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Full Length Research Paper

Effects of ATP and Zn^{2+} on degradation of the large subunit of ribulose-1, 5-bisphosphate carboxylase/oxygenase in wheat leaves

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Ribulose-1, 5-bisphosphate carboxylase/oxygenase (Rubisco) degradation is a complex process involving a multitude of proteolytic pathways, and its mechanism is still unclear now. We previously confirmed that a new senescence-associated protease in the stroma of chloroplasts from senescing leaves was suggested to be involved in the appearance of a 51 kDa fragment of Rubisco. In this study, the 51-kDa fragment was also detected when the crude extracts of mature leaves were incubated in Tris-HCl buffer (pH 7.5) with 1 mM ATP or the chloroplast lysates of mature leaves were incubated in the same buffer with 1 mM ATP and 1 µM Zn^{2+} for 1.5 h. However, the special degradation of large subunits (LSU) in the crude extracts of senescing leaves seemed to not to be affected by ATP and Zn^{2+} presence. Furthermore, the 51-kDa fragment could be detected when chloroplast lysates of senescing leaves were incubated in pH 7.5 Tris-HCl buffer containing 1 mM ATP and 1 µM Zn^{2+} only for 0.5 h, but it would be at least for 1 h if the buffer did not contain 1 mM ATP and 1 µM Zn^{2+}. The results from this study implied that there would be two types of protease, which could specially degrade LSU into 51-kDa fragment, in cytoplasm or vacuole and in chloroplast of mature leaves respectively before leaf senescence.

Key words: ATP, Zn^{2+}, Rubisco, protein degradation, wheat.

INTRODUCTION

Ribulose-1, 5-bisphosphate carboxylase/oxygenase (Rubisco; EC 4.1.1.39), the most abundant protein in plants, is present at very high levels in photosynthesizing cells. It is a hexadecamer composed of eight chloroplast-encoded large subunits (LSU) and eight nuclear-encoded small subunits (SSU). Rubisco is also considered as a protein reserve that can be mobilized during leaf senescence or when the plants are deficits of either nitrogen or carbohydrates. As such, environmental stresses impact Rubisco concentration and mobilization. The degradation of Rubisco is closely related to the rate of photosynthesis as well as nitrogen economy in leaves (Feller and Fischer, 1994; Liu et al., 2008). An understanding of degradation mechanism of Rubisco is thus important to establish the optimal utilization of leaf nitrogen as the major amino acid source of seed storage proteins in crops. The decrease in Rubisco holoenzyme is accompanied by a decline in the transcript level of the enzyme as well as of other photosynthetic proteins (Crafts-Brandner et al., 1996). The different size fragments of LSU degradation under different condition have been reported. For example, the LSU from wheat could be modified and become more susceptible to

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Abbreviations: AEBSF, 4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride; **ATP,** adenosine-triphosphate; **CBB,** Coomassie Brilliant R-250; **E-64,** transepoxysuccinyl-L-leucylamido (4-guanidino) butane; **LSU,** large subunits; **Rubisco,** ribulose-1,5-bisphosphate carboxylase/oxygenase ; **SSU,** small subunits.

proteolysis by reactive oxygen species, where 37 and 16-kDa fragments could be detected (Ishida et al. 1997, 1998); and 20 to 43-kDa's fragments of LSU under chilling stress were identified by proteomics analysis (Yan et al., 2006). Furthermore, the involvement of endopeptidases in Rubisco catabolism (at least under certain conditions) was validated by several laboratories. Such as, a metallo-endopeptidase may play an important role in Rubisco degradation within intact pea chloroplasts (Bushnell et al., 1993), and a DNA-binding aspartic protease which can degrade Rubisco in senescent leaves of tobacco *(Nicotiana tabacum)* (Kato et al., 2004, 2005). Additionally, vacuole contains a number of protein hydrolases and has the functions being similar to the animal lysosome (Yoshida and Minamikawa, 1996). So it has been studied how the proteases in vacuole interact with Rubisco in chloroplast and how they might affect each other. Minamikawa et al. (2001) reported that the chloroplasts were transferred into the vacuole in senescing French bean (*Phaseolus vulgaris*) leaves and the LSU was degraded by vacuolar enzymes. However, Chiba et al. (2003) reported that Rubisco was excluded from chloroplasts by specific bodies in naturally senescing wheat leaves. Recently, Ishida et al. (2008) reported that Rubisco and stroma-targeted fluorescent proteins could be mobilized to the vacuole through an ATG gene-dependent autophagic process without prior chloroplast destruction. However, the particular mechanism of Rubisco degradation in leaves, especially in the chloroplast, remains to be elucidated (Houtz and portis, 2003).

Many energy-dependent proteases (Shanklin et al., 1995; Ostersetzer et al., 1996; Roulin and Feller, 1997) and ATP-dependent degradation of specific proteins (Reinbothe et al., 1995; Desimone et al., 1998) have been reported. Some proteases have even been partially purified and biochemically characterized (Musgrove et al., 1989; Huffaker, 1990; Kuwabara et al., 1992). Whatever, it is generally thought that the initial step of Rubisco degradation in leaves occurs within the chloroplasts. Recently, in our laboratory a 51-kDa fragment of LSU was detected and should be a product of a senescence-associated protease in the stroma of wheat chloroplast (Rui and Xu, 2004; Zhang et al., 2005, 2007). However, its particular mechanism is still unclear. In this study, effects of ATP and Zn^{2+} on degradation of LSU in mature wheat leaves and senescing wheat leaves were investigated. The results from this study implied that there might be two types of protease which could specially degrade LSU into 51-kDa fragment, in cytoplasm or vacuole and in chloroplast of the mature wheat leaves, respectively.

MATERIALS AND METHODS

Materials

Wheat (*triticum aestivum* L. cv. Yangmai 158) seeds were planted in pots with sandy clay and watered frequently with Hoagland's complete nutrient solution. Plants were grown in natural environment. Samples were harvested approximately four weeks after planting. The wheat seedlings with five leaves were sampled. The leaves from down to up were named as 1st, 2nd, 3rd, 4th and 5th leaf according to Zhang et al. (2001). The fourth fully expanded leaves were used as samples. For dark-induced senescence leaves, the 4th leaves were cut into small segments (1 cm in length), then the leaf segments were put in the beakers covered with wet cloth and stored in dark at room temperature for 48 h.

Leaf crude extracts

The leaf crude extracts of mature or dark-induced senescing leaves were prepared by grinding at a ratio of 1 g leaves: 5 ml Tris-HCl buffer (50 mM Tris-HCl, pH 7.5) containing 3 mM β-mercaptoethanol, 1% PVP and some sand quarts in ice bath. Homogenates were centrifuged at 10,000 × g for 30 min at 4°C without filtration. The supernatants were used as the leaf crude extracts.

Isolation and identification of intact chloroplasts

Isolation of intact chloroplast was carried out according to Miyadai et al. (1990) with minor modifications. Mature leaves or senescing leaves (10 g) were cut into small pieces, and homogenized with 50 ml of a semi-frozen buffer containing 50 mM Hepes-KOH (pH 7.6 at 4°C), 0.33 mol/L sorbitol, 2 mM Na_2-EDTA, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 2 mM $NaNO_3$, 1 mM NaH_2PO_4 and 2 mM Na-isoascorbate (grinding buffer). The homogenate was passed through four layers of cheesecloth, and centrifuged for 60 s at 500 g with a high speed refrigerated centrifuge (Beckman).

The supernatant was used and re-centrifuged for 60 s at 1800×g. The pellets were washed twice with 2 ml grinding buffer, the precipitates were suspended in the 2 ml grinding buffer, and further purified by centrifugation. The pellets were subsequently mixed with a solution containing 50% (V/V) Percoll (Sigma), 3% (W/V) PEG6000, 1% (W/V) Ficoll (Sigma) and the same ingredients as the grinding buffer. The mixture was centrifuged at 30,000 g for 30 min and the lower green band was recovered. This fraction was diluted with grinding buffer and centrifuged at 2,000 g for 10 min. The pellet containing intact chloroplasts was re-suspended in the grinding buffer. All procedures were carried out between 0 and 4°C. The integrity of chloroplasts was examined by the ferricyanide photo-reduction assay and phase-contrast microscopy (Walker et al., 1987). The result of isolation yield, 80% recovery of intact chloroplasts on average, was verified by both methods above.

Thermolysin treatment of isolated chloroplasts was carried out according to Miyadai et al. (1990), and then the chloroplasts treated in 1 ml of 1 mol/L glucose were collected by centrifugation at 1,800 g for 50 s.

Separation of stroma and membrane fraction of chloroplasts

The intact chloroplasts were ruptured in a hypotonic buffer (HEPES–NaOH, pH 7.5) containing chloramphenicol (50 µg ml^{-1}), and the solutions of ruptured chloroplast were used as the chloroplast lysates. Stroma and membrane fractions were separated from the lysates by ultracentrifugation at 100,000 g for 60 min according to Kokubun et al. (2002). The membrane fraction was washed twice and suspended in the grinding buffer.

Different ATP or/and Zn^{2+} treatments

The leaf crude extracts or chloroplast lysates were incubated in pH

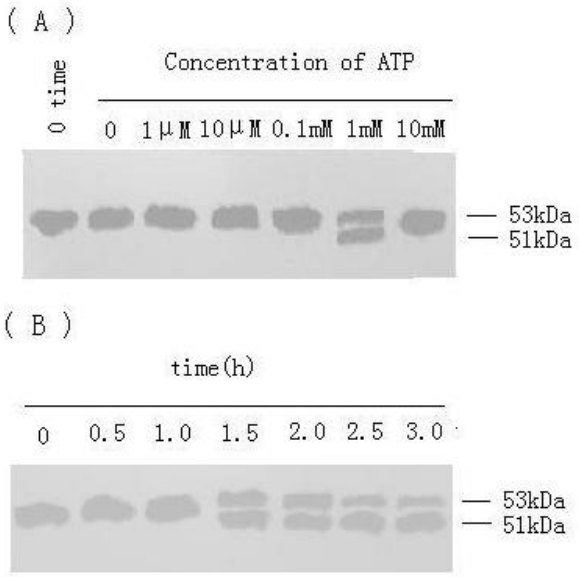

Figure 1. Effects of ATP on the LSU degradation in the leaf crude extracts of mature leaves. The leaf crude extracts of mature leaves were incubated at 35°C (A) in pH 7.5 buffers containing different concentration of ATP: 0 µM, 1 µM, 10 µM, 0.1 mM, 1 mM and 10 mM, for 1.5 h. (B) in pH 7.5 buffers containing 1mM ATP for 0, 0.5, 1.0, 1.5, 2.0, 2.5 and 3.0 h, respectively.

7.5 buffers with different concentrations of ATP or/and Zn^{2+} as follows: the leaf crude extracts were treated for 1.5 h in pH 7.5 buffers containing 1, 10, 100 µM, 1 and 10 mM ATP. The chloroplast lysates were also treated in pH 7.5 buffers containing different concentrations of ATP the same as above, or containing 1, 10, 100 µM, 1 and 10 mM Zn^{2+}, or containing 1 mM ATP and 1, 10, 100 µM, 1 and 10 mM Zn^{2+} for 0, 3, 6, 9 and 12 h.

Different pH treatments

To test effects of different pH on the LSU degradation, the leaf crude extracts were incubated at 35°C for 1.5 h in buffers containing 1 mM ATP at pH 5.5, 6.0, 6.5, 7.0, 7.5, 8.0 and 8.5, respectively, or the chloroplast lysates were incubated at 35°C for 6 h in buffers containing 1 mM ATP and 1 µM Zn^{2+} at pH 5.5, 6.0, 6.5, 7.0, 7.5, 8.0 and 8.5.

Different temperature treatments

Effects of temperature on the formation of the degradation product were examined by treating the leaf crude extracts in pH 7.5 buffers containing 1 mM ATP for 1.5 h at 25, 30, 35, 40, 45 and 50°C, or the chloroplast lysates in pH 7.5 buffers containing 1 mM ATP and 1 µM Zn^{2+} for 6 h at 25, 30, 35, 40, 45 and 50°C.

Different class-specific protease inhibitor treatments

Five protease inhibitors were used to examine the type of the protease triggering the LSU degradation in the leaf crude extracts or chloroplast lysates. The following inhibitors were used. 4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride (AEBSF: 10 mM, Sigma, St Louis. MO, USA), transepoxysuccinyl-L-leucylamido (4-guanidino) butane (E-64: 25 µM, Sigma), leupeptin (10 µM, Sigma), pepstatin (25 µM, Sigma), 1, 10-phenanthroline (10 mM, Sigma). After these inhibitors were added to same volume of leaf crude extracts or chloroplast lysates respectively, the mixtures were pre-incubated on ice for 30 min according to the method of Michaud et al. (1993) and then incubated at the indicated conditions as described above prior to electrophoresis.

SDS-PAGE and immunoblotting

According to Laemmli et al. (1970), the running gel and stacking gel contained 12.5% and 4.5% acrylamide, respectively. After electrophoresis, the gels were subjected to staining with Coomassie Brilliant R-250 (CBB), or to immunoblotting. For immunoblotting, the separated polypeptides on the SDS-PAGE gel were electrophoretically transferred to a nitrocellulose membrane with an electroblotting apparatus (Biometra B33). The membrane was reacted with antiserum raised against the Rubisco, and further reacted with the secondary antibody with horseradish peroxidase-conjugated goat anti-rabbit IgG (Sigma).

Tricine-SDS-PAGE

According to the method of Schagger et al. (1987) with minor modification, the separating gel, “spacer” gel and stacking gel contained 16.5, 10 and 4% acrylamide, respectively. Besides, 6 mol/L urea was used to increase the resolution effect below 5-kDa. After electrophoresis, the gels were fixed, stained with Coomassie Brilliant Blue R-250 (CBB) and distained rapidly to avoid the elution of small molecular polypeptides.

Purification of Rubisco and preparation of anti-LSU antiserum

Rubisco was purified from the mature leaves as described previously (Desimone et al., 1996). Anti-LSU antiserum was prepared according to the method of Miyadai et al. (1990).

RESULTS

LSU degradation in the crude extracts of mature leaves

When the crude extracts of mature leaves were incubated at 35°C for 1.5 h in Tris-HCl buffer (pH 7.5) with 1, 10 µM, 0.1, 1 and 10 mM ATP, respectively, the 51 kDa fragment of LSU was detected only when the reaction system contained 1 mM ATP (Figure 1A). Subsequently, when the leaf crude extracts were incubated in the same buffer with 1 mM ATP for 0.5, 1.0, 1.5, 2.0, 2.5 and 3.0 h, the 51-kDa fragment existed only after the leaf crude extracts were incubated for 1.5 h, and the decrease of 53-kDa LSU amount and increase of 51-kDa fragment formation were simultaneously observed during the time course of Rubisco degradation (Figure 1B). However, the 51-kDa fragments were not detected when the crude extracts were pre-treated at 100°C for 5 min and then incubated in the same buffer with 1 mM ATP for more than 1.5 h (data not shown). The

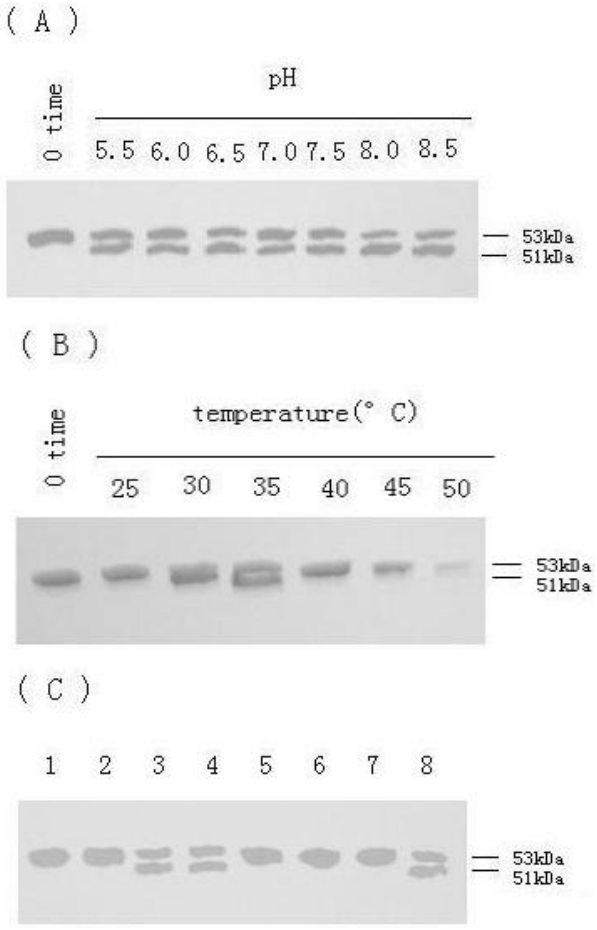

Figure 2. Effects of pH, temperature and different protease inhibitors on the appearance of the 51-kDa fragment in the leaf crude extracts of mature leaves. Degradation product of Rubisco was detected by immunoblotting after SDS-PAGE. Lane 1 in A and B: control. Lanes 2-8 in A represent the leaf crude extracts of mature leaves were incubated in pH 5.5, 6.0, 6.5, 7.0, 7.5, 8.0 and 8.5 buffers containing 1mM ATP at 35°C for 1.5 h, respectively. Lanes 2-8 in B represent the leaf crude extracts of mature leaves were incubated in pH 7.5 buffers containing 1 mM ATP at 25, 30, 35, 40, 45 and 50°C for 1.5 h respectively. Lanes in C represent the leaf crude extracts were incubated in pH 7.5 buffers containing 1mM ATP for 1.5 h after the leaf crude extracts were pre-incubated on ice for 30 min with different inhibitors. Lane 1, no inhibitor and without incubation; lane 2, no inhibitor; lane 3, 1, 10-phenanthroline (10 $mmolL^{-1}$); lane 4, E-64 (25 $\mu molL^{-1}$); lane 5, AEBSF (10 $mmolL^{-1}$); lane 6, leupeptin (10 $\mu molL^{-1}$); lane 7, pepstatin (25 $\mu molL^{-1}$).

results suggested that there would be a protease, which could specifically degrade 53-kDa LSU to 51-kDa fragment in pH 7.5 Tris-HCl buffer under appropriate concentration of ATP, in the mature leaves.

To further characterize the protease degrading LSU into 51-kDa fragment, effects of pH, temperature and different class-specific protease inhibitors on the appearance of the 51-kDa fragment in the mature leaf extracts were tested. It was found that the 51-kDa fragments were detected in the buffers of pH values from 5.5 to 8.5 at 35°C (Figures 2A and B), and the optimal temperature was about 35°C (Figure 2B). Effects of different class-specific protease inhibitors on the appearance of the 51-kDa fragment were shown in Figure 2C, the results showed that E-64 (a cysteine proteinase inhibitor), AEBSF (a serine proteinase inhibitor) and leupeptin (a serine and cysteine proteinase inhibitor) could inhibit the appearance of the 51-kDa fragment.

LSU degradation in the crude extracts of dark-induced senescing leaves

When the leaf crude extracts of dark-induced senescing leaves were incubated at 35°C for 1.5 h in the pH 7.5 buffers containing different concentrations of ATP as described above, the 51 kDa fragments of LSU were detected in the all experiments, and there were no obvious differences between control (0 µM ATP) and those treated with different concentrations of ATP (Figure 3A). Furthermore, the leaf crude extracts of dark-induced senescing leaves were incubated at 35°C in pH 7.5 buffers without or with 1 mM ATP for 0, 0.5, 1.0, 2.0 and 3.0 h. It was also found that the 51 kDa fragments of LSU were detected in the all experiments, there were no obvious differences between control (0 mM ATP) and those treated with different concentrations of ATP (Figure 3B). The results indicated that the LSU degradation seemed not to be affected by ATP presence in the crude extracts of dark-induced senescing leaves.

LSU degradation in the chloroplast isolated from mature leaves

The chloroplast lysates of mature leaves were incubated at 35°C for 6 h in pH 7.5 buffers with 1, 10 µM, 0.1, 1 and 10 mM ATP, or in pH 7.5 buffers with 1 mM ATP for 0, 3, 6, 9 and 12 h, respectively. It showed that the presence of 1 µM to 10 mM ATP had no effect on the LSU degradation in chloroplast lysates incubated even for 12 h (Figures 4A and B). Subsequently, the chloroplast lysates were incubated at 35°C in pH 7.5 buffers containing 1 mM ATP and 1 , 10, 100µM, 1 and 10 mM Zn^{2+}, or in pH 7.5 buffers with 1 mM ATP and 1 µM Zn^{2+} for 0, 3, 6, 9 and 12 h, respectively. The results showed that 51-kDa fragment of LSU was detected only when the reaction system contained 1mM ATP and 1µM Zn^{2+} synchronously after 6 h incubation (Figures 4C and D). However, no matter whether Zn^{2+} was added or not, the 51-kDa fragment of LSU was not detected in the reaction system without 1 mM ATP, and the amount of 51-kDa LSU decreased rather quickly in the reaction system when the concentration of Zn^{2+} was over 1 mM (Figure 4E).

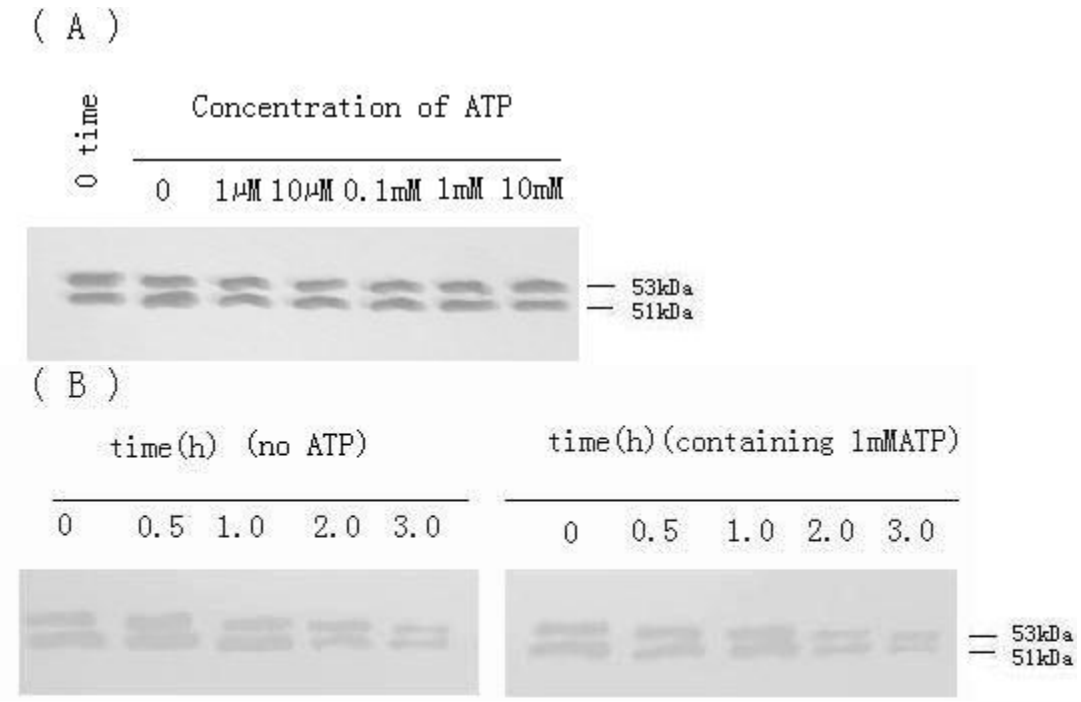

Figure 3. Effect of ATP on the LSU degradation in the leaf crude extracts of senescing leaves. The leaf crude extracts of senescing mature leaves were incubated at 35°C, (A) Lane 1 in A : control. Lanes 2-8 in A represent the leaf crude extracts of senescing leaves were incubated in pH 5.5, 6.0, 6.5, 7.0, 7.5, 8.0 and 8.5 buffers (B) in pH 7.5 buffers for 0, 0.5, 1.0, 2.0 and 3.0 h or in pH 7.5 buffers with adding 1 mM ATP for 0, 0.5, 1.0, 2.0 and 3.0 h, respectively. After incubation, the leaf crude extracts of senescing mature leaves were analyzed by immunoblotting following SDS-PAGE.

Effects of pH, temperature and different class-specific protease inhibitors on the degradation of the LSU in chloroplast lysates of mature leaves were also investigated. It was found that there was no 51-kDa fragment in the reaction system at pH below 7.0, but the fragment appeared at the alkalescence pH (7.5 to 8.5) (Figure 5A). The optimal temperature was 30-35°C. However, the degradation speed of both LSU and 51-kDa fragment, especially the latter 51-kDa fragment, increased when the incubation temperature was above 40°C (Figure 5B). The sensitivities of LSU degradation in the chloroplast lysates to different class-specific protease inhibitors were also examined. The results showed that not only AEBSF, or leupeptin, but also 1, 10-phenanthroline (a metallo-protease inhibitor) inhibited the appearance of the 51-kDa fragment in the chloroplast lysates. However, E-64 had no inhibition to this reaction (Figure 5C).

Furthermore, the chloroplast lysates from mature leaves were separated into stroma and membrane fraction by ultracentrifugation. The stroma and membrane fractions supplemented with purified Rubisco were incubated at 35°C in pH 7.5 buffers with 1 mM ATP and 1 μM Zn^{2+} for 0, 3, 6 and 9 h. After incubation, the samples were analyzed by SDS–PAGE and then immunoblotted following the SDS–PAGE. It was observed that LSU was degraded to the 51-kDa fragment only in the stroma fractions as did in the lysates, but the fragment was not found when the membrane fractions containing purified Rubisco were incubated at the all indicated condition (Figure 6). The results above suggest that the protease specially degrading LSU to the 51-kDa fragment could be located in the stroma of chloroplasts.

LSU degradation in the chloroplast isolated from senescing leaves

When the chloroplast lysates isolated from senescing leaves were incubated at 35°C in pH 7.5 buffers for 0, 0.5, 1.0 and 2.0 h, respectively, the 51-kDa fragment of LSU could be detected in the chloroplast lysates incubated after 1 h (Figure 7A). In addition, the 51-kDa fragment was also found in the chloroplast lysates when the intact chloroplasts were pre-treated with thermolysin (data not shown). The results were the same as in previously reported research in our laboratory (Zhang et al., 2005, 2007). Subsequently, the chloroplast lysates from senescing leaves were incubated at 35°C for 1.5 h in the pH 7.5 buffers with 0, 1, 10 μM, 0.1, 1 and 10 mM ATP, respectively. The results also showed that the 51-kDa fragments of LSU were detected in the all experiments,

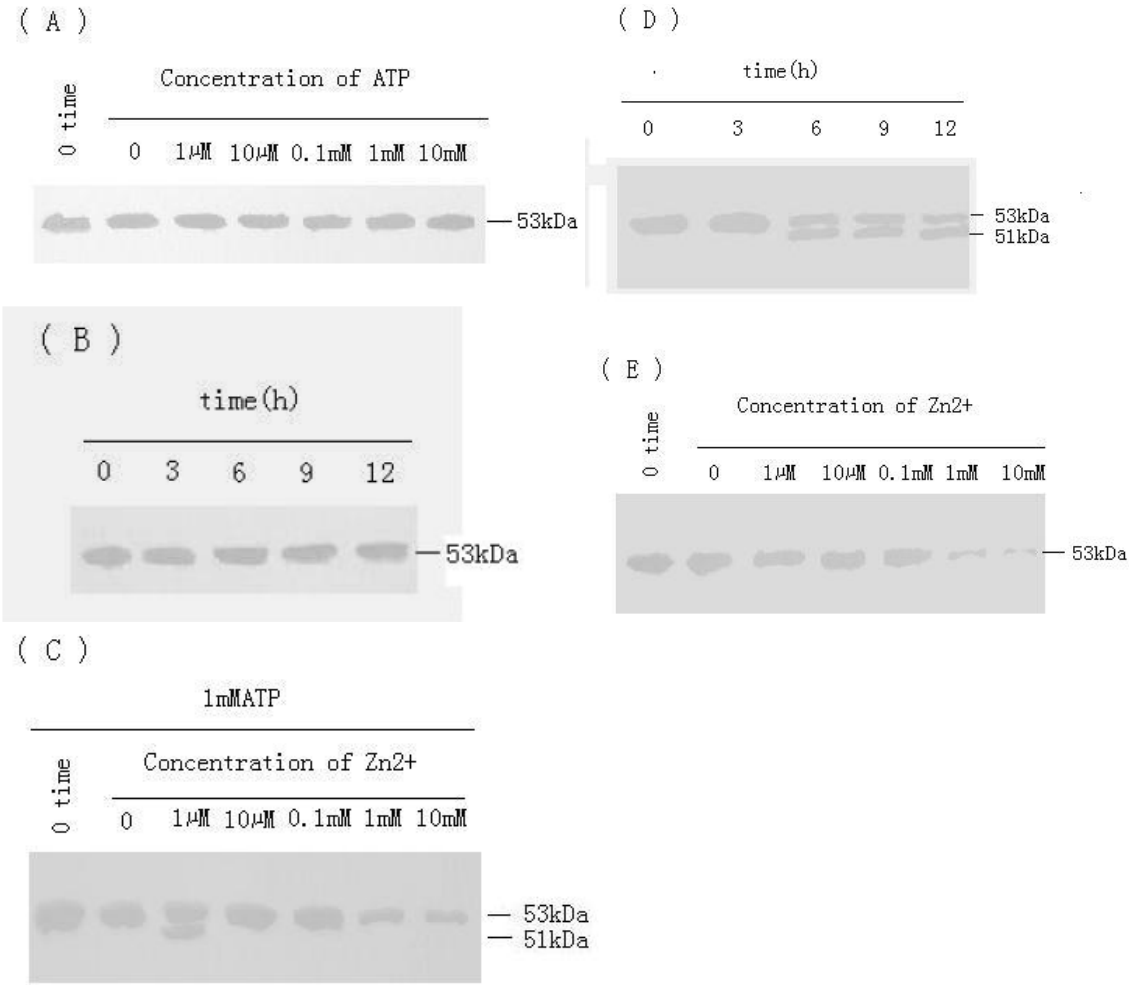

Figure 4. LSU degradation in the chloroplast lysates of mature leaves. The chloroplast lysates of mature leaves were incubated at 35°C, (A) in pH 7.5 buffers with different concentration of ATP: 0, 1, 10 µM, 0.1, 1 and 10 mM for 6 h, (B) in pH 7.5 buffers with adding 1 mM ATP for 0, 3, 6, 9 and 12 h, (C) for 6 h in pH 7.5 buffers with adding 1 mM ATP and different concentration of Zn^{2+}: 0, 1, 10, 100 µM, 1 and 10 mM, (D) in pH 7.5 buffers with 1 mM ATP and 1 µM Zn^{2+} for 0, 3, 6, 9 and 12 h, (E) for 6 h in pH 7.5 buffers with adding different concentration of Zn^{2+}: 0, 1, 10µM, 0.1, 1 and 10 mM, respectively. After incubation, the chloroplast lysates of mature leaves were analyzed by immunoblotting following SDS-PAGE.

and there were no obvious differences between control (0 µM ATP) and those treated with different concentrations of ATP (Figure 7B). Furthermore, the chloroplast lysates from senescing leaves were incubated with different concentrations of Zn^{2+}. The results showed that the 51-kDa fragments could also be detected in chloroplast lysates without Zn^{2+} and with Zn^{2+} concentrations from 1 µM to 0.1 mM (Figure 7C). However, the 51-kDa fragment totally disappeared when the concentration of Zn^{2+} was over 1 mM (Figure 7C). These results indicated that ATP or Zn^{2+} seemed to be ineffective in the specific degradation of LSU in the chloroplast lysates from senescing leaves. However, when the chloroplast lysates from senescing leaves were incubated with 1 mM ATP and 1 µM Zn^{2+} synchronously for 0, 0.5, 1 and 2 h, the 51-kDa fragment could be detected only within 0.5 h (Figure 7D). However, it would need at least 1 h if the chloroplast lysates did not contain 1 mM ATP and 1 µM Zn^{2+} (Figure 7A).

Identification of another small polypeptide from LSU

Tricine-SDS-PAGE system with urea was used to investigate whether there would be another small polypeptide besides the 51-kDa fragment in the reaction system because of its sensitivity to the resolution of small polypeptides in the range from 1 to 100-kDa. However, the result showed that there was no any 1 or 2-kDa polypeptides detected on the separating gel (Figure 8) or on the nitrocellulose membrane by the western blotting (data not shown).

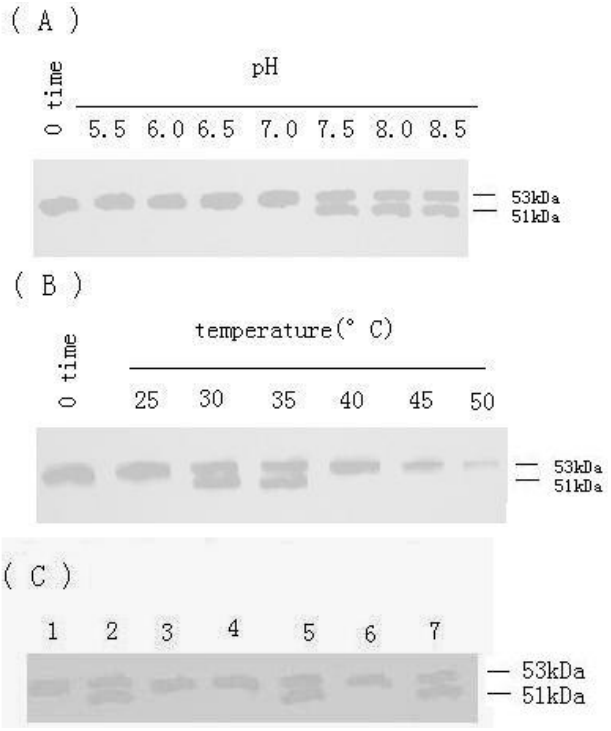

Figure 5. Effects of pH, temperature and different protease inhibitors on the appearance of the 51-kDa fragment in the chloroplast lysates of mature leaves. Product of Rubisco degradation was identified by immunoblotting after SDS-PAGE. Lane 1 in A and B: control. Lanes 2-8 in A represent the chloroplast lysates of mature leaves were incubated for 6 h at 35°C in pH 5.5, 6.0, 6.5, 7.0, 7.5, 8.0 and 8.5 buffers containing 1 mM ATP and 1 µM Zn^{2+}, respectively. Lanes 2-8 in B represent the chloroplast lysates of mature leaves were incubated for 6 h in pH 7.5 buffers containing 1 mM ATP and 1 µM Zn^{2+} at 25, 30, 35, 40, 45 and 50°C, respectively. Lanes in C represent the chloroplast lysates were incubated for 6 h at 35°C in pH 7.5 buffers containing 1 mM ATP and 1 µM Zn^{2+} after the chloroplast lysates were pre-incubated on ice for 30 minutes with different inhibitors. Lane 1, no inhibitor and without incubation; lane 2, no inhibitor; lane 3, 1,10-phenanthroline (10 $mmolL^{-1}$); lane 4, AEBSF (10 $mmolL^{-1}$); lane 5, E-64 (25 µmol L^{-1}); lane 6, leupeptin (10 µmol L^{-1}); lane 7, pepstatin (25 $µmolL^{-1}$).

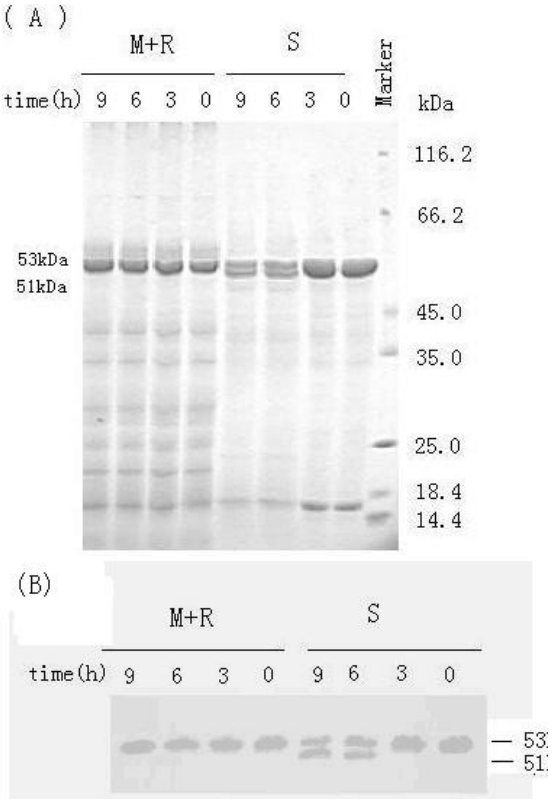

Figure 6. LSU degradation in sub-chloroplastic fractions. The stroma fraction (S) and the membrane fraction supplemented with purified Rubisco (M + R) were incubated at 35°C in pH 7.5 buffers for 0, 3, 6 and 9 h. After incubation, the samples were analyzed by SDS–PAGE (12.5%) (A), and immunoblotting following SDS–PAGE (B).

DISCUSSION

The degradation of Rubisco is of great physiological significance since it provides additional amino acids for mobilization to the reproductive organs as well as to newly growing tissues. Once Rubisco is degraded, the amino acids can be reutilized in these leaves for the synthesis of special vacuolar proteins in the paraveinal mesophyll (Feller et al., 2008). It is generally thought that the initial step of Rubisco degradation in leaves occurs within the chloroplasts. A 44-kDa fragment of Rubisco was found in the lysates of chloroplasts isolated from naturally senescing wheat leaves. However, the 51-kDa fragment is the largest one detected in wheat LSU degradation so far (Zhang et al., 2005, 2007). It might be postulated that the 51-kDa fragment was the first product of LSU degradation. The protease(s) might be the key protease(s) for Rubisco breakdown

The present study showed that the LSU could be degraded into the 51-kDa fragment when the leaf crude extracts of mature leaves were incubated at 35°C in pH 7.5 buffer with 1 mM ATP or the chloroplast lysates of mature leaves were incubated at 35°C in pH 7.5 buffer with 1 mM ATP and 1 µM Zn^{2+}, synchronously. It implied that the target protease(s) specifically degrading LSU to 51-kDa fragment in mature leaves might be ATP-dependent. ATP-dependent proteases in vacuole have not been reported. But ATP-dependent degradation of some specific proteins has been detected in chloroplasts (Reinbothe et al., 1995). ATP is the universal energy carrier in nature, and is fairly heat-stable. However, ATP is usually not very stable in solutions containing plant material. Therefore, the concentration of ATP is lower than that used of this experiment. Since it now appears unlikely that the ubiquitin pathway will be

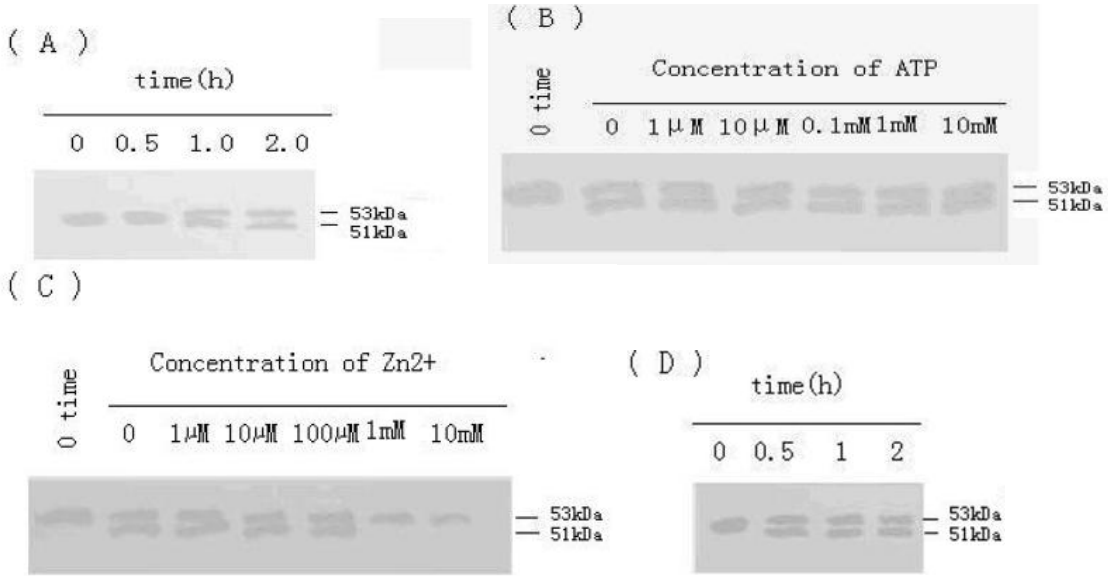

Figure 7. LSU degradation in the chloroplast of senescing leaves. The chloroplast lysates of senescing leaves were incubated at 35°C, (A) in pH 7.5 buffers for 0, 0.5, 1.0 and 2.0 h, (B) in pH 7.5 buffers with 0, 1, 10 μM, 0.1, 1 and 10 mM ATP for 1.5 h, (C) in pH 7.5 buffers with 0, 1, 10 μM, 0.1, 1 and 10 mM Zn^{2+} for 1.5 h, (D) in pH 7.5 buffers containing 1 mM ATP and 1 μM Zn^{2+} for 0, 0.5, 1.0 and 2.0 h, respectively. After incubation, the chloroplast lysates of senescing leaves were analyzed by immunoblotting following SDS-PAGE.

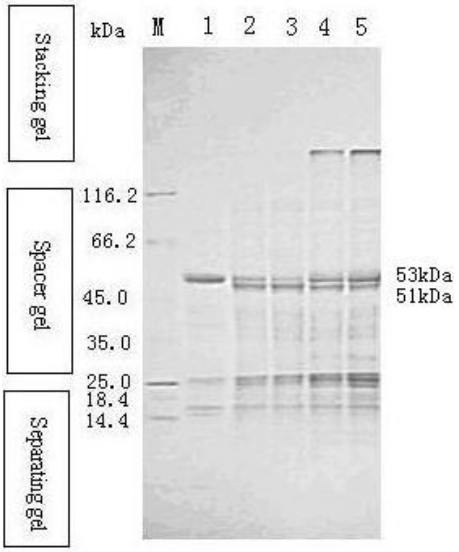

Figure 8. Detection of small fragment of LSU degradation by Tricine-SDS-PAGE Lane 1: the crude extract of mature leaves was kept in the ice bath; Lane 2: the crude extract of mature leaves in pH 7.5 buffer containing 1mM ATP was incubated at 35°C for 1.5 h; Lane 3: the crude extract of senescing leaves in pH 7.5 buffer was incubated at 35°C for 1.5 h; Lane 4: the chloroplast lysate of mature leaves in pH 7.5 buffer containing 1 mM ATP and 1 μM Zn^{2+} was incubated at 35°C for 6 h; Lane 5: the chloroplast lysate of senescing leaves in pH 7.5 buffer was incubated at 35°C for 1.5 h.

involved in proteolysis in chloroplasts (Vierstra, 1996), other proteolysis mechanism involving an ATP-dependent protease is more likely. Up to now, ATP-dependent known proteases in chloroplasts were known as CLp, FtsH and Ion (Adam et al., 2002). However, it has not been reported that CLp and Ion in chloroplasts required Zn^{2+} for proteolytic activity. ATP and Zn^{2+}-dependent FtsH family is bound to thylakoid membrane whereas the protease detected in this study is confined to chloroplast stroma. Additionally, EP1, a Zn^{2+}-dependent protease purified from the pea chloroplast, could hydrolyze the LSU to 36-kDa fragment (Bushnell et al., 1993). But it was not reported that EP1 required ATP for its proteolytic activity.

At the same time, it was found that ATP had no effect on the LSU degradation in leaf crude extracts and in the chloroplast lysates of dark-induced senescing leaves. It was widely accepted that the initial step of Rubisco degradation in leaves occurred within the chloroplast (Huffaker, 1990). So the main question is whether the protease in the chloroplast of mature leaves is the same as the protease in the chloroplast of senescing leaves. If they are the same proteases, why has the protease in mature leaves no hydrolytic activity to LSU under normal condition, and how would the protease be activated during leaf senescing process? If the proteases in mature and senescing leaves are not the same protease, would the protease be newly synthesized during the leaf senescence? It is very interesting to know more about the protease(s). We have ever tried to purify the protease, but it was rather difficult to separate it from Rubisco

because the protease(s) were tight bound to Rubisco. Additionally, it was also found that when the purified Rubisco was incubated in appropriate condition, the 51-kDa fragment of LSU could also be detected. Anyway, the purification of this protease(s) is being carried out in our laboratory. In addition, there may be more than one LSU degrading protease from the susceptibility to inhibitors of various types of proteases. That is to say, the cleavage site may be susceptible to various proteases.

Tricine-SDS-PAGE system (16.5% T, 6% C) with 6 mol/L urea was used to investigate whether the protease(s) just hydrolyzed LSU to the 51-kDa fragment and a 2-kDa polypeptide, because of the method sensitivity to the resolution of polypeptides in the range from 1 to 100-kDa. The results in this study showed that there was no 1-kDa or 2-kDa polypeptides detected on the separating gel. So the target protease(s) might cleave LSU to a 51-kDa fragment and several other smaller peptides below 1-kDa, or the small polypeptides could be degraded rapidly to amino acids or other smaller peptides by the other peptidases in leaf crude extracts or chloroplast lysates, or the target protease(s) might be an exopeptidase. Examining sequentially the cleavage character of the protease(s) will be valuable to understand the particular mechanism of Rubisco degradation. So further research about the protease(s) will be the emphasis in future research, and the purification of this protease(s) is to be especially required in our laboratory.

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REFERENCES

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

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

Crafts-Brandner SJ, Klein RR, Klein P, Holzer R, Feller U (1996). Coordination of protein and mRNA abundances of stromal enzymes and mRNA abundances of the Clp protease subunit during senescence of Phaseolus vulgaris L. leaves. Planta, 200: 312-318.

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.

Desimone M, Henke A, Wanger 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.

Desimone M, Wanger 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.

Feller U, Anders I, Mae T (2008). Rubiscolytics: fate of Rubisco after its enzymatic function in a cell is terminated. J. Exp. Bot., 59: 1615-1624.

Huffaker RC (1990). Proteolytic activity during senescence of plants. New Phytol., 116: 199-231.

Houtz RL, Portis AR Jr (2003). The life of ribulose-1,5-bisphosphate carboxylase/oxygenase-posttranslational facts and mysteries. Arch. Biochem. Biophys., 414: 150-158.

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.

Ishida H, Shimizu S, Makino A, Mae T (1998). Light-dependent fragment of the large subunit of ribulose-1,5-bisphos-phate carboxylase/oxygenase in chloroplasts isolated from wheat leaves. Planta, 204: 305-309.

Ishida H, Yoshimoto K, Izumi M, Reisen D, Yano Y, Makino A, Ohsumi Y, Hanson M, Mae T (2008). Mobilization of Rubisco and Stroma-Localized Fluorescent Proteins of Chloroplasts to the Vacuole by an *ATG* Gene-Dependent Autophagic Process. Plant Physiol., 148: 142-155.

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.

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: 1390-1394.

Kuwabara T (1992). Characterization of a prolyl endopeptidase from spinach thylakoids. FEBS Lett., 300: 127-130.

Laemmli UK (1970). Cleavage of structural proteins during the assay of the head of bacteriophage T4. Nature, 277: 680-685.

Liu J, Wu YH, Yang JJ, Liu YD, Shen FF (2008). Protein Degradation and Nitrogen Remobilization during leaf senescence. J. Plant Biol., 51: 11-19.

Michaud D, Faye L, Yelle S (1993). Electrophoretic analysis of plant cysteine and serine proteinase using gelatin containing polyacrylamide gels and class-specific proteinase inhibitors. Electrophoresis, 14: 94-98.

Minamikawa 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.

Miyadai K, Mae T, Makino A, Ojima K (1990). Characteristics of ribulose-1,5-bisphosphate carboxylase/oxygenase degradation by lysates of mechanically isolated chloroplasts from wheat leaves. Plant Physiol., 92: 1215-1219.

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

Ostersetzer O, Tabak S, Yarden O, Shapira R, Adam Z (1996). Immunological detection of proteins similar to bacterial proteases in higher plant chloroplasts. Eur. J. Biochem., 236: 932-936.

Reinbothe C, Apel K, Reinbothe S (1995). A light-induced protease from barley plastids degrades NADPH: protochlorophyllide oxidoreductase complexed with chlorophyllide. Mol. Cell Biol., 15: 6206-6212.

Roulin S, Feller U (1997). Light-induced proteolysis of stromal proteins in pea (*Pisum sativum L.*) chloroplasts: requirement for intact organelles. Plant Sci., 128: 31-41.

Rui Q, Xu LL (2004). Degradation of ribulose-1,5-bisphosphate carboxylase/oxygenase in wheat leaves during dark-induced senescence. Acta Bot. Sin., 46: 137-141.

Schagger H, Jagow G (1987). Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa. Anal. Biochem., 166: 368-379.

Shanklin J, Dewitt ND, Flanagan JM (1995). The stroma of higher plant plastids contain ClpP and ClpC, functional homologous of Escherichia coli ClpP and ClpA: An archetypal two-component ATP-dependent protease. Plant Cell, 7: 1713-1722.

Vierstra RD (1996). Proteolysis in plants: mechanisms and functions. Plant Mol. Biol., 32: 275-302.

Walker DA, Cerovic ZG, Robinson SP (1987). Isolation of intact chloroplasts: general principles and criteria of integrity. Meth. Enzymol., 148: 145-157.

Yan SP, Zhang QY, Tang ZC, Su WA, Sun WN (2006). Comparative Proteomic analysisi provides new insights into chilling stress responses. Mol. Cell. Proteomics, 5: 484-496.

Yoshida T, Minamikawa T (1996). Successive aminoterminal 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.

Zhang LF, Rui Q, Xu LL (2005). Degradation of the large Subunit of Ribulose-1,5 -bisphosphate Carboxylase/oxgenaseb in wheat leaves. J. Integr. Plant Biol., 47: 60-66.

Zhang LF, Rui Q, Zhang P, Wang XY, Xu LL (2007). A novel 51-kDa fragment of the Large Subunit of Ribulose-1,5-bisphosphate Carboxylase/oxgenase was formed in the stroma of chloroplast in Dark-induced Senescing wheat leaves. Physiol. Plant, 3: 64-71.

Zhang ZG, Rui Q, Xu LL (2001). Relationship between endopeptidases and H2O2 during wheat leaves aging. Acta. Bot. Sin., 43: 127-131.