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TOTAL AND RUBISCO SPECIFIC PROTEOLYTIC ACTIVITY DURING DARK INDUCED SENESCENCE OF BARLEY SEEDLINGS

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Summary. Six-day-old seedlings of barley (*Hordeum vulgare* L., cv. Hemus) were placed in darkness in order to induce senescence-like changes. The symptoms of senescence and the reversibility of the processes were followed by analyses of the content of leaf pigments, the levels of total soluble and Rubisco immunoreactive protein and total proteolytic activity with casein as a substrate. Rubisco specific degradation was estimated applying highly sensitive avidin-biotin ELISA method. The results do not support an involvement of metallo protease or ATP-dependent protease in Rubisco degradation in leaf extracts during dark treatment. It is suggested that enzyme modifications, aggregation and/or association with membranes may occur prior to Rubisco degradation.

Key words: *Hordeum vulgare* L., Rubisco specific proteolytic activity, senescence, total proteolytic activity

Abbreviations: biotin NHS – N-hydroxysuccinimidobiotin**;** DEAE – diethylaminoethyl; EDTA – ethylene diamine tetraacetic acid; ELISA – enzyme-linked immunosorbent assay; LS – Rubisco large subunit; OPD – o-phenylenediamine dihydrochloride; PBS – 20 mM phosphate buffered saline pH 7.2; PMSF – phenyl-methyl sulphonyl fluoride; Rubisco – ribulose-1,5-bisphosphate carboxylase/oxygenase, EC 4.1.1.39; SS – Rubisco small subunit

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Introduction

Leaf senescence is a complex highly regulated developmental phase characterized by co-ordinated ordered degradation of macromolecules and the subsequent mobilization of components to other parts of the plant. In addition to the programmed type of leaf senescence which takes place during plant development, the degradation of macromolecules and mobilization of cellular components from leaves occurs also as a response to external environmental factors. The senescence of leaves is provoked or accelerated by different factors: light limitation, mineral deficiency, wound and drought stress, pathogen infection etc. (Buchanan-Wollaston, 1997). It is assumed that the biochemical degradation processes during senescence or under different kinds of stress take place through mechanisms, which are probably similar (Desimone et al., 1996).

Rubisco is the major leaf protein and the key enzyme of photosynthesis, accounting for about 40–50% of soluble protein during leaf expansion. This enzyme, which has been reported to have negligible turnover in mature leaves, undergoes a dramatic change in turnover rate during early senescence (Crafts-Brandner et al., 1990). The decrease in Rubisco holoenzyme is accompanied by a decline in the transcripts level of the enzyme as well as of other photosynthetic proteins (Crafts-Brandner et al., 1996). In addition to the reduced synthesis, the rapid loss of Rubisco is ascribed to selective proteolysis. It is assumed that during senescence, the breakdown of this enzyme is of great physiological significance since it provides additional amino acids for mobilization to the reproductive organs. Nevertheless, the mechanisms of Rubisco degradation during senescence remain to be elucidated (Huffaker, 1990; Roulin and Feller, 1998).

Changes in total proteolytic activity during experimentally induced and natural senescence has been attributed to vacuolar enzymes (Wittenbach, 1978, 1979; Peoples et al., 1980). More than 90% of the proteolytic activity in the leaf extracts is due to vacuolar proteases with acid pH optimum. However, the role of the vacuoles in general protein breakdown remains still unclear. A more specialized function for these organelles is not excluded, for example defence against pathogens and parasites, or during the final stage of plant senescence after rupture of the vacuolar membrane (Vierstra, 1996). In senescing leaves the chloroplasts remain apparently intact and are disintegrated only in the last stage, hence the specific degradation of Rubisco is probably located in the chloroplasts, not in the vacuoles (Peoples et al., 1980; Martinoia et al., 1983). Neutral proteolytic activities in the stroma and membrane fractions of chloroplasts have been described by some authors (Bushnell et al., 1993; Shanklin et al., 1995; Sokolenko et al., 1997) but little is known about their functions, activity patterns, regulation and natural substrates. Shanklin et al. (1995) found the highest levels of the ATP-dependent chloroplast Clp protease complex (homologous to the prokaryotic ATP-dependent protease Clp system) in expanding leaves and the lowest levels in senescing leaves. Thus, Clp protease could have an important function in non-senes-

cing photosynthetically active chloroplasts. Recently, some data concerning the role of this protease in senescence have been published (Nakabayashi et al., 1999). Bushnell et al. (1993) purified and characterized Mg^{2+} or Ca^{2+}-activated metalloprotease from pea which was able to attack LS of Rubisco under physiological conditions (pH 7.7) and assumed that this might be the stromal located protease responsible for the start of Rubisco degradation during senescence. It could be expected that Rubisco specific proteases have higher affinity to the Rubisco molecule as compared to exogenous substrates generally used for detection of proteolytic activity. Therefore, it is necessary to apply, instead of the colorimetric methods, other more sensitive methods for estimating Rubisco-specific proteolytic activity, using Rubisco as a substrate. Otto and Feierabend (1994) introduced an assay system with fluorescent or [^{14}C]-labelled Rubisco SS and Rubisco holoenzyme as substrates for investigation of holoprotein assembly. Similar sensitivity was obtained using ELISA assay on the basis of avidin-biotin interactions for collagenase (Wilkinson et al., 1990).

The aim of the present investigation was to induce senescence-like symptoms in barley seedlings by transferring them to dark conditions in order to study certain possibilities for Rubisco specific degradation in these conditions applying highly sensitive ELISA method with biotinylated Rubisco based on avidin-biotin interactions.

Materials and Methods

Plant material. Six-day-old barley seedlings (*Hordeum vulgare* L., cv. Hemus) grown in tap water under 12/12 h day/night at 63 $W.m^{-2}$ (27/22 °C) were induced to senescence by placing them in the dark according to the model system of dark-induced senescence applied by Wittenbach (1978) to intact wheat seedlings. The reversibility of the processes was controlled by transferring the seedlings again from darkness to light for recovery. Leaf material was taken 24 and 72 h after the transfer from light to darkness and 72 h after the retransfer of the seedlings to light after their dark treatment.

Leaf extracts. Two grams of leaf material were homogenised with 8 ml of ice-cold 100 mM Tris-HCl pH 8 containing 20 mM $MgCl_2$, 10 mM $NaHCO_3$, 12.5% glycerol (v/v), 20 mM β-mercaptoethanol, 120 mg Polyclar and centrifuged at 12 000×g for 15 min. The supernatant was stored at -20 °C and used for determination of proteolytic activity. For estimation of total soluble protein and Rubisco the same extraction buffer was used containing 1 mM EDTA and 2 mM PMSF in addition.

Rubisco-specific proteolytic activity. New avidin-biotin test system was used according to Simova-Stoilova and Demirevska-Kepova (2000). Briefly, avidin 0.5 µg in 100 µl per well was immobilised one night at 8 °C on microtiter plate (plate A). Non-occupied places were blocked with 1% ovalbumin in PBS pH 7.2 for one hour at 30 °C. Incubations for proteolytic activity were performed on another plate (plate

B), blocked with 1% ovalbumin, washed and dried. The extracts were diluted in buffers with different pH: 200 mM phosphate-citrate buffer pH 6, 200 mM Tris-HCl pH 7, 250 mM borate buffer pH 8 and pH 9, each buffer supplemented with 40 mM $MgCl_2$. Biotinylated Rubisco (20 µg/ml) was mixed (1:1, v/v) with leaf extracts (0.5 mg/ml) or with 1% ovalbumin as a control. A standard curve with different concentrations of biotinylated Rubisco (2.5 to 10 µg/ml with carrier protein 0.5% ovalbumin in 250 mM borate buffer pH 8) for estimation of the quantity of undegraded biotinylated Rubisco was made on the same plate B. After incubation for one hour at 30 °C protease inhibitors from stocks were added to all wells to a final concentration 10 mM EDTA, 2 mM PMSF, 10 µM leupeptin and 10 µM DL-norleucine. The samples were transferred to the plate A with immobilized avidin (100 µl per well). After 1 hour incubation at 30°C avidin-peroxidase (diluted 1:3000) was added to the wells. The sandwich avidin-peroxidase–non-degraded biotinylated Rubisco–immobilized avidin was visualized by a colour reaction for peroxidase using 2 mg/ml OPD and 0.015% hydrogen peroxide in phosphate-citrate buffer pH 5. The reaction was stopped by adding 6 N H_2SO_4 and the optical density at 490 nm was registered by ELISA reader.

Total proteolytic activity. The total proteolytic activity was analysed using the method of Saleemuddin et al. (1980) modified for barley leaf extracts. A standard curve with casein as a substrate (0–100 µg in 50 µl test sample) was prepared. Total proteolytic activity was studied at various pH using 200 mM phosphate-citrate buffer pH 6, 200 mM Tris-HCl pH 7 and 250 mM borate buffer pH 8 and pH 9. The assay mixture contained a sample with protein quantity 120 µg, casein 400 µg (from stock 5 $mg.ml^{-1}$) and buffer with different pH in a total volume of 200 µl. After incubation for 3 hours at 30 °C, the reaction was stopped by adding the reactive of Bradford (see below). The optical density at 595 nm was registered spectrophotometrically.

Determination of protein quantity was done spectrophotometrically according to Bradford (1976).

Determination of leaf pigment quantity was according to Arnon (1949) and calculated using the formula of Mc Kinney .

Quantitative determination of Rubisco in leaf extracts from barley was carried out immunochemically by sandwich ELISA using rabbit polyclonal antibodies specific against barley Rubisco and goat anti-rabbit IgG-peroxidase conjugate (Metodiev and Demirevska-Kepova, 1992).

Results

Changes in leaf pigments, total soluble protein and Rubisco immunoreactive protein were analysed in primary barley leaves from intact seedlings kept for various periods

in darkness in order to induce symptoms typical of senescence. The reversibility of the processes was controlled by the same analyses in barley seedlings retransferred from darkness to light for recovery. Generally, induced senescence was studied in fully expanded leaves. According to our preliminary data on protein quantity in leaf extracts, the primary leaves of intact barley seedlings entered the phase of full expansion by the day eighth after sowing. The phase of natural senescence provoked by the development of the second leaf was reached by the day twelfth. In order to follow the recovery processes after the light deprivation before the onset of natural senescence symptoms, the dark treatment was applied from the day sixth after sowing.

The data on the content of leaf pigments are presented in Fig. 1. Dynamic changes are observed in the content of chlorophyll *a*, chlorophyll *b* and carotenoids. The content of all pigments decreased rapidly by approximately 50% in the first 24 h. Later, the content of chlorophyll *a* and *b* decreased only slightly but the carotenoids began to increase. In the first 24 h the loss of pigments was completely recoverable, whereas after 72 h – not fully recoverable for chlorophyll *a* and *b* and completely recoverable for carotenoids. The chl *a* to chl *b* ratio decreased slightly from 2.7 to 2.1 in the first 24 h but this change was recoverable. Thereafter the chl *a* to chl *b* ratio remained almost constant. The chl *a*+*b* to carotenoides ratio decreased only after 7 h light depriv-

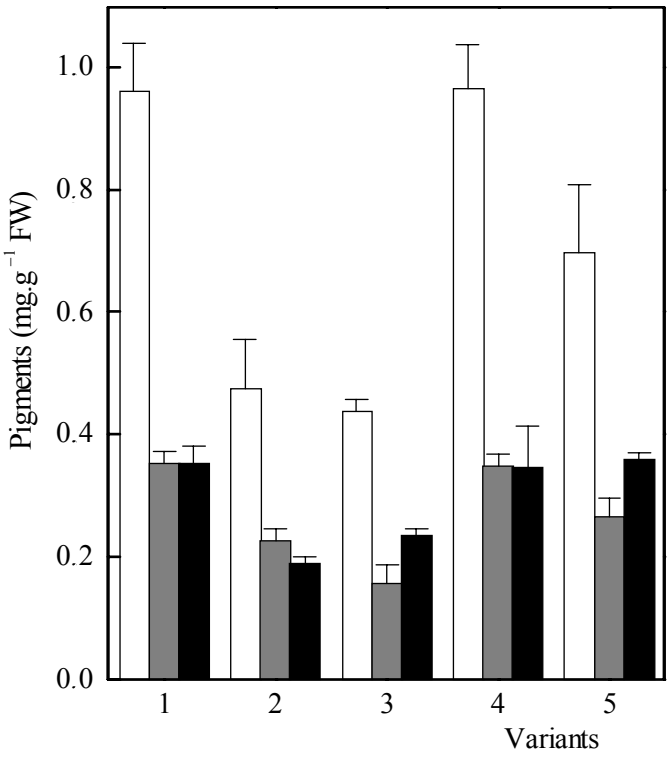

Fig. 1. Content of leaf pigments in green plants (1), plants after transfer to the dark for 24 h (2) and 72 h (3) and after 72 h recovery from 24 h (4) and 72 h (5) dark-treatment. White columns – chlorophyll *a*, gray columns – chlorophyll *b* and black columns – carotenoids. Vertical bars – standard deviations.

ation from 3.7 to 2.5 and this change was not recoverable.

The content of total soluble protein did not change during the dark treatment (Fig. 2A). A slight decrease (approximately 19%) in total soluble protein was observed only during the recovery from 72 h treatment in darkness. Similarly to the decrease in pigments content, Rubisco quantity declined by 36% in the first 24 h and by 49% later as compared to the Rubisco content in green leaves (Fig. 2B). In percent of total soluble protein the loss of Rubisco was recoverable (Fig. 2C). This loss was specific during the 24–72 h dark treatment. On the other hand, during the recovery from 72 h light deprivation Rubisco quantity decreased to the same extent as the total soluble protein.

At a biochemical level the decline in leaf pigments, total soluble protein, and especially Rubisco are usually considered as senescence progression indices and a measure for the reversibility of the processes. In the model system used in the present study we observed the typical rapid and selective loss of Rubisco. That is why we investigated the Rubisco selective proteolytic activity in comparison with the total proteolytic activity in leaf extracts from dark-treated barley seedlings.

Total proteolytic activity with casein as a substrate (caseinolytic activity) was investigated at various pH in the presence of 20 mM $MgCl_2$ (Fig. 3). Because of the appearance of precipitates in the samples during incubation, pH 5 was not included. The caseinolytic activity in green plants (Fig. 3A) was highest at pH 6. The total proteolytic activity in the first 24 h of dark treatment decreased from 30 to 50% at pH

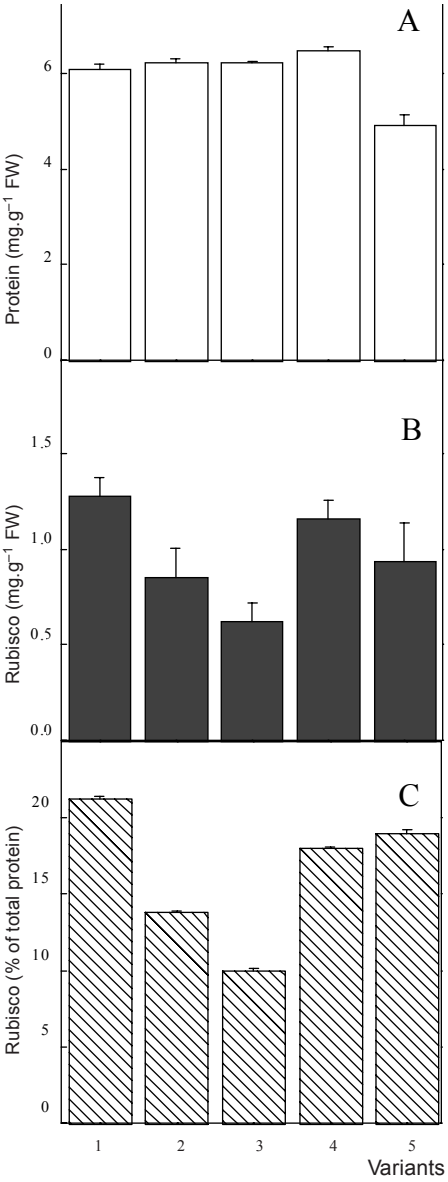

Fig. 2. Content of the total soluble protein in mg.g^{-1} FW (A), Rubisco content in mg.g^{-1} FW and Rubisco as a percent of the total soluble protein (C) in green plants (1), plants after transfer to the dark for 24 h (2) and 72 h (3) and after recovery from 24 h (4) and 72 h (5) treatment in darkness. Vertical bars represent standard deviations.

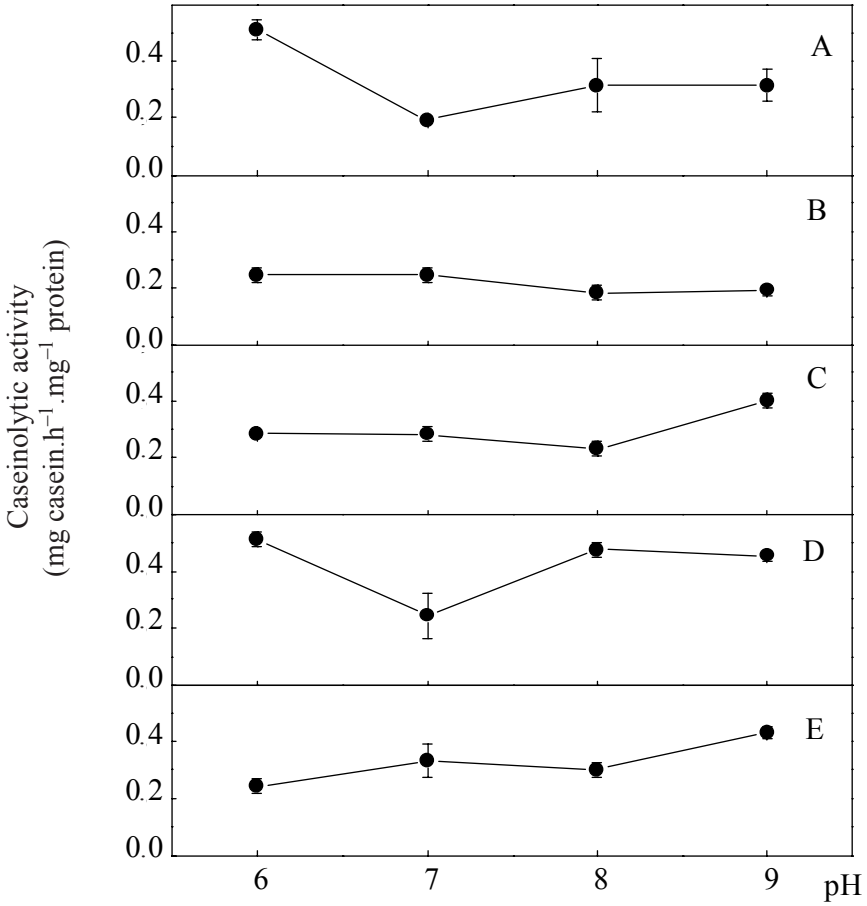

Fig. 3. Caseinolytic activity in green plants (A), plants after transfer to the dark for 24 h (B) and 72 h (C) and after recovery from 24 h (D) and 72 h (E) darkness in the presence of 20 mM $MgCl_2$. The results are expressed as mg degraded casein for one hour per mg leaf protein. Vertical bars – standard deviations.

6, 8 and 9, while at pH 7 it remained almost unchanged compared to green plants. After 72 h in darkness the caseinolytic activity measured at pH 9 increased and remained unchanged during recovery. The pH profile of caseinolytic activity after recovery from the first 24 h was similar to the profile of the green plants. After recovery from 72 h of dark treatment the pH profile was similar to the profile of the plants treated for 72 h in darkness, indicating the irreversibility of the processes. The decrease in caseinolytic activity during the first 24 h in darkness was not connected with the specific loss of Rubisco.

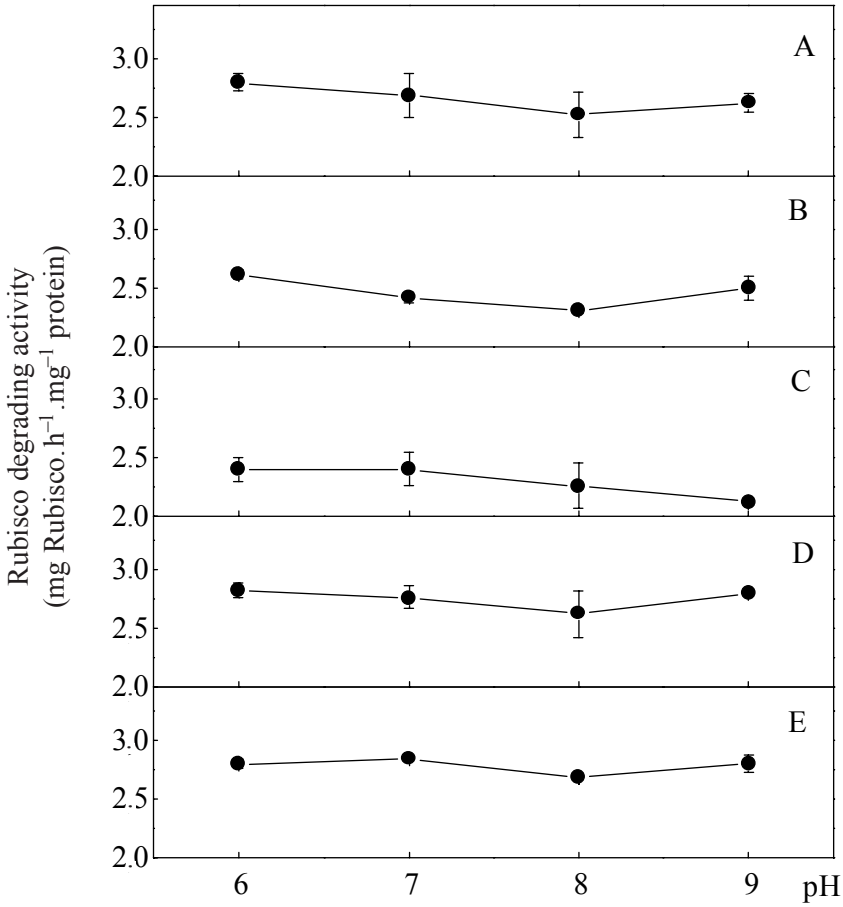

Fig. 4. Rubisco degrading activity in green plants (A), plants after transfer to the dark for 24 h (B) and 72 h (C) and after recovery from 24 h (D) and 72 h (E) darkness in the presence of 20 mM $MgCl_2$. The results are expressed as µg degraded Rubisco for one hour per mg leaf protein. Vertical bars – standard deviations.

The dependence of the quantity of degraded biotinylated Rubisco on pH is presented in Fig. 4. In these analyses Mg^{2+} was added to the buffers. During dark treatment and recovery periods evident differences in Rubisco specific proteolytic activity were not observed. Only after 72 h in darkness Rubisco specific proteolytic activity at pH 9 decreased slowly. Probably, the avidin-biotin test for Rubisco specific proteolytic activity detects a certain kind of constitutive protease. The influence of ATP addition at pH 7.5 on Rubisco specific proteolysis was also studied and the same levels of proteolytic activity were obtained (data not presented).

Discussion

Light deprivation is a convenient tool for inducing senescence-like changes in most of the plant species under controlled and reproducible conditions. Similar changes in chlorophyll, total protein and rapid selective loss of Rubisco were observed in seedlings placed in the dark and at the onset of senescence in the leaves of field grown wheat (Wittenbach, 1978, 1979). However, a difference from the natural and programmed senescence during plant development which is an irreversible process, is that in the cases of senescence, induced by environmental factors, the process may be reversible if the stress conditions are relieved before senescence has progressed beyond a certain point (Buchanan-Wollaston, 1997). According to Wittenbach (1978) during dark-induced senescence of fully expanded primary leaves in intact wheat seedlings, the processes were completely reversible when plants were transferred to the light during the first two days, but thereafter became irreversible. During the reversible stage of senescence the loss of total soluble protein was almost entirely due to the loss of Rubisco while in the irreversible stage Rubisco quantity decreased to the same extent as total soluble protein. In our experimental system these two stages were not so obvious. We obtained non selective loss of Rubisco only during the recovery from 72 h in darkness. Species-specific differences in Rubisco stability and in degradation characteristics have already been observed (Esquivel et al., 1998). Probably this is one of the reasons for the different results. Another reason may be the leaves used in our experiments were not fully expanded. Regardless of the leaf age, the typical selective loss of Rubisco is an early event during light deprivation.

During its life span, a leaf undergoes three phases of development – growth with rapid protein synthesis, maturity with protein turnover at consistently low level, and senescence with predomination of protein degradation (Buchanan-Wollaston, 1997). In order to study the recovery processes, before the beginning of the natural senescence processes we have started the dark treatment from the day sixth after sowing. By that time the primary leaves were not fully expanded. This fact may explain the differences in the results on pigment and protein quantity compared with those obtained during dark-induced senescence of fully expanded leaves.

We found a rapid decrease of chlorophyll content in the first 24 h of light deprivation and a slow decrease thereafter. Chl *a* content declined faster than that of chl *b* during the first 24 h in darkness and at the same rates as chl *b* later. On the other hand, the quantity of carotenoids remained almost unchanged. Such a sharp decrease of chlorophylls was not observed when seedlings with fully expanded primary leaves were transferred to darkness. A statistically significant change of chlorophyll quantity in expanded leaves appeared only at the fourth day of the dark treatment and these changes were rather small (our unpublished results). Such differences between expanding and mature leaves placed in darkness were surprising but reproducible. The chlorophylls to carotenoids ratio in fully expanded leaves was 1:5–1:7 and was similar

to that in the foliage of barley during spring in green house soil cultures (inpublished data). The ratio 1:3 reported in the present experiments may be due to the fact that at day sixth the primary barley leaves were not fully expanded. In general, chlorophyll and soluble proteins were degraded simultaneously during dark-induced senescence (Wittenbach, 1978). In our experiments the content of total soluble protein remained almost unchanged during dark treatment, but Rubisco level decreased in parallel to the loss of chlorophyll. Probably the loss of Rubisco was accompanied by the synthesis of other proteins. Another possibility was that the quantity of Rubisco was only 20% of the total protein in this barley variety at day sixth after sowing. Hence, the changes in Rubisco quantity could not reflect strongly the changes in the total protein content. In this barley variety the quantity of Rubisco reached almost 45% of total soluble protein in fully expanded primary leaves (Metodiev and Demirevska-Kepova, 1992). Regardless of some differences in senescence indices comparing the model systems of dark-induced senescence, it may be considered that the observed rapid selective loss of Rubisco makes this model system appropriate for investigation of the mechanisms of selective Rubisco degradation.

Vacuolar proteases with acid pH optimum are not likely to be responsible for Rubisco degradation *in vivo* early in senescence. An increase of proteolytic activity at pH 9 was observed late in natural senescence (Fisher and Feller, 1994). In our investigations there was no increase in total proteolytic activity with casein as a substrate at pH 6–9 in the first 24 h of the dark treatment. Increased total proteolytic activity at pH 8–9 was observed after 72 h in darkness, when the loss of Rubisco was not selective.

The avidin-biotin ELISA test for Rubisco specific proteolytic activity was developed by analogy with the method of Wilkinson et al. (1990) for collagenolytic activity with collagen as biotinylated substrate. In principle, every protein could be biotinylated conserving the high affinity and specificity of avidin-biotin interaction, so that this method is applicable to a wide variety of proteins. Our method differs from the original one in the type of ELISA used – sandwich instead of inhibitory, and in the maintenance of the proteolytic digestion in solution. The sensitivity of the method for Rubisco specific proteolytic activity is hundreds times greater than the caseinolytic activity assay. The avidin-biotin test system applied in this study served for estimation of native Rubisco degradation level in solution in the presence of Mg^{2+}. Our results did not confirm the participation of metalloprotease or ATP-dependent protease in Rubisco breakdown in dark-induced senescence. It is possible that high background levels of vacuolar proteolytic enzymes existed and masked in some manner the Rubisco specific proteolytic activity (Huffaker, 1990). This effect could be overcome using purified chloroplasts instead of leaf extracts. The specificity of Rubisco degradation could not be controlled by high affinity for Rubisco as a substrate but more likely by compartmentation of proteases. Besides, some modification of Rubisco could possibly occur prior to enzyme degradation during senescence. Data exist about oxi-

dation and/or aggregation and/or association with the membranes under different stress conditions, particularly oxidative stress (Mehta et al., 1992; Desimone et al., 1996) but it is not clear yet if these conditions exist in chloroplasts during senescence. Thylakoid-bound Rubisco degrading activity was described by Otto and Feierabend (1994) in chloroplasts from six-day-old seedlings of rye. Probably the Rubisco specific proteases induced in senescence do not function in solution but they are rather membrane-bound enzymes. Further investigations are under way to verify some of these presumptions.

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References

Arnon, D. I., 1949. Copper enzymes in isolated chloroplasts: polyphenoloxidase in *Beta vulgaris*. Plant Physiol., 24, 1–15.

Bradford, M. M., 1976. A rapid and sensitive method for the quantification of microgram quantities of proteins utilising the principle of protein-dye binding. Anal. Biochem., 72, 248–254.

Buchanan-Wollaston, V., 1997. The molecular biology of leaf senescence. J. Exp. Bot., 48, 307, 181–199.

Bushnell, T. P., D. Bushnell, A. T. Jagendorf, 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, S. J., M. E. Salvucci, D. E. Egli, 1990. Changes in ribulosebisphosphate carboxylase/oxygenase and ribulose 5-phosphate kinase abundances and photosynthetic capacity during leaf senescence. Photosynth. Res., 23, 223–230.

Crafts-Brandner, S. J., R. R. Klein, P. Klein, R. Holzer, U. Feller, 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.

Desimone, M., A. Henke, E. Wagner, 1996. Oxidative stress induced partial degradation of the large subunit of Ribulose-1,5-bisphosphate carboxylase/oxygenase in isolated chloroplasts of barley. Plant Physiol., 111, 789–796 .

Esquivel, M. G., R. B. Ferreira, A. R. Teixeira, 1998. Protein degradation in C_3 and C_4 plants with particular reference to Ribulose-1,5-bisphosphate carboxylase/oxygenase and glycolate oxidase. J. Exp. Bot., 49**,** 807–816 .

Fisher A., U. Feller, 1994. Senescence and protein degradation in leaf segments of young winter wheat: influence of leaf age. J. Exp. Bot., 45, 270, 103–109.

Huffaker, R. C.,1990. Proteolytic activity during senescence of plants. New Phytol., 116, 199–231.

Martinoia, E., U. Heck, M. J. Dalling, Ph. Matile, 1983. Changes in chloroplast number and chloroplast constituents in senescing barley leaves. Biochem. Physiol. Pflanzen, 178, 147–155.

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

Metodiev, M., K. Demirevska-Kepova, 1992. Rubisco quantitation in different barley varieties by enzyme-linked immunosorbent assay. J. Exp. Bot., 43, 155–158.

Nakabayashi K., T. Kiyosue, K. Shinozaki, A. Watanabe, 1999. Identification of *clp* genes expressed in senescing *Arabidopsis* leaves. Plant Cell Physiol., 40(5), 504–514.

Otto, S., J. Feierabend, 1994. Assay and comparative characterization of the proteolytic degradation of isolated small subunit and holoenzyme of ribulose-1,5-bisphosphate carboxylase/oxygenase in chloroplasts from rye leaves. J. Plant Physiol., 144, 26–33.

Peoples, M. B., V. C. Beilharz, S. P. Waters, R. J. Simpson, M. J. Dalling, 1980. Nitrogen restribution during grain growth in wheat (*Triticum aestivum* L.). Planta, 149, 241–251.

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

Saleemuddin, M., H. Ahmad, A. Husain, 1980. A Simple, rapid and sensitive procedure for the assay of endoproteases using Coomassie Brilliant Blue G-250. Anal. Biochem., 105, 202–206.

Shanklin, J., N. D. DeWitt, J. M. Flanagan, 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.

Simova-Stoilova, L., K. Demirevska-Kepova, 2000. Sensitive avidin-biotin test system for Rubisco specific proteolytic activity. Compt. rend. Acad. bulg. Sci., 52(5), 97–100.

Sokolenko, A., L. Altschmied, R. G. Herrmann, 1997. Sodium dodecyl sulphate-stable proteases in chloroplasts. Plant Physiol., 115, 827–832.

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

Wilkinson, M. J., L. C. Cohen, M. A. Shuman, 1990. A nonradioactive assay for type IV collagen degradation. Anal. Biochem., 185, 294–296.

Witenbach, V. A., 1978. Breakdown of ribulose bisphosphate carboxylase and changes in proteolytic activity during dark-induced senescence of wheat seedlings. Plant Physiol., 62, 604–608.

Wittenbach, V. A., 1979. Ribulose bisphosphate carboxylase and proteolytic activity in wheat leaves from anthesis through senescence. Plant Physiol., 64, 884–887.