Protoplasma (2001) 218: 144–153

PROTOPLASMA

Degradation of ribulose-bisphosphate carboxylase by vacuolar enzymes of senescing French bean leaves: immunocytochemical and ultrastructural observations

T. Minamikawa[1], **K. Toyooka**[1,**], **T. Okamoto**[1,*], **I. Hara-Nishimura**[2], and **M. Nishimura**[3]

[1] Department of Biological Sciences, Tokyo Metropolitan University, Hachioji, Tokyo, [2] Graduate School of Science, Kyoto University, Kyoto, and [3] Department of Cell Biology, National Institute of Basic Biology, Okazaki

Received March 20, 2001
Accepted July 10, 2001

Summary. The possible involvement of vacuolar cysteine proteinases in degradation of ribulose-bisphosphate carboxylase (Rubisco) in senescing French bean leaves was studied by ultrastructural and immunocytochemical analyses with antibodies raised against the large subunit (LSU) of Rubisco and SH-EP, a cysteine proteinase from *Vigna mungo* that is immunologically identical to one of the major proteinases of French bean plants. Primary leaves of 10-day-old plants were detached and placed at 25 °C in darkness for 0, 4, and 8 days to allow their senescence to proceed. The leaves at each senescence stage were subjected to the conventional electron microscopic and immunocytochemical studies. The results indicated that the chloroplasts of senescing French bean leaves were separated from the cytoplasm of the cell periphery and taken into the central vacuole and that the Rubisco LSU in the chloroplasts was degraded by vacuolar enzymes such as an SH-EP-related cysteine proteinase that developed in senescing leaves. The present results together with the results of previous biochemical studies using vacuolar lysates support the view that Rubisco is degraded through the association of chloroplasts with the central vacuole during the senescence of leaves that were detached and placed in darkness.

Keywords: Cysteine proteinase; Degradation; Ribulose-bisphosphate carboxylase; Senescence; Vacuole.

Introduction

Ribulose-bisphosphate carboxylase (Rubisco; EC 4.1.1.39) is a bifunctional enzyme residing in the stroma or soluble fraction of chloroplasts. In addition to its enzymatic activities of photosynthetic carbon dioxide fixation and photorespiratory carbon oxidation, Rubisco in senescing leaves of plants is the major nitrogen source providing amino acids for developing organs, since the protein comprises as much as half of the total soluble proteins in mesophyll cells of fully expanded leaves (Peoples and Dalling 1988, Feller and Fischer 1994). An understanding of the catabolic mechanism of Rubisco in senescing leaves is thus important to establish the optimal use of leaf nitrogen as the major amino acid source of seed storage proteins in crops such as cereals and legumes (Mae et al. 1984, Peoples and Dalling 1988, Feller and Fischer 1994).

The degradation of Rubisco in senescing leaves has been interpreted from two points of view. One is that the vacuole is the major intracellular compartment involved in the degradation of Rubisco. Lin and Wittenbach (1981) demonstrated localization of Rubisco-degrading proteases in the vacuoles of mesophyll protoplasts of wheat and corn leaves. In ultrastructural studies, Wittenbach et al. (1982) observed the movement of chloroplasts toward the center of the protoplasts from senescing wheat primary leaves and concluded that this implied a physical interaction between the chloroplasts and the vacuole during senescence. Miller and Huffaker (1982) and Thayer and Huffaker (1984) reported the hydrolysis of Rubisco by two vacuolar proteinases from senescing barley leaves. Bhalla and Dalling (1986) also reported the degradation of the large subunit (LSU) of Rubisco

* Correspondence and reprints: Department of Biological Sciences, Tokyo Metropolitan University, Minami-ohsawa 1-1, Hachioji, Tokyo 192-0397, Japan.
** Present address: Department of Infectious Diseases and Tropical Medicine, Research Institute International Medical Center of Japan, Shinjuku, Tokyo, Japan.

by lysed vacuolar preparations from wheat primary leaves. The other viewpoint is that Rubisco is hydrolyzed by proteolytic enzymes inside the chloroplast with the digestion products being exported. Proteolytic activities that reside in the stroma of chloroplasts were observed in leaves of a number of plant species (Dalling et al. 1983, Liu and Jagendorf 1984, Malek et al. 1984, Mae et al. 1989, Musgrove et al. 1989). Ragster and Chrispeels (1981) found proteolytic activity that degraded the LSU of Rubisco in isolated soybean chloroplasts. Mitsuhashi et al. (1992) detected degradation products derived from the Rubisco LSU after incubation of intact pea chloroplasts. Bushnell et al. (1993) purified an LSU-hydrolyzing zinc protease from the stroma of pea chloroplasts. Recently, Desimone et al. (1996, 1998) and Ishida et al. (1997) reported the involvement of active oxygen in the chloroplastic degradation of the LSU of Rubisco. The results of these studies, however, were not necessarily consistent. Experiments using senescing wheat primary leaves (Ono et al. 1995) led to the conclusion that chloroplast proteins are mobilized in two different ways during leaf senescence: by gradual degradation of the proteins inside the chloroplast, and by the successive disappearance of a small population of whole chloroplasts.

Our previous biochemical studies (Yoshida and Minamikawa 1996) provided evidence that the Rubisco LSU of 53 kDa was degraded to produce a 41 kDa polypeptide when incubated with vacuolar lysates prepared from senescing French bean primary leaves. The degradation took place successively through three intermediates of 50, 48, and 42 kDa, which were formed by removal of a small amino-terminal peptide. It was also suggested that the degradation step from the 53 kDa LSU to the 48 kDa polypeptide is mediated by a cysteine proteinase that is the same type of enzyme as SH-EP, the major vacuolar proteinase from the *Vigna mungo* cotyledon (Mitsuhashi and Minamikawa 1989). On the basis of these enzymatic studies, the present ultrastructural and immunocytochemical analyses were designed to examine the possible involvement of vacuolar proteinases in the degradation of the Rubisco LSU with antibodies to the LSU and SH-EP in regard to the interaction between the vacuole and the chloroplasts. The results supported the view that, in senescing French bean leaves detached and placed in darkness, Rubisco is massively degraded through the association of chloroplasts with the central vacuole.

Material and methods

Plant material and incubation of detached leaves

French bean (*Phaseolus vulgaris* L. cv. Goldstar) plants were grown in a phytotron at 25 °C with a photoperiod of 14 h (190 mol photons per $m^2 \cdot s$), and primary leaves of 10-day-old plants were used for experiments. The detached leaves were placed on wet filter paper at 25 °C in darkness for 0, 4, or 8 days.

Gel electrophoresis and immunoblotting

Ten discs (1.1 mm in diameter) from fresh or detached and incubated leaves were homogenized with 2 ml of sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer. The homogenate was boiled for 3 min and centrifuged at 7000 **g** for 15 min. The supernatant was analyzed by SDS-PAGE and immunoblotting as described elsewhere (Tanaka et al. 1993).

Immunocytochemistry and ultrastructural analysis

Fresh or detached and incubated leaves were cut into pieces of ca. 1 mm^2 and fixed with 4% paraformaldehyde and 2% glutaraldehyde in 0.1 M potassium phosphate buffer, pH 7.4, for 4 h at 4 °C. After dehydration of the leaf pieces in a graded methanol series, the pieces were further dehydrated in acetone-methanol (1 : 1), absolute acetone, acetone-methanol (1 : 1), and absolute methanol. Ultrastructural analysis was conducted essentially as described (Hara-Nishimura et al. 1993) with a transmission electron microscope (model 1010EX; JEOL) at 80 kV. For immunochemical analysis, the dehydrated pieces were embedded in the hard formulation of LR White resin, and the ultrathin sections were mounted on nickel grids and blocked with 10% fetal bovine serum as described previously (Toyooka et al. 2000). The sections were then labeled with polyclonal antibodies to LSU (diluted 1 : 100) and affinity-purified 33 kDa SH-EP (1 : 1) or a monoclonal antibody to 33 kDa SH-EP (1 : 1). Antibodies to LSU and SH-EP were prepared in our previous studies (Yoshida and Minamikawa 1996, Toyooka et al. 2000).

Results

Changes in shape and structure of chloroplasts in senescing French bean leaves

When primary leaves of 10-day-old French bean plants were detached and placed on layers of wet filter paper at 25 °C, their senescence proceeded more rapidly than that of the attached leaves, irrespective of illumination (Yoshida and Minamikawa 1996). On days 0, 4 and 8 of the incubation, leaf sections were stained with toluidine blue. Sections from freshly detached (day 0) leaves exhibited cells in which a thin rim of cytoplasm surrounded a large central vacuole (Fig. 1 A). A lot of the chloroplasts were located in the thin layer of cytoplasm. The chloroplasts of day 0 leaves contained starch granules. On day 4 of incubation, the number of chloroplasts in a cell evidently decreased and the chloroplasts became rounded in shape (Fig. 1 B). The relative population of chloroplasts per cell was

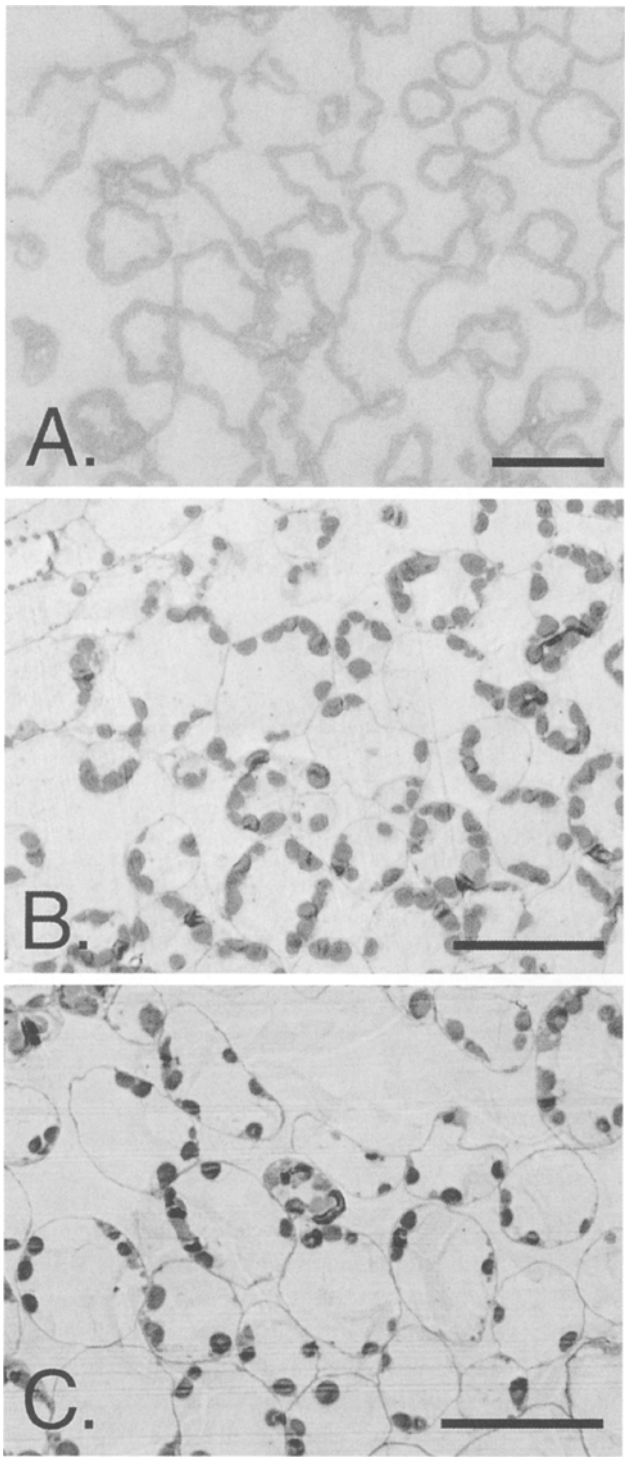

Fig. 1 A–C. Toluidine blue staining of sections from senescing leaves of French bean. Primary leaves were collected from 10-day-old plants, and the detached leaves were placed in darkness for 0 (**A**), 4 (**B**), or 8 days (**C**) as described in Material and methods. **A** The chloroplasts of freshly detached (day 0) leaves located in the thin layer of the cytoplasm and were not directly associated with the vacuole. **B** On day 4, the number of chloroplasts decreased and they became round. **C** On day 8, the number of chloroplasts per cell was further decreased and their shape became globular. Bars: 50 μm

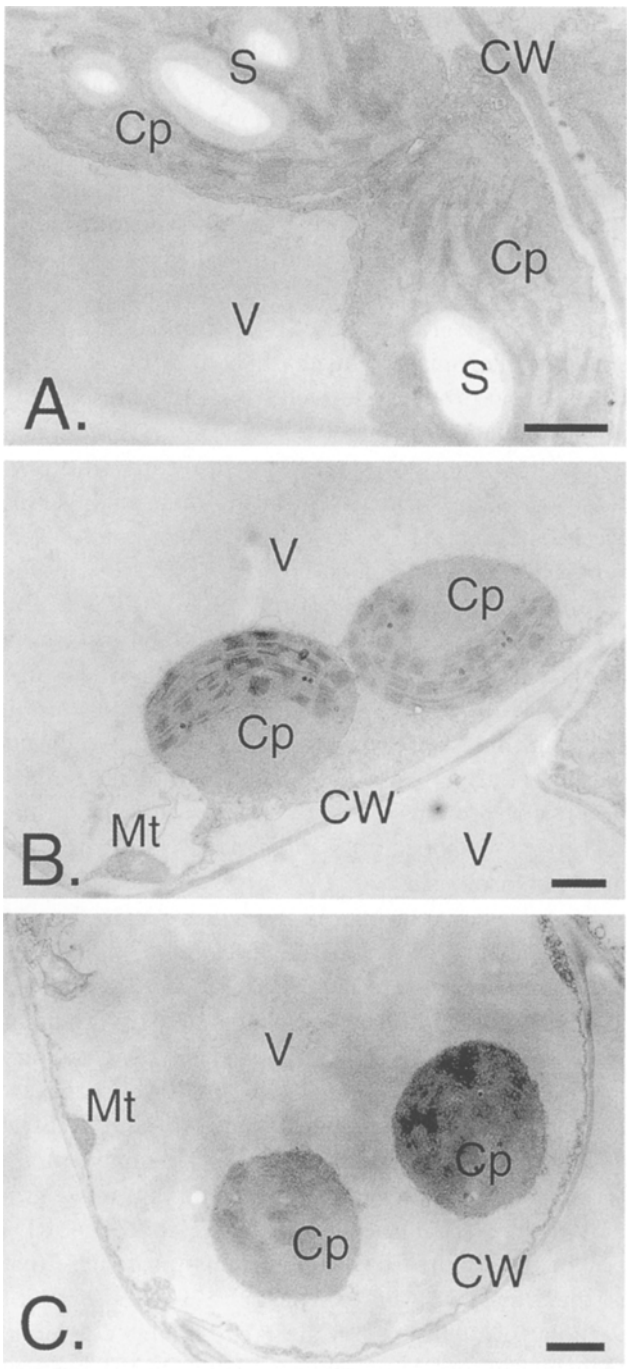

Fig. 2 A–C. Conventional electron micrographs showing changes in the shape and structure of the chloroplasts of senescing French bean leaves. The detached primary leaves collected from 10-day-old plants were placed for 0 (**A**), 4 (**B**), or 8 days (**C**) in darkness. **A** Chloroplasts of day 0 leaves were located in the thin cytoplasm between the large vacuole and the cell periphery. Each chloroplast contained starch granules (*S*). **B** Chloroplasts of day 4 leaves kept in touch with the cytoplasm along the cell periphery but turned roundish. No starch granules were present in the chloroplasts. **C** On day 8, the chloroplasts became globular. Note that the chloroplasts at this senescence stage seemed to have been separated from the cytoplasm and taken into the vacuole. *Cp* Chloroplast; *V* vacuole; *CW* cell wall; *Mt* mitochrondrion. Bars: A and B, 500 nm; C 1 μm

counted to be about 70% with reference to the population on day 0. On day 8 of the incubation, these tendencies became more obvious; the relative population of chloroplasts per cell further decreased to about 45% and the shape of chloroplasts became globular (Fig. 1C). The outer membrane of the chloroplasts might be degraded, but the boundary between the vacuole and the cytoplasm rim could be observed, showing that the tonoplast remained intact.

High-magnification views more clearly showed the structural changes in the chloroplasts during leaf senescence. Chloroplasts of day 0 leaves were enclosed by a thin layer of cytoplasm between the large vacuole and the cell periphery, and each chloroplast contained starch granules (Fig. 2A). Chloroplasts of day 4 leaves still kept in touch with the cytoplasm along the cell periphery but turned roundish (Fig. 2B). The chloroplasts contained grana and thylakoid membrane, but the inner structures were one-sided within the chloroplasts. No starch granules were present in the chloroplasts of day 4 leaves. On day 8 of the incubation, the chloroplasts became globular, and more importantly some of them were found to have been separated from the cytoplasm and taken into the vacuole (Fig. 2C). About 10% of chloroplasts were observed to have been taken into the vacuole in toluidine blue-stained sections from day 8 leaves. The membrane structure in the chloroplasts was maintained but appeared to be disordered.

Apparent internalization of chloroplasts into the vacuole

An apparent sequence in which the chloroplasts were taken into the vacuole is shown in Fig. 3. A globular chloroplast of a day 8 leaf as shown in Fig. 3A still resided within the thin cytoplasm along the cell periphery. The inner structure of the chloroplast remained but was evidently disordered. A chloroplast shown in Fig. 3B looked to be separated from the cytoplasm which contained a mitochondrion. The surface of the outer membrane of the chloroplast seemed to be rough as compared with that of the chloroplast of day 0 leaves (see Fig. 2A). The chloroplast shown in Fig. 3C was distinctly separated from

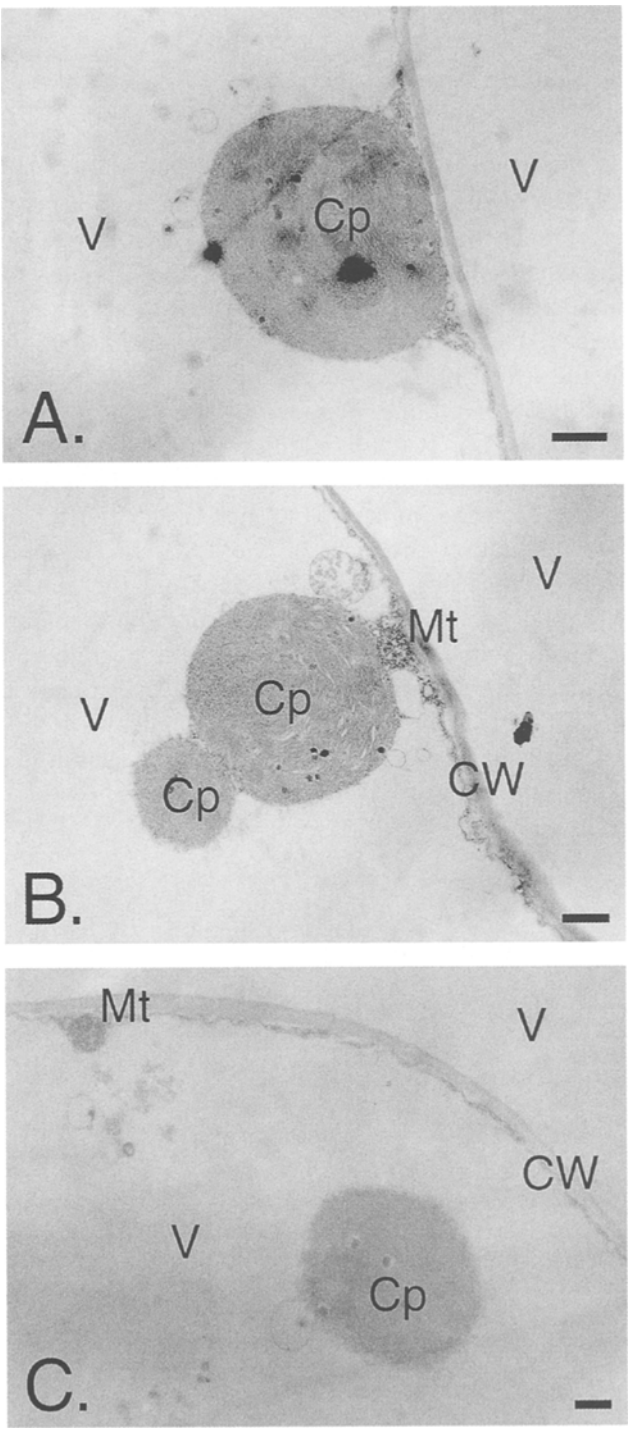

Fig. 3A–C. Electron micrographs showing apparent internalization of chloroplasts into the vacuole in day 8 senescing French bean leaves. The apparent sequence of chloroplasts being taken into the vacuole is reconstructed. **A** A globular chloroplast of day 8 leaf was still present in the thin layer of cytoplasm along the cell periphery. The inner structure of the chloroplast remained, but it was evidently disordered. **B** A chloroplast was about to be separated from the cytoplasm which contained a mitochondrion. The surface of the chloroplast seemed to be rough as compared with that of the chloroplast shown in **A**. **C** The chloroplast taken into the vacuole had a rough surface, showing that the outer membrane had been exposed to degradation by vacuolar enzymes. *Cp* Chloroplast; *V* vacuole; *CW* cell wall; *Mt* mitochondrion. Bars: 500 nm

the thin rim of cytoplasm which contained a mitochondrion. The chloroplast taken into the vacuole had a rough surface, indicating that the outer membrane had been subjected to degradation by vacuolar enzymes. Fragments probably formed from the chloroplasts or other organelles were also contained in the vacuolar matrix (Fig. 3C).

SDS-PAGE and immunoblotting analysis of Rubisco and SH-EP-related cysteine proteinase in senescing leaves

Five or more major polypeptides including those corresponding in size to the 53 kDa Rubisco LSU and 15 kDa SSU were detected in extracts from senescing French bean leaves by SDS-PAGE followed by Coomassie blue staining (Fig. 4A). The amounts of these polypeptides all decreased as the senescence of detached leaves proceeded. The decreases in amounts of the LSU and SSU during senescence were more clearly observed on immunoblots with the respective antibody (Fig. 4B, C). In addition to the 53 kDa LSU, at least five polypeptides smaller than the LSU were detected on the immunoblot. These were thought to be degradation products of the LSU. In the previous study using vacuolar lysates from the leaves, we reported immunological detection and amino-terminal amino acid sequences of four degradation products formed from the LSU with molecular masses from 41 to 50 kDa (Yoshida and Minamikawa 1996). The amount of the SSU was similarly reduced in the senescing leaves. In addition to the 15 kDa SSU, a small amount of a 33 kDa polypeptide appeared on the immunoblot with the antibody to the SSU (Fig. 4C). This immunoreactive polypeptide was thought to be a conjugate of the SSU and another protein.

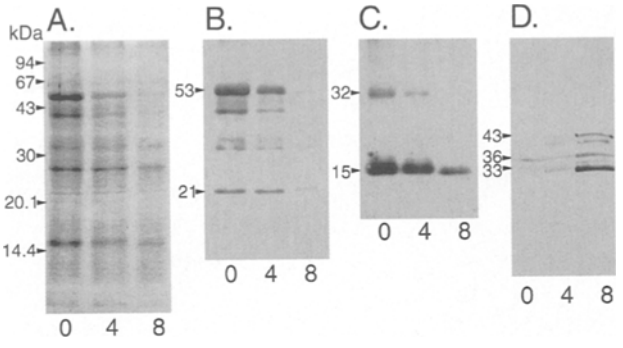

Fig. 4A–D. SDS-PAGE and immunoblotting of extracts from senescing French bean leaves. The detached leaves were placed for 0, 4, or 8 days in darkness, and leaf extracts (2 µl per lane in **A–C**; 20 µl per lane in **D**) were analyzed by SDS-PAGE on 15% (**A–C**) or 12.5% (**D**) gel. The gel was stained with Coomassie blue (**A**). After the gel electrophoresis, the proteins in the gel were blotted on a membrane, which was then probed with antibodies to the Rubisco LSU (**B**) and SSU (**C**) or a monoclonal antibody to 33 kDa SH-EP (**D**)

One of the major cysteine proteinases from French bean, termed EP-C1, is distributed in pods of maturing fruits and various organs of the young plants and is immunologically cross-reactive to SH-EP, the major proteinase isolated from *Vigna mungo* (Tanaka et al. 1993). Both enzymes share 96% homology at the amino acid sequence level and show analogous post-translational processing (Yamauchi et al. 1992). Therefore, we postulated that EP-C1 is one of the major vacuolar enzymes acting to degrade the LSU of Rubisco and used antibodies to SH-EP to detect EP-C1 and related proteinases. With the monoclonal antibody to 33 kDa mature SH-EP, a 43 kDa precursor as well as 36 and 39 kDa intermediates of SH-EP were visualized on the immunoblot in addition to the 33 kDa mature form (Fig. 4D). In contrast to Rubisco, the amounts of these SH-EP-related polypeptides increased during the leaf senescence with the highest level on day 8 of the incubation. As a result of these immunoblot analyses, the antibody to the LSU, the monoclonal antibody to 33 kDa SH-EP, and the polyclonal antibody to 33 kDa SH-EP were used for the following immunogold labeling experiments.

Distribution of LSU and SH-EP-related vacuolar cysteine proteinase in cells of senescing leaves

Immunogold labeling of cells of freshly detached (day 0) leaves indicated that the gold particles conjugated with the antibody to the LSU were specifically distributed in the stroma but not in the thylakoid membranes of the chloroplasts (Fig. 5A). In cells of day 4 leaves, the LSU was also nearly exclusively distributed in the chloroplast which had become rounded as compared to day 0 chloroplasts, but the specific association of the antibody to the LSU with the stroma was not observed probably because of the partial disorder of the inner structure of the chloroplast of the senescing leaf (Fig. 5B). These results would exclude the possibility of nonspecific labeling of leaf cells with the antibody to the LSU used in the present study.

In cells of day 4 leaves labeled with the polyclonal antibody to SH-EP, gold particles were seen in the vacuoles, while a smaller number of the gold particles were localized in the chloroplasts (Fig. 6A). On

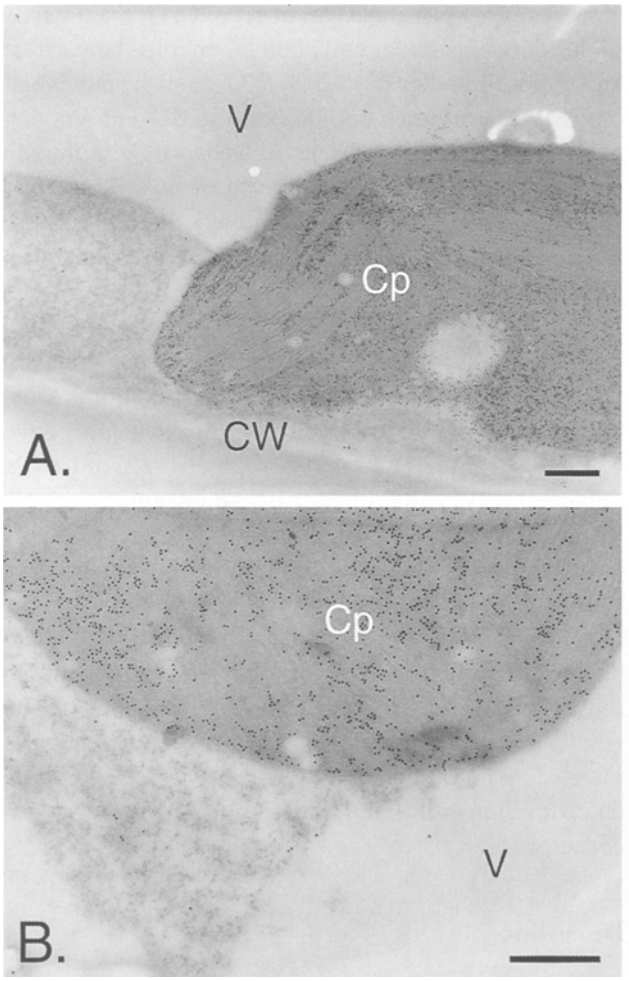

Fig. 5 A, B. Immunocytochemical localization of the Rubisco LSU in the cells of day 0 and day 4 senescing French bean leaves. **A** The stroma, but not the thylakoid membranes, of the chloroplasts of a day 0 leaf were specifically immunogold labeled with the antibody to the LSU. No gold particles were found to be associated with the vacuole. **B** In a cell of a day 4 leaf, the LSU was also nearly exclusively distributed in the chloroplast which had become rounded as compared to the day 0 chloroplast, but the labeling with the antibody to the LSU was not specifically distributed in the stroma probably because of the partial disorder of the chloroplast interior of the senescing leaf. *Cp* Chloroplast; *V* vacuole; *CW* cell wall. Bars: 200 nm

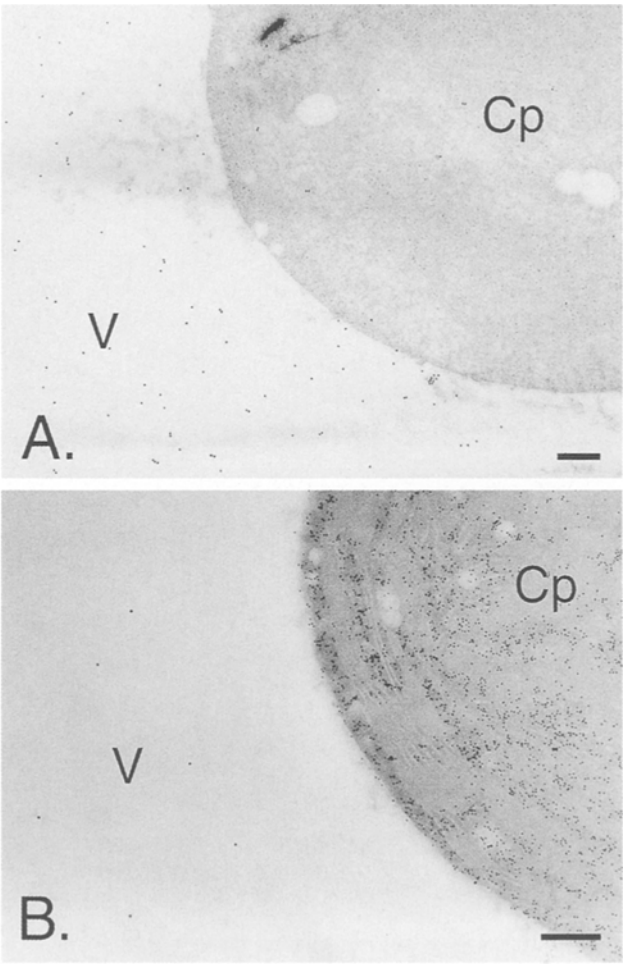

Fig. 6. Electron micrographs showing chloroplasts and vacuoles of day 4 senescing French bean leaves immunogold labeled with polyclonal antibody to 33 kDa SH-EP (**A**) and double labeled with polyclonal antibody to the LSU (10 nm diameter particles) and monoclonal antibody to 33 kDa SH-EP (15 nm diameter particles) (**B**). **A** With the polyclonal antibody to SH-EP, gold particles were seen in the vacuole and a smaller number were localized in the chloroplast. **B** On double immunogold labeling with the antibody to the LSU and the monoclonal antibody to SH-EP, labeling with the antibody to SH-EP was distributed in both the vacuole and the chloroplast. The vacuole was not labeled with the antibody to the LSU. *Cp* Chloroplast; *V* vacuole. Bars: 200 nm

double immunogold-labeling with the antibody to the LSU and the monoclonal antibody to SH-EP (Fig. 6B), the gold particles were distributed in both the vacuole and the choloroplast, although the number of gold particles in the vacuole was much smaller than in that in Fig. 6A. In the following immunocytochemical observations, this double labeling with antibodies to the LSU and SH-EP was exclusively used.

Conventional micrographs indicated that in most cells of day 4 leaves the inner structure of chloroplasts was partly fragmented and taken into the vacuole (Fig. 7A). The fragments in the vacuole were strongly stained with gold particles conjugated with the antibody to the LSU (Fig. 7B, C). In the same micrographs, some labeling with the monoclonal antibody to SH-EP was seen to be localized in the chloroplast in addition to the vacuole. In cells of day 8 leaves, the outer membrane of a globular chloroplast which had been taken into the vacuole was wholly disrupted (see Fig. 3C), and labeling with the monoclonal antibody to SH-EP was frequently distributed in both the vacuole and the chloroplast (Fig. 8A). In a high-magnification

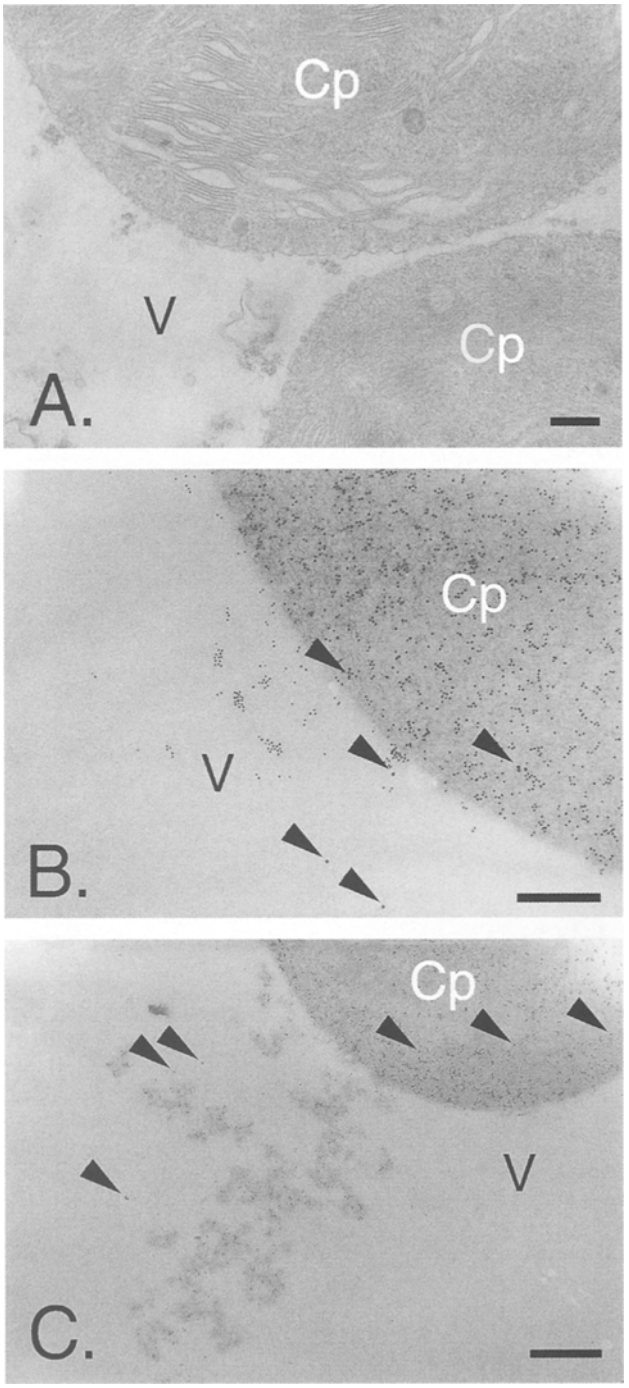

Fig. 7A–C. Electron micrographs showing partly degraded chloroplasts of day 4 senescing French bean leaves. **A** Ultrastructural analysis showed that the chloroplast was partly fragmented and associated with the vacuole. **B** and **C** Immunogold labeling with the antibodies to the LSU (10 nm diameter particles) and SH-EP (15 nm diameter particles). Labeling with the antibody to the LSU was observed in the vacuole as clusters near the surface of the chloroplast (**B**) and on the fragments apparently released from the chloroplast (**C**). Labeling with the antibody to SH-EP was seen to be localized in the chloroplast in addition to the vacuole. Arrowheads indicate 15 nm gold particles. *Cp* Chloroplast; *V* vacuole. Bars: 200 nm

view, the inner structure was observed to remain partly in the chloroplast that had been taken into the vacuole and exposed to digestion (Fig. 8B). In such cells, labeling with the monoclonal antibody to SH-EP was distributed in the chloroplast as well as in the vacuole. In chloroplasts where the breakdown of the interior had proceeded further, few labeling with the antibody to the LSU were localized in the inside of the chloroplast (Fig. 8C).

To confirm the localization of SH-EP-related vacuolar enzymes in the chloroplasts of senescing leaves, the gold particles conjugated with the monoclonal antibody to SH-EP in chloroplasts and vacuoles were counted in four to eight micrographs. In cells of freshly detached leaves, the amount of the enzymes was at a low level (Fig. 4D), and the numbers of the gold particles per square micrometer were smaller than 0.5 in both the chloroplast and the vacuole. On day 4 of the incubation, however, the values increased to 2.2 and 0.9 in the chloroplast and the vacuole, respectively. On day 8, these values further increased to 4.0 and 1.5. The results clearly indicate that SH-EP-related enzymes increase in amount and are localized noticeably in the chloroplast in addition to the vacuole as the leaf senescence proceeds.

Discussion

Distinct changes in morphological features observed in cells of senescing French bean leaves were a decrease in the population of chloroplasts per cell and a turn of the chloroplasts into globular shape (Figs. 1 and 2). The relative chloroplast populations of day 4 and day 8 leaves were about 70% and 45%, respectively, with reference to the population of freshly detached leaves. The structural disorder of the chloroplasts might reflect their change into a globular shape and further the one-sided disposition of inner membranes in the chloroplasts of the senescing leaves. A further noteworthy observation was that about 10% of the chloroplasts in cells of day 8 senescing leaves were separated from the thin layer of the cytoplasm along the cell periphery and taken into the large vacuole (Fig. 2). This observation is consistent with an earlier ultrastructural observation of Wittenbach et al. (1982) demonstrating that, since chloroplasts moved toward the center of mesophyll protoplasts in senescing wheat leaves, they apparently moved into invaginations of the vacuole or were taken into the vacuole. These observations are also supported by the evidence that

decreases in the amount of Rubisco and in the rate of photosynthesis were, to some extent, accompanied by a decrease in the number of chloroplasts per cell during senescence of leaves (Kura-Hotta et al. 1990, Ono et al. 1995). The present results indicated that, under the described experimental conditions, the amount of the LSU seemed to decrease more markedly than the relative population of chloroplasts (Figs. 1 and 4). An apparent process by which the chloroplasts were taken into the vacuole was reconstructed in the present study (Fig. 3). The chloroplast taken into the vacuole had a rough surface, indicating that it came into contact with contents of the vacuole and was subjected to digestion by vacuolar enzymes.

Immunocytochemical analysis using antibodies to the Rubisco LSU and SH-EP more evidently demonstrated the degradation of LSU by vacuolar cysteine proteinases. In these experiments, a monoclonal antibody to 33 kDa mature SH-EP was used together with an antibody to the LSU for double immunogold-labeling of cells of senescing French bean leaves, since SH-EP, a vacuolar cysteine proteinase isolated from *Vigna mungo* cotyledons (Mitsuhashi and Minamikawa 1989), is immunologically identical to EP-C1, one of the major proteinases of French bean plants (Tanaka et al. 1993). In freshly detached leaves, labeling with the antibody to the Rubisco LSU was exclusively distributed in the stroma of chloroplasts (Fig. 5A), but this specific distribution of the LSU became disrupted as leaf senescence proceeded (Fig. 7). In cells of senescing leaves, the chloroplast and its probable fragments taken into the vacuole were immunogold labeled with the antibody to SH-EP in addition to that to the LSU. However, mostly gold particles conjugated with the antibody to SH-EP and

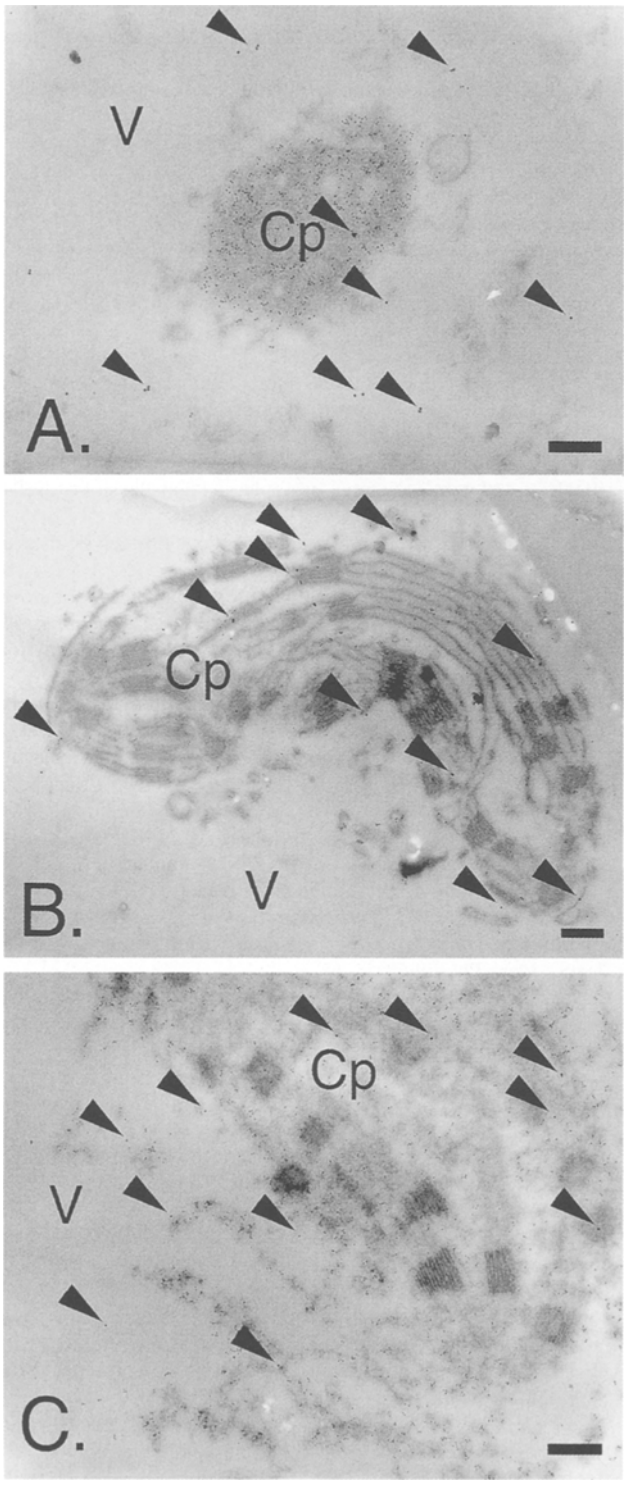

Fig. 8A–C. Electron micrographs showing the distributions of immunogold particles conjugated with the antibody to the LSU (10 nm diameter particles) and the monoclonal antibody to SH-EP (15 nm diameter particles) in the disrupted chloroplasts and the surrounding vacuole of day 8 senescing French bean leaves. **A** In addition to the vacuole, a substantial number of immunogold particles conjugated with the antibody to SH-EP were localized in a chloroplast which was being degraded as a consequence of chloroplast invagination into the vacuole. Labeling with the antibody to the LSU appeared to be specifically localized in the chloroplast. **B** Labeling with the antibody to SH-EP was present in a chloroplast which was flattened probably due to the disrupted outer membrane. A smaller number of gold particles conjugated with the antibody to the LSU were distributed in the chloroplast as compared to the dense labeling observed in **A**. This is interpreted as a result of the release and vacuolar digestion of the stromal LSU. **C** In a cell in which the vacuolar degradation had proceeded, labeling with the antibody to the LSU was present in the disrupted chloroplast and also in fragments probably released from the chloroplast. In such a cell, both the chloroplast and the vacuole were immunogold labeled with the antibody to SH-EP. Arrowheads indicate 15 nm gold particles. *Cp* Chloroplast; *V* vacuole. Bars: 200 nm

only a few particles conjugated with the antibody to the LSU were associated with the chloroplast where further proteolytic degradation took place (Fig. 8). The quantification of the distribution of the gold particles also indicated that the level of SH-EP-related enzymes increased more evidently in the chloroplast than in the vacuole during 8 day leaf senescence.

Our previous biochemical analyses (Yoshida and Minamikawa 1996) showed that SH-EP-related vacuolar proteinase(s) acted to successively remove small amino-terminal peptides from the Rubisco LSU to produce several polypeptides with molecular masses from 41 to 50 kDa when the LSU was incubated with vacuolar lysates prepared from senescing French bean leaves. The present immunocytochemical and ultrastructural observations together with the results of the previous biochemical studies indicate that the chloroplasts of the senescing French bean leaves were separated from the cytoplasm of the cell periphery and were taken into the central vacuole and that Rubisco in the chloroplasts was degraded by vacuolar enzymes such as EP-C1, an SH-EP-related cysteine proteinase, that develop in senescing leaves. Leaf senescence in the whole plant is not a passive catabolic process but a positive process to mobilize the nitrogen sources to the sink in other parts of the plant (Feller and Fischer 1994). The mechanism of the massive degradation of Rubisco in senescing leaves may differ from that of the steady-state turnover of Rubisco in growing or fully expanded leaves, although the present study provided no information on Rubisco catabolism that occurs inside the chloroplast. Further studies using leaves attached to growing plants will be needed to generalize our view on the involvement of the vacuolar proteinases in degrading Rubisco in senescing leaves.

Acknowledgments

We are grateful to Dr. Maki Kondo, National Institute for Basic Biology, for useful discussion and technical advice in electron microscopy. We also thank Dr. Akeo Kadota of our department for valuable discussions.

References

Bhalla PL, Dalling MJ (1986) Endopeptidase and carboxypeptidase enzymes of vacuoles prepared from mesophyll protoplasts of the primary leaf of wheat seedlings. J Plant Physiol 122: 289–302

Bushnell TP, Bushnell D, Jagendorf AT (1993) Purified zinc protease of pea chloroplasts, EP1, degrades the large subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase. Plant Physiol 103: 585–591

Dalling MJ, Tang A, Huffaker RC (1983) Evidence for the existence of peptide hydrolase activity associated with chloroplasts isolated from barley mesophyll protoplasts. Z Pflanzenphysiol 111: 311–318

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

– Wagner E, Johanningmeier U (1998) Degradation of active-oxygen-modified ribulose-1,5-bisphosphate carboxylase/oxygenase by chloroplastic proteases requires ATP-hydrolysis. Planta 205: 459–466

Feller U, Fischer A (1994) Nitrogen metabolism in senescing leaves. Crit Rev Plant Sci 13: 241–273

Hara-Nishimura I, Takeuchi I, Inoue K, Nishimura M (1993) Vesicle transport and processing of the precursor to 2S albumin in pumpkin. Plant J 4: 793–800

Ishida H, Nishimori Y, Sugisawa M, Makino A, Mae T (1997) The large subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase is fragmented into 37-kDa and 16-kDa polypeptides by active oxygen in the lysates of chloroplasts from primary leaves of wheat. Plant Cell Physiol 38: 471–479

Kura-Hotta M, Hashimoto H, Satoh K, Katoh S (1990) Quantitative determination of changes in the number and size of chloroplasts in naturally senescing leaves of rice seedings. Plant Cell Physiol 31: 33–38

Lin W, Wittenbach VA (1981) Subcellular localization of proteases in wheat and corn mesophyll protoplasts. Plant Physiol 67: 969–972

Liu X-Q, Jagendorf AT (1984) ATP-dependent proteolysis in pea chloroplasts. FEBS Lett 166: 248–252

Mae T, Kai N, Makino A, Ohira K (1984) Relation between ribulose bisphosphate carboxylase content and chloroplast number in naturally senescing primary leaves of wheat. Plant Cell Physiol 25: 333–336

– Kamei C, Funaki K, Miyadai K, Makino A, Ohira K, Ojima K (1989) Degradation of ribulose-1,5-bisphosphate carboxylase/oxygenase in the lysates of the chloroplasts isolated mechanically from wheat (*Triticum aestivum* L.) leaves. Plant Cell Physiol 30: 193–200

Malek L, Bogorad L, Ayers AR, Goldberg AL (1984) Newly synthesized proteins are degraded by an ATP-stimulated proteolytic process in isolated pea chloroplasts. FEBS Lett 166: 253–257

Miller BL, Huffaker RC (1982) Hydrolysis of ribulose-1,5-bisphosphate carboxylase by endoproteinases from senescing barley leaves. Plant Physiol 69: 58–62

Mitsuhashi W, Minamikawa T (1989) Synthesis and posttranslational activation of sulfhydryl-endopeptidase in cotyledons of germinating *Vigna mungo* seeds. Plant Physiol 89: 274–279

– Crafts-Brandner SJ, Feller U (1992) Ribulose-1,5-bis-phosphate carboxylase/oxygenase degradation in isolated pea chloroplasts incubated in the light or in the dark. J Plant Physiol 139: 653–658

Musgrove JE, Elderfield PD, Robinson C (1989) Endopeptidases in the stroma and thylakoids of pea chloroplasts. Plant Physiol 90: 1616–1621

Ono K, Hashimoto H, Katoh S (1995) Changes in the number and size of chloroplasts during senescence of primary leaves of wheat grown under different conditions. Plant Cell Physiol 36: 9–17

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

Ragster LE, Chrispeels MJ (1981) Autodigestion in crude extracts of soybean leaves and isolated chloroplasts as a measure of proteolytic activity. Plant Physiol 67: 104–109

Tanaka T, Minamikawa T, Yamauchi D, Ogushi Y (1993) Expression of an endopeptidase (EP-C1) in *Phaseolus vulgaris* plants. Plant Physiol 101: 421–428

Thayer SS, Huffaker RC (1984) Vacuolar localization of endoproteinases EP_1 and EP_2 in mesophyll cells. Plant Physiol 75: 70–73

Toyooka K, Okamoto T, Minamikawa T (2000) Mass transport of proform of a KDEL-tailed cysteine proteinase (SH-EP) to protein storage vacuoles by endoplasmic reticulum-derived vesicle is involved in protein mobilization in germinating seeds. J Cell Biol 148: 453–463

Wittenbach VA, Lin W, Habert RR (1982) Vacuolar localization of proteases and degradation of chloroplasts in mesophyll protoplasts from senescing primary wheat leaves. Plant Physiol 69: 98–102

Yamauchi D, Akasofu H, Minamikawa T (1992) Cysteine endopeptidase from *Vigna mungo*: gene structure and expression. Plant Cell Physiol 33: 789–797

Yoshida T, Minamikawa 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