Vacuolar cysteine proteases of wheat (*Triticum aestivum* L.) are common to leaf senescence induced by different factors

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Abstract

Cellular proteins are extensively degraded during leaf senescence, and this correlates with an up-regulation of protease gene expression, particularly cysteine proteases. The objectives of this work were (i) to detect cysteine proteases associated with senescence of wheat leaves under different conditions and (ii) to find out their subcellular location. Activity labelling of cysteine proteases with the biotinylated inhibitor DCG-04 detected five bands at 27, 36, 39, 42, and 46 kDa in leaves of wheat senescing under continuous darkness. In-gel activity assays showed that these proteases are only active in an acid milieu (pH 4), and their activity increased several-fold in senescing leaves. Fractionation experiments showed that the senescence-associated cysteine proteases of 36, 39, and 42, and 46 kDa localize to a vacuolar-enriched fraction. The vacuolar cysteine proteases of 36, 39, and 42 kDa increased in activity in attached flag leaves senescing naturally during post-anthesis, and in attached leaves of plants subjected to a period of water deficit. Thus, the activity of these vacuolar cysteine proteases is associated with developmental (post-anthesis) senescence and with senescence induced by stress factors (i.e. protracted darkness or drought). This suggests that vacuoles are involved in senescence-associated cellular degradation, and that different senescence-inducing factors may converge on a single degradation pathway.

Key words: Cysteine proteases, leaf senescence, protein degradation, vacuole, wheat.

Introduction

Senescence represents the last phase in the ontogeny of a leaf, and it is characterized by a marked decline in the assimilatory capacities of the leaf (e.g. photosynthesis) associated with massive degradation of cellular proteins (Krupinska and Humbeck, 2004). Chloroplast proteins appear to be the main target for degradation in senescing leaves, and the loss of the stromal proteins of the carbon reduction cycle is often the first detectable event of senescence in many plants (Krupinska and Humbeck, 2004). Given the importance of cellular degradation during senescence, it is not surprising that 7% of the about 2500 genes expressed in senescing leaves code for various types of hydrolases, including proteases (Gepstein, 2004).

Many senescence-associated genes (SAGs), i.e. genes whose expression appears de novo or increases during senescence, code for proteases. There is an increase in the expression of genes representative of the different mechanistic classes of proteases, for example, aspartic-, cysteine-, serine-, and metalloproteases (Guo et al., 2004), although cysteine- (Cys-) proteases are the class most frequently detected among protease SAGs (Andersson et al., 2004; Guo et al., 2004). The identification of senescence-associated proteases has been largely based on studies of the changes in their mRNA levels. However, the activity of some proteases appears to be under post-translational control, with maturation and activation often depending on pH (Wiederanders et al., 2003). For example, RD21, a Cys-protease expressed in leaves under water-stress or during senescence (Koizumi et al., 1993; Yamada et al., 2001), is synthesized as an inactive 57 kDa...
pro-protease, which is cleaved to the mature, active 33 kDa form in the acidic environment of the central vacuole (Yamada et al., 2001). Protease activity can also be modulated by changes in the concentration of specific inhibitors, as in the case of Cys-proteases involved in soybean programmed cell death which are regulated by cellular levels of cystatins, a group of specific endogenous protease inhibitors (Solomon et al., 1999). Thus, changes in mRNA levels may not necessarily result in parallel changes in activity. Searching for senescence-associated proteases based on changes in their activity might help to overcome some of the uncertainties inherent to studies based solely on mRNA levels.

Interestingly, few of the protease SAGs discovered so far code for proteases targeted to the chloroplast (Nakabayashi et al., 1999), although most of the protein broken down during senescence is located in the plastid. Finding out the function of the different proteases will require information about their subcellular location. A number of proteases associated with senescence appear to localize to the vacuole (Hensel et al., 1993; Drake et al., 1996), where most of the protease activity of senescing cells is found (Wittenbach et al., 1982). Vacuolar processing enzymes, a subset of Cys-proteases involved in maturation of vacuolar proteins, are also up-regulated during senescence of leaves (Smart et al., 1995; Kinoshita et al., 1999; Page et al., 2001). This suggests that vacuolar proteases might play a role in cellular degradation during senescence. SAG12, a senescence-specific Cys-protease (Lohman et al., 1994) localizes to a novel class of senescence-associated vacuoles clearly distinguishable from the central vacuole of mesophyll cells (Otegui et al., 2005). The localization of most other senescence-associated proteases remains unknown.

The choice of experimental system can further complicate the identification of proteases involved in cellular degradation during senescence. Although leaf senescence progresses in a developmentally programmed manner during plant ontogeny, several environmental and stress factors (e.g. water and nutrient deficiencies, light deprivation, etc) modulate the rate of senescence. The signalling pathways that control senescence in these different scenarios, and the hydrolytic enzymes involved may be quite different. For example, salicylic acid is required for the normal progression of senescence in Arabidopsis leaves senescing ‘naturally’, attached to the plant, but not for senescence of excised leaves under continuous darkness (Buchanan-Wollaston et al., 2005). Similarly, the expression of SAG12 is a diagnostic marker of ‘natural’ senescence of attached leaves, but SAG12 is undetectable in excised leaves induced to senesce by dark incubation (Buchanan-Wollaston et al., 2005).

Since Cys-proteases are consistently associated with senescence over a wide spectrum of different species, an attempt was made to detect Cys-proteases whose activity increases during the senescence of wheat leaves, to identify their subcellular location, and to determine if similar sets of proteases are associated with protein degradation in leaves where senescence is induced, or accelerated, by different stimuli.

**Materials and methods**

**Plant material and growing conditions**

For most experiments, seeds of wheat (Triticum aestivum L.) cv. Buck Poncho were planted in pots filled with perlite and watered daily with complete Hoagland nutrient solution. Plants were grown in a growth room at 24 °C, with a 12 h photoperiod and 400 μmol m⁻² s⁻¹ of photosynthetic photon flux density. Three weeks after planting, senescence was induced by transferring the pots to continuous darkness at 24 °C. Samples from naturally senescing leaves attached to the plant were taken from a field plot planted with cv. Buck Poncho in the experimental farm of Facultad de Ciencias Agrarias y Forestales, Universidad Nacional La Plata (La Plata, Argentina, 34°54’ S). The field was planted and managed under standard agricultural practices, and received non-limiting rainfall during the growing season. Samples of the flag leaf were taken for analysis at regular intervals starting at anthesis. Flag leaves from the same field plot were also excised at anthesis, transported to the laboratory, and incubated in continuous darkness at 24 °C to follow the changes associated with dark-induced senescence of detached leaves.

To analyse protease activity in leaves of water-stressed plants, seeds of cv. Buck Poncho were sown in 1.0 l plastic pots filled with a loamy soil and grown in a growth room under the conditions described above. Pots were initially maintained at field capacity for 2 weeks. Water deficit was imposed by withholding watering until the soil water potential reached −0.6 MPa. Soil water potential was monitored daily by weighing the pots. The relationship between gravimetric soil water content and water potential measured with an HR-33T Dew Point Microvoltmeter and PST-55 soil probes (Wescor, Inc.) was determined before the start of the experiment.

**Leaf area and chlorophyll content**

The area of each leaf was measured with a Li-Cor LI-3000 leaf area meter. Chlorophyll content was estimated with a portable SPAD 502 chlorophyll meter (Minolta Co, Japan) or spectrophotometrically after extraction with N,N-dimethyl formamide (Inskeep and Bloom, 1985).

**Activity-based detection of Cys-proteases**

Cys-proteases were labelled with DCG-04 as in Greenbaum et al. (2000). DCG-04 is an analogue of the irreversible Cys-protease inhibitor E-64, with a biotin residue added as a tag. Thus, DCG-04 can bind to the active site of Cys-proteases irreversibly, and the biotin residue can conveniently be used to detect DCG-04 bound proteases with a streptavidin-based detection system. Briefly, leaves were homogenized in distilled water at 4 °C and centrifuged at 13 000 g for 15 min. One volume of the supernatant was mixed with 3 vols of 5 μM DCG-04 dissolved in 25 mM Na-acetate buffer, 10 mM cysteine, pH 5, and the mixture was incubated at room temperature with gentle shaking for 5 h. Proteins were precipitated with chilled (−20 °C) acetone, resuspended in Laemmli’s buffer (25 mM TRIS pH 6.8, 2% w/v SDS, 10% v/v glycerine) and boiled for 1 min before loading on an SDS–PAGE gel. To control for the specificity of the DCG-04 labelling, competition
experiments with E-64 were carried out on parallel aliquots of the protein extract. These samples were preincubated with 100 μM E-64 in buffer (25 mM sodium acetate, 10 mM cysteine, pH 5) for 1 h, and then treated with DCG-04 as described above. DGC-04 labelled proteins were separated in 12% acrylamide SDS–PAGE gels and electro-transferred to nitrocellulose. After blocking with 10% w/v non-fat milk dissolved in PBS-T (8.0 mM potassium phosphate buffer pH 7.4, 150 mM NaCl, 0.02% v/v Tween-20). DCG-04 labelled proteins were detected with an streptavidin–peroxidase conjugate (1:2000 v/v, Ultrasensitive Streptavidin–Peroxidase conjugate, Sigma Chemical Corp., St Louis, MO, USA) and a chemiluminescence detection kit (Western Lightning, Perkin-Elmer, Boston, MA, USA).

**SDS-PAGE and protease zymograms**

Soluble proteins were extracted by grinding leaves in buffer (25 mM TRIS pH 7.5, 2 mM cysteine, 1% w/v insoluble polyvinylpyrrolidone), and centrifuged at 10 000 g for 15 min. SDS-PAGE analysis was run in 12% acrylamide concentration gels, and proteins were stained with Coomassie Blue. For zymogram analysis of protease activity, gelatin (0.04% w/v) was added to the resolving gel of a normal SDS–PAGE, and electrophoresis was run at 4 °C. After electrophoresis, gels were washed in 75 mM Na-acetate pH 4.5, 2% v/v Triton X-100, 8 mM DTT (wash buffer) for 1 h with gentle shaking to remove SDS. Gels were then transferred to incubation buffer (75 mM Na-acetate pH 4.5, 8 mM DTT) and developed at 37 °C for 16 h. Activity bands were detected as clear zones after staining with Coomassie Blue. Na-acetate (pH 4.0–5.5) or TRIS-based (pH 7.5) buffers were used as wash and incubation buffers in experiments to determine the pH-dependency of protease activity. To assign activity bands to mechanistic classes of proteases, protein samples were incubated with class-specific inhibitors (20 μM E-64, 1 mM PMSF, 40 μM pepstatin or 2 mM EDTA, for Cys-erine-, aspartic-, and metalloproteases, respectively) at 0 °C for 30 min before electrophoresis, and inhibitors were also included in wash and incubation buffers. Where the stock inhibitor solutions were made with solvents other than water (e.g. methanol for PMSF or DMSO for pepstatin), control samples without inhibitor were prepared with the corresponding amounts of methanol or DMSO.

**Vacuole isolation**

Vacuoles were isolated from senescing leaves essentially as described in Bednarek and Raikhel (1991). Leaves were cut into small pieces (about 1 mm²), infiltrated with suspension buffer (25 mM MES, 0.5 M betaine, pH 5.5) under vacuum for 3 min, and incubated for 2 h at room temperature with digestion buffer (25 mM MES, 0.5 M betaine, 0.1% w/v cellulase, 0.03% w/v pectolyase, pH 5.5). The digestion buffer was then removed and protoplasts were released by shaking the leaf pieces gently for 30 min in suspension buffer with 0.01% (w/v) neutral red (a marker for acidic organelles). Protoplasts were collected by centrifugation at 50 g for 10 min, resuspended in buffer (25 mM HEPES pH 7, 0.5 M betaine, 10% w/v Ficoll) and pipetted gently to promote evagination. The vacuolar fraction was purified by flotation. The sample (composed of vacuoles, evacuated protoplasts, and intact protoplasts) was placed in the bottom of a centrifuge tube, and was overlaid with 8, 6, 2, and 0% Ficoll (w/v) dissolved in buffer (25 mM HEPES pH 7, 0.5 M betaine). Tubes were centrifuged at 10 000 g for 20 min and vacuoles were collected from the 6–8% Ficoll interface. Vacuolar proteins were concentrated with 10 kDa cutoff Nanosep filters (Pall Corp., Ann Arbor, MI, USA). To assess the degree of contamination of our vacuolar preparation by other organelles, markers for the central vacuole (vacuolar ATPase and aleurain/SAG2), chloroplasts (the D1 protein of PSII), mitochondria (cytochrome C), peroxisomes (catalase), the Golgi apparatus (UTPG), and endoplasmic reticulum (BiP) were detected by Western blotting with specific antibodies.

**Results**

**Cys-proteases in senescing leaves of wheat**

Cys-proteases constitute the mechanistic class of proteases most consistently associated with senescence of leaves, therefore an attempt was made to detect senescence-associated Cys-proteases in wheat leaves. Activity-based labelling with DCG-04 was previously used to identify senescence-associated proteases in Arabidopsis thaliana (van der Hoorn et al., 2004). In non-senescent, mature leaves of wheat, DCG-04 detected Cys-protease bands with apparent molecular masses of 36, 39, 42, and 44 kDa (Fig. 1). The 42 kDa and 44 kDa bands in Fig. 1 appear as a doublet. In leaves senescing in darkness, 60% of the Chl was lost in 3–4 d of incubation, and this was accompanied by important changes in the pattern of Cys-proteases detected with DCG-04 (Fig. 1). The intensity of the Cys-protease band at about 42 kDa appeared to decrease, whereas the bands at 36 kDa and 39 kDa strongly increased in abundance in senescing leaves. Interestingly, two new proteases, at about 27 kDa and 46 kDa appeared de novo associated with senescence. Pre-incubation of leaf extracts with E-64 completely inhibited labelling of these proteases.

Fig. 1. Cysteine proteases profiling in mature and senescing wheat leaves. Cysteine proteases were extracted from 10 cm² of leaf tissue, biotinylated with DCG-04 and detected in western blots with streptavidin-HRP. To determine the specificity of the DGC-04 labelling, an excess of E-64 (+) was added to control samples before biotinylation. Arrows indicate cysteine proteases (27, 36, 39, 42, and 46 kDa), arrowheads show non-specific DCG-04 binding. NS, non-senescing, mature leaves; S, senescing leaves. Figures on the left represent the position of molecular mass markers (kDa).
bands, indicating that they represent Cys-proteases, rather
than non-specific labelling of SH-groups by DCG-04.

**Senescence-associated Cys-proteases are active at low pH**

The activity of the Cys-proteases detected in senescing leaves was assayed in zymograms incubated at different pH values (Fig. 2). Most activity bands were detected only at very low pH values (i.e. pH 4). Bands at 36, 39, 42, and 46 kDa were clearly seen in zymograms incubated at pH 4, and all these bands matched the apparent size of proteins labelled by DCG-04. Compared with non-senescing leaves, the activity of the proteases of 36, 39, 42, and 46 kDa increased several-fold in leaves that had lost 60% of their Chl (Fig. 3). Other activity bands at higher apparent molecular mass, not labelled by DCG-04 (Fig. 1), were detected in zymograms (Figs 2, 3). A 49 kDa band was active at pH 4, and its activity increased with leaf senescence. Two additional senescence-associated proteases with apparent masses around 66 kDa were active over a wider range of pH, with an optimum around pH 5 (Figs 2, 3).

**Mechanistic classes of senescence-associated proteases**

Class-specific inhibitors were used to assign the senescence-associated activity bands to the different mechanistic types of proteases (Fig. 4). Consistent with their labelling by DCG-04 (Fig. 1), activity of the Cys-proteases in the range of 36–46 kDa was completely inhibited by E-64 (Fig. 4A). Other types of protease inhibitors had no effect on the activity of these bands. The two bands at around 66 kDa were insensitive to inhibitors of metallo- and Cys-proteases (Fig. 4B), which is consistent with their lack of labelling with DCG-04 (Fig. 1). Their activities were completely abolished by either PMSF (an inhibitor of serine-proteases) or pepstatin (an inhibitor of aspartic-proteases), which prevented an unambiguous assignment of these two proteases to a mechanistic class.

**Vacuolar localization of senescence-associated cysteine proteases**

The low pH optimum for activity of the senescence-associated Cys-proteases suggested that they might be active in the acid environment of vacuoles. To find out if these proteases localize to the central vacuole, a vacuolar-enriched fraction was prepared through floatation centrifugation of evacuolated protoplasts (Fig. 5A). Control experiments showed that the vacuolar-enriched fraction was completely devoid of contamination by chloroplasts, mitochondria, peroxisomes, endoplasmic reticulum, or the Golgi apparatus (Fig. 5B). The vacuolar fraction was strongly enriched in SAG2/Aleu and V-ATPase, which are markers for the vacuolar lumen and tonoplast, respectively (Fig. 5B). Similarly, the protease bands of about 36, 39, 42, and 46 kDa were substantially enriched...
in the vacuolar fraction (Fig. 5C), indicating that these cysteine proteases localize to the central vacuole.

Proteases in wheat leaves senescing during the reproductive period or under water stress

Incubation of leaves under protracted darkness is a convenient experimental system to accelerate senescence, but dark-induced senescence might differ from senescence of leaves attached to the plant under a light/dark regime in terms of the mechanism and regulation of cellular degradation. Therefore, the pattern of active proteases in leaves incubated in darkness, and in attached leaves senescing during the post-flowering period of wheat plants growing in the field under standard agricultural conditions, were compared. The flag leaf of wheat lost about 22% of Chl in the first 20 d after anthesis, whereas barely 40% of the Chl remained 7 d thereafter (Fig. 6A). These changes in Chl content accompanied a substantial drop in protein content (Fig. 6A). As with detached leaves kept in darkness, proteases with apparent molecular masses of 36, 39, 42, and 46 kDa were non-detectable (or barely detectable) at anthesis, but their activity increased dramatically during post-anthesis senescence of the flag leaf (Fig. 6B). These proteases were all active at pH 4, and inhibited by E-64 (data not shown). No additional protease bands specific for the flag leaf senescing during the reproductive period, or water-stressed leaves, could be detected with the zymograms employed here, even at neutral pH (data not shown).

Discussion

Cysteine proteases are associated with developmental senescence and senescence induced by stress factors

Senescence is a normal part of the ontogeny of a leaf, however, the timing and the rate of senescence-associated protein degradation are modulated by the effects of hormones (e.g. ethylene) and environmental stresses (e.g. nutrient and water deficit, light deprivation, etc) superimposed on this developmental programme. Exposure to continuous darkness can induce senescence of mature leaves, and this represents a convenient experimental system to synchronize and accelerate senescence. However, transcriptomic analysis and other studies have pointed out a number of differences between natural and dark-induced senescence in terms of gene expression and the metabolic pathways up-regulated in each case. Some of these differences relate to the fate of the products of cellular degradation in these different scenarios. In leaves senescing attached to the plant, amino acids released from protein degradation are converted into glutamine for export to other parts of the plant, and this requires increased expression of glutamine synthetase genes (Kamachi et al., 1992; Buchanan-Wollaston et al., 2005). By contrast, glutamate dehydrogenase is up-regulated in leaves senescing in continuous darkness, possibly to deaminate glutamate and channel the carbon skeletons of amino acids into the Krebs cycle for energy production. There are also differences in the expression of proteases; for example, SAG12, a cysteine protease gene specifically expressed during senescence of leaves attached to the plant, is not up-regulated in leaves where senescence is induced by dark incubation (Weaver et al., 1999). Likewise, the proteases up-regulated during ‘natural’ developmental senescence might not be the same as those which increase in activity in leaves senescing under the influence of various stress factors. The NtCP1 cysteine protease of tobacco is up-regulated during developmental senescence, but not in leaves of plants under drought (Beyene et al., 2006). The pattern of proteases that increase in activity during developmental senescence is different from senescence induced by N deprivation in clover (Kingston-Smith et al., 2005). However, a cysteine protease of barley (HvSF42) is up-regulated during developmental and dark-induced senescence (Scharrenberg et al., 2003). Similarly,
in our study, the cysteine protease activities at 36, 39, 42, and 46 kDa also increased during senescence of excised leaves in darkness and during developmental post-anthesis senescence. DCG-04 labelling and in-gel activity assays detected the same cysteine protease bands, suggesting that our in-gel activity assays did not miss any major Cys-protease, and that these are the most abundant cysteine proteases in senescing leaves of wheat. Thus, developmental and dark-induced senescence may differ in the expression of mRNAs for less abundant Cys-proteases, but the most active cysteine proteases may be the same. However, comparing developmental senescence and water stress, the 46 kDa Cys-protease did not increase in activity in leaves senescing under water deficit. In leaves senescing during the grain-filling period, the activity of the 46 kDa protease increases at an advanced stage of senescence, i.e. after the leaves have lost more than half of their Chl (Fig. 6). The 46 kDa Cys-protease may become detectable only at an advanced stage in senescence, and leaves in the water-deficit treatment (which lost only 27% of their initial chlorophyll) might not have reached that stage at the time of sampling. Alternatively, senescence induced by water deficit may not involve the activity of this protease. In any event, developmental senescence and senescence induced by light deprivation or water stress shared the activity of the majority of the Cys-proteases detected in this work.

It is likely that the pathways regulating senescence vary in different situations (e.g. developmental senescence, light deprivation, nutrient or water deficiencies, etc), but the different senescence-inducing factors may all converge on a common degradative pathway involving the same hydrolytic enzymes. In support of this idea, ‘stay green’ mutations of soybean (*Glycine max* L. Merrill) and *Festuca* inhibit chlorophyll and thylakoid protein degradation to a similar extent in leaves senescing attached to the plant, and in detached leaves incubated in darkness (Thomas and Smart, 1993) or detached leaves treated with various senescence-accelerating hormones (Guiamet et al., 1991, Guiamet and Giannibelli, 1994).

**Senescence-associated cysteine proteases localize to the vacuole**

The central vacuole of plants is thought to be analogous to the animal lysosome in that it contains most of the hydrolytic activity of the cell (De, 2000). In cell suspension proteases in the vacuolar (V) fraction through labelling with DCG-04. The far left lane corresponds to samples of senescing protoplasts preincubated with E-64 before labelling with DCG-04 to control for non-specific labelling. Arrows show the proteases of 36, 39, 42, and 46 kDa. Arrowheads show non-specific labelling. Figures on the left represent the position of molecular mass markers (kDa).

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**Fig. 5.** Localization of senescence-associated cysteine proteases in a vacuolar-enriched fraction. (A) Protoplasts (P) were isolated from senescing leaves, and vacuoles (V) were released by gentle pipetting and purified by floatation centrifugation. Vacuoles are stained by the acidotropic dye Neutral Red. (B) The vacuolar fraction (V) was devoid of contamination by chloroplasts (D1), mitochondria (cytochrome c), peroxisomes (catalase), endoplasmic reticulum (BiP), and the Golgi apparatus (UDP-glucose:protein transglucosylase, UTPG), whereas the vacuolar markers Sag2 and V-ATPase concentrated in the vacuolar-enriched fraction. Equal amounts of protein corresponding to the protoplast and vacuolar preparations were analysed by western blot with specific antibodies. (C) Detection of senescence-associated cysteine proteases in the vacuolar (V) fraction through labelling with DCG-04.
cultures of *Acer* the central vacuole is involved in recycling of cytosolic proteins and degradation of abnormally synthesized proteins (Canut et al., 1985, 1986). The cysteine proteases detected with DCG-04 in senescing leaves of wheat are enriched in the vacuolar fraction, which is consistent with the vacuolar localization of the senescence-associated cysteine proteases SENU3 and SENU4 of tomato (Drake et al., 1996), and AtAleu/SAG2 of *Arabidopsis* (Hensel et al., 1993). Our data show that the activity of several vacuolar cysteine proteases is associated with leaf senescence under different conditions. This points to a crucial role of vacuolar cysteine proteases in protein degradation during senescence. Vacuolar hydrolases might participate in cell protein degradation in a variety of ways. The final step of tracheary cell differentiation involves rupture of the tonoplast with the release of vacuolar proteases into the cytosol, which brings about degradation of cellular components leading to cell death (Dahiya, 2003). However, digestion of cell contents by loss of tonoplast integrity and mixing of vacuolar proteases with the cytosol is unlikely in senescing leaves because the integrity of the vacuolar membrane is retained until quite late during leaf senescence (Inada et al., 1998). The retention of cell viability in leaves that have lost most of their chlorophyll and protein (Zavaleta-Mancera et al., 1999) suggests that digestion of cellular contents is not brought about by loss of vacuole compartmentation and random degradation of proteins by vacuolar proteases released into the cytosol (Matile, 1997). Furthermore, in our in-gel activity assays wheat cysteine proteases are active at low pH values, suggesting that these proteases may work only under the acid conditions of the intact vacuole. However, vacuolar proteases might bring about wholesale digestion of residual proteins once the tonoplast collapses immediately prior to cell death (Matile, 1997).

Vacuolar proteases might participate in an autophagic pathway, where parts of the cytosol are engulfed in autophagosomes and then delivered to the central vacuole for degradation (Thompson and Vierstra, 2005). The expression of several autophagy genes (e.g. *ATG7*, *ATG8*) increases during senescence (Doelling et al., 2002). Under conditions of nutrient starvation knock out lines for *ATG5*, *ATG7*, and *ATG9* show reduced survival, which suggests that the ATG7-dependent pathway is essential to recycle cytosolic proteins (Doelling et al., 2002; Hanaoka et al., 2002; Thompson et al., 2005). Thus, an autophagic pathway might operate in the degradation of cytosolic proteins in senescing leaves. There is also some evidence suggesting that vacuolar proteases might function in the breakdown of chloroplast proteins. Chloroplasts and immunologically detectable Rubisco were also seen in the central vacuole of chemically fixed sections taken from senescing leaves of French bean (Minamikawa et al., 2001). However, degradation of chloroplasts by the central vacuole would require the operation of a hitherto unknown pathway for internalization of chloroplasts or chloroplast fragments within the vacuole, or for transport of chloroplast proteins to the vacuole.

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