DEGRADATION OF RUBISCO AND OTHER CHLOROPLAST PROTEINS UNDER ABIOTIC STRESS

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Summary. Abiotic stress (e.g. drought, heat, hypoxia, heavy metal pollution) can strongly affect senescence and the degradation of chloroplast proteins. In general, such a stress causes an earlier or accelerated senescence. Amino acids deriving from protein catabolism may be redistributed within the plant via the phloem and serve as a basis for protein synthesis in other plant parts. Under certain conditions amino acids may accumulate to high levels in leaves. Besides the onset and the velocity of senescence, the sequence of events may be altered under abiotic stress. Chloroplasts are dismantled in an early phase of senescence, while other subcellular compartments (e.g. mitochondria) are still functional. Rubisco is the most abundant protein on earth and contributes up to 50 % of the soluble proteins and up to 30 % of total leaf nitrogen in leaves of C_3 plants. Therefore, the degradation of Rubisco and the reutilization of the amino acids liberated are important for the nitrogen budget of plants. During natural senescence, often only the bands representing the intact large and small subunits (but no fragments) are visible on stained gels or immunoblots. However, under abiotic stress inducing a rapid net degradation of Rubisco fragments of the large subunit may become detectable on immunoblots. Site-specific

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antibodies are helpful tools to characterize the fragmentation. From such experiments it became evident that under certain conditions (e.g. oxidative stress in isolated chloroplasts) small peptides at the C-terminus were released, while under other conditions (e.g. in leaf segments under hypoxia in darkness) the first cut(s) might occur near the N-terminus.

Key words: abiotic stress, antibodies, *Phaseolus vulgaris*, proteolysis, Rubisco degradation, *Triticum aestivum*.

Abbreviations: Rubisco - Ribulose-1,5-bisphosphate carboxylase/oxygenase; LSU – large subunit of Rubisco.

INTRODUCTION

Protein synthesis and protein degradation are equally important for changes in the protein pattern and are of fundamental importance for the normal development, homeostasis and final death of a plant cell (Vierstra, 1996). Proteolysis in plants is a complex process involving many enzymes and multifarious proteolytic pathways in various cellular compartments (Grudkowska and Zagdańska, 2004). Therefore proteolysis is an important process in maintaining functional chloroplasts under optimal and stress conditions. Recent progress in genome information revealed various plastidial plant proteases that are involved in the degradation (gradual degradation to oligopeptides and amino acids) of proteins especially in response to environmental conditions (Sakamoto, 2006). ATP-independent and ATP-dependent proteolytic pathways are involved in plant proteolysis (Callis, 1995). It has been proposed that chloroplast proteins may be degraded by vacuolar proteases, via the ubiquitin pathway in the cytosol and also by the plastidial Clp system (Shanklin et al., 1995; Vierstra, 1996). Conformational changes of chloroplasts proteins may open them and make them accessible for different types of proteases.

Chloroplasts are a major site of protein degradation during senescence (Mae et al., 1984). Rubisco is the most abundant protein on earth and contributes a high percentage to the total leaf nitrogen in C_3 plants (Ellis,

1979; Feller et al., 2008). During early stages of senescence, Rubisco accounts for about 90 % of the degraded proteins (Miller and Huffaker, 1985). Roberts et al. (2003) reported a serine protease activity in senescing wheat leaves for which Rubisco was a target protein. A net degradation of Rubisco and other chloroplast proteins can be observed during endogenously initiated leaf senescence as well as during or after abiotic stress phases and allows the reutilization of the nitrogen in other organs after the transfer via the phloem (Crafts-Brandner and Egli, 1987; Crafts-Brandner et al., 1998; Herrmann and Feller, 1998; Demirevska-Keopva et al., 2004, 2005; Thoenen et al., 2007; Feller et al., 2008). Not only the velocity of Rubisco degradation, but also the mechanisms involved may depend on the environmental conditions (Feller et al., 2008).

LSU fragments can be detected on immunoblots from isolated pea chloroplasts incubated in the light (several bands in the range of 30 – 45 kDa) or in darkness (one major band at about 37 kDa) for several hours (Mitsuhashi et al., 1992; Roulin and Feller, 1998a, b). Since these fragments were detected by antibodies against the whole enzyme as well as by antibodies against the first 25 amino acids of the mature LSU, it can be concluded that the N-terminus was still present and smaller pieces were removed from the C-terminus. This fragmentation was most likely catalyzed by a metalloendopeptidase inside the chloroplasts (Roulin and Feller, 1998a). It has been reported that LSU may also be non-enzymatically cleaved by reactive oxygen species (Ishida et al., 1997, 1999; Luo et al., 2002; Nakano et al., 2006). Reactive oxygen species may directly cleave this protein or modify it in a manner making it more susceptible to proteolytic cleavage (Desimone et al., 1996, 1998; Ishida et al., 1997, 1999). Increased levels of reactive oxygen species may be caused by high light at low temperature (Nakano et al., 2006) or by excessive supply of some heavy metals (Demirevska-Kepova et al., 2004). Rubisco aggregates can be formed within chloroplasts as a result of osmotic or oxidative stress (Ferreira and Shaw, 1989, Desimone et al., 1996). The cross-linked LSU has been found to be insolubilized and more sensitive to proteases (Mehta et al., 1992). Evidence for the involvement of a cysteine endopeptidase under certain conditions has been presented, too (Minamikawa et al., 2001; Thoenen et al., 2007; Yoshida and Minamikawa, 1996). Increased cysteine

endoproteinase activities suggest that vacuolar enzymes are potentially involved in the response to stress conditions.

The levels of many plastidial and extraplastidial enzyme proteins besides Rubisco respond to abiotic stress (Cushman et al., 1989; Dizengremel, 2001; Meloni et al., 2004; Sahu et al., 2001; Van Herwaarden et al., 1998; Wang et al., 2007; Xu and Yu, 2006). Although the general effects of stress conditions on plant growth are well known, the primary effects at the plastidial and extraplastidial enzyme protein levels in comparison with Rubisco are not yet clear.

For example, enzyme proteins may differ in their susceptibility to modifications or to proteolytic attack. Rubisco activase, that catalyzes the activation of Rubisco, is highly sensitive to elevated temperature (reversible and irreversible effects), while Rubisco is far more stable under such conditions (Eckardt and Portis, 1997; Feller at. al., 1998). Furthermore, interactions with solutes may influence the degradation of enzyme proteins by peptide hydrolases (Houtz and Mulligan, 1991). Nitrogen deficiency, hypoxia and darkness belong to the most important abiotic stresses.

The aim of the work reported here was to elucidate the fate of Rubisco during abiotic stress (nitrogen deficiency, oxygen deficiency and darkness) in comparison to other stromal and extraplastidial enzyme proteins. Site-specific antibodies against well defined regions of LSU were raised in rabbits and represent helpful tools in this context allowing the identification of N-terminal and C-terminal fragments.

MATERIALS AND METHODS

Plant Material

Wheat (*Triticum aestivum* L., "Arina") and bean (*Phaseolus vulgaris* L., "Saxa") were germinated on wet tissue paper in darkness for 2 days and afterwards grown hydroponically in a light/dark cycle (14 h light, 50 µE m^{-2} s^{-1}, 24 °C; 10 h darkness, 19 °C). A standard nutrient medium containing all nutrients as described by Hildbrand et al. (1994) was used for the beans. Detached fully expanded bean leaves were incubated in darkness for 0, 5 and 10 days (either "+O_2" floating on deionized water, or "-O_2" submerged

in deionized water to exclude oxygen). Wheat plants grew on deionized water until day 13 and were then transferred to a nutrient medium lacking mineral nitrogen or to the same medium supplemented with 5 mM NH_4NO_3. The first and second leaves of wheat plants were collected 13 (transfer to nutrient medium with or without mineral nitrogen), 18 and 26 days after imbibition. The samples were stored frozen prior to the extraction.

Immunoblotting analysis

Proteins were extracted as reported previously (Thoenen et al., 2007). After gel electrophoresis, proteins were blotted onto nitrocellulose and the intact proteins as well as fragments deriving from them were detected with specific antibodies using the PAP complex and 4-chloro-1-naphtol for visualization (Herrmann and Feller, 1998). Polyclonal antibodies against glycolate oxidase were produced in rabbits as described by Mitsuhasi and Feller (1992). Specific polyclonal antibodies against the N-terminus and C-terminus of LSU were produced in rabbits after immunization with synthetic peptides representing the first 17 amino acids (N-terminus; N-terminal proline acetylated as reported by Mulligan et al., 1988) or of the last 18 amino acids (C-terminus) of the mature LSU of wheat.

RESULTS AND DISCUSSION

Nitrogen deficiency is a frequent abiotic stress. The net degradation of chloroplast proteins is in general well coordinated under such conditions (Fig. 1). From the results presented in Fig. 1 it became evident that in nitrogen deficient plants (-N) stromal proteins were degraded in the oldest leaf (leaf 1) and the amino acids were used for the synthesis of proteins in the younger leaf (leaf 2) after the translocation via the phloem, while the protein levels increased in both leaves of plants with an adequate nitrogen supply (+N). Nitrite reductase represents an interesting enzyme in this context. No band was visible on day 13 (plant growth depended on the reserves in the caryopsis and no external nitrate was available). After supplying mineral nitrogen (+N, days 18 and 26), this enzyme was detected on immunoblots, but it was still absent in plants grown without nitrate

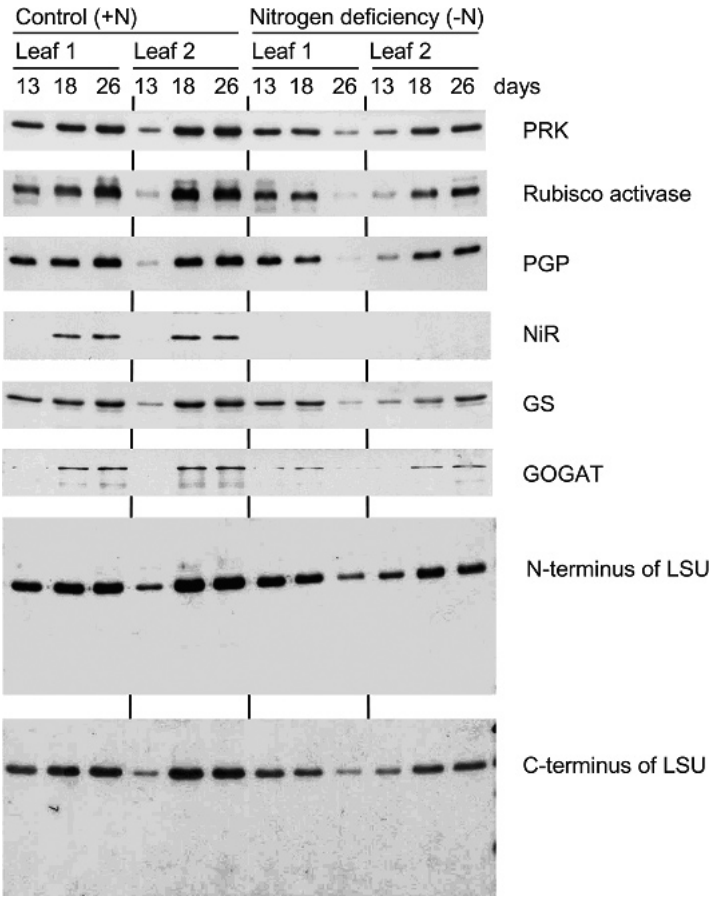

Fig. 1. Effects of nitrogen deficiency on the levels of Rubisco and other chloroplast proteins in wheat leaves. All lanes for a blot were loaded with an equal percentage of a leaf to allow a direct comparison (0.3 % of a leaf lamina for the Rubisco blots and 0.6 % of a leaf lamina for the other proteins). Phosphoribulokinase (PRK; EC 2.7.1.19), rubisco activase, phoshoglycolate phosphatase (PGP; EC 3.1.3.18), nitrite reductase (NiR; EC 1.7.7.1), glutamine synthetase (GS; EC 6.3.1.2) and glutamate synthase (ferredoxin GOGAT; EC 1.4.7.1) were detected on immunoblots with specific antibodies developed in rabbits. Two types of antibodies raised in rabbits were used to detect LSU (large subunit of Rubisco, EC 4.1.1.39) and LSU fragments: antibodies against a synthetic peptide representing the first 17 amino acids of the mature wheat LSU (N-terminus of LSU) and antibodies against a synthetic peptide representing the last 18 amino acids (C-terminus of LSU) of wheat LSU.

(-N). Only the intact LSU, but no fragments were detected on immunoblots developed with specific antibodies against the N- or C-terminus of LSU, even in leaves characterized by a net degradation of this protein (-N, leaf 1). Tsai et al. (1991) suggested that the decreased abundance of Rubisco polypeptides during growth of maize at low soil nitrogen was a result of enhanced proteolysis. From our results it can be concluded that fragments produced by a first cleavage were rapidly degraded to small peptides and free amino acids which were no longer detected on immunoblots. In contrast to previous observations concerning intact leaves incubated in darkness under oxygen deficiency (Feller et al., 2008) or isolated chloroplasts incubated in the light (Roulin and Feller, 1998a, b), no large LSU fragments produced by the removal of small pieces from the N- or C-terminus were detected. Kingston-Smith et al. (2004) reported an increase in cysteine protease activity when plants were stressed by withholding nitrate. The authors suggested that nitrogen limitation induced an early, reversible stage of senescence in which perturbations in protease activity facilitated the degradation of non-essential proteins in order to increase the chances of plant survival.

An accumulation of two large LSU fragments still containing the C-terminus was detected in bean leaves incubated in darkness under hypoxia (Feller et al., 2008). However, other plastidial as well as extraplastdial proteins were degraded in darkness upon $+O_2$ and $-O_2$ incubations without a major accumulation of fragments (Fig. 2). Cytosolic glutamine synthetase (Fig. 2, weak band below the plastidial form) was maintained longer than the chloroplast enzyme. Glutamate dehydrogenase (mitochondrial protein) was also maintained for a longer period than the stromal proteins. The level of this enzyme protein increased even during the first days upon $+O_2$ incubations. An interesting response was detected for phosphoglycolate phosphatase. This protein was degraded on one hand and a new immunoreactive band above the intact subunit was detected after incubation under hypoxia ($-O_2$) on the other hand. The nature of this band is not yet clarified. It could derive from a stable cross-linkage of the subunit or of a fragment with another polypeptide as detected previously for Rubisco activase (Feller et al., 1998). Apparently the accumulation of large amounts of fragments under hypoxia was specific for Rubisco and not a general process.

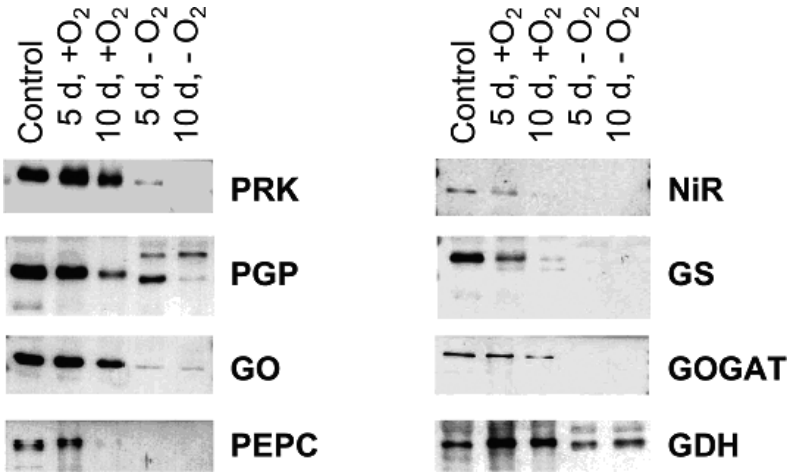

Fig. 2. Net degradation of plastidial and extraplastdial proteins in bean leaves incubated under oxygen deficiency in darkness. The lanes were loaded with an equal percentage of a leaf to allow a direct comparison (0.6 % of a primary leaf). Phosphoribulokinase (PRK, plastidial), nitrite reductase (NiR, plastidial), phoshoglycolate phosphatase (PGP, plastidial), glutamine synthetase (GS, major band plastdidial), glycolate oxidase (GO, peroxisomal; EC 1.1.3.15), glutamate synthase (GOGAT, plastidial), phosphoenolpyruvate carboxylase (PEPC, cytosolic; 4.1.1.31) and NADH-glutamate dehydrogenase (GDH, mitochondrial; EC 1.4.1.2) were detected on immunoblots with specific antibodies developed in rabbits.

The accumulation of different Rubisco fragments under various abiotic stress conditions is summarized in Fig. 3. Several fragments still containing the N-terminus (removal of smaller fragments at the C-terminus) were detected on immunoblots from isolated chloroplasts incubated in the light (Roulin and Feller, 1998a, b). It has been demonstrated that Rubisco can be degraded in intact chloroplasts (Mitsuhashi et al., 1992). Reactive oxygen species (Nakano et al., 2006) and a metalloendopeptidase (Roulin and Feller, 1998a) must be considered important factors in this context. In contrast, in detached leaves incubated under hypoxia in darkness other fragments (still containing the C-terminus, but missing the N-terminus) accumulated (Hildbrand et a., 1994; Feller et al., 2008). Large LSU fragments still containing the C-terminus were also observed in wheat leaf segments

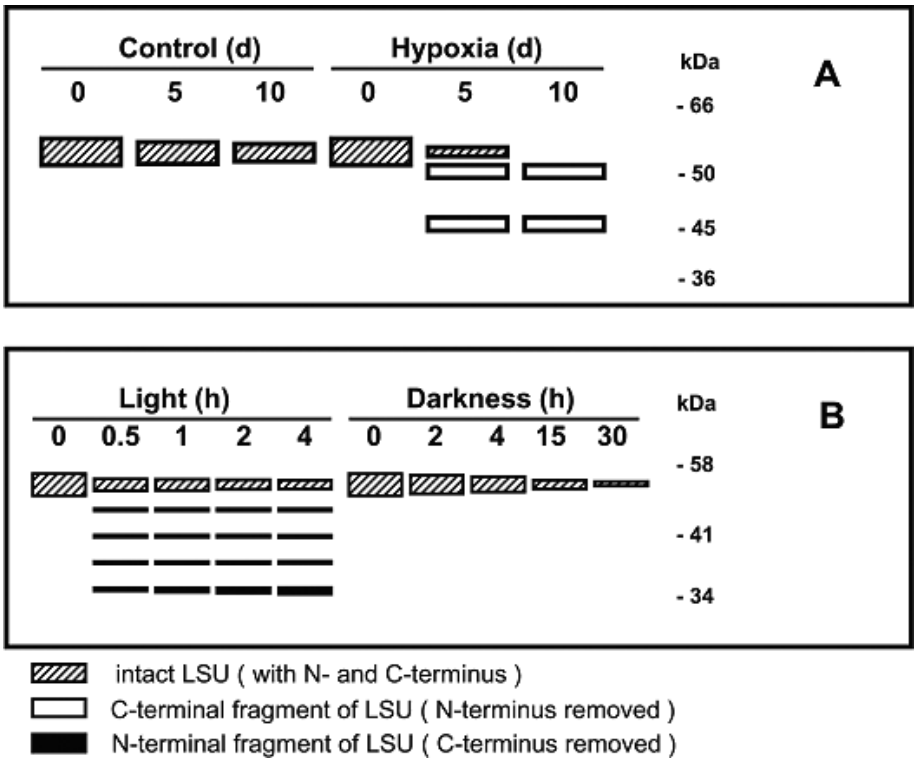

Fig. 3. Scheme representing different types of LSU fragmentation under abiotic stress conditions. No LSU fragments were detectable on immunoblots of detached bean leaves incubated for up to 10 days floating on deionized water in darkness, while large amounts of two LSU fragments containing the C-terminus (N-terminus removed) accumulated under hypoxia in darkness (A). In isolated chloroplasts incubated in the light, various Rubisco fragments containing the N-terminus (C-terminus removed) accumulated in the range of 30 - 45 kDa (B).

incubated under a limiting energy supply (Thoenen et al., 2007), but such fragments were not found in isolated chloroplasts. Additional studies suggest that a cysteine endopeptidase (presumably vacuolar) is involved in Rubisco degradation and that the first cleavage of LSU can occur when it is still present in the holoenzyme together with the other large and with the small subunits (Thoenen et al., 2007).

CONCLUSIONS

Rubisco represents an interesting enzyme protein with respect to its catabolism. Rubisco degradation depends on the actual conditions. Different mechanisms might be relevant under various stress conditions (Luo et al., 2002; Demirevska-Kepova et al., 2004; Thoenen et al., 2007; Feller et al., 2008). The accumulation of immunoreactive fragments may allow the identification of different mechanisms involved in Rubisco degradation (Zhang et al., 2007; Feller et al., 2008). Specific antibodies raised against defined sequences of LSU might allow more detailed investigations. The various steps in Rubisco degradation, the proteolytic enzymes involved and the subcellular compartmentation of these processes under various abiotic stress conditions remain to be elucidated in the future.

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References

Callis, J., 1995. Regulation of protein degradation. Plant Cell, 7, 845-857.

Crafts-Brandner, S.J., D.B. Egli., 1987. Sink removal and leaf senescence in soybean. Cultivar effects. Plant Physiol., 85, 662-666.

Crafts-Brandner, S.J., R. Hölzer, U. Feller, 1998. Influence of nitrogen deficiency on senescence and the amounts of RNA and proteins in wheat leaves. Physiol. Plant., 102, 192-200.

Cushman, J.C., G. Meyer, C.B. Michalowski, J.M. Schmitt, H.J. Bohnert, 1989. Salt stress leads to differential expression of two isogenes of phosphoenolpyruvate carboxylase during Crassulacean acid metabolism induction in the common ice plant. Plant Cell, 1, 715-725.

Demirevska-Kepova, K., R. Hölzer, L. Simova-Stoilova, U. Feller, 2005.

Heat stress effecects on Rubisco, Rubisco binding protein and Rubisco activase in wheat leaves, Biol. Plant., 49, 521-525.

Demirevska-Kepova, K., L. Simova-Stoilova, Z. Stoyanova, R. Hölzer, U. Feller, 2004. Biochemical changes in barley plants after excessive supply of copper and manganese. Environ. Exp. Bot., 52, 253-266.

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

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

Dizengremel, P., 2001. Effects of ozone on the carbon metabolism of forest trees. Plant Physiol. Biochem., 39, 729-742.

Eckardt, N.A., A.R. Portis, 1997. Heat denaturation profiles of ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) and Rubisco activase and the inability of Rubisco activase to restore activity of heat-denatured Rubisco, Plant Physiol., 113, 243-248.

Ellis, R.J., 1979. The most abundant protein in the world. Trends Biochem. Sci., 4, 241-244.

Feller, U., I. Anders, T. Mae, 2008. Rubiscolytics: fate of Rubisco after ist enzymatic function in a cell is terminated. J. Exp. Bot., (*in press*).

Feller, U., S.J. Crafts-Brandner, M.E. Salvucci, 1998. Moderately high temperatures inhibit ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) activase-mediated activation of Rubisco. Plant Physiol., 116, 539-546.

Ferreira, R.B., N.M. Shaw, 1989. Effect of osmotic stress on protein turnover in *Lemna minor* fronds. Planta, 179, 456-465.

Grudkowska, M., B. Zagdańska, 2004. Multifunctional role of plant cysteine proteinases. Acta Biochim. Pol., 51, 609-624.

Herrmann, B., U. Feller, 1998. CO_2, light and temperature influence senescence and protein degradation in wheat leaf segments, Physiol. Plant., 103, 320-326.

Hildbrand, M., A. Fischer, U. Feller, 1994. Protein catabolism in bean leaf discs: accumulation of a soluble fragment of ribulose-1,5-

bisphosphate carboxylase/oxygenase under oxygen deficiency, J. Exp. Bot., 45, 1197-1204.

Houtz, R.L, R.M. Mulligan, 1991. Protection of tryptic-sensitive sites in the large subunit of ribulosebisphosphate carboxylase/oxygenase by catalysis. Plant Physiol., 96, 335-339.

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

Ishida, H., A. Makino, T. Mae, 1999. Fragmentation of the large subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase by reactive oxygen species occurs near Gly-329. J. Biol. Chem., 274, 5222-5226.

Kinngston-Smith, A., A. Bollard, F. Minchin, 2004. Stress-induced changes in protease composition are determined by nitrogen supply in non-nodulating white clover. J. Exp. Bot., 56, 745-753.

Luo, S., H. Ishida, A. Makino, T. Mae, 2002. Fe^{+2}-catalyzed site-specific cleavage of the large subunit of ribulsoe 1,5-bisphosphate carboxylase close to the active site. J. Biol. Chem., 277, 12383-12387.

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

Mehta, R.A., T.W. Fawcett, D. Porath, A.K. Mattoo, 1992). Oxidative stress causes rapid membrane translocation and *in vivo* degradation of ribulose-1,5-bisphosphate carboxylase/oxygenase. J. Biol. Chem., 267, 2810-2816.

Meloni, D. A., M. R. Gulotta , C. A. Martínez, M. A. Oliva, 2004. The effects of salt stress on growth, nitrate reduction and praline and glycinebetaine accumulation in *Prosopis alba*. J. Plant Physiol., 16, 39-46.

Miller, B.L., R.C. Huffaker, 1985. Differential induction of endoproteinases during senescence of attached and detached barley leaves. Plant Physiol., 78, 442-446.

Minamikawa, T., K. Toyooka, T. Okamoto, I. Hara-Nishimura, M. Nishimura, 2001. Degradation of ribulose-bisphosphate carboxylase by vacuolar

enzymes of senescing French bean leaves: immunocytochemical and ultrastructural observations. Protoplasma, 218, 144-153.

Mitsuhashi, W., S.J. Crafts-Brandner, U. Feller, 1992. Ribulose-1,5-bisphosphate carboxylase/oxygenase degradation in isolated pea chloroplasts incubated in the light or in the dark. J. Plant Physiol., 139, 653-658.

Mitsuhashi, W., U. Feller, 1992. Effects of light and external solutes on the catabolism of nuclear-encoded stromal proteins in intact chloroplasts isolated from pea leaves. Plant Physiol., 100, 2100-2105.

Mulligan, R.M., R.L. Houtz, N.E. Tolbert, 1988. Reaction-intermediate analogue binding by ribulose bisphosphate carboxylase/oxygenase causes specific changes in proteolytic sensitivity: the amino terminal residue of the large subunit is acetylated proline. Proc. Natl. Acad. Sci. USA, 85, 1513-1517.

Nakano, R., H. Ishida, A. Makino, T. Mae T., 2006. *In vivo* fragmentation of the large subunit of ribulose-1,5-bisphosphate carboxylase by reactive oxygen species in an intact leaf of cucumber under chilling-light conditions. Plant Cell Physiol., 47, 270-276.

Roberts, I.N., P.F. Murray, C.P. Caputo, S. Passeron, A.J. Barneix, 2003. Purification and characterization of a subtilisin-like serine protease induced during the senescence of wheat leaves. Physiol. Plant., 118, 483-490.

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

Roulin, S., U. Feller, 1998b. Dithiothreitol triggers photooxidative stress and fragmentation of the large subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase in intact pea chloroplasts. Plant Physiol. Biochem., 36, 849-856.

Sahu, A.C., S.K. Sahoo, N. Sahoo, 2001. NaCl-stress induced alteration in glutamine synthetase activity in excised senescing leaves of a salt-sensitive and a salt-tolerant rice cultivar in light and darkness. Plant Growth Regul., 34, 287-292.

Sakamoto, W., 2006. Protein degradation machineries in plastids. Annu. Rev. Plant Biol., 57, 599-621.

Shanklin, J, N.D. DeWitt, J.M. Flanagan, 1995. The stroma of higher plant plastids contain ClpP and ClpC, functional homologues of *Escherichia coli* ClpP and ClpA: and archetypal two component ATP-dependent protease. Plant Cell, 7, 1713-1722.

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

Tsai, C.Y., D.M. Huber, H.L. Warren, A. Lyznic, 1991. Nitrogen uptake and redistribution during maturation of maize hybrids. J. Sci. Food Agr., 57, 175-187.

Van Herwaarden, A.F., J.F. Angus, R.A., Richards, G.D. Farquhar, 1998. "Haying-off", the negative grain yield response of dryland wheat to nitrogen fertilizer – II. Carbohydrate and protein dynamics. Aust. J. Agric. Res., 49, 1083-1093.

Vierstra, R.D., 1996. Proteolysis in plants: mechanisms and functions. Plant Mol. Biol., 32, 275-302.

Wang, Z-Q., Y-Z. Yuan, J-Q. Ou, Q-H. Lin, C-F. Zhang, 2007. Glutamine synthetase and glutamate dehydrogenase contribute differentially to proline accumulation in leaves of wheat (*Triticum aestivum*) seedlings exposed to different salinity. J. Plant Physiol., 164, 695-701.

Xu, Z.Z., Z.W. Yu, 2006. Nitrogen metabolism in flag leaf and grain of wheat in response to irrigation regimes. J. Plant Nutr. Soil Sci., 169, 118-126.

Yoshida, T., T. Minamikawa, 1996. Successive amino terminal proteolysis of the large subunit of ribulose-1,5-bisphsopahe carboxylase/oxygenase by vacular enzymes from French bean leaves. Eur. J. Biochem., 238, 317-324.

Zhang, L.F., Q. Rui, P. Zhang, X.Y. Wang, L.L. Xu, 2007. A novel 51-kDa fragment of the large subunit of ribulose-1,5.bisphosphate carboxylase/oxygenase formed in the stroma of chloroplasts in dark-induced senescing wheat leaves. Physiol. Plant., 131, 64-71.