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ORIGINAL PAPER

Senescence in wheat leaves: is a cysteine endopeptidase involved in the degradation of the large subunit of Rubisco?

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Abstract In wheat (*Triticum aestivum* L.), leaf senescence can be initiated by different factors. Depending on the plant system (intact plants or detached leaves) or the environmental conditions (light, nutrient availability), the symptoms of senescence differ. The aim of this work was to elucidate the catabolism of ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco, EC. 4.1.1.39) under various senescence-inducing conditions. Leaf senescence was initiated in intact plants by darkness or by N-deprivation and in leaf segments by exposure to light or darkness. Depending on the treatment, a 50 kDa fragment of Rubisco was observed. The formation of this fragment was enhanced by leaf detachment and low light. In segments exposed to high light and in intact plants induced to senesce by N-deprivation, the fragment was essentially absent. Since an antibody against the N-terminus of a large subunit of Rubisco (LSU) did not cross-react with the fragment, it appears likely that a smaller fragment was removed from the N-terminus of LSU. Inhibitor studies suggest that a cysteine endopeptidase was involved in the formation of the 50 kDa fragment. Non-denaturing-PAGE followed by SDS-PAGE revealed that the fragment was produced while LSU was integrated in the holoenzyme complex, and that it remained there after being produced. It remains open how the putative endopeptidase reaches the stromal protein Rubisco. The results indicate that depending on the senescence-inducing conditions, different proteolytic enzymes may be involved. The involvement of vacuolar proteases must be considered as occurring during LSU degradation, which takes place in darkness, low light or under carbon limitation.

Keywords Compartmentation · Fragment · Light · Nitrogen starvation · Proteolysis · Rubisco · Stromal proteins · *Triticum aestivum* (L.) · Vacuolar proteases

Abbreviations

E-64	*trans*-epoxysuccinyl-L-leucylamido-(4-guanidino)butane
GO	glycolate oxidase
GOGAT	glutamate synthase
LHCII	light-harvesting chlorophyll *a,b* binding complex
LSU	large subunit of Rubisco
N-LSU	N-terminal part of LSU
PAR	photosynthetically active radiation
PEPC	phosphoenolpyruvate carboxylase
SSU	small subunit of Rubisco

Introduction

Leaf senescence is the final stage of development during which controlled degradation and remobilization of cell components take place. The initiation of leaf senescence is regulated by several environmental factors, such as shading, drought, or nutrient deficiency, and internal factors including reproductive development, phytohormone levels, or age (Noodén

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et al. 1997; Brouquisse et al. 2001). Various models have been used to investigate senescence. By transferring plants to darkness or by withdrawing nitrogen, senescence can be artificially initiated. Also, detaching of plant parts may lead to senescence (Feller 1983; Cuello et al. 1984; Parrott et al. 2005). Depending on the plant system or the experimental conditions used, senescence-associated genes are differently expressed, thus altering the progress of the senescence process (Park et al. 1998; Weaver et al. 1998). Moreover, senescence in detached leaves is only partially representative of natural senescence in intact plants, since stress-related genes may play a role in excised leaves (Becker and Apel 1993). Differences among the various types of senescence may also be related to biochemical and physiological changes caused by the experimental conditions used (Brouquisse et al. 2001; Demirevska-Kepova et al. 2005). It must be considered that phloem transport to other plant parts is no longer possible in detached leaves (Feller and Fischer 1994; Parrott et al. 2005). Therefore, carbohydrates accumulate in the light and might contribute to the acceleration of senescence (Herrmann and Feller 1998). Catabolic processes in plant tissues may be influenced by sugar accumulation as well as by a depletion of soluble sugars (Brouquisse et al. 2001; Parrott et al. 2005; Roulin and Feller 2001; Wälti et al. 2002).

There are, however, hallmarks of leaf senescence. Rapid net protein degradation, changes in the enzyme pattern and, in general, also chlorophyll catabolism are observed under various senescence-inducing conditions (Feller 2004; Parrott et al. 2005). A large fraction of leaf nitrogen is localized in the chloroplasts, mainly in the form of proteins (Makino and Osmond 1991). The most prominent protein in chloroplasts of C3 plants is ribulose 1,5-bisphosphate carboxylase/oxygenase (Rubisco). The degradation of this enzyme during senescence is therefore of special interest. Rubisco degradation has been attributed to both plastidial and vacuolar proteases (Hörtensteiner and Feller 2002). Since hydrolytic enzymes of the cytosol or the vacuole are spatially separated from plastidial proteins, it is tempting to suggest that plastidial proteins become degraded inside the chloroplast by plastidial peptide hydrolases (Brouquisse et al. 2001; Hörtensteiner and Feller 2002). Fragmentation of Rubisco has frequently been shown to occur in chloroplasts and chloroplast lysates (Mitsuhashi et al. 1992; Desimone et al. 1996; Ishida et al. 1997, 1998). A plastidial zinc protease able to hydrolyze Rubisco has been reported by Bushnell et al. (1993). Further evidence for the involvement of a zinc-containing metalloprotease in the degradation of stromal proteins inside the plastids was obtained from experiments with intact chloroplasts isolated from pea leaves (Roulin and Feller 1998a). A role for activated oxygen species in the degradation of Rubisco in intact chloroplasts seems likely. Both stimulation of Rubisco degradation under oxidative conditions (Desimone et al. 1996; Roulin and Feller 1998b) and direct fragmentation of the enzyme by hydroxyl radicals (Ishida et al. 1998) have been reported. The most prominent degradation product of LSU in chloroplasts or their lysates had a molecular mass in the range of 36–37 kDa (Desimone et al. 1996; Mitsuhashi et al. 1992; Ishida et al. 1997; Roulin and Feller 1998b). This fragment likely contains the N-terminus of Rubisco (Ishida et al. 1997, 1998; Roulin and Feller 1998b), although another fragment without the N-terminus of LSU was mentioned by Desimone et al. (1996). The direct cleavage of Rubisco in vitro by active oxygen species resulted in the formation of an N-terminal 37-kDa product and a 16-kDa fragment bearing the C-terminus (Ishida et al. 1998, 1999). A contamination of chloroplasts with vacuolar proteases resulted in a different degradation pattern of Rubisco (Miyadai et al. 1990). More recently, such a fragmentation of the large subunit of Rubisco by reactive oxygen species was also detected in intact cucumber leaves incubated in light at 4°C (Nakano et al. 2006). Redox-sensitive modifications, an insolubilization of Rubisco under stress, and the role of two cystein residues in this context were recently elucidated in *Clamydomonas reinhardtii* using site-directed mutagenesis as a tool (Marín-Navarro and Moreno 2006).

High levels of endopeptidases and carboxypeptidases are localized in vacuoles (Brouquisse et al. 2001; Huffaker 1990; Otegui et al. 2005). Recently, the possibility has been discussed that vacuolar proteases are involved in the degradation of plastidial proteins during senescence of purple nutsedge (Fischer et al. 1998). Beside this, mRNAs encoding for cysteine proteases were increased or were retained during senescence (Hensel et al. 1993; Lohmann et al. 1994; Smart et al. 1995). It has been shown that Rubisco degrading proteases exist in the vacuole (Lin and Wittenbach 1981; Thayer and Huffaker 1984; Bhalla and Dalling 1986). Yoshida and Minamikawa (1996) suggested the involvement of at least two proteases in the degradation of purified Rubisco by vacuolar lysates. In the first step, a cysteine protease catalyzed the degradation at the N-terminus of LSU leading to a 48-kDa fragment through the 50 kDa intermediate, while in the second step, the 48-kDa polypeptide was converted by a serine protease through the 42-kDa intermediate to the 41-kDa product.

The aim of this work was to elucidate the catabolism of the predominant stromal protein Rubisco under

various senescence-inducing conditions. The proteolytic enzymes involved, the timing, the compartmentation, and the control mechanisms may partially depend on the factors initiating or accelerating senescence. Some controversial findings reported previously might be related to the conditions used to induce senescence.

Materials and methods

Plant matersial

Wheat (*Triticum aestivum* L.cv. Arina) grains were germinated for 4 days on wet paper in darkness and then for 3 days on well-watered coarse sand in a day/night cycle of 14/10 h at 25°C. Seven days after germination, the seedlings were transferred to a half-strength nutrient solution, according to Hildbrand et al. (1994). On day 11, the nutrient solution was exchanged with a full strength solution and the grains were removed from the plant. The plants were left on the pots until day 17, when the incubation of segments was started. The photosynthetically active radiation (PAR) was 80 μmol m^{-2} s^{-1}, measured at the level of leaf 2.

Experiments with leaf segments

Leaf segments (2 × 5 cm) were cut from the middle part of the fully expanded leaf 2 and incubated for the indicated times in plastic beakers containing 100 ml of deionized water, sucrose (50 mM), sorbitol (50 mM), or KCl (25 mM). In some experiments, the cysteine protease inhibitor (E-64) was used. Segments were incubated on 25 ml of deionized water or KCl (25 mM) with or without E-64 (100 μM), a cysteine protease inhibitor. During incubation, PAR was 0, 25, or 80 μmol m^{-2} s^{-1}, measured at the level of the segments. When segments were separated into different zones, the first 0.5 cm from both cut ends (zone A), the next 1 cm at both sides (zone B), and the middle 2 cm (zone C) were separated, immediatly frozen in liquid nitrogen, and then stored at –80°C. In one experiment (±CO_2), the leaf material was prepared and incubated as described previously (Herrmann and Feller 1998).

Experiments with intact plants

For the nitrogen starvation experiment, the nutrient solution was replaced on day 11 by either a N-containing (3.5 mM) or a N-depleted full-strength nutrient solution containing 800 g m^{-3} KH_2PO_4, 750 g m^{-3} $MgSO_4 \cdot 7H_2O$, 198 g m^{-3} $CaCl_2 \cdot 2H_2O$, and 70 g m^{-3} sequestren. The micronutrients were as described in Hildbrand et al. (1994). On day 13, the experiment was started. When intact plants were incubated in darkness, the nutrient solution was changed at the beginning of the experiment (day 17) and the plants were placed in darkness. Samples (5-cm segments) from the middle part of leaf 2 (dark experiment) or leaf 1 (N experiment) were stored at –80°C prior to analysis.

Preparation of leaf extracts

Leaf samples were powdered under liquid nitrogen and were then extracted with a Polytron mixer (PT 1200 Kinematica, Luzern, Switzerland) for 10 s at 3/4 speed and for 5 s at full speed. Unless stated otherwise, 10 cm (total length of two segments) plant material was extracted in 1 ml phosphate buffer (20 mM, pH 7.5) containing 1% [w/v] PVPP;and 0.1% [v/v] *β*-mercaptoethanol. The crude extract was filtered through Miracloth (Calbiochem, La Jolla, USA).

Incubation of extracts

The segments were incubated for 3 days at either 80 μmol m^{-2} s^{-1} in water or at 25 μmol m^{-2} s^{-1} PAR in water or KCl (25 mM). The leaf extracts were prepared from the inner part (zone B and C) of the segments. Duplicates of three segments (4 cm leaf length per segment) were homogenized in 600 μl of either a phosphate buffer (pH 7.5) or an acetate buffer (pH 5.4), each containing 1% [w/v] PVPP and 0.1% [v/v] *β*-mercaptoethanol. The crude extract was passed through Miracloth and was centrifuged at 4°C for 10 min at 20,000*g*. Samples of 120 μl of the supernatant were incubated for 0, 2, 4, or 8 h at 30°C.

Quantification of chlorophylls, proteins, and carbohydrates

The Miracloth filtrate was used to measure chlorophylls (Strain et al. 1971). For the determination of soluble proteins and soluble carbohydrates, the filtrate was centrifuged for 10 min at 20,000*g* at 4°C. Soluble proteins were measured in the supernatant according to Bradford (1976) using bovine serum albumin (PIERCE, Rockford, IL, USA) as the standard. The soluble carbohydrates were determined according to Stieger and Feller (1994). Sucrose was used as the standard.

SDS-PAGE and immunoblotting

Gel electrophoresis was carried out in a Mini Protean II Dual Slab Cell (Bio Rad, Glattbrugg, Switzerland)

according to Laemmli (1970). For Coomassie Blue-stained gels and for immunoblots developed with antibodies against the whole denatured LSU or against a synthetic version of the first 25 amino acids of the N-terminal region of LSU (N-LSU), 9% slab gels (0.75 mm) were used. For immunoblots developed with antibodies against other enzymes, 12% slab gels (0.75 mm) were used. Samples from either two or three replicates were combined and mixed before analysis by SDS-PAGE. Each lane was loaded with aliquots containing equal amounts of leaf length (0.67 mm), unless stated otherwise. After electrophoresis, the gels were stained with Coomassie Brilliant Blue R-250 for visualizing the protein pattern or blotted onto nitrocellulose membranes (0.45 mm supported, Bio Rad) for the detection of specific proteins (Mitsuhashi and Feller 1992). The primary polyclonal antibodies were kindly provided by S. Gepstein (Technion-Israel Institute of Technology, Haifa, Israel) against the whole denatured large subunit of Rubisco (Anti-LSU; Pauncz et al. 1992), by R. L. Houtz (University of Kensington, Lexington, USA) against the synthetic version of the first 25 amino acids from the N-terminal region of LSU of spinach Rubisco (Anti-N-LSU), by R. M. Wallsgrove (IACR-Rothamsted, Harpenden) against ferredoxin-dependent glutamate synthase (GOGAT) from barley (Marquez et al. 1988), by S. Crafts-Brandner (Western Cotton Research Laboratory, Phoenix, Arizona, USA) against purified recombinant tobacco Rubisco activase (Feller et al. 1998), and by T. Sugiyama (Nagoya University, Nagoya, Japan) against phosphoenolpyruvate carboxylase (PEPC) from maize (Sugiyama et al. 1984). Antibodies against purified glycolate oxidase (GO) from sugar beet were raised as reported previously (Mitsuhashi and Feller 1992).

Non-denaturing PAGE

For non-denaturing PAGE, the proteins were extracted from plant tissue as described above with the following modifications. The leaf extracts were prepared from the inner part (zone B and C) of segments incubated for 0 or 7 days in water at 25 μmol m^{-2} s^{-1}. Triplicates of four segments were homogenized (16 cm plant material in 600 μl phosphate buffer), combined, and mixed with half the volume of a sample buffer (196 mM Tris–HCl pH 6.8, 32% (v/v) glycerol, 0.02% (w/v) bromphenol blue). Gel elctrophoresis was carried out at 4°C in a Mini Protean II Dual Slab Cell (Bio Rad, Glattbrugg, Switzerland) according to Laemmli (1970) using 6% slab gels (1.5 mm). SDS was omitted from the gels and the running buffer. Each lane was loaded with an aliquot containing an equal amount of leaf length (3.6 mm). Proteins were detected by Coomassie-Blue staining (40% Methanol, 0.1% Comassie Brilliant Blue R-250). A piece containing Rubisco was excised from the gel and crushed with a plastic pistil in the presence of 40 μl of a 1:2 diluted sample buffer (196 mM Tris–HCl pH 6.8, 6.3% (w/v) SDS, 16% (v/v) *β*-mercaptoethanol, 32% (v/v) glycerol, 0.02% (w/v) bromphenol blue). After centrifugation at 20,000*g* for 10 min at 4°C, the supernatant was boiled for 5 min. Supernatant of 10 μl was loaded on a 9% slab gel (1.5 mm). SDS-PAGE was carried out as described above.

Results

Depending on the senescence-inducing treatment, different time courses for chlorophyll and protein degradation were observed (Table 1). For intact plants induced to senesce by nitrogen deficiency or darkness, protein degradation was more pronounced than chlorophyll degradation. In segments with increasing light intensities, proteolysis was retarded compared to chlorophyll degradation.

The level of carbohydrates was strongly influenced by the senescence-inducing conditions (Table 1). It differed markedly between segments exposed to low light or darkness and segments incubated at the higher light intensity. After 2 days of exposure to high light, the amount of soluble carbohydrates was fivefold higher than in segments incubated in low light. In dark-incubated segments or dark-incubated intact plants, the carbohydrate content decreased rapidly to a very low level. Senescence induced by nitrogen deprivation coincided with an increase of soluble carbohydrates.

The catabolism of proteins in senescing segments is shown in Fig. 1a. During the incubation of segments in low light, LSU was quite stable; however, a fragment of about 50 kDa appeared on day 7 (Fig. 1a, b). The same fragment was also present after 4 days of dark incubation, but it was essentially absent in segments exposed to high light. The 50 kDa band was identified with antibodies against whole denatured LSU, but it could not be detected by an antibody against the synthetic version of the first 25 amino acids of the N-terminal region of LSU (Fig. 1b). The 50 kDa cleavage product was also observed in intact plants (Fig. 2a, b). After 2 days of darkness, it was clearly visible, although it did not accumulate to such high levels as detected in segments. In nitrogen-starved leaves, LSU was continuously degraded, but until day 13, the 50 kDa fragment did not accumulate (Fig. 3) and also a longer period of N-starvation did not lead to the

Table 1 Chlorophyll, soluble protein and carbohydrate contents in extracts of senescing wheat leaves or leaf segments

Treatment	Time of incubation (day)	Chlorophyll (μg/segment)	Soluble proteins (μg/segment)	Soluble carbohydrates (μg/segment)
Leaf segments (leaf 2)				
PAR: 80 μmol m^{-2} s^{-1}	0	43	377	150
	2	47	531	2,740
	4	38	442	4,433
	7	8	183	3,463
PAR: 25 μmol m^{-2} s^{-1}	0	43	377	150
	2	41	361	538
	4	40	344	644
	7	30	210	371
PAR: 0 μmol m^{-2} s^{-1}	0	43	377	150
	2	36	234	55
	4	25	124	54
	7	11	35	19
Dark-incubated intact plants (leaf 2)				
Light	0	36	294	104
	2	50	431	139
	4	57	415	163
	7	62	353	165
Dark	0	36	294	104
	2	44	294	48
	4	37	139	26
	7	25	103	26
Nitrogen-starved intact plants (leaf 1)				
+Nitrogen	0	48	419	153
	4	45	438	154
	8	45	206	146
	13	46	228	204
–Nitrogen	0	59	394	141
	4	36	262	132
	8	41	105	273
	13	23	57	304

Senescence was induced by incubating segments (5 cm) in darkness or in light (25 or 80 μmol m^{-2} s^{-1}) or by incubating intact plants either in darkness or at N-limiting conditions. Duplicates of two or three (N-deficiency) segments were analyzed

formation of this band (data not shown). The catabolism of Rubisco in leaf segments was influenced by temperature and the level of CO_2 (Fig. 4). During the incubation of segments at 25°C, LSU was catabolized. The formation of the 50 kDa polypeptide was strongly stimulated by low CO_2 levels, whereas the influence of light was less important. In contrast, at 35°C, LSU remained more stable. Only at the lower light intensity, especially in the absence of CO_2, LSU declined, however, without any accumulation of the 50 kDa polypeptide. This result is consistent with the findings that Rubisco itself is not very susceptible to elevated temperature (Demirevska-Kepova and Feller 2004 and references therein).

During the incubation of segments, it became obvious that the inner part of the segment senesced differently from the outer part. To examine if the cutting of segments and a partial destruction of cells are responsible for this specific degradation of Rubisco, wheat segments were separated into different zones after incubation. The first, 0.5 cm at both ends (zone A), the next, 1.0 cm at both sides (zone B), and the inner 2 cm of the segment (zone C) were analyzed separately (Fig. 5). The catabolism of proteins was comparable in zone B and C, but it was different from zone A where an altered polypeptide pattern became visible. Furthermore, the 50 kDa polypeptide accumulated to higher levels in zone B and C than at the ends of the segment in zone A.

A cysteine protease mediating the first step in the catabolism of LSU was described by Yoshida and Minamikawa (1996). We therefore tested the influence of the cysteine protease inhibitor E-64 in our system (Fig. 6). In the presence of the inhibitor, the possible fragment of Rubisco at 50 kDa did not accumulate in the inner part of segments exposed to low light, although the main subunit declined. An enhancement of LSU degradation was achieved by incubating segments either with low light in KCl or in darkness in water. Again, the formation of the 50 kDa band was decreased by the addition of E-64. The influence of the cysteine protease inhibitor on the catabolism of other enzymes than Rubisco was also studied in order to distinguish between Rubisco-specific and more general

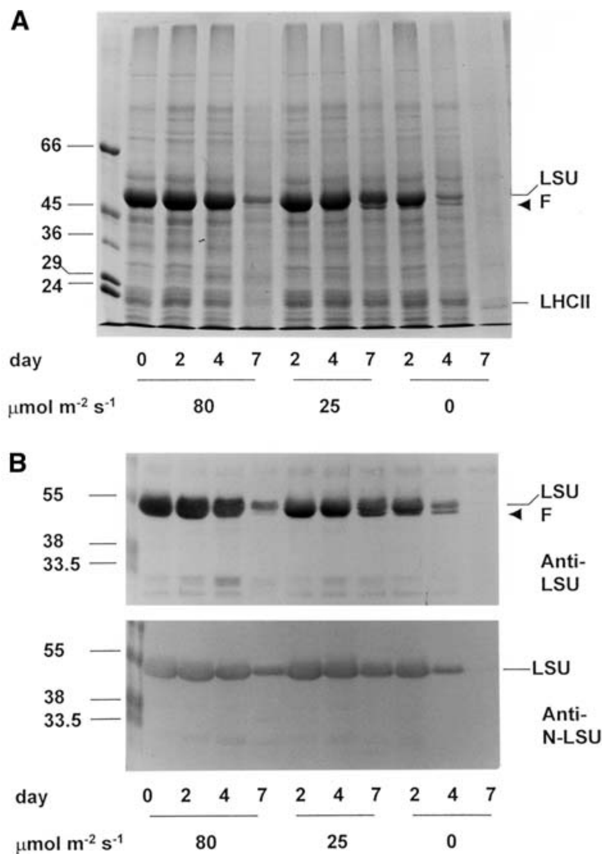

Fig. 1 Effects of light on the degradation of proteins in senescing wheat leaf segments. The segments were incubated in light (25 or 80 µmol m^{-2} s^{-1}) or in darkness (0 µmol m^{-2} s^{-1}). *LSU* and *LHCII* were visualized by Coomasie Blue-staining (**a**). A fragment of *LSU (F)* was detected at about 50 kD. *LSU* was immunologically detected (**b**) with specific antibodies against the whole denatured subunit (Anti-LSU) or against the N-terminus of LSU (Anti-N-LSU)

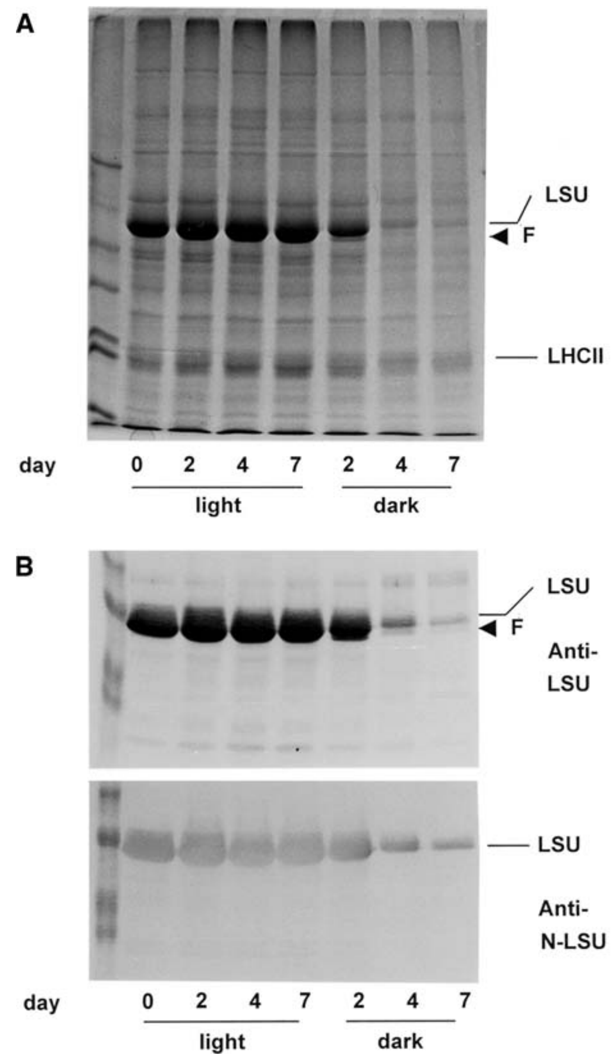

Fig. 2 Degradation of proteins in the second leaf of intact plants incubated in a light/dark cycle (*light*) or in permanent darkness (*dark*). *LSU* and *LHCII* were visualized on a Comassie Blue-stained gel. A fragment (*F*) at about 50 kD was detected on the gel (**a**). Immunoblots (**b**) were decorated with antibodies against the whole denatured LSU (Anti-LSU) or with antibodies against the N-terminus (Anti-N-LSU). The fragment was recognized by Anti-LSU but not by Anti-N-LSU

effects (Fig. 6). Rubisco activase (Activase), an enzyme present in the chloroplast, was very susceptible to degradation. The addition of E-64 to the incubation medium retarded the catabolism of this protein on low light, in the presence and absence of KCl, and in darkness. Another plastidial protein, glutamate synthase (GOGAT), was much more stable than Activase during incubations. Due to this stability, a protective effect of E-64 was less clearly visible. Enzymes localized in other compartments than the chloroplast were analyzed for comparison. Phosphoenolpyruvate carboxylase (PEPC) present in cytosol was relatively stable. An effect of the protease inhibitor was therefore hardly visible. The peroxisomal protein glycolate oxidase (GO), however, was rapidly degraded. The catabolism of this enzyme was only slightly affected by E-64, but the data indicated that GO was protected weakly during light incubations. The effect of E-64 on protein catabolism during senescence of segments exposed to higher light was tested in addition (Fig. 7). Except PEPC, all proteins tested were essentially not influenced by the presence of the inhibitor.

An incubation of leaf extracts, prepared from the inner part of segments (zones B and C), revealed a pH dependence of the proteolytic event leading to the formation of the 50 kDa fragment (Fig. 8). At pH 5.4, LSU was cleaved and the prominent fragment transiently accumulated already after 2 h of incubation. An additional weak band below this fragment became visible on the gel after 2 h of

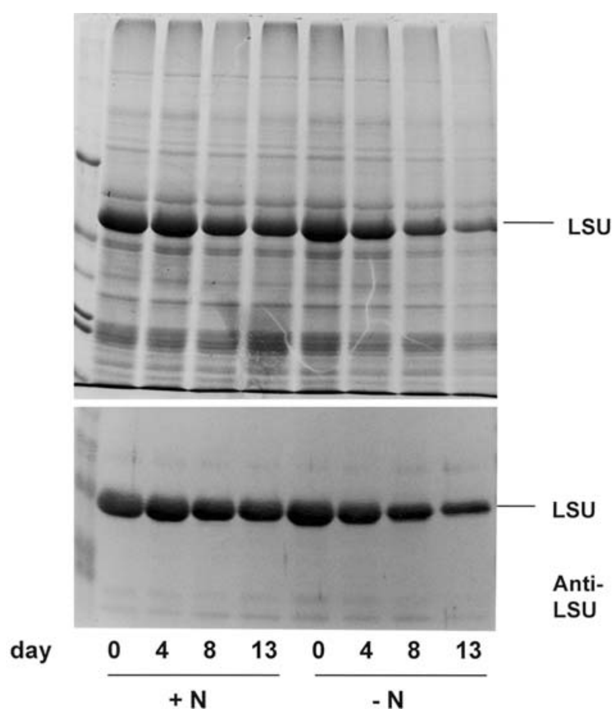

Fig. 3 Degradation of LSU in the first leaf of intact wheat plants under nitrogen deficiency. Two days after supplying either a nitrogen-containing (+*N*) or a nitrogen-depleted (–*N*) nutrient solution, the experiment was started (day 0). *LSU* was visualized on a Coomassie Blue-stained gel and on an immunoblot incubated with anti-LSU

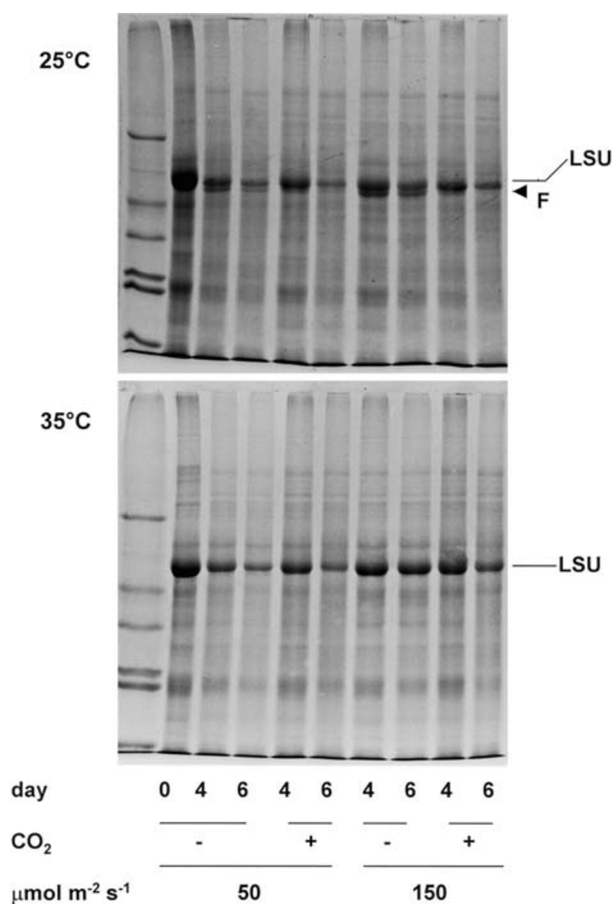

Fig. 4 Effect of CO_2 on the degradation of LSU under two irradiances in senescing wheat leaf segments. Segments of leaf 1 were incubated at 25 or 35°C in ambient (+) or CO_2-depleted (–) air. The PAR was 50 or 150 µmol m^{-2} s^{-1}. LSU and a possible *LSU fragment* (*F*) in the range of 50 kD were visualized on a Coomasie Blue-stained gel. Each lane was loaded with an amount of sample equivalent to 0.7 mm of a leaf segment

incubation. The intensity of this band depended on the preincubation conditions of the plant material. The band did not cross-react with antibodies against LSU. At a higher pH (7.5), Rubisco was less affected. Even after 8 h of incubation, LSU was not markedly reduced.

Non-denaturing-PAGE followed by SDS-PAGE was used to determine if the 50 kDa polypeptide was generated while LSU was still integrated in the holoenzyme, and whether it remained there after proteolytic cleavage (Fig. 9a, b). On non-denaturing PAGE from control plant material (day 0), only one sharp, intense band was detected. Analysis of the senescing plant material (day 7) revealed one sharp, intense band slightly below the band from control plants (Fig. 9a). From the intensity of this band and from the comparison of its migration properties with those of molecular weight markers, it was concluded that this predominant band represents Rubisco holoenzyme from control and senescing leaves. Additionally, the subsequent analysis by SDS-PAGE showed the presence of LSU and SSU (Fig. 9b). When the holoenzyme of the senescing leaves was analyzed by SDS-PAGE, an additional band below LSU at 50 kDa was present.

Discussion

The relative rates of chlorophyll and protein degradation have been demonstrated to vary according to the senescence-inducing conditions applied. In addition, distinctly different degradation patterns of LSU became evident depending on the senescence-inducing condition. In segments incubated in darkness or in low light and in intact dark-incubated plants, a degradation product of about 50 kDa accumulated while LSU declined. Yoshida and Minamikawa (1996) described a similar fragment of 50 kDa in incubated French bean leaf extracts. In contrast to our results, they could not detect the fragment in incubated detached leaves. Since there are differences in the response of monocotyledonous and dicotyledonous plants to detachment (Feller and Fischer 1994), such conflicting data may arise. In oat leaf slices, a senescence-like response was

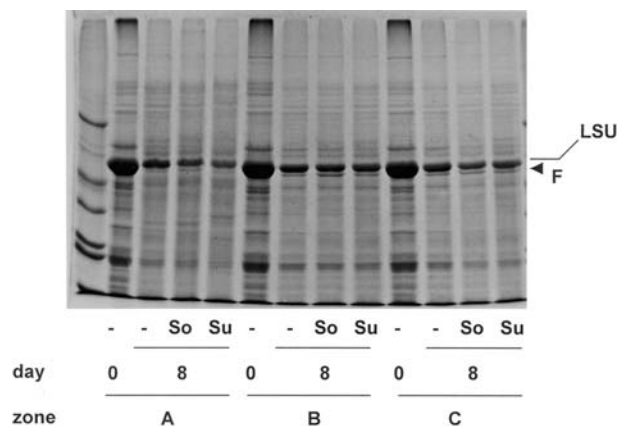

Fig. 5 Proteolysis in different zones of senescing wheat leaf segments. The segments were incubated in water (–), 50 mM sorbitol (*So*), or 50 mM sucrose (*Su*) in low light (25 µmol m^{-2} s^{-1}). After incubation, the segments were separated into zone A (0.5 cm at both ends), B (next 1.0 cm at both sides) and C (2.0 cm in the center). Proteins were visualized on a Coomassie Blue-stained gel. Especially in zone *B* and *C*, a possible LSU fragment (*F*) appeared below LSU at about 50 kD

achieved by incubation with the host-selective toxin victorin (Navarre and Wolpert 1999). It resulted in the formation of an LSU cleavage product of 53 kDa in darkness, whereas in light, LSU was degraded without any accumulation of degradation products. Parrott et al. (2005) also detected a band of this size in extracts of excised (but not of attched) barley leaves after initiating senescence. Yoshida and Minamikawa (1996) further reported that Rubisco LSU is degraded by at least two types of vacuolar proteases. A protease, which belongs to the same cysteine protease class as SH-EP, hydrolyzes LSU in the first step. In wheat extracts, a similar fragment of 50 kDa has been described by Ishida et al. (1997). The cleavage of LSU was impaired in the presence of the cysteine protease inhibitor E-64, in this system. Moreover, it has been reported by Navarre and Wolpert (1999) that in victorin-treated leaf slices, the formation of the 53 kDa fragment could be prevented in a similar manner by the addition of two different cysteine protease inhibitors (E-64 and leupeptin). In intact chloroplasts and chloroplast lysates, the most prominent cleavage products of LSU had molecular weights of about 36 and 16-kDa, but no 50 kDa product was described (Desimone et al. 1996; Ishida et al. 1997; Roulin and Feller 1998b). Degradation products of about 50 kDa could be detected in lysates only if chloroplasts were contaminated with vacuolar proteases (Miyadai et al. 1990). In our experiments, the protease inhibitor E-64 prevented the formation of the 50 kDa polypeptide in leaf segments incubated in low light and under senescence-promoting

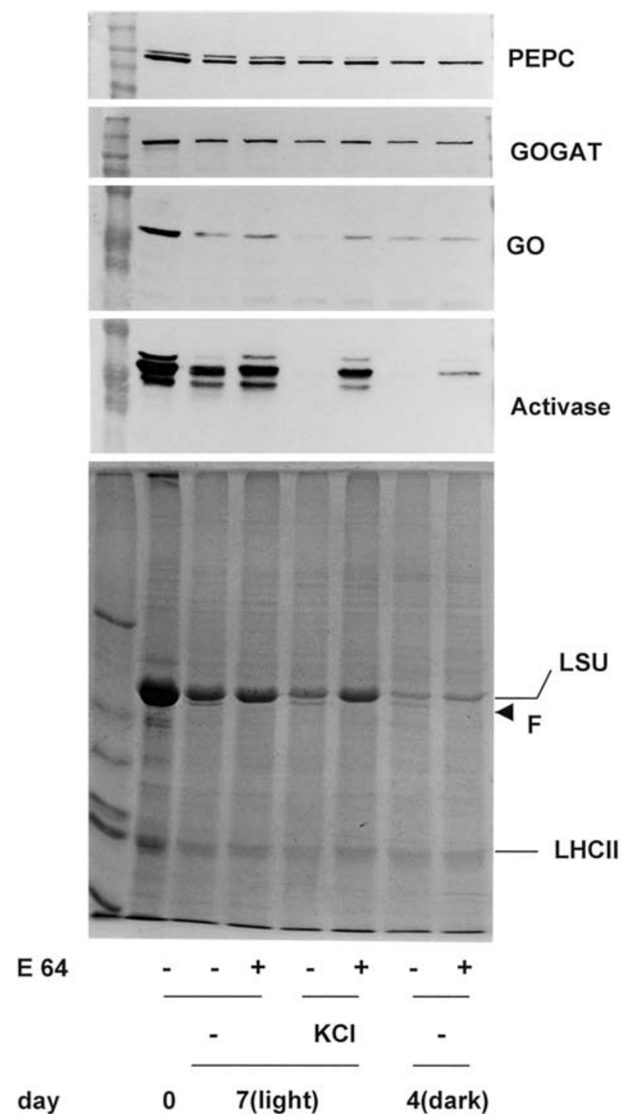

Fig. 6 Effect of E-64 (100 µM) on the degradation of selected proteins in senescing wheat leaf segments incubated for 7 days in water (–) or 25 mM KCl at 25 µmol m^{-2} s^{-1} (*light*) or for 4 days in water, in darkness (*dark*). After incubation, 0.5 cm at both ends of the segments was removed and the remaining part was analyzed. *LSU*, the possible *LSU fragment* (*F*), and *LHCII* were visualized on a Coomassie Blue-stained gel. *PEPC*, *GOGAT*, *GO*, and *Activase* were identified with specific antibodies

conditions. Moreover, the proteolytic process leading to the 50 kDa fragment in incubated extracts was clearly stimulated at pH 5.4 as compared to pH 7.5. A low pH (5.4) would be adequate for vacuolar enzymes, but it would be far below the pH in the stroma of functional chloroplasts. These results prompted us to suggest that a vacuolar protease functions to degrade Rubisco under conditions where the 50 kDa fragment is formed. This conclusion is consistent with the observation that such a fragment was formed in crude

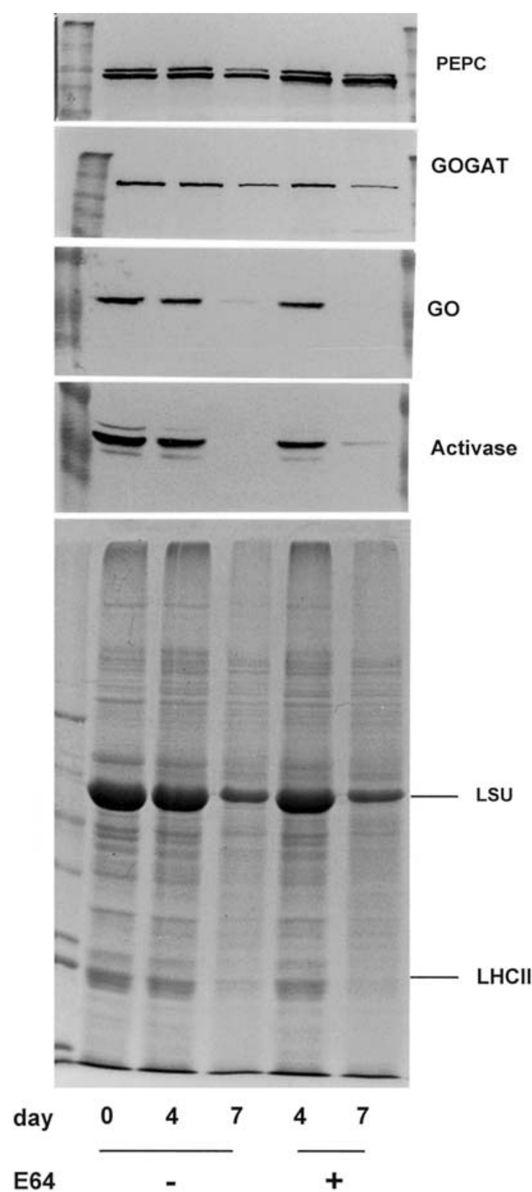

Fig. 7 Degradation of selected proteins in senescing wheat leaf segments exposed to light (80 μmol m^{-2} s^{-1}) in the presence or absence of 100 μM E-64. After incubation, 0.5 cm at both ends of the segments was removed and the remaining part was analyzed. *LSU* and *LHCII* were visualized on a Coomasie Blue-stained gel. *PEPC*, *GOGAT*, *GO*, and *Activase* were identified with specific antibodies

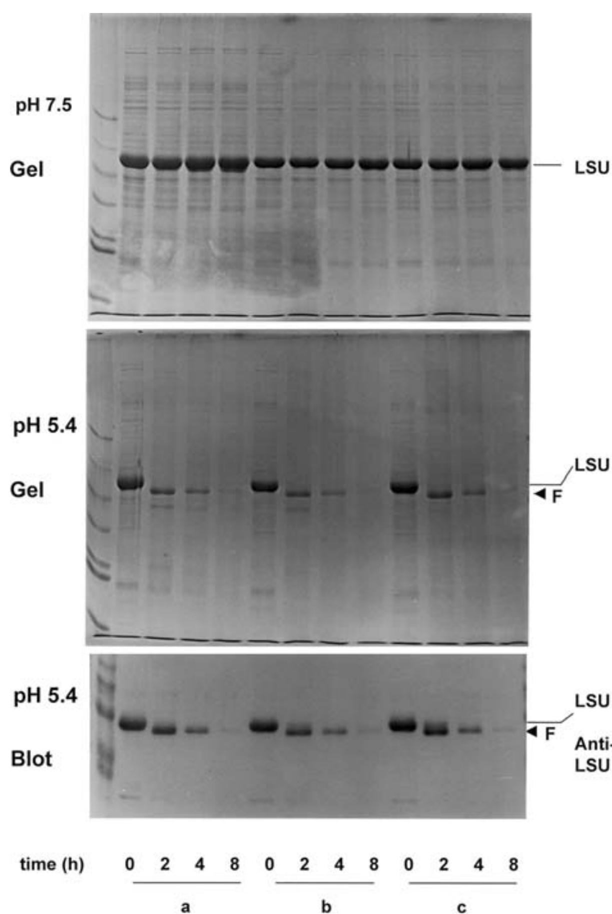

Fig. 8 Rubisco degradation in incubated wheat leaf extracts. Wheat segments were preincubated for 3 days at 80 μmol m^{-2} s^{-1} in water (**a**) or at 25 μmol m^{-2} s^{-1} in water (**b**) or 25 mM KCl (**c**). After preincubation, 0.5 cm at both ends of the segments was removed and the remaining part was extracted with either an acetate buffer (pH 5.4) or a phosphate buffer (pH 7.5). After centrifugation, the supernatants were incubated at 30°C. *LSU* and a *fragment (F)* at about 50 kD were detected on Coomassie Blue-stained gels and on the immunoblot incubated with specific antibodies against whole denatured LSU

extracts from young wheat leaves, but not in chloroplast lysates from the same source (Zhang et al. 2006). A comparison of Rubisco with other proteins showed that beside LSU, Rubisco activase was stabilized by the protease inhibitor E-64. Other enzymes were either not affected or were too stable to show an inhibition of proteolysis. However, E-64 was unable to completely inhibit protein degradation during the time course. In high light, E-64 had no obvious effect, although proteins were degraded. Therefore, at least two different mechanisms, E-64-sensitive and E-64-insensitive, may have taken place under the different conditions used (high light, low light, darkness).

A senescence-associated formation of special vacuoles containing a senescence-specific cysteine protease has been detected in leaves of *Arabidopsis* and soybean (Otegui et al. 2005). Ricinosomes (precursor protease vesicles) containing large amounts of a papain-type cysteine endopeptidase develop at the beginning of programmed cell death in the nucellus of *Ricinus communis* (Gietl and Schmid 2001; Greenwood et al. 2005). The ricinosomes bud from the endoplasmatic reticulum (Schmid et al. 2001). The

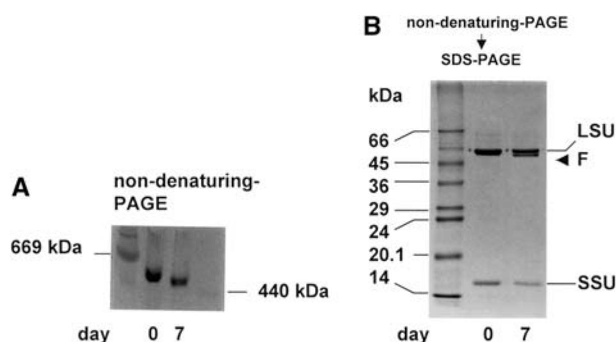

Fig. 9 Rubisco holoenzyme isolated from a non-denaturing gel. Wheat segments incubated for 0 or 7 days in water in low light (25 μmol m^{-2} s^{-1}) were analyzed by non-denaturing PAGE (**a**). Rubisco holoenzymes and two marker proteins, thyroglobulin (669 kDa) and ferritin (440 kDa), were visualized by a Coomassie Blue-staining (without acetic acid). Each lane was loaded with an amount of sample equivalent to 3.6 mm of a segment. The band corresponding to Rubisco holoenzyme was excised from the gel and further analyzed by SDS-PAGE (**b**). *LSU*, *SSU*, and the possible *LSU fragment (F)* were visualized together with a molecular weight marker in the range of 14–66 kDa on a Coomassie Blue-stained gel

cysteine endopeptidase precursor in these vesicles contains a C-terminal KDEL motif (Schmid et al. 2001). It remains open to debate whether this type of endopeptidases was involved in the E-64-sensitive degradation of Rubisco reported in this paper or another type of cysteine-endopeptidase was involved.

In our system, the 50 kDa fragment was not or only weakly detectable in segments exposed to high light and in intact plants induced to senescence by nitrogen deprivation, although the level of LSU declined. In excised leaf segments incubated in higher light, carbohydrates markedly increased (Table 1), and in senescing leaves of N-stressed plants, a twofold increase of carbohydrates could be observed. On the other hand, both detachment and low light intensities seem to enhance the accumulation of the 50 kDa fragment. Additionally, removing CO_2 during incubations of segments in high light led to low carbohydrate levels and to accumulation of the possible LSU fragment at 50 kDa. These results indicated that the C-status of the cell could play a regulatory role in the degradation of Rubisco and in the accumulation of the 50 kDa polypeptide. Feeding of sucrose during incubations in low light, however, did not prevent the formation of the 50 kDa polypeptide in the inner part of the segments. LSU was degraded and the possible 50 kDa fragment accumulated, although the carbohydrate level was increased about threefold compared to controls (data not shown). Individual sugars or other metabolites and their compartmentation might be more relevant in this context than the overall level of carbohydrates in a leaf segment. We can only speculate about the regulatory mechanisms leading to the 50 kDa polypeptide and the involvement of assimilate levels or of the energy status of the cell.

Since the formed 50 kDa fragment was not recognized by a specific antibody raised against the N-terminus of LSU, it appears likely that a smaller fragment was removed from the N-terminus of LSU. Consistent with this result, the cleavage product reported by Ishida et al. (1997) also did not cross-react with anti-N-LSU. The cleavage site was further characterized and located at the 14th amino acid from the N-terminus (Yoshida and Minamikawa 1996; Navarre and Wolpert 1999). LSU seems to be especially vulnerable at this site. Other investigators described that during in vitro treatment of Rubisco with trypsin and the endoproteinase Lys C, proteolysis occurred at Lys 14 (Gutteridge et al. 1986; Mulligan et al. 1988; Houtz et al. 1989). This proteolytic event caused a loss of carboxylase and oxygenase activities without destroying the quaternary structure and without disrupting the substrate binding (Gutteridge et al. 1986; Mulligan et al. 1988). Proteolytic inactivation of Rubisco was drastically reduced under catalytic conditions (Houtz and Mulligan 1991).

The 50 kDa fragment accumulated, while LSU was still integrated in the holoenzyme complex. Assuming that the cysteine protease involved is located in the vacuole, the question arises, how Rubisco as a holoenzyme would reach the protease (or vice versa). Transfer of vesicles, autophagic processes or loss of membrane integrity represent possible mechanisms (Greenwood et al. 2005; Hörtensteiner and Feller 2002). It has been shown in *Chlamydomonas reinhardtii* that soluble plastidial proteins can be transferred via protrusions from the chloroplast to vacuoles (Park et al. 1999). A mass exodus from chloroplasts of senescing soybean leaves has been proposed by Guiamet et al. (1999). For higher plant cells subjected to carbon deprivation, an autophagic process leading to the disintegration of plastids has been reported (Aubert et al. 1996). This mechanism provides a possibility for plants to compensate for the energy demand under carbon starvation by dissimilating lipids and proteins instead of sugars.

For future work, it has to be considered that different proteolytic events may take place under various senescence conditions and that a wide range of proteolytic enzymes may contribute to the catabolism of leaf proteins during senescence (Bhalerao et al. 2003). The involvement of vacuolar proteases in the degradation of plastidial proteins should be considered as a possibility, especially under low light intensity or under carbon limitation.

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