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Accumulation of pathogenesis-related proteins in barley leaf intercellular spaces during leaf senescence

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Abstract

Accumulation of the pathogenesis-related (PR) proteins localised in intercellular spaces of barley primary leaves, chlorophyll content, structure of chloroplasts, and photosynthesis were examined during natural and *in vitro* induced leaf senescence (cultivation of whole plants in the dark or detached leaves under nutrient deficiency). Some of PR proteins accumulated during natural senescence, but their accumulation pattern was different from those of pathogen-induced as well as during *in vitro*-induced senescence, which indicate different molecular bases of these processes. Photosynthetic rate and chlorophyll content indicate that natural senescence of barley primary leaves began from 15th day after sowing. In 35-d-old first leaves, the chloroplasts showed typical characteristics of senescence as significant decrease of size, greater grana, and prominent plastoglobuli. The chloroplasts contained more grana under *in vitro* induced senescence and they had reduced length in the dark. Correspondingly, accumulation of PR proteins was detectable on about the 15th day but the content of some PR proteins increased in later stages of senescence.

Additional key words: chlorophyll content, chloroplast structure, *Hordeum vulgare*, intercellular washing fluid, photosynthetic rate, powdery mildew.

Introduction

Senescence is the latest phase of leaf development, where nutrients are recycled to the other, younger parts of the plant. The leaf cells undergo highly co-ordinated changes in their structure, metabolism, and gene expression (for reviews see Smart 1994, Gan and Amasino 1997). Decrease of chlorophyll content, photosynthesis and

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Abbreviations: DAS - days after sowing; IWF - intercellular washing fluid; PR - pathogenesis-related.

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changes in chloroplast structure are common characteristics of senescent leaves. Increase in size and mostly also in number of plastoglobuli seems to be the earliest change while thylakoids and chlorophyll content remain unchanged (Wrischer 1994). Also, large grana formation and destruction of some stroma thylakoids may represent initial signs of chloroplast senescence. Further changes accompanying chloroplast senescence are decrease of organelle volume, and change of their shape, dilation of the stroma and later also of the grana thylakoids. The last steps include loss of parallel arrangement of thylakoids, gradual reduction of thylakoid membranes, simultaneous with growth of plastoglobuli, up to the destruction of the chloroplast envelope (Kutík 1985).

The expression of most of the genes is down-regulated, whereas the expression of some genes is up-regulated during leaf senescence (Lohman *et al.* 1994, John *et al.* 1997). These up-regulated genes encode proteins with specific functions in the senescence process, for example mainly degradative enzymes such as proteases (Hensel *et al.* 1993) and glutamine synthetase (Kamachi *et al.* 1991) which function in re-mobilisation of nitrogen from proteins. Lipases and enzymes involved in the glyoxylic acid pathway play a key role in the lipid breakdown (Gut and Matile 1988). Senescence specific RNase has been identified in *Arabidopsis* (Taylor *et al.* 1993). Enzymes involved in chlorophyll breakdown are strongly activated during senescence and one of them occurs exclusively in senescent leaves (Matile *et al.* 1996). The senescence-specific metallothionein-like protein may have a function in re-mobilisation or de-toxification of released metals (Buchanan-Wollaston 1994). Hanfrey *et al.* (1996) identified an increased expression of genes encoding pathogenesis-related (PR) proteins during leaf senescence in *Brassica napus*.

The common characteristics of PR proteins are selective extractability at low pH, high resistance to proteases, low molecular mass and localisation in intercellular spaces (Van Loon 1985). Recently, they have been classified into eleven groups (Van Loon *et al.* 1994). For some of them their role in the host defence mechanism has been identified (Mauch and Staehelin 1989).

The aim of the present study was to compare the accumulation of PR proteins in the intercellular spaces of barley primary leaves, induced by powdery mildew infection, during natural senescence, and *in vitro* induced senescence.

Materials and methods

Plants: Barley (*Hordeum vulgare* L. cv. Ricardo) with the *Mla3* powdery mildew resistance gene was grown in a growth chamber at a light/dark temperature of 20/16 °C and a 16-h photoperiod [irradiance 70 μmol(photon) m^{-2} s^{-1}; 400-700 nm]. Seven-day-old first leaves were inoculated with an avirulent race of powdery mildew (*Erysiphe graminis* f. sp. *hordei*) under 16-h photoperiod or intact plants were incubated in the dark, or detached and incubated in agar without nutrients for 3 d, to induce senescence. Natural senescence of the first leaf was analysed 7 d (first leaf developed only), 10 d (very small second leaf), 15 d (second leaf fully developed), 20 d (very small third leaf), 25 d (third leaf developed), 30 d (fourth leaf in midway

state) and 35 d (fully developed fourth and very small fifth leaves) after sowing (DAS). The data represent the mean value of three completely independent experiments.

Protein purification: Intercellular washing fluid (IWF) was isolated according to Rohringer *et al.* (1983). The cut leaves were rinsed with distilled water and infiltrated with 100 mM citrate-phosphate buffer, pH 2.8, containing 15 mM mercaptoethanol in a desiccator using a vacuum pump, for 1 min. The IWF was recovered after centrifugation of the infiltrated leaves, at 750 *g* for 10 min. After passing through *Sephadex G25* the IWF was lyophilised. The proteins were separated under non-denaturating conditions on 15 % slab polyacrylamide gels using the discontinuous buffer system (Laemmli 1970) and visualised by silver staining (Heukesloven and Dernick 1985). Proteins were quantified with bovine serum albumin as a standard by the method of Bradford (1976).

Chlorophyll assay: Leaf segments (1 cm long) were excised from the middle of the first leaves and soaked overnight in 0.5 cm^3 of 80 % acetone. Extracts were diluted with 2 cm^3 of 100 % acetone. Chlorophyll concentrations were measured spectrophotometrically at 646 and 663 nm and calculated by the equation of Hill *et al.* (1985).

Photosynthetic rate: Similar leaf segments were transferred into de-aerated 10 mM phosphate buffer, pH 6.5. O_2 evolution was measured polarographically at 25 °C using Clark type oxygen electrode. The irradiance was 110 $\mu mol\ m^{-2}\ s^{-1}$.

Electron microscopy: Segments cut from the middle of the first leaf on 7 (control) and 35 (natural senescence) DAS as well as on the third day of induced senescence, were fixed with 1 % glutaraldehyde and postfixed with 1 % osmium tetroxide both buffered with 0.1 M Na cacodylate buffer, pH 7.2. After dehydration with an ethanol series and propylene oxide the samples were embedded in *Epon-Araldite* mixture. Sections stained with uranyl acetate and Pb citrate were examined in *Tesla BS500* electron microscope (Brno, Czech Republic).

Results

Eight major pathogen-induced apoplastic proteins were identified in intercellular spaces of barley first leaves 72 h post-inoculation with the avirulent races of powdery mildew (Fig. 1). Some PR proteins accumulated in the same barley leaves during senescence without any pathogen infection. Photosynthesis and chlorophyll content indicate that natural senescence of barley primary leaves begins on about the 15th day after sowing, when rapid decrease of chlorophyll content and decline in photosynthetic activity were detected (Fig. 2). Accumulation of PR proteins in the intercellular spaces were also started at this ontogenetical stage of the leaves (Fig. 3; lane 15) and increased during the following 10 d (Fig. 3; lanes 20,25). However, accumulation of one of the PR proteins was detectable already 10 DAS (Fig. 3;

lane 10). The highest amounts of PR proteins were detected 30 DAS when the leaves already showed visible yellowing (Fig 3; lane 30). One of the PR proteins remained at the increased level after 35 DAS while the accumulation of the others decreased. At this stage of development, desiccated parts of the first leaves were already visible. The accumulation of PR proteins was significant in spite of the fact that total protein content of the IWF decreased since the 15 DAS (Fig. 4*B*). In contrast to natural senescence, the IWF protein concentration of the leaves inoculated with the avirulent race of powdery mildew was increased (Fig. 4*A*).

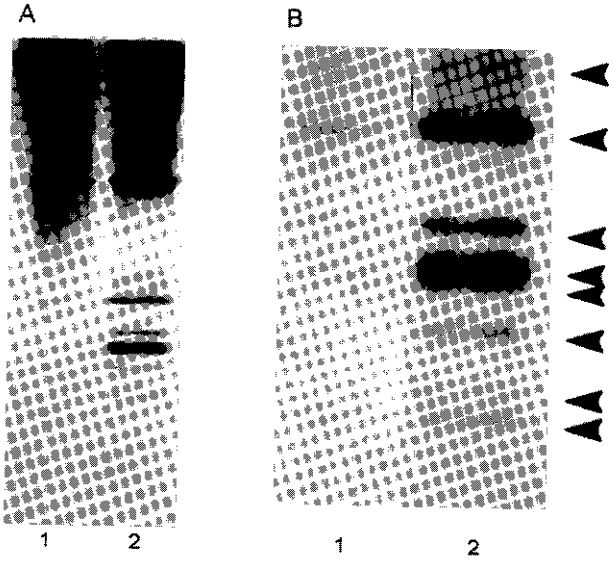

Fig. 1. Anodic PAGE patterns of barley extracellular PR proteins, 72 h post-inoculation with an avirulent races of powdery mildew. Lane 1 - control plants; lane 2 - avirulent race (*A*). Detail of the pathogen-induced proteins indicated by arrows (*B*).

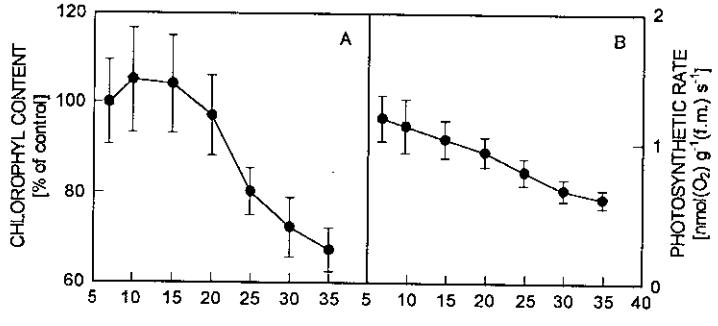

Fig. 2. Chlorophyll content (*A*) and photosynthetic rate (*B*) in the first leaves from the top during development of barley seedlings (control - 7-d-old non-inoculated plant).

The chlorophyll content and photosynthetic rate decreased both in the detached and dark incubated leaves (Fig. 5). These results were similar to naturally senescent leaves (Fig. 2). Similarity was observed also in analyses of IWF protein concentration

(quantity) under natural and induced senescence. Compared to the control plants, protein content of IWF isolated from first leaves decreased significantly in dark incubated and particularly in detached leaves 3 d after treatment (Fig. 4*C*). As far as quality of the stress-induced apoplastic proteins concerns there were clear differences between natural and induced senescence revealed by PAGE. The patterns of PR protein accumulation in dark incubated and detached leaves were different from that of naturally senescent leaves (Fig. 6). Higher accumulation of PR proteins occurred in cut leaves than in dark incubated ones.

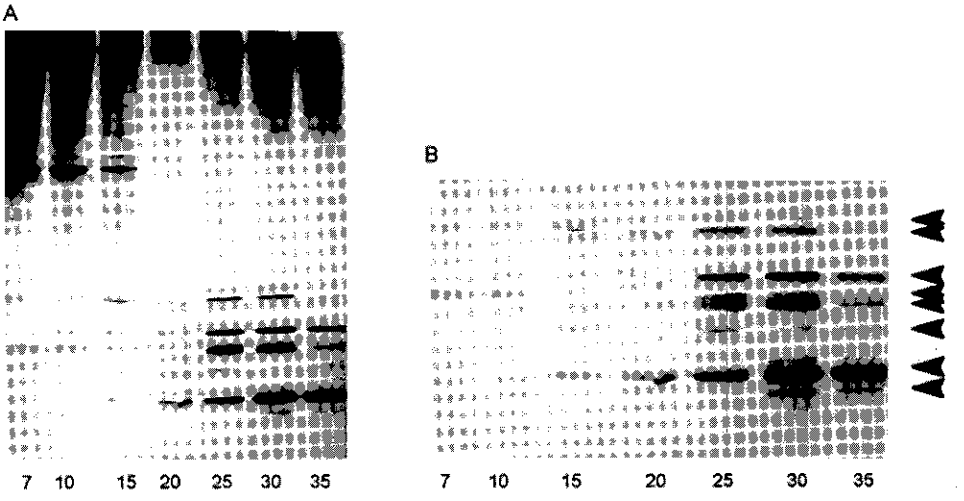

Fig. 3. Anodic PAGE patterns of barley IWF proteins during natural leaf senescence (*A*). Detail of PR proteins. The numbers of the lanes indicate DAS. PR proteins accumulated during senescence are indicated by an arrow (*B*).

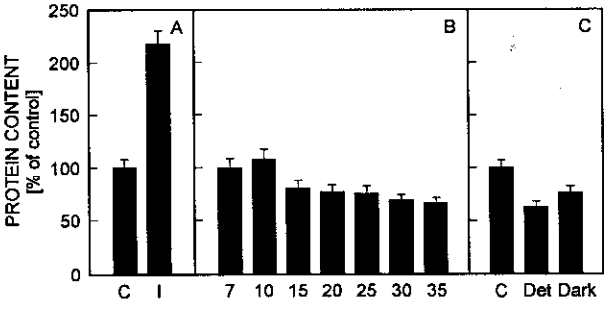

Fig. 4. Protein concentration in IWF of the first barley leaves: *A* - 3 d after inoculation with avirulent race of powdery mildew (I) and control plants (C); *B* - naturally senescing for 7 to 35 d; *C* - control (C), detached (Det) and incubated in dark (Dark) for 3 d.

In the control, 7-d-old first leaves the chloroplasts showed typical structures of the normal fully developed organelles (Fig 7*A*). After 3 d of senescence induction either

in detached (Fig. 7*B*) or in dark-incubated (Fig. 7*C*) leaves the structure of chloroplasts did not change markedly. Only slightly larger grana and, under dark

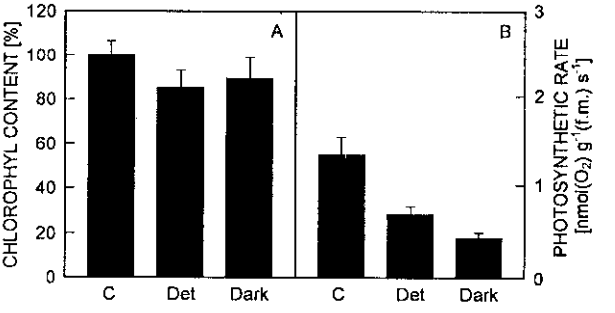

Fig. 5. Chlorophyll content (*A*) and photosynthetic rate (*B*) of detached first leaves incubated in the dark for 3 d.

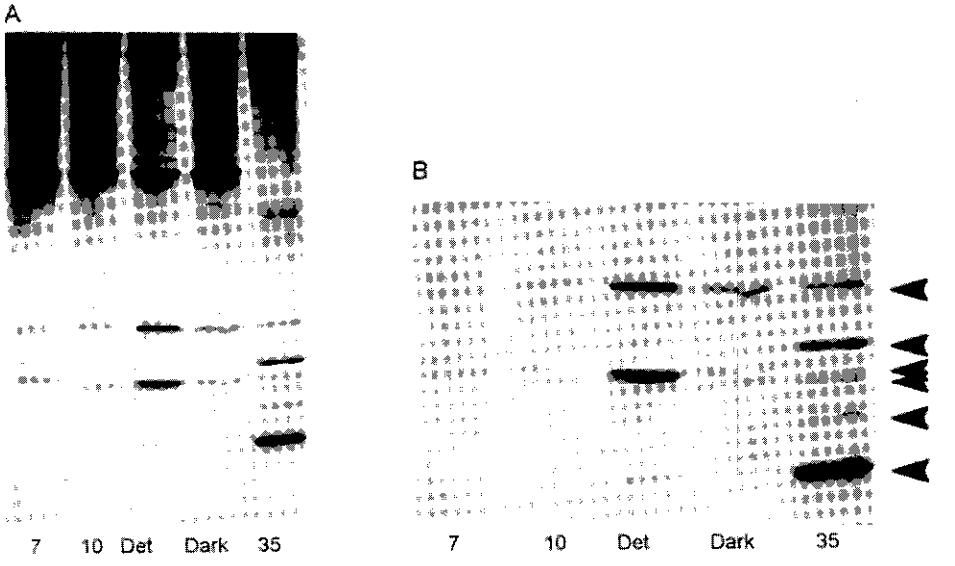

Fig. 6. Anodic PAGE patterns of barley IWF proteins of detached (Det) and dark incubated (Dark) first leaves. The numbers under the lanes indicate days after sowing (*A*). Detail of PR proteins. The PR proteins accumulated in the intercellular spaces are indicated by arrows (*B*).

condition also a decrease in chloroplast length (Table 1) characterised the symptoms of chloroplast senescence. Larger grana and plastoglobuli, and a significant decrease in chloroplast size (Table 1) occurred in the chloroplast of the naturally senescing 35-d-old plants (Fig. 7*D*).

Table 1. Length and width of chloroplasts of the first leaf in the 7-d-old seedlings (control), after senescence induced in detached leaves by cultivation under nutrient deficiency or in the dark, and after natural senescence 35 DAS. Means ± SD, $n = 16$, * - statistically significant at $P < 0.01$.

	7 DAS	Detached	Dark	35 DAS
Length [μm]	5.65 ± 1.39	5.47 ± 1.21	4.20 ± 0.35*	3.31 ± 0.37*
Width [μm]	2.28 ± 0.24	2.43 ± 0.45	2.37 ± 0.39	1.48 ± 0.47*

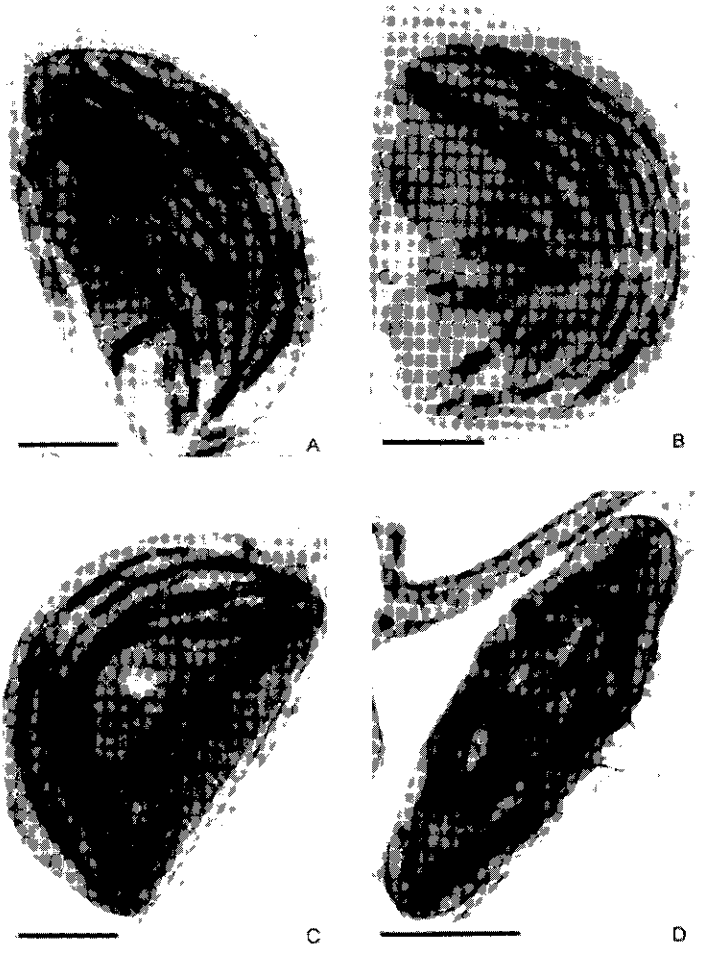

Fig. 7. Chloroplasts from the first leaves of barley. Control, 7-d-old leaf (*A*). Chloroplasts in senescing leaves with larger grana (*arrowheads*) and plastoglobuli (*arrows*) (*B,C,D*). Senescence induced in detached leaves on agar medium without nutrients (*B*). Senescence induced by cultivation in the dark (*C*). Natural senescence 35 DAS (*D*). Bars represent 1 μm.

Discussion

Structural characteristics of the chloroplasts from the first barley leaves indicate natural or artificially induced senescence. Following 3 d of senescence induction by either nutrient deficiency or darkness the formation of larger grana and some decrease in chloroplast length corresponded with very early structural changes accompanying natural organelle senescence (Kutík 1985). While the amount of chlorophyll declined in dark incubated barley leaves, the thylakoid membranes were not affected under these conditions. The degradation of chlorophyll is a significant feature of barley leaf segments floated in the dark, which is accompanied by an increase of chlorophyllase activity (Rodrígues *et al.* 1987). It has been shown that chlorophyll degradation may occur earlier than the disintegration of the thylakoid membrane system (Biswal *et al.* 1983). The structural manifestations of senescence including size diminution, larger grana and plastoglobuli were most evident in the 35-d-old first leaves. These correlated well with decreases in photosynthesis and protein content, similar to the senescing leaves of rice (Hashimoto *et al.* 1989).

Natural senescence of the first leaf in barley seedlings started on about day 15. At this stage of plant development, the second leaf was fully developed. One of the PR proteins accumulated already 10 DAS, while accumulation of other PR proteins were detectable only 15 DAS. Similar differences in accumulation of PR proteins were observed by Hanfrey *et al.* (1996) in mature green leaves of *Brassica napus*. In their system, PR-1 like protein accumulated before any decreases in chlorophyll content were detectable, while protein with a sequence similar to chitinase accumulated only in the later stage of the leaf development, when the first indication of chlorophyll loss was already detectable. The accumulation of PR proteins before any changes either in chlorophyll content, chloroplast structure or in protein concentration are detectable, indicates that senescence is a highly regulated process of leaf development, starting with co-ordinated expression of specific genes. Recently several senescence-related genes were cloned. They were activated or inhibited in the early stage of senescence, and therefore they can be used as molecular markers for leaf senescence (John *et al.* 1997). Analysis of senescence-associated genes in *Arabidopsis* also revealed that their expression preceded reductions in both photosynthesis and expression of photosynthesis-associated genes (Hensel *et al.* 1993)

Unlike similar decrease in photosynthesis, chlorophyll content and protein concentration in the IWF during natural and *in vitro* induced senescence, different proteins accumulated. Becker and Apel (1993) observed that one of the three cDNA clones isolated from dark-induced senescing barley leaves was not expressed during natural senescence. The differences in accumulation of some proteins during natural and artificially induced senescence indicate different molecular bases of these processes.

Two senescence-related cDNAs representing genes that are expressed early in radish leaf senescence, showed similarities to genes for PR proteins (Hanfrey *et al.* 1996). The genes for these two cDNA clones showed increased level of expression after salicylic acid treatment. In our previous work some of the barley PR proteins were also activated by salicylic acid and phosphate treatment (Tamás and Huttová

1996). Here, we demonstrate that some of the pathogen-induced apoplastic proteins are also induced during the senescence of the first barley leaf. It is possible that the role of the PR proteins was to increase resistance against pathogen invasion of the more sensitive senescent first leaf, during the time when nutrients are recycled to the younger parts of a plant. This could be a new function of PR proteins associated with the terminal developmental phase of the leaves, in the non-stressed plants.

It is well known that the expressions of genes for PR proteins are induced by several stresses (Ohashi and Ohshima 1992). The accumulation of some of the barley PR proteins are induced, *e.g.*, by metals, such as Cu, Al, Cd and Co, and by cold (Tamás *et al.* 1997). Accumulation of PR proteins during several stresses and in some developmental stages, *e.g.*, accumulation of PR proteins in the tobacco flower during flowering (Lotan *et al.* 1989) suggest their general role in plants. It is a question if one protein has many functions or, if different isoforms of PR proteins exist, which exhibit specific functions under different stresses.

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