

Exclusion of Ribulose-1,5-bisphosphate Carboxylase/oxygenase from Chloroplasts by Specific Bodies in Naturally Senescing Leaves of Wheat

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Immunocytochemical electron-microscopic observation indicated that ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco, EC 4.1.1.39) and/or its degradation products are localized in small spherical bodies having a diameter of 0.4–1.2 μm in naturally senescing leaves of wheat (*Triticum aestivum* L.). These Rubisco-containing bodies (RCBs) were found in the cytoplasm and in the vacuole. RCBs contained another stromal protein, chloroplastic glutamine synthetase, but not thylakoid proteins. Ultrastructural analysis suggested that RCBs had double membranes, which seemed to be derived from the chloroplast envelope, and that RCBs were further surrounded by the other membrane structures in the cytoplasm. The appearance of RCBs was the most remarkable when the amount of Rubisco started to decrease at the early phase of leaf senescence. These results suggest that RCBs might be involved in the degradation process of Rubisco outside of chloroplasts during leaf senescence.

Keywords: Chloroplast — Protein degradation — Ribulose-1,5-bisphosphate carboxylase/oxygenase (EC 4.1.1.39) — Senescence — Wheat (*Triticum aestivum*).

Abbreviations: anti-LSU, antibodies against the large subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase; APG, the gene essential for autophagy; LHC II, light-harvesting chlorophyll *ab* protein of PSII; LSU, large subunit; RCB, ribulose-1,5-bisphosphate carboxylase/oxygenase-containing body; Rubisco, ribulose-1,5-bisphosphate carboxylase/oxygenase; SSU, small subunit.

Introduction

Ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco, EC 4.1.1.39) catalyzes two competing reactions, photosynthetic CO₂ fixation and photo respiratory carbon oxidation, in the stroma of chloroplasts. This enzyme is the most abundant protein, accounting for 12–35% of total leaf protein in C₃ plants (Evans and Seemann 1989). In addition, the amount of Rubisco can be a limiting factor for light-saturated photosynthesis under the present atmospheric air conditions (Makino et al. 1984, Makino et al. 1985). Rubisco is mainly synthesized during leaf expansion and its content reaches its maximum soon after full

expansion of the leaf. Thereafter, during leaf senescence, Rubisco is degraded rapidly and its nitrogen is translocated into growing organs (Mae et al. 1983). Therefore, Rubisco degradation is closely related to photosynthesis and nitrogen economy in plants. However, little is known about the degradation process of Rubisco in senescing leaves (for reviews, see Noodén et al. 1997, Hörtensteiner and Feller 2002).

Most or all of the proteolytic activities against Rubisco in mature leaves have been found in the vacuole (Miller and Huffaker 1981, Wittenbach et al. 1982, Thayer and Huffaker 1984, Yoshida and Minamikawa 1996). Wittenbach et al. (1982) proposed that whole chloroplasts are taken up into the vacuole and that Rubisco is then degraded by vacuolar proteases during dark-induced senescence in wheat. Recently, Minamikawa et al. (2001) have reported a similar phenomenon in French bean leaves that were detached and placed in darkness. On the other hand, many previous reports have shown that the decrease of Rubisco content is much faster than the decrease in the number of chloroplasts in leaves during natural or dark-induced senescence (Martinoia et al. 1983, Mae et al. 1984, Wardley et al. 1984, Ford and Shibles 1988, Ono et al. 1995). Thus, it is generally thought that most Rubisco must be degraded into small nitrogenous molecules such as oligopeptides and/or amino acids within the chloroplasts at the early to middle stage of senescence and that the rest is then degraded by vacuolar proteases in the process of autolysis in the last stage of senescence. It is now known that chloroplasts contain a number of different types of proteases and that some of them are able to degrade their various own constituents (for reviews, see Anderson and Aro 1997, Adam et al. 2001). However, chloroplast-localized proteases that are able to degrade Rubisco have not yet been identified and characterized at the molecular level. Indeed, we were able to find only few or weak proteolytic activities against Rubisco in chloroplast fractions isolated from senescing leaves of wheat (Mae et al. 1989, Miyadai et al. 1990, Ishida et al. 1997, Kokubun et al. 2002). It is still not known whether Rubisco is actually degraded into oligopeptides and/or amino acids within the chloroplast.

Recently, Guimét et al. (1999) found that numerous plastoglobuli containing chlorophyll or chlorophyll-derivatives protruded through the chloroplast envelope and emerged into the cytoplasm in senescing soybean leaves. In *Chlamydomonas reinhardtii*, Park et al. (1999) reported the localization of some

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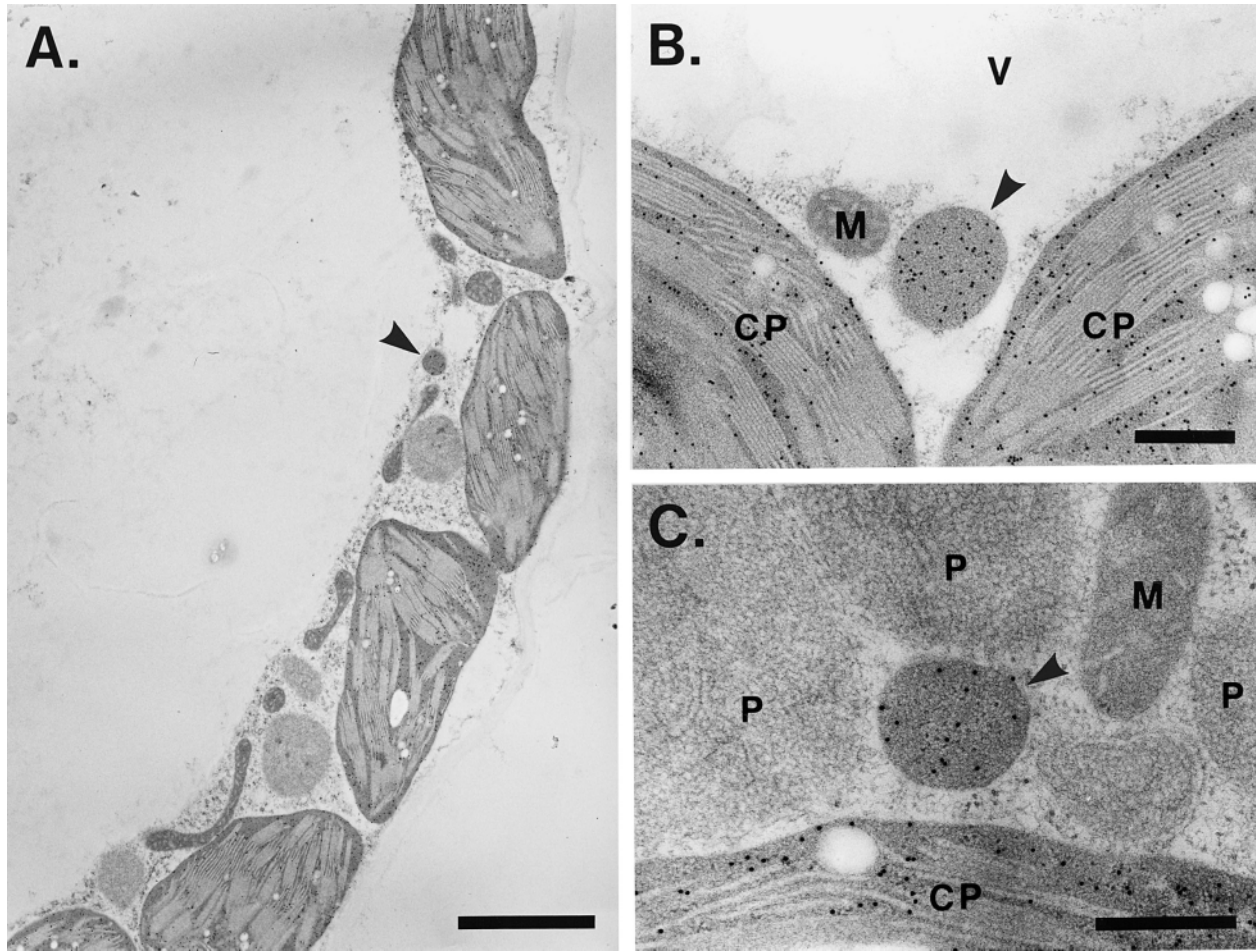


Fig. 1 Immunolocalization of Rubisco-LSU in the mesophyll cells of 21-d-old primary leaves of wheat. Specimens were fixed by the chemical fixation method. (A) Overview of a mesophyll cell. Note that chloroplasts and an RCB (arrowhead) were labeled with gold particles. Bar = 2.0 μm (B, C) RCB has a spherical body and it appears to contain only a stromal portion. Bars = 0.5 μm . CP, chloroplast; M, mitochondrion; P, peroxisome.

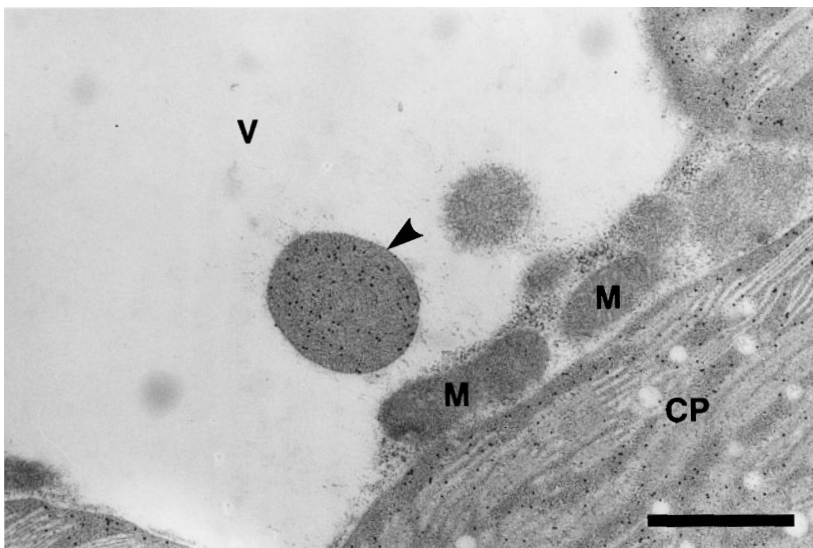


Fig. 2 Localization of the RCB in the vacuole of the mesophyll cell of 21-d-old primary leaf. The specimen was fixed by the chemical fixation method. An arrowhead indicates the RCB. Bar = 1 μm . CP, chloroplast; M, mitochondrion; V, vacuole.

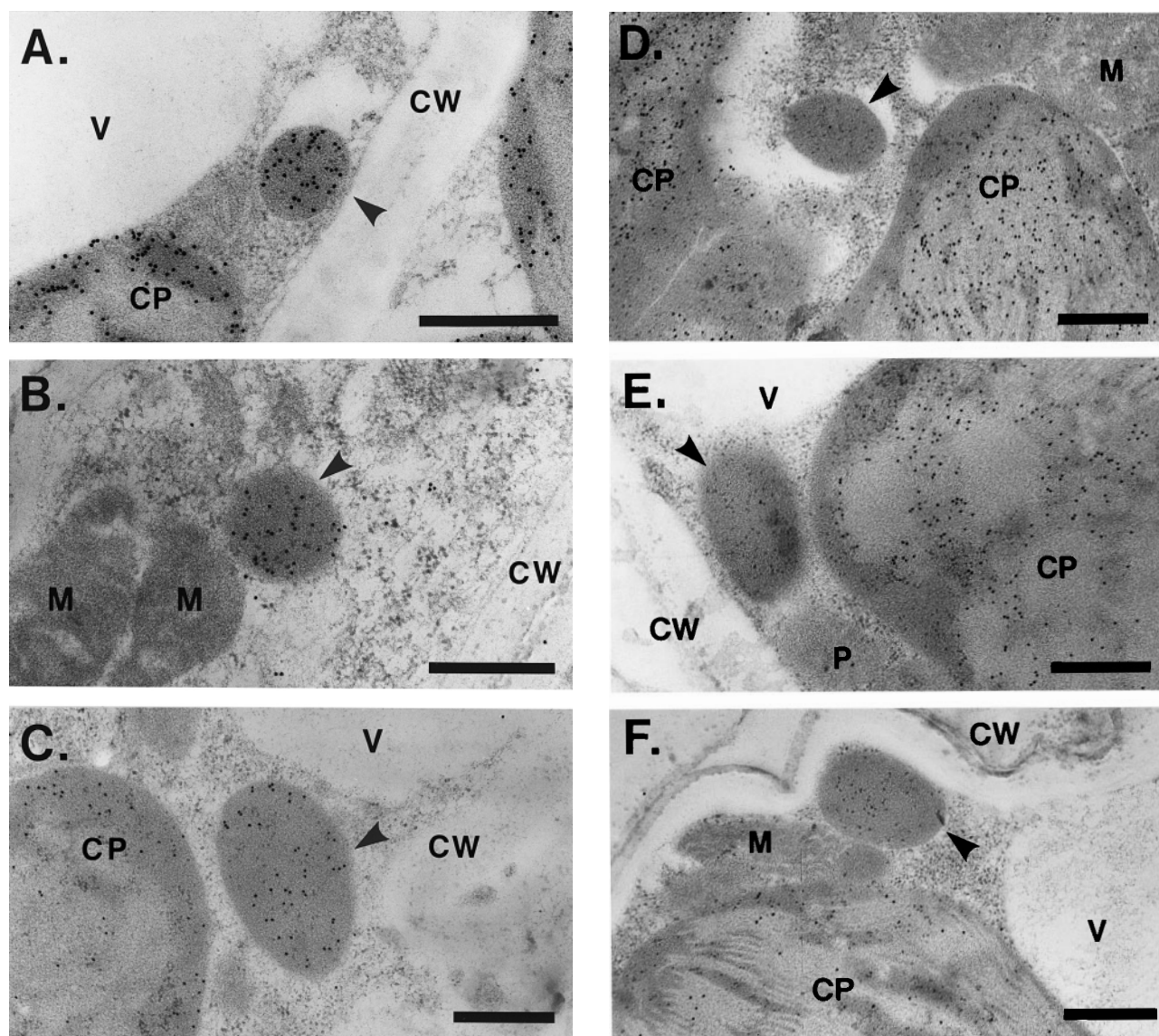


Fig. 3 RCBs existed in the cryofixed leaves. Specimens of 24-day-old primary leaves were prepared by the use of high-pressure freezing and freeze substitution methods and were subjected to immunocytochemistry with specific antibodies against Rubisco-LSU (A), Rubisco-SSU (B), chloroplastic glutamine synthetase (C), LHC II (D), α,β -subunits of coupling factor 1 (ATPase) (E), and cytochrome *f* (F). Arrowheads indicate RCBs. Bars = 0.5 μm . CP, chloroplast; CW, cell wall; M, mitochondrion; P, peroxisome; V, vacuole.

chloroplastic proteins, including Rubisco, in the vacuole. They observed the protrusion of the outer membrane of the envelope that enclosed the stroma. These reports suggest the existence of unknown pathways that exclude chloroplast components to the cytoplasm or to the vacuole for the degradation before the destruction of whole chloroplasts. Therefore, we were interested in whether Rubisco is excluded from chloroplasts in leaves for the degradation during natural senescence and investigated the localization of Rubisco in mesophyll cells of naturally senescing wheat leaves using immunocytochemical electron microscopy. Herein, we show that Rubisco is localized in small spherical bodies located in the cytoplasm and in the vacuole.

Results

Localization of Rubisco in small spherical bodies

The localization of Rubisco in the mesophyll cells of senescent leaves was first examined by immunocytochemical electron microscopy using a standard chemical fixation method (Fig. 1, 2). Antibodies against the large subunit (LSU) of Rubisco (anti-LSU) strongly cross-reacted with the chloroplast stroma as expected (Fig. 1A). In addition, interestingly enough, careful observation revealed that anti-LSU also reacted with the small spherical bodies existed in the cytoplasm (indicated by arrowheads in Fig. 1). These Rubisco-containing bodies were

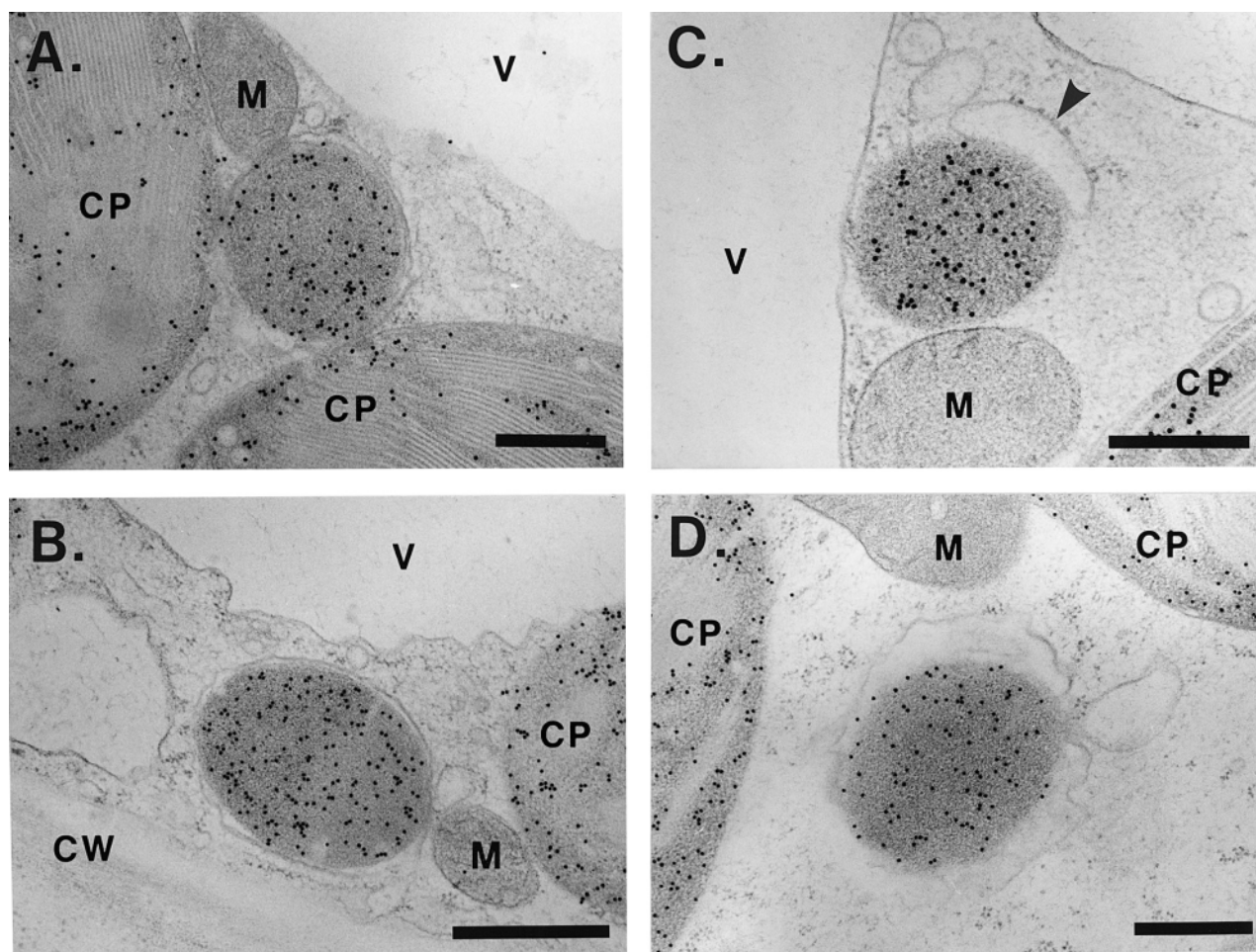


Fig. 4 RCBs existed in the cryofixed leaves post-fixed with osmium tetroxide. The 24-day-old leaves were post-fixed with osmium tetroxide following cryofixation and then were subjected to immunocytochemistry with anti-LSU. (A, B) RCBs surrounded by the other membrane. (C) The membrane structure (arrowhead) surrounding the RCB has ribosomes on its surface. (D) The enlarged form of the membrane structure surrounding the RCB. Bars = 0.5 μm . CP, chloroplast; CW, cell wall; M, mitochondrion; V, vacuole.

named RCBs. Gold signals were rarely seen in other cell compartments such as the cell wall, cytosol, mitochondria, or peroxisomes (Fig. 1). RCBs were also found in the vacuole (Fig. 2). No signals were found when pre-immune sera of rabbits were used as the primary antibody (data not shown). RCBs were 0.4–1.2 μm in diameter and had a staining density similar to that of the stroma and did not include thylakoid structures.

It is well known that cryofixation methods are advantageous for preserving the labile structures and constituents in living cells and for minimizing the creation of artifacts during fixation. When high-pressure freezing and freeze substitution methods were used, the RCB was also found in the cytoplasm (Fig. 3A). The result strongly supported that RCBs were not artificially created during the process of chemical fixation. RCBs were recognized by antibodies against a small subunit (SSU) of Rubisco (Fig. 3B), as well as against chloroplastic glutamine synthetase (Fig. 3C). No signals were found in RCBs when antibodies against thylakoid proteins such as light-

harvesting chlorophyll *alb* protein of PSII (LHC II), α,β -subunits of coupling factor 1 (ATPase), and cytochrome *f* were used as the primary antibodies (Fig. 3D–F). These results clearly suggested that RCB contains only the stromal portion of chloroplasts. Frequently, RCBs were surrounded with a membrane structure in cryofixed specimens that were freeze-substituted with acetone containing 4% (w/v) osmium tetroxide (Fig. 4A, B). It was also found that the membrane structure surrounding RCB has ribosomes on its surface (Fig. 4C) and was enlarged, distorting the enclosed RCB (Fig. 4D). Chloroplast envelopes fixed by the freeze-substitution method did not show dilation within the double membranes usually observed by chemical fixation method due to the artifact (that is, the double membranes created during chemical fixation). Therefore we carried out the ultrastructural analysis of mesophyll cells to observe the double membrane of chloroplast and RCB by the use of specimens chemically fixed with osmium tetroxide (Fig. 5). RCBs themselves had double membranes (arrowheads) as

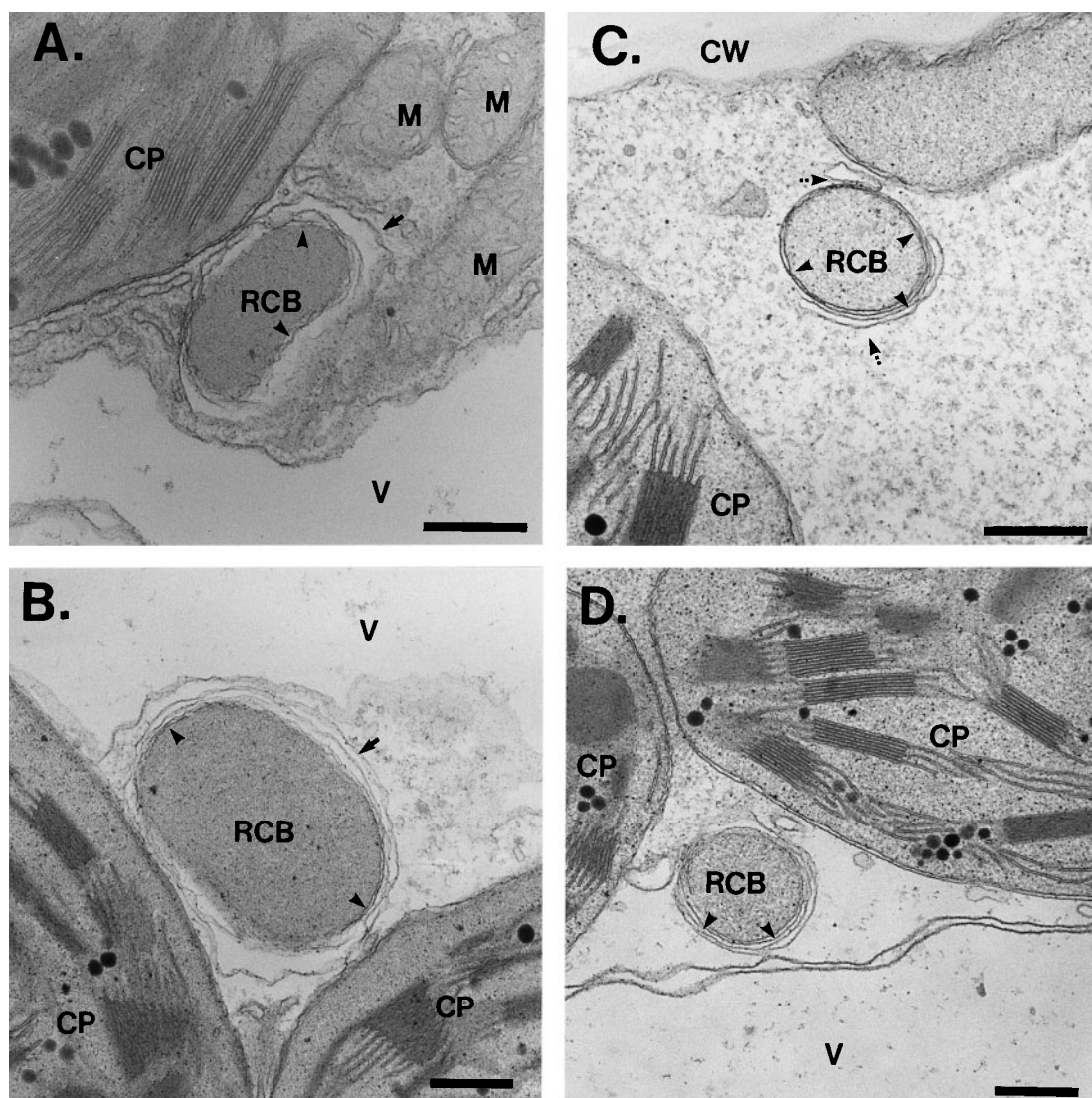


Fig. 5 The ultrastructure of RCB. Specimens of 21-day-old primary leaves (A), the fourth leaves 6 d after the full expansion (B), and the fourth leaves of dark-treated plants (C, D) were prepared by the chemical fixation method with osmium tetroxide. Arrowheads indicate the double membranes of RCB. Arrows indicate membrane structures that surround RCB. Dashed arrows indicate tubular structures that surround RCB. Bars = 0.5 μ m. CP, chloroplast; CW, cell wall; M, mitochondrion; V, vacuole.

the chloroplast had. RCBs were further surrounded by the other membrane structures (arrows in Fig. 5A, B) or tubular structures (dashed arrows in Fig. 5C). Fig. 5D also showed that small vesicles sequester a portion of the cytoplasm including a RCB and the enclosed cytoplasm was degraded, suggesting the activity of autophagy in mesophyll cells of dark-treated plants.

Changes in the number of RCBs during leaf development

Changes in the amounts of Rubisco, chlorophyll, and LHC II and in the number of RCBs during leaf development were investigated (Fig. 6). The amounts of Rubisco, chlorophyll, and LHC II increased in a similar manner during leaf expansion

and reached their maxima at 17 d after sowing. The amount of Rubisco then started to decline rapidly. Amounts of chlorophyll and LHC II remained at the same level until 25 d after sowing and then started to decline. In contrast, the number of RCBs remained at a low level until 17 d after sowing, drastically increased at 21 d after sowing, and then declined rapidly.

Discussion

This is the first report showing the existence of Rubisco (and/or its degradation products)-containing bodies in the cytoplasm and the vacuole of mesophyll cells of higher plants. Although the possible existence of a pathway which excludes

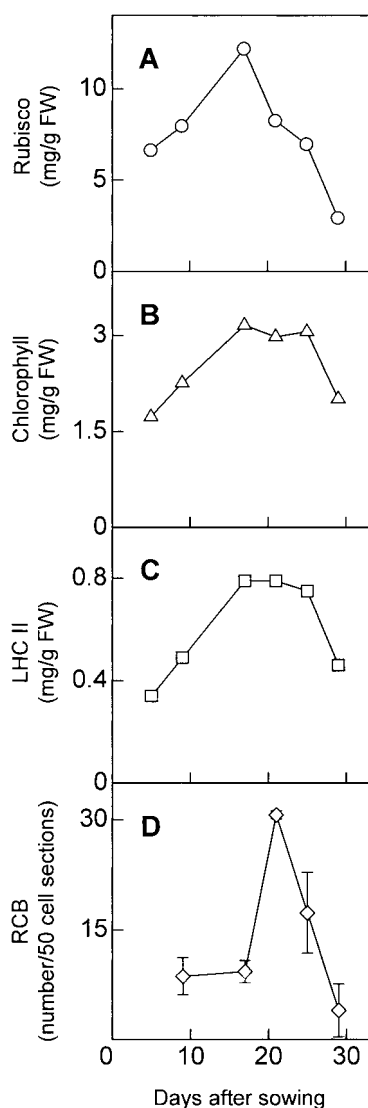


Fig. 6 Changes in the amounts of Rubisco (A), chlorophyll (B), LHC II (C), and in the number of RCBs (D) in the primary leaves of wheat throughout leaf development. In (D), each value is a mean of three different parts of the leaf, \pm SD.

proteins from chloroplasts for their degradation has been hypothesized, no direct evidence has been obtained (Mae et al. 1984, Hörtensteiner and Feller 2002). In this study, we found RCBs in wheat leaves by immunocytochemical electron microscopy using both the chemical fixation method and the high-pressure freezing and freeze substitution method (Fig. 1–4). RCBs seemed to be derived from chloroplasts because they had double membranes as the chloroplasts had (Fig. 5). Moreover, the existence of the other membrane structures surrounding RCBs clearly indicates that the RCB is different from an image corresponding to the end of the chloroplast or stroma-containing protuberances (Köhler et al. 1997, Bourett et al. 1999).

The number of RCBs peaked when the amount of Rubisco started to decline (Fig. 6). RCBs also contained another stromal protein, chloroplastic glutamine synthetase, but did not contain thylakoid membrane proteins such as LHC II, cytochrome *f*, and α , β -subunits of coupling factor 1 (ATPase) (Fig. 3). It is known that the amounts of these stromal proteins, Rubisco and glutamine synthetase, decrease in a similar fashion during leaf senescence (Kamachi et al. 1991). It is reasonable to postulate that RCBs may be responsible for the degradation of stromal proteins during the early stage of leaf senescence. If chloroplasts were taken up into the vacuole, chloroplastic proteins should be immediately degraded into oligopeptides or into amino acids by vacuolar proteases. However, the decrease of Rubisco content was much faster than the decrease of LHC II content during senescence (Fig. 6). This result suggests that these two stromal and thylakoid proteins are degraded in different pathways or manners in mesophyll cells as previously suggested (Mae et al. 1993).

In yeast and in animals, autophagy has been studied extensively as a process for vacuolar or lysosomal degradation of cytoplasmic components under nutrient-deprived conditions (for review, see Klionsky and Ohsumi 1999). The same phenomenon was also observed in cultured plant cells especially under carbon-starved conditions (Chen et al. 1994, Aubert et al. 1996, Moriyasu and Ohsumi 1996), in the cotyledon cells of *Vigna mungo* (Toyooka et al. 2001), and in the root cells of rice (Nishizawa and Mori 1980). The ultrastructural analysis suggests that the autophagy of RCBs is structurally similar to yeast macroautophagy mediated by autophagosome (Fig. 4, 5). However, there have been few reports showing that a part of the organelle is sequestered by an autophagosome, although it is well known that whole organelles such as mitochondria and peroxisomes are also substrates for autophagy in yeast and plants. Recently, *Arabidopsis* mutants having a T-DNA insertion within APGs, which are essential for macroautophagy in yeast, have been characterized (Doelling et al. 2002, Hanaoka et al. 2002). In these APG mutants, the degradation of chlorophyll and chloroplastic proteins including Rubisco was not inhibited, but rather more stimulated than that in the wild type. In addition, it was shown that the abundance of mRNAs and protein for APGs increased in the later stage of senescence when most Rubisco had already been degraded in the wild type. Their results clearly show that chloroplastic components may be degraded by some unknown pathways other than a process that depends on APG genes. Although morphological and immunocytochemical analysis of APG mutants by electron microscopy has not been reported, it is of particular interest whether RCBs are present in senescing leaves of APG mutants.

Rubisco degradation directly affects the photosynthetic ability and nitrogen economy in plants. Therefore, it can be speculated that plants have several pathways for the degradation of Rubisco and that each pathway is strictly regulated throughout the life span of the leaf and under various environmental conditions. The autophagy of the RCB might be one

possible such pathway, and it is important to investigate how much this pathway contributes to Rubisco degradation under various situations in a future study.

Materials and Methods

Plant material

Wheat (*Triticum aestivum* L. cv. Aoba) seeds were planted on a plastic net floating on tap water adjusted to pH 5.5. Seedlings were grown in a phytotron with a day/night temperature of 20°C/18°C and 70% relative humidity. The photoperiod was 12 h, with a quantum flux density of 300 $\mu\text{mol quanta m}^{-2} \text{ s}^{-1}$ (before transplantation) and 500 $\mu\text{mol quanta m}^{-2} \text{ s}^{-1}$ (after transplantation) at plant height. Seven d after sowing, seedlings were transplanted into 3.5 liter pots (10 plants per pot) containing a nutrient solution following that by Makino and Osmond (1991). The nutrient solution was renewed once a week. A half concentration of the nutrient solution was used only for the first week. For the dark treatment, whole plants in their pots were placed for 3 d in the same chamber without illumination 3 d after full expansion of fourth leaves. The Rubisco content of fourth leaves of dark-treated plants declined significantly.

Electron microscopy

Sections of the wheat leaves of about 1 cm at each age were taken 3 cm from the leaf tip. For standard chemical fixation methods, sections were vacuum-infiltrated with a fixative that consisted of 4% (w/v) paraformaldehyde and 1% (v/v) glutaraldehyde in 50 mM Na-cacodylate buffer (pH 7.4) for immunocytochemistry or 2.5% (v/v) glutaraldehyde in the same buffer for the ultrastructural analysis. The sections were then cut into small pieces (1×1 mm) and fixed with a fixative overnight. After they had been washed with the cacodylate buffer, the samples for immunocytochemistry were dehydrated in a graded dimethylformamide series at -20°C and embedded in LR-White resin (London Resin, Berkshire, U.K.). The samples for ultrastructural observation were post-fixed with 1% (w/v) osmium tetroxide for 2 h, dehydrated in a graded ethanol series, replaced to propylene oxide, and embedded in Spurr resin. In addition to chemical fixation, high-pressure freezing and freeze substitution methods were also used. Leaf sections were cut into small pieces, sandwiched between carriers, set into a holder, and then frozen with a high-pressure freezing machine (HPM010S, BAL-TEC, Balzers, FL, U.S.A.). For the freeze substitution, samples were kept for 2 d at -80°C in either acetone containing 4% (w/v) osmium tetroxide or in only acetone, and then the temperature was gradually raised to the room temperature or -20°C, respectively. The former samples were washed with acetone and placed in propylene oxide. These samples were then embedded in Spurr resin. The latter samples were placed in dimethylformamide at -20°C and embedded in LR-White resin. The LR-White resin was polymerized in an ultraviolet polymerizer (TUV-200; Dosaka EM, Kyoto, Japan) at -20°C for 24 h. Ultrathin sections were prepared using an ultramicrotome and mounted on nickel grids. The sections were treated with blocking solution, namely, phosphate-buffered saline containing 1% (w/v) bovine serum albumin and 0.1% (w/v) sodium azide, for 30 min at room temperature. They were then incubated with primary antibodies in blocking solution at 4°C overnight. Sections were washed and then incubated with secondary antibodies (goat anti-rabbit IgG 15 nm gold conjugate, Biocell, Cardiff, U.K.) in blocking solution at room temperature for 30 min. The sections were washed and then stained with 2% (w/v) uranyl acetate and lead citrate. All sections were examined under a transmission electron microscope (H-8100, Hitachi Ltd., Tokyo, Japan) operated at 100 kV.

Antibodies

Anti-LSU and anti-SSU of Rubisco antibodies were prepared from rabbit anti-rice Rubisco antiserum (Makino et al. 1983) by affinity-purification using the LSU and the SSU of Rubisco purified from wheat leaves as ligands (Ishida et al. 1997). Purified anti-LSU and anti-SSU antibodies specifically cross-reacted with the LSU and the SSU in crude extracts from wheat leaves separated by SDS-PAGE, respectively. Rabbit anti-maize plastidic glutamine synthetase antiserum was a gift from Dr. Hitoshi Sakakibara, RIKEN (Sakakibara et al. 1992). Antibodies were affinity-purified from this antiserum using chloroplastic glutamine synthetase purified from wheat leaves (Ishida et al. 2002). These purified anti-chloroplastic glutamine synthetase antibodies cross-reacted with both chloroplastic and cytosolic isoforms of glutamine synthetase in crude extracts from wheat leaves separated by SDS-PAGE. However, the band intensity of the cytosolic isoform (a 41-kDa polypeptide) was approximately one hundredth of that of the chloroplastic isoform (a 44-kDa polypeptide) in an immunoblot of crude extracts of wheat leaves following SDS-PAGE. Antibodies against LHC II, α , β -subunits of coupling factor 1 (ATPase), and cytochrome *f* were as previously described (Hidema et al. 1991, Hidema et al. 1992).

Quantification of chlorophyll, proteins, and RCB

Three-centimeter leaf segments encompassing the sections used for the specimens of electron microscopy were homogenized in 50 mM Na-phosphate buffer (pH 7.2) containing 2 mM mono-iodoacetic acid, 0.7% (w/v) 2-mercaptoethanol and 5% (w/v) glycerol in a chilled mortar and pestle. Chlorophyll was determined by the method of Arnon (1949). Rubisco and LHC II were determined by the method of Makino et al. (1986) using purified wheat Rubisco (Ishida et al. 1997) for Rubisco and bovine serum albumin for LHC II as standards, respectively. The number of RCB per 50 cell sections was counted with ultrathin sections viewed in the transmission electron microscope. To distinguish RCB from other organelles, ultrathin sections were immunostained with anti-LSU as described above. We defined a spherical body that contains the LSU and that has similar electron density to the chloroplast stroma and no thylakoid structures as RCB.

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