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Senescence and protein degradation in leaf segments of young winter wheat: influence of leaf age

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Abstract

Leaf senescence in intact wheat plants can be strongly influenced by altered source/sink relations. Interactions with other plant parts are no longer possible in detached leaves and therefore differences in their senescence behaviour reflect the physiological status of the leaf before cutting. The net degradation of chlorophylls and of selected enzyme proteins (detected by SDS–PAGE and immunoblotting) was delayed in detached young leaves as compared to senescing or mature leaves excised from the same field-grown wheat plants. The physiological leaf age was therefore decisive for the velocity of artificial senescence. Net degradation rates of the enzymes investigated varied in detached leaves. The protein quantities of plastidial glutamine synthetase, phosphoribulokinase and phosphoglycolate phosphatase decreased more rapidly than those of ferredoxin-dependent glutamate synthase and nitrite reductase. Differences were also detected between two enzymes involved in the same metabolic pathway (photorespiratory carbon cycle) but located in different subcellular compartments: the plastidial enzyme phosphoglycolate phosphatase was lost more rapidly than glycolate oxidase (peroxisomal enzyme).

Key words: Detached leaves, senescence, proteolysis, leaf age, wheat.

Introduction

Steep senescence gradients can be observed in cereals on a whole plant level as well as on an individual leaf level. The leaf directly below the ear (flag leaf) is normally the last leaf to senesce in maturing wheat plants (Feller, 1990). Within an individual leaf, natural senescence starts at the tip and proceeds towards the base (Feller, 1990). Characteristic metabolic changes have been described during this phase of development. Photosynthesis and assimilatory enzymes were shown to decrease in an early phase, while respiration remained active longer (Feller and Erismann, 1978; Feller, 1990). Aminopeptidases reached high activities in expanding and mature wheat leaves and decreased during senescence, while a carboxypeptidase was found to remain active longer (Feller, 1986; Fröhlich and Feller, 1992). Endopeptidases (especially a form with a slightly alkaline pH optimum) were shown to reach maximal activities late during senescence (Feller, 1986; Fröhlich and Feller, 1992).

The described senescence pattern can be strongly influenced by changes in the source–sink interactions. For example, a very rapid protein and chlorophyll degradation was initiated by steam-girdling wheat flag leaves at their base (Fröhlich and Feller, 1991, 1992). Free amino groups and buffer-soluble carbohydrates accumulated in such leaves. When the phloem was interrupted below the flag leaf node, proteins and chlorophylls were degraded earlier in the flag leaf than in the second leaf from the top (Schenk and Feller, 1990).

Leaf segments are completely isolated from interactions with other plant parts. The senescence pattern may therefore be different from that of attached leaves. An accumulation of amino acids and of carbohydrates has been shown in detached leaf material (Thimann et al., 1974; Krapp et al., 1991; Peeters and van Laere, 1992). Since leaf segments are easy to handle and to incubate under controlled conditions, they have often been used to study different aspects of the senescence process (Thomas, 1978; Malik, 1983; Düggelin et al., 1988; Hilditch et al., 1989). The influence of light and of different effectors like phytohormones and amino acids on senescence of leaf segments has been extensively studied (Martin and Thimann, 1972; Shibaoka and Thimann, 1970; Weidhase et al., 1987a, b; Okada et al., 1992). However, the leaf age and the growth conditions have generally not been considered in this context. Since, in segments, interactions

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on the whole plant level are no longer possible, differences in the senescence behaviour can be related to the physiological status of the leaves prior to cutting. In the experiments reported here, detached leaves of different age cut from young, vegetative, field-grown winter wheat plants were analysed with respect to artificial senescence under controlled conditions.

Materials and methods

Plant material

Winter wheat plants (*Triticum aestivum* L. cv. Arina) were grown in a field near Bern. Whole shoots were collected after the restart of rapid growth in Spring 1991 (on 27 March and 10 April) and transported to the laboratory on ice. Leaf laminae were separated from the rest of the shoots and extracted immediately. For incubation experiments with leaves and leaf segments, the plants were harvested and were transported to the laboratory with their root systems standing in deionized water.

Incubation of detached leaves

Whole leaf laminae were cut into segments of approximately 4 cm length and incubated for 24 h in a culture room (13 h light, 100 μE m^{-2} s^{-1}/10 h darkness/1 h light, 21–25 °C). The segments were floated on a feeding solution in Petri dishes. The feeding solution consisted of 100 mmol m^{-3} methionine containing 0.1 μCi cm^{-3} ^{35}S-methionine in order to label newly synthesized proteins. In addition, intact leaf laminae standing with the cut xylem in test tubes containing the feeding solution were incubated under the same light and temperature conditions.

Extraction

The extraction medium contained 20 mol m^{-3} sodium phosphate buffer pH 7.5, 1% (w/v) polyvinylpolypyrrolidone and 0.1% (v/v) β-mercaptoethanol. Fifteen freshly harvested leaf laminae were extracted in 9 cm^3 extraction medium for older leaves (laminae 1 to 3) or 18 cm^3 for the larger upper leaves (laminae 4 to 7) with a Polytron mixer (Kinematica, Littau/Luzern) for 20 s at medium and for 5 s at full speed. Three laminae incubated for 24 h or segments from three laminae incubated for 24 h were homogenized in 2 cm^3 extraction medium. The crude extracts were filtered through Miracloth (Calbiochem, San Diego).

Quantification of proteins and chlorophylls

The Miracloth filtrate was mixed with acetone (final concentration 80%) and centrifuged (5 min at 2300 g) before photometry at 649 and 665 nm. Chlorophyll was calculated according to Strain *et al.* (1971). Total proteins in the Miracloth filtrate were determined according to Bradford (1976) using bovine gamma globulin as the standard.

Activities of peptide hydrolases

Freshly prepared extracts were centrifuged for 10 min at 2300 g. Aminopeptidases were detected in micro–titration plates using the method described previously (Blättler and Feller, 1988). L-amino acid-p-nitroanilides (final concentration in the substrate solution: 1 mol m^{-3}) were dissolved in dimethylsulphoxide (final concentration: 2% v/v) and then mixed with buffer (100 mol m^{-3} *bis-tris* propane and 50 mol m^{-3} sodium acetate adjusted to the desired pH value with HCl). Hydrolysis of the

different substrates was measured at slightly different pH values, according to the optima determined in a preliminary experiment. Carboxypeptidase activity was detected in micro–titration plates according to Salgó and Feller (1987) using the N-masked dipeptide N-carbobenzoxy-L-phenylalanine-L-alanine as substrate. Endopeptidase activities were analysed according to Fröhlich and Feller (1992) using azocasein as substrate. The extracts were desalted as reported previously (Feller *et al.*, 1977) by centrifugation through Sephadex G-25 equilibrated with 20 mol m^{-3} sodium phosphate buffer pH 7.5 and 0.1% (v/v) β-mercaptoethanol. The eluate (400 mm^3) was mixed with 400 mm^3 substrate solution (1% azocasein in 200 mol m^{-3} acetate buffer pH 5.0, 200 mol m^{-3} imidazole buffer pH 7.5 or 200 mol m^{-3} *bis-tris* propane buffer pH 9.0) and incubated for 3 h at 37 °C. Trichloroacetic acid-soluble products were determined photometrically (Fröhlich and Feller, 1992).

SDS–PAGE and immunoblotting

Samples were prepared from the Miracloth filtrate and separated by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS–PAGE) in a Mini Protean II Dual Slab Cell (BIORAD, Richmond) according to Laemmli (1970). For visualization of total proteins gels were stained with Coomassie Brilliant Blue R-250. Immunoblotting was performed according to Tijssen (1985) as described by Fröhlich and Feller (1991). All primary antibodies used were developed in rabbit. Glutamine synthetase was detected with an antibody directed against the plastidial isoenzyme from mustard (Höpfner *et al.*, 1988). Phosphoribulokinase (Crafts-Brandner *et al.*, 1990), nitrite reductase (Ida, 1987), ferredoxin-dependent glutamate synthase (Marquez *et al.*, 1988), phosphoglycolate phosphatase (Baldy *et al.*, 1989) and glycolate oxidase (Mitsuhashi and Feller, 1992) were identified on immunoblots with specific antibodies. The bands were visualized with goat-anti-rabbit-IgG for bridging and peroxidase-anti-peroxidase soluble complex (Mitsuhashi and Feller, 1992). The peroxidase substrate was 4-chloro-1-naphthol. The whole protocol from the primary antibodies to the colour reaction was repeated for the detection of nitrite reductase, phosphoglycolate phosphatase and glycolate oxidase in order to amplify the bands.

Fluorography

In order to detect the incorporation of ^{35}S-methionine into proteins during the incubation of leaf segments, the extracted proteins were separated by SDS–PAGE as mentioned above. The gels were slightly stained with Coomassie Brilliant Blue, treated with EN^{3}HANCE (DuPont, Boston) and exposed to a preflashed X-ray film (Fuji RX) at −80 °C (Laskey, 1984).

Results

The four oldest leaves were senescing or starting to senesce between 27 March and 10 April, as judged by changes in protein and chlorophyll levels (Table 1). A considerable net decrease of various proteins (such as ribulose-1,5-*bis*phosphate carboxylase/oxygenase, Rubisco) was detected in these leaves on Coomassie Blue-stained SDS–PAGE gels (Fig. 1). Net protein losses were between 30% and 50% during this period (Table 1). The fifth leaf was still growing and two additional leaves were formed. The lamina of the seventh leaf was just emerging on 10 April and was quite unequally developed in different

Table 1. *Protein and chlorophyll degradation during the incubation of detached leaves*

Protein and chlorophyll contents were determined in leaf laminae from control plants freshly harvested on 27 March or on 10 April (Cont) and in leaf laminae incubated for 1 d in a culture room. The leaf blades were incubated either standing intact in test tubes (Inc1) or floating as segments in Petri dishes (Inc2). Leaves 6 and 7 were not yet expanded on 27 March. The values for controls were calculated from four independent samples (each containing 15 leaf laminae), and the values for the incubations were determined from one sample (containing three leaf laminae).

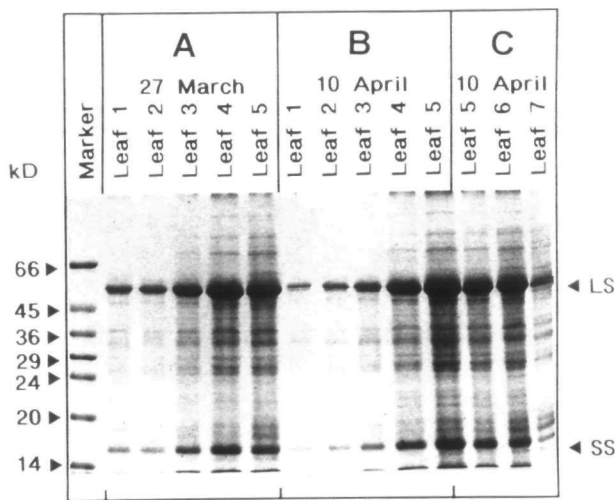| Leaf number	Protein content (mg leaf lamina^{-1})					Chlorophyll content (μg leaf lamina^{-1})				
	27 March		10 April			27 March		10 April		
	Cont	Inc1	Cont	Inc1	Inc2	Cont	Inc1	Cont	Inc1	Inc2
1	3.1	2.0	1.5	0.9	1.0	59	47	40	22	27
2	3.3	1.7	2.2	1.1	1.0	59	36	56	39	29
3	5.1	3.1	3.0	1.4	1.3	107	78	91	49	50
4	11.6	7.1	8.5	3.9	3.9	231	202	224	135	139
5	11.9	9.1	13.0	7.2	6.8	169	215	320	266	230
6			27.0	20.5	21.6			445	477	477
7			7.4	3.4	5.9			123	72	190

Fig. 1. Protein pattern in freshly collected leaves of different age. Leaf laminae were analysed on 27 March and on 10 April by SDS–PAGE and staining with Coomassie Brilliant Blue R-250. Each lane was loaded with a sample equivalent to 0.250% (A, B) or 0.135% (C) of a leaf blade. Leaves 6 and 7 were not yet expanded on 27 March, while senescence was already initiated in leaf 1 (oldest leaf). Molecular weight markers are shown on the left side, while the large (LS) and small (SS) subunits of Rubisco are indicated on the right side.

plants. The main endopeptidase activity was detected at pH 5.0 (Table 2). The activities detected in the alkaline pH range are low. They may therefore represent the tail of the pH response curve of the major endopeptidase(s) with a pH optimum around 5, and are not necessarily due to a different endopeptidase form with a high pH optimum which has been detected in senescing flag leaves (Feller and Keist, 1986). The endopeptidase activity found in leaf 1 on 27 March was about 200% of the activity present in whole shoots (with three leaves) a few weeks before (Fischer and Feller, 1993). For the comparison of enzyme activities in different leaves it must be borne in mind that the lowest leaves (1 to 3) were smaller

than the upper leaves, as indicated by protein and chlorophyll contents (Table 1). Taking this fact into consideration, it becomes evident that endopeptidase activity was high in old leaves and remained low in expanding leaves (Table 2). Aminopeptidase activities reached high levels before endopeptidase activities in the course of leaf development (Tables 2, 3). Only minor variations were detected between aminopeptidase activities measured with different substrates (Table 3). Several aminopeptidase forms differing in their substrate specificities have been identified in various plants (Feller, 1986; Blättler and Feller, 1988). However, one aminopeptidase form may hydrolyse a series of substrates with different rates (Blättler and Feller, 1988, and references therein). It remains open how many aminopeptidase forms contributed to the hydrolysis of the substrates listed in Table 3.

The changes detected in detached leaves incubated for only 24 h in a culture room were much more rapid than the changes in the field plants (Table 1). Net protein and chlorophyll losses were quite comparable between whole leaf laminae standing with the cut xylem in test tubes and leaf segments floating on the solution in Petri dishes (Table 1). Proteins and chlorophylls were rapidly degraded when the incubated material was taken from senescing and mature leaves (leaves 1–4 on 27 March, leaves 1–5 on 10 April), but they decreased much more slowly when the incubated material was cut from young leaves (leaf 5 on 27 March, leaves 6 and 7 on 10 April). The large variations visible for leaf 7 are due to the unequal development of this not yet fully expanded leaf at the time of excision.

The differences in net degradation between leaves of different physiological age were visible for all enzymes identified by SDS–PAGE (Rubisco) or by immunoblotting with specific antibodies (Figs 2, 3). All these enzyme proteins were much more stable in young leaves. The enzymes examined by immunoblotting were phosphoribu-

Table 2. *Endopeptidase activities in leaf laminae collected in the field on 27 March or on 10 April*
At the first sampling date senescence was already initiated in the oldest leaf (leaf 1), while leaf 7 was not yet expanded. Azocasein solutions buffered at three different pH values were used as substrates. Means and standard deviations of four independent samples (each containing 15 leaf laminae) are shown.

pH	Endopeptidase activity (μg leaf lamina^{-1} h^{-1})						
	27 March			10 April			
	Leaf 1	Leaf 3	Leaf 5	Leaf 1	Leaf 3	Leaf 5	Leaf 7
5.0	212±8	150±17	113±9	186±7	192±13	317±26	94±37
7.5	71±13	39±4	46±3	56±9	48±23	79±18	33±8
9.0	34±16	8±5	11±4	21±5	7±1	15±2	12±4

Table 3. *Exopeptidase activities in leaf laminae collected in the field on 27 March or on 10 April*
At the first sampling date senescence was already initiated in the oldest leaf (leaf 1), while leaf 7 was not yet expanded. Carboxypeptidase (CP) was measured using an N-masked dipeptide as substrate, and aminopeptidases (AP) were detected using p-nitroanilides of the amino acids indicated. Means and standard deviations of four independent samples (each containing 15 leaf laminae) are shown.

Peptide hydrolase	Enzyme activity (nkat leaf lamina^{-1})			
	Leaf 1	Leaf 3	Leaf 5	Leaf 7
27 March				
CP	5.36±0.72	5.71±0.90	6.02±1.34	
Ala-AP	0.93±0.05	1.49±0.11	3.21±0.22	
Gly-AP	0.13±0.01	0.23±0.03	0.50±0.01	
Lys-AP	0.82±0.05	1.40±0.14	2.35±0.21	
Leu-AP	0.74±0.06	1.21±0.09	2.31±0.18	
Met-AP	0.38±0.02	0.60±0.04	1.14±0.07	
Pro-AP	0.17±0.01	0.18±0.01	0.26±0.01	
Phe-AP	0.30±0.06	0.43±0.07	0.47±0.01	
10 April				
CP	4.76±0.77	6.14±0.38	12.86±1.42	2.69±0.79
Ala-AP	0.68±0.05	1.26±0.06	4.67±0.15	2.48±0.56
Gly-AP	0.17±0.03	0.21±0.01	0.81±0.10	0.56±0.15
Lys-AP	0.54±0.04	1.00±0.03	3.54±0.15	1.71±0.39
Leu-AP	0.47±0.04	0.89±0.02	3.05±0.12	1.76±0.43
Met-AP	0.27±0.03	0.45±0.02	1.56±0.15	1.14±0.28
Pro-AP	0.14±0.02	0.17±0.01	0.46±0.17	0.64±0.16
Phe-AP	0.33±0.12	0.47±0.04	0.75±0.10	0.50±0.11

Fig. 2. Changes in the protein pattern during incubation of detached leaves. Laminae were collected in the field on 10 April, cut into segments and incubated in Petri dishes for 1 d (13 h light, 10 h dark, 1 h light). Freshly harvested (C) and incubated (Inc) leaves were analysed by SDS–PAGE and staining with Coomassie Brilliant Blue R-250. Each lane was loaded with a sample equivalent to 0.135% of a leaf blade. Molecular weight markers are shown on the left side, while the large (LS) and small (SS) subunits of Rubisco are indicated on the right side.

lokinase (another important Calvin cycle enzyme besides Rubisco); nitrite reductase (a stromal enzyme, essential for the assimilation of inorganic nitrate); glutamine synthetase and ferredoxin-dependent glutamate synthase (stromal enzymes involved in the assimilation of inorganic nitrate and in the re-assimilation of ammonia liberated by glycine decarboxylase in photorespiration); phosphoglycolate phosphatase (stromal enzyme involved in the photorespiratory carbon cycle) and glycolate oxidase (peroxisomal enzyme, also involved in the photorespiratory carbon cycle). Differences in the net degradation of these enzymes were detected in leaves 4 and 5: The protein quantities of glutamine synthetase, phosphoribulokinase and phosphoglycolate phosphatase decreased more rapidly than those of glutamate synthase and nitrite reductase (Fig. 3). Thus, the net degradation of enzymes present in the sam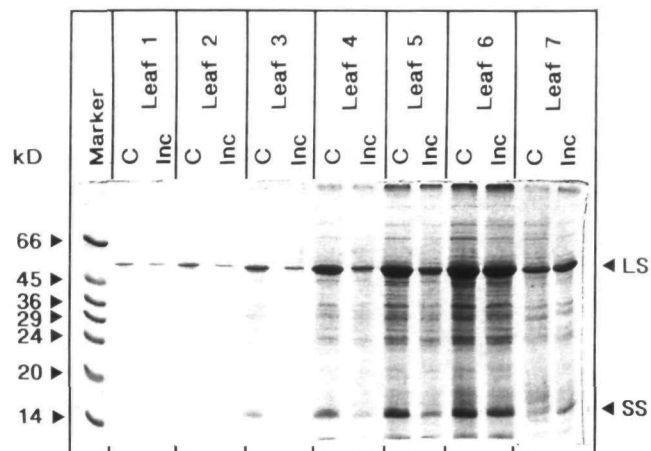e subcellular compartment (chloroplast) was different. Since phosphoglycolate phosphatase (plastidial enzyme) was lost more rapidly than glycolate oxidase (peroxisomal enzyme), there were also differences between enzymes involved in the same metabolic pathway (photorespiratory carbon cycle) but located in different subcellular compartments.

Protein synthesis was detected in leaf segments by the incorporation of ^{35}S-methionine (Fig. 4). It was high in the youngest leaves (leaves 6 and 7) and low in mature and senescing leaves. Consequently, the fact that net protein degradation was low in segments cut from young leaves may be due, in part, to protein synthesis still going on in these leaves. The incorporation of radioactivity into leaf proteins was always very low when intact leaf blades were fed through the cut xylem. Possibly methionine was removed from the transpiration stream before reaching the mesophyll cells. A xylem-to-phloem transfer has been shown to be important for several amino acids (Feller and Hölzer, 1991).
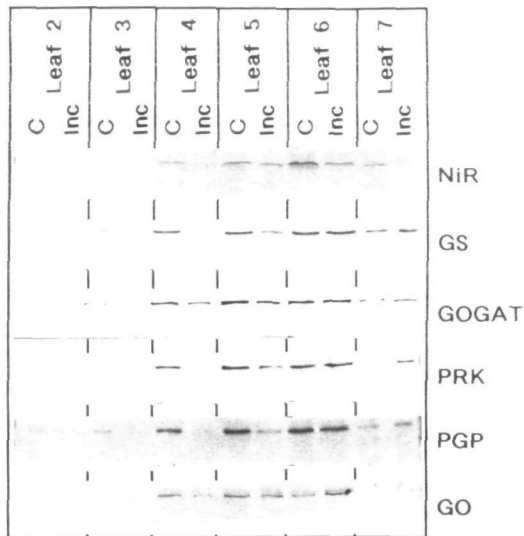
Fig. 3. Changes in the amount of selected enzyme proteins in leaves of different age. Leaves were collected in the field on 10 April. Leaf 1 (oldest leaf) was omitted since the quantities of the enzymes investigated were already below the detection limit before incubation. Detached laminae were cut into segments, incubated in Petri dishes for 1 d (13 h light, 10 h dark, 1 h light), and analysed by SDS–PAGE and immunoblotting. Samples equivalent to the same percentage of a leaf blade (0.250%) were loaded on each lane. Protein quantities were analysed in freshly harvested (C) and in incubated (Inc) leaves with specific antibodies against nitrite reductase (NiR), plastidial glutamine synthetase (GS), ferredoxin-dependent glutamate synthase (GOGAT), phosphoribulokinase (PRK), phosphoglycolate phosphatase (PGP) and glycolate oxidase (GO).
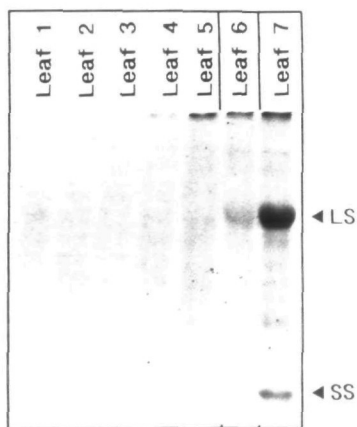

Fig. 4. Incorporation of ^{35}S-methionine into proteins in leaves of different age. Lanes 1 (oldest leaf) to 7 (youngest leaf) were collected in the field on 10 April. The laminae were cut into segments, incubated in Petri dishes with labelling solution for 1 d (13 h light, 10 h darkness, 1 h light) and analysed by SDS–PAGE followed by fluorography. Each lane was loaded with a sample equivalent to 1.22% of a lamina for leaves 1 to 5 and 7, but only 0.41% of a lamina could be loaded for leaf 6 (largest leaf). The large (LS) and the small (SS) subunits of Rubisco are indicated on the right side.

Discussion

Leaf senescence (as judged by protein and chlorophyll degradation and the activities of some peptide hydrolases)

in young, vegetatively growing wheat was, in general, similar to the senescence of flag leaves during the period of grain filling (Fröhlich and Feller, 1991, 1992). A major difference was detected in the endopeptidase pattern. The main activity detected in this study was always below pH 7, although it was reported that an endopeptidase form with a high pH optimum becomes more active during later phases of leaf senescence in maturing wheat plants (Feller and Keist, 1986; Fröhlich and Feller, 1992). The significance of the altered endopeptidase pattern for protein degradation is not yet clear.

Differences in the net degradation of stromal enzyme proteins were found in incubated leaf segments. It is still open to discussion how the degradation of various proteins in the same subcellular compartment with its given set of proteolytic activities can be selectively regulated. The activities of peptide hydrolases and the susceptibilities of their potential substrate proteins may be influenced by solutes (Kurlandsky *et al.*, 1988; Feller, 1990; Fischer *et al.*, 1992). An exchange of nutrients and assimilates with other plant parts is no longer possible in leaf segments. This may indirectly affect the exchange of solutes between the stroma and the cytosol and, as a consequence, proteolytic processes inside the chloroplasts. Different degradation rates of nuclear-encoded stromal proteins were found in isolated intact pea chloroplasts where *de novo* synthesis was no longer possible (Mitsuhashi and Feller, 1992). It was also shown in this system that the degradation of plastidial proteins can be influenced by solutes in the incubation medium of the chloroplasts (Mitsuhashi and Feller, 1992).

A strong influence of the physiological leaf age on net protein and chlorophyll degradation was detected in detached leaves. These differences became obvious although interactions with other plant parts were interrupted for segments from young leaves as well as for segments from mature and senescing leaves. Leaf age was important for the degradation of all investigated enzyme proteins, regardless of the differences observed between them. A redistribution of limiting phloem-mobile nutrients to young leaves (sinks) prior to cutting may be important in this context. Preliminary experiments suggest that the nutritional status of the intact plant can influence the behaviour of leaf segments during artificial senescence. Further investigations considering several environmental factors (light, temperature, nutrients) during growth of the experimental plants may allow a deeper insight into the regulatory network in senescing wheat leaf segments.

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