation of the large subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase in isolated chloroplasts of barley. Plant Physiol 111:789–796

Ding L, Wang KJ, Jiang JM, Liu MZ, Niu SL, Gao LM (2005) Post-anthesis changes in photosynthetic traits of maize hybrids released in different years. Field Crops Res 93:108–115

Doelling JH, Walker JM, Friedman EM, Thompson AR, Vierstra RD (2002) The APG8/12-activating enzyme APG7 is required for proper nutrient recycling and senescence in *Arabidopsis thaliana*. J Biol Chem 277:33105–33114

Drake R, John I, Farrell A, Cooper W, Schuch W, Grierson D (1996) Isolation and analysis of cDNAs encoding tomato cysteine proteases expressed during leaf senescence. Plant Mol Biol 30:755–767

Droillard MJ, Bates NJ, Rothstein SJ, Thompson JE (1992) Active translation of the D1 protein of photosystem II in senescing leaves. Plant Physiol 99:589–594

Epimashko S, Meckel T, Fischer-Schliebs E, Lüttge U, Thiel G (2004) Two functional different vacuoles for static and dynamic purposes in one plant mesophyll leaf cell. Plant J 37:294–300

Evans M, Rus AM, Belanger EM, Kimoto M, Brusslan JA (2010) Dismantling of *Arabidopsis thaliana* mesophyll cell chloroplasts during natural leaf senescence. Plant Biol 12:1–12

Feller U (2004) Proteolysis. In: Noodén LD (ed) Plant cell death processes. Academic, San Diego, pp 107–123

Gepstein S (1988) Photosynthesis. In: Noodén LD, Leopold AC (eds) Senescence and aging in plants. Academic, San Diego, pp 85–109

Gietl C, Schmid M (2001) Ricinosomes: an organelle for developmentally regulated programmed cell death in senescing plant tissues. Naturwissenschaften 88:49–58

Giunta F, Motzo R, Pruneddu G (2008) Has long-term selection for yield in durum wheat also induced changes in leaf and canopy traits? Field Crops Res 106:68–76

Grbic V (2003) SAG2 and SAG12 protein expression in senescing Arabidopsis plants. Physiol Plant 119:1–7

Greenbaum D, Medzihradsky K, Burlingame A, Bogyo M (2000) Epoxide electrophiles as activity-dependent cysteine protease profiling and discovery tools. Chem Biol 7:569–581

Gregersen PL, Holm PB (2007) Transcriptome analysis of senescence in the flag leaf of wheat (*Triticum aestivum* L.). Plant Biotechnol J 5:192–206

Gregersen PL, Holm PB, Krupinska K (2008) Leaf senescence and nutrient remobilization in barley and wheat. Plant Biol 10:37–49

Guiamet JJ, Tyystjärvi E, Tyystjärvi T, John I, Kairavuo M, Pichersky P, Noodén LD (2002) Photoinhibition and loss of photosystem II reaction center proteins during senescence of soybean leaves. Enhancement of photoinhibition by the "stay-green" mutation *cytG*. Physiol Plant 115:468–478

Gunning BES (2005) Plastid stromules: video microscopy of their outgrowth, retraction, tensioning, anchoring, branching, bridging and tip shedding. Protoplasma 225:33–42

Guo Y, Cai Z, Gan S (2004) Transcriptome of Arabidopsis leaf senescence. Plant Cell Environ 27:521–549

Hanaoka H, Noda T, Shirano Y, Kato T, Hayashi H, Shibata D, Tabata S, Ohsumi Y (2002) Leaf senescence and starvation-induced chlorosis are accelerated by the disruption of an Arabidopsis autophagy gene. Plant Physiol 129:1181–1193

Hanson MR, Satterzadeh A (2011) Stromules: recent insights into a long neglected feature of plastid morphology and function. Plant Physiol 155:1486–1492

Hensel LL, Grbic V, Baumgarten DA, Bleecker AB (1993) Developmental and age-related processes that influence the longevity and senescence of photosynthetic tissues in Arabidopsis. Plant Cell 5:553–564

Hidema J, Makino A, Mae T, Ojima K (1991) Photosynthetic characteristics of rice leaves aged under different irradiances from full expansion through senescence. Plant Physiol 97:1287–1293

Hidema J, Makino A, Kurita Y, Mae T, Ojima K (1992) Changes in the levels of chlorophyll and light-harvesting chlorophyll *a/b* protein of PSII in rice leaves aged under different irradiances from full expansion through senescence. Plant Cell Physiol 33:1209–1214

Holloway PJ, Maclean DJ, Scott KJ (1983) Rate-limiting steps of electron transport in chloroplasts during ontogeny and senescence of barley. Plant Physiol 72:795–801

Hörtensteiner S, Feller U (2002) Nitrogen metabolism and remobilization during senescence. J Exp Bot 53:927–937

Humbeck K, Krupinska K (2003) The abundance of minor chlorophyll a/b binding proteins CP29 and LHCI of barley (*Hordeum vulgare* L.) during leaf senescence is controlled by light. J Exp Bot 54:375–383

Humbeck K, Quast S, Krupinska K (1996) Functional and molecular changes in the photosynthetic apparatus during senescence of flag leaves from field-grown barley plants. Plant Cell Environ 19:337–344

Hwang Y, Bethke PC, Gubler F, Jones RL (2003) cPrG-HCl a potential H+/Cl− symporter prevents acidification of storage vacuoles in aleurone cells and inhibits GA-dependent hydrolysis of storage proteins and phytate. Plant J 35:154–163

Ishida H, Yoshimoto K, Izumi M, Reisen D, Yano Y, Makino A, Ohsumi Y, Hanson MR, Mae T (2008) Mobilization of Rubisco and stroma-localized fluorescent proteins of chloroplast to the vacuole by an ATG gene-dependent autophagic process. Plant Physiol 148:142–155

Kamachi K, Yamaya T, Hayakawa T, Mae T, Ojima K (1992) Changes in cytosolic glutamine synthetase polypeptide and its mRNA in a leaf blade of rice plants during natural senescence. Plant Physiol 98:1323–1329

Kapri-Pardes E, Naveh L, Adam Z (2007) The thylakoid lumen protease DegP1 is involved in the repair of photosystem II from photoinhibition in Arabidopsis. Plant Cell 19:1039–1047

Kato Y, Murakami S, Yamamoto Y, Chatani H, Kondo Y, Nakano T, Yokota A, Sato F (2004) The DNA-binding protease, CND41, and the degradation of ribulose-1,5-bisphosphate carboxylase/oxygenase in senescent leaves of tobacco. Planta 220:97–104

Kato Y, Yamamoto Y, Murakami S, Sato F (2005) Post-translational regulation of CND41 protease activity in senescent tobacco leaves. Planta 222:643–651

Kato Y, Miura E, Ido K, Ifuku K, Sakamoto W (2009) The variegated mutants lacking chloroplastic FtsHs are defective in D1 degradation and accumulate reactive oxygen species. Plant Physiol 151:1790–1801

Keech O, Pesquet E, Ahad A, Askne A, Nordrvall D, Vodnala SM, Tuominen H, Hurry V, Dizengremel P, Gardeström P (2007) The different fates of mitochondria and chloroplasts during dark-induced senescence in Arabidopsis leaves. Plant Cell Environ 30:1523–1534

Killingbeck KT (2004) Nutrient resorption. In: Noodén LD (ed) Plant cell death processes. Academic, San Diego, pp 215–226

Köhler RH, Cao J, Zipfel WR, Webb WW, Hanson MR (1997) Exchange of protein molecules through connections between higher plant plastids. Science 276:2039–2042

Kokubun N, Ishida H, Makino A, Mae T (2002) The degradation of the large subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase into the 44 kDa fragment in the lysates of chloroplasts incubated in darkness. Plant Cell Physiol 43:1390–1395

Krupinska K (2007) Fate and activities of plastids during leaf senescence. In: Wise RR, Hoober JK (eds) The structure and function of plastids. Springer, Netherlands, pp 433–449

Kusaba M, Ito H, Morita R, Iida S, Sato Y, Fujimoto M, Kawasaki S, Tanaka R, Hirochika H, Nishimura M, Tanaka A (2007) Rice NON-YELLOW COLORING 1 is involved in light-harvesting complex II and grana degradation during leaf senescence. Plant Cell 19:1362–1375

Lamattina L, Pont-Lezica R, Conde R (1985) Protein metabolism in senescing wheat leaves. Plant Physiol 77:587–590

Lim PO, Kim HJ, Nam HG (2007) Leaf senescence. Annu Rev Plant Biol 58:115–136

Lohman KN, Gan S, John LM, Amasino RM (1994) Molecular analysis of natural leaf senescence in *Arabidopsis thaliana*. Physiol Plant 92:322–328

Lu C, Lu Q, Zhang J, Kuang T (2001) Characterization of photosynthetic pigment composition, photosystem II photochemistry and thermal energy dissipation during leaf senescence of wheat plants grown in the field. J Exp Bot 52:1805–1810

Mae T (2004) Leaf senescence and nitrogen metabolism. In: Noodén LD (ed) Plant cell death processes. Academic, San Diego, pp 157–168

Martínez DE, Bartoli CG, Grbic V, Guiamet JJ (2007) Vacuolar cysteine proteases of wheat (*Triticum aestivum* L.) are common to leaf senescence induced by different factors. J Exp Bot 58:1099–1107

Martínez DE, Costa ML, Gomez FM, Otegui MS, Guiamet JJ (2008a) "Senescence-associated vacuoles" are involved in the degradation of chloroplast proteins in tobacco leaves. Plant J 56:196–206

Martínez DE, Costa ML, Guiamet JJ (2008b) Senescence-associated degradation of chloroplast proteins inside and outside the organelle. Plant Biol 10:15–22

Masclaux-Daubresse C, Reisdorf-Cren M, Orsel M (2008) Leaf nitrogen remobilisation for plant development and grain filling. Plant Biol 10:23–36

Matile P (1997) The vacuole and cell senescence. Adv Bot Res 25:87–112

May JD, Killingbeck KT (1992) Effects of preventing nutrient resorption on plant fitness and foliar nutrient dynamics. Ecology 73:1868–1878

Miersch I, Heise J, Zelmer I, Humbeck K (2000) Differential degradation of the photosynthetic apparatus during leaf senescence in barley (*Hordeum vulgare* L.). Plant Biol 2:618–623

Minimikawa T, Toyooka K, Okamoto T, Hara-Nishimura I, Nishimura M (2001) Degradation of ribulose-bisphosphate carboxylase by vacuolar enzymes of senescing French bean leaves: immunocytochemical and ultrastructural observations. Protoplasma 218:144–153

Müntz K (2007) Protein dynamics and proteolysis in plant vacuoles. J Exp Bot 58:2391–2407

Nakabayashi K, Ito M, Kiyosue Y, Shinozaki K, Watanabe A (1999) Identification of clp genes expressed in senescing Arabidopsis leaves. Plant Cell Physiol 40:504–514

Nakano T, Nagato N, Kimura T, Sekimoto M, Kawaide H, Murakami S, Kaneko Y, Matsushima H, Kamiya J, Sato F, Yoshida S (2003) CND41, a chloroplast nucleoid protein that regulates plastid development, causes reduced gibberellin content and dwarfism in tobacco. Physiol Plant 117:130–136

Nakashima K, Kiyosue T, Yamaguchi-Shinozaki K, Shinozaki K (1997) A nuclear gene, *erd1*, encoding a chloroplast targeted Clp protease regulatory subunit homolog is not only induced by water stress but also developmentally upregulated during senescence in *Arabidopsis thaliana*. Plant J 12:851–861

Niwa Y, Kato T, Tabata S, Seki M, Kobayashi M, Shinozaki K, Moriyasu Y (2004) Disposal of chloroplasts with abnormal function into the vacuole in *Arabidopsis thaliana* cotyledon cells. Protoplasma 223:229–232

Noodén LD (1988) The phenomena of senescence and aging. In: Noodén LD, Leopold AC (eds) Senescence and aging in plants. Academic, San Diego, pp 1–50

Noodén LD, Schneider MJ (2004) Light control of senescence. In: Noodén LD (ed) Plant cell death processes. Academic, San Diego, pp 375–383

Noodén LD, Guiamet JJ, John I (2004) Whole plant senescence. In: Noodén LD (ed) Plant cell death processes. Academic, San Diego, pp 227–244

Otegui M, Noh Y-S, Martínez D, Vila-Petroff M, Staehelin A, Amasino R, Guiamet JJ (2005) Senescence-associated vacuoles with intense proteolytic activity develop in senescing leaves of Arabidopsis and soybean. Plant J 41:831–844

Parrott DL, Martin JM, Fischer AM (2010) Analysis of barley (*Hordeum vulgare* L.) leaf senescence and protease gene expression: a family C1A cysteine protease is specifically induced under conditions characterized by high carbohydrate, but low to moderate nitrogen levels. New Phytol 187:313–331

Peoples MB, Dalling MJ (1988) The interplay between proteolysis and amino acid metabolism during senescence and nitrogen reallocation. In: Nooden LD, Leopold AC (eds) Senescence and aging in plants. Academic, San Diego, pp 181–217

Roulin S, Feller U (1998) Light-independent degradation degradation of stromal proteins in intact chloroplasts isolated from *Pisum sativum* L.: requirement for divalent cations. Planta 205:297–304

Sakamoto W (2006) Protein degradation machineries in plastids. Annu Rev Plant Biol 57:599–621

Schmid M, Simpson D, Kalousek F, Gietl C (1998) A cysteine endopeptidase with a C-terminal KDEL motif isolated from castor bean endosperm is a marker enzyme for the ricinosome, a putative lytic compartment. Planta 206:466–475

Schmid M, Simpson D, Gietl C (1999) Programmed cell death in castor bean endosperm is associated with the accumulation and release of a cysteine endopeptidase from ricinosomes. Proc Natl Acad Sci 96:14159–14164

Schmid M, Simpson D, Sarioglu H, Lottspeich F, Gietl C (2001) The ricinosomes of senescing plant tissue bud from the endoplasmic reticulum. Proc Natl Acad Sci 98:5353–5358

Schmutz D, Wyss H-R, Brunold C (1983) Activity of sulfate-assimilating enzymes in primary leaves of *Phaseolus vulgaris* L. during dark-induced senescence. Z Pflanzenphysiol 110:211–219

Senatore A, Trobacher CP, Greenwood JS (2009) Ricinosomes predict programmed cell death leading to anther dehiscence in tomato. Plant Physiol 149:775–790

Shanklin J, DeWitt ND, Flanagan JM (1995) The stroma of higher plant plastids contain ClpP and ClpC functional homologs of *Escherichia coli* ClpP and ClpA: an archetypal two components ATP-dependent protease. Plant Cell 7:1713–1722

Srivalli B, Bharti S, Khanna-Chopra N (2001) Vacuolar cysteine proteases and ribulose-1,5-bisphosphate carboxylase/oxygenase degradation during monocarpic senescence in cowpea leaves. Photosynthetica 39:87–93

Sun X, Peng L, Guo J, Chi W, Ma J, Lu C, Zhang L (2007) Formation of Deg5 and Deg8 complexes and their involvement in the degradation of photodamaged photosystem II reaction center D1 protein in Arabidopsis. Plant Cell 19:1347–1361

Sun X, Fu T, Chen N, Guo J, Ma J, Zou M, Lu C, Zhang L (2010) The stromal chloroplast Deg7 protease participates in the repair of photosystem II after photoinhibition in Arabidopsis. Plant Physiol 152:1263–1273

Swanson SJ, Bethke PC, Jones RL (1998) Barley aleurone cells contain two types of vacuoles: characterization of lytic organelles by means of fluorescent probes. Plant Cell 10:685–698

Tang Y, Wen X, Lu C (2005) Differential changes in degradation of chlorophyll-protein complexes of photosystem I and photosystem II during flag leaf senescence of rice. Plant Physiol Biochem 43:193–201

Thoenen M, Herrmann B, Feller U (2007) Senescence in wheat leaves: is a cysteine endopeptidase involved in the degradation of the large subunit of Rubisco? Acta Physiol Plant 29:339–350

Thomas H, Howarth CJ (2000) Five ways to stay green. J Exp Bot 51:329–337

Thompson AR, Doelling JH, Suttangkakul A, Vierstra RD (2005) Autophagic nutrient recycling in Arabidopsis directed by the ATG8 and ATG12 conjugation pathways. Plant Physiol 138:2097–2110

Uauy C, Distelfeld A, Fahima T, Blechl A, Dubcosky J (2006) A NAC gene regulating senescence improves grain protein, zinc and iron content in wheat. Science 314:1298–1301

van der Hoorn RAL, Leeuwenbergh MA, Bogyo M, Joosten MHA, Peck SC (2004) Activity profiling of papain-like cystein proteases in plants. Plant Physiol 135:1170–1178

Wada S, Ishida H, Izumi M, Yoshimoto K, Ohsumi Y, Mae T, Makino A (2009) Autophagy plays a role in chloroplast degradation during senescence in individually darkened leaves. Plant Physiol 149:885–893

Wagner R, Aigner H, Pruzinská A, Johanson Jänkänpää H, Jansson S, Funk C (2011) Fitness analysis of *Arabidopsis thaliana* mutants depleted of FtsH metalloproteases and characterization of three FtsH6 deletion mutants exposed to high light stress, senescence and chilling. New Phytol. 191:449–458

Weaver LM, Froehlich JE, Amasino RM (1999) Chloroplast-targeted ERD1 protein declines but its mRNA increases during senescence in Arabidopsis. Plant Physiol 119:1209–1216

Woo HR, Chung KM, Park J-H, Oh SA, Ahn T, Hong SH, Jang SK, Nam HG (2001) Ore 9, an F-box protein that regulates leaf senescence in Arabidopsis. Plant Cell 13:1779–1790

Xiong Y, Contento AL, Bassham DC (2005) AtATG18a is required for the formation of autophagosomes during nutrient stress and senescence in *Arabidopsis thaliana*. Plant J 42:535–546

Yoshida T, Minimikawa T (1996) Successive amino-terminal proteolysis of the large subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase by vacuolar enzymes from French bean leaves. Eur J Biochem 238:317–324

Zaltsman A, Ori N, Adam Z (2005) Two types of FtsH subunits are required for chloroplast biogenesis and Photosystem II repair in Arabidopsis. Plant Cell 17:2782–2790

Zavaleta-Mancera HA, Franklin KA, Ougham HJ, Thomas H, Scott IM (1999) Regreening of senescent Nicotiana leaves. I. Reappearance of NADPH-protochlorophyllide oxidoreductase and light-harvesting chlorophyll a/b-binding protein. J Exp Bot 50:1677–1682

Zelisko A, Jackowski G (2004) Senescence-dependent degradation of Lhcb3 is mediated by a thylakoid membrane-bound protease. J Plant Physiol 161:1157–1170

Zelisko A, García-Lorenzo M, Jackowski G, Jansson S, Funk C (2005) AtFtsH6 is involved in the degradation of the light-harvesting complex II during high-light acclimation and senescence. Proc Natl Acad Sci 102:13690–13704

Zheng B, MacDonald TM, Sutinen S, Hurry V, Clarke AK (2006) A nuclear-encoded ClpP subunit of the chloroplast ATP-dependent Clp protease is essential for early development in *Arabidopsis thaliana*. Planta 224:1103–1115