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ENDOPEPTIDASES DURING THE DEVELOPMENT AND SENESCENCE OF *LOLIUM TEMULENTUM* LEAVES

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Key Word Index—*Lolium temulentum*; Gramineae; leaf development; senescence; cysteine endopeptidases.

Abstract—The endopeptidase activity of *Lolium temulentum* leaf tissue was measured using azocasein as a substrate. The enzyme increased with leaf age, and also during senescence of excised leaf tissue. There were at least two distinct endopeptidase activities, characterized by different pH optima. The predominant form in leaves of intact plants was maximally active at pH 5. In detached leaves during the later stages of senescence this activity was replaced by an enzyme with an optimum at pH 8. An antibody raised against the non-glycosylated cysteine endopeptidase papain cross-reacted with polypeptides in protein preparations of *L. temulentum* leaf tissue. The correlation between enzyme activity and the pattern of immunoreactive polypeptides suggested that the polyclonal antibody was able to recognize the *Lolium* homologues of papain. The switch from pH 5 to pH 8 enzyme in detached leaves was associated with an evident decrease in the M_r values of papain-like antigens detected on western immunoblots, from *ca* 60000 to 30000. It is possible that the alkaline activity is derived from the acid form, perhaps by limited autolysis in protein-depleted tissue at an advanced stage of senescence. On the other hand, the response of protease activation to treating leaf tissue with inhibitors of protein biosynthesis is more consistent with *de novo* appearance of a different form of the enzyme in late senescence.

INTRODUCTION

The leaves of many plant species are rich sources of proteolytic enzymes [1-3], but the physiological functions of the major proteases are not clear. Although there are exceptions, protease activities in most leaves increase with age, and particularly when foliar senescence is promoted by stress or excision. Protein mobilization is characteristic of senescing tissues, but it has proved difficult to establish a direct connection with increased levels of protease. In cereals, grasses and many other species, most of the measurable proteolytic activity of leaves is accounted for by a small number of acid endopeptidases [1,4-6]. Consistent with the low pH optimum is the observation that the acid endopeptidase activity of mesophyll cells is located predominantly in the vacuole [7-11]. For these enzymes to play a direct part in proteolysis during senescence there must be some kind of interchange between the vacuole and the chloroplast, which is the site of most of the mobilizable protein of leaf cells [12, 13]. Such contacts between the two organelles have never been convincingly demonstrated.

As well as acid endopeptidases, activities at neutral-alkaline pH values have been detected [3, 14-16]. The alkaline protease of oat leaves is located outside the vacuole [11, 16]. Experiments with inhibitors indicate that senescence-related increases in protease activity require protein synthesis [2]. Recently, cDNAs encoding proteases with homology to cysteine endopeptidases have been isolated from *Arabidopsis* [17] and maize [18] and used to show increased abundance of the corresponding mRNAs during senescence. It seems likely, therefore, that both transcription and translation are necessary for protease activation in ageing leaf tissue.

Lolium temulentum has been used extensively for studies of leaf growth and senescence [19-21]. Detached leaves of this species [15] initially accumulated acid protease activity and subsequently replaced it with a neutral-alkaline protease, the dominant enzyme in late senescence. We have re-examined *L. temulentum* leaf proteases with the object of characterizing the pH response in more detail, applying an immunological approach to visualizing qualitative changes in protease complement, and establishing the degree to which these changes depend on current protein synthesis.

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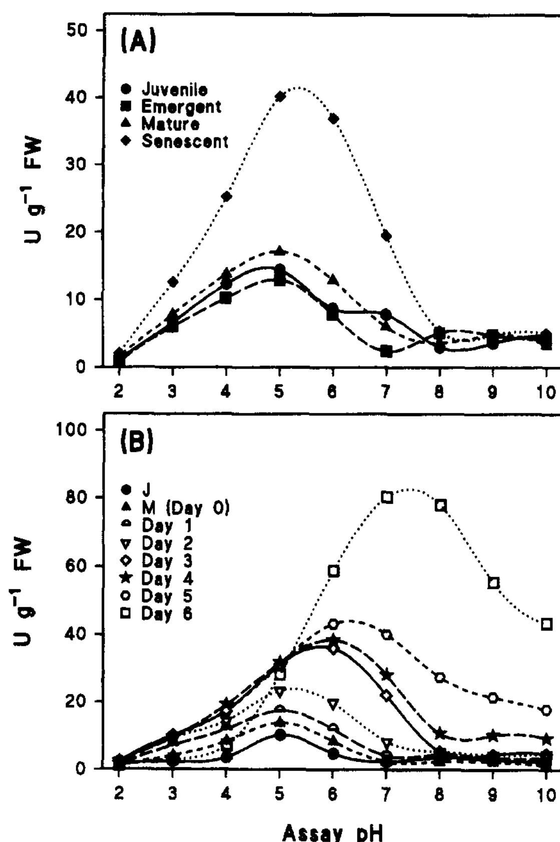

Fig. 1. Endopeptidase activity profiles for leaves of *Lolium temulentum*. The endopeptidase activity of attached (A) or excised (B) leaves was determined with azocasein as substrate as described in the Experimental section.

RESULTS AND DISCUSSION

Endopeptidase activity of attached and detached leaf tissue

Crude desalted protein extracts of mature *L. temulentum* leaf tissue released trichloroacetic acid-soluble chromogen when incubated with azocasein solution. The pH optimum for this enzyme activity was *ca* 5.0 (Fig. 1A). Juvenile (J) and emergent (E) leaf tissue yielded slightly lower activities than mature (M) leaves, whereas senescent (S) leaves gave extracts with high activity (Fig. 1A). During induced senescence of excised leaves, the endopeptidase activity increased and there was a progressive shift in the pH optimum towards pH 8.0 (Fig. 1B). Similar observations were reported in ref. [15], where it was concluded that there were two enzyme activities, one an acid endopeptidase, the other with a neutral–alkaline pH optimum, whose activities were reciprocally related. By assaying at pH 5 and 8 the relative contributions of the two enzymes to protein hydrolysis could be assessed. In the intact plant the shift in the pH optimum of endopeptidase activity was negligible (Fig. 1A). In terms of chlorophyll and protein content and endopeptidase activity, S-stage leaves are equivalent to excised tissue after about three days of dark incubation, before the sharp increase in pH 8 activity has commenced.

Detached leaves have been widely exploited as models of senescence [15, 22, 23]. They have the experimental convenience of accelerated uniform senescence, independence from the hormonal and nutritional influences of other organs and ease of treatment with chemicals and other exterior influences, but there are well-recognized disadvantages of using isolated tissue. These include the possible induction of wound responses and associated altered gene expression [2, 24]. Incubation of tissue in darkness may also cause metabolic changes related more to the light–dark transition than to specifically senescence-related processes [24–26]. Nevertheless, while not overlooking the possibility of stress-induced protease induction, we have no reason to suppose that the underlying basis of changes in proteolysis occurring in senescing detached tissue is fundamentally different from that of leaves from intact plants [27].

Polypeptides with proteolytic activity in *Lolium* leaf extracts were visualized by SDS–PAGE and a protein substrate (casein) followed by *in situ* renaturation and protein staining (Fig. 2). Clear bands represent zones where the substrate has been hydrolysed by protease. A different complement of enzymes was distinguishable at the two pH values. At pH 5 a prominent band at a M_r intermediate between the 66 000 and 45 000 markers was apparent four days after excision and declined thereafter. Two higher M_r bands were prominent in six-day tissue. At pH 8 the intensity and number of bands increased strongly over the period four to six days. While the use of substrate-containing gels is informative in the detection of proteases, only tentative conclusions as to the apparent M_r values of these can be drawn. This is because the casein substrate in the gel is likely to interfere

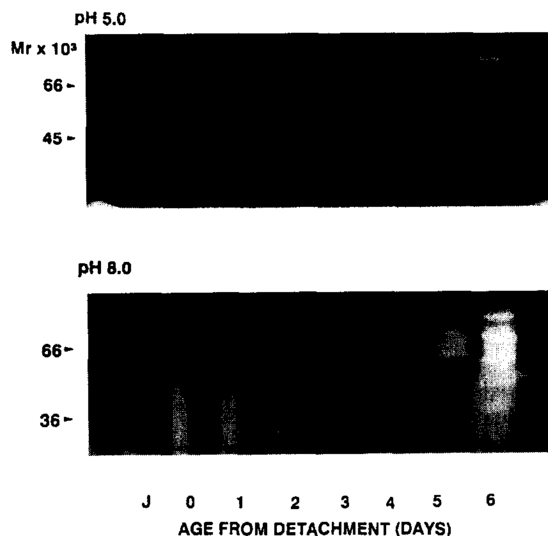

Fig. 2. Substrate-containing gel electrophoresis of proteins from excised leaves of *L. temulentum*. Soluble proteins were separated without prior denaturation by SDS-PAGE on casein-containing gels. Gels were washed subsequently in 1% (v/v) Triton, and then incubated overnight at the pH shown. Following staining with Coomassie Blue R any proteins demonstrating proteolytic activity were seen as clear bands against a blue background.

with electrophoretic migration, and differently with respect to the M_r standards and the plant proteins since the protein extracts applied to each gel were not denatured and, although SDS was present during electrophoresis, disaggregation and separation of the subunits of oligomeric enzymes was probably incomplete. This may account for the complexity of the pattern, particularly assayed at pH 8 for leaf sections six days from detachment. Here, the 'smearing' suggests that, despite adopting the methodology we have found previously will prevent proteolysis of leaf proteins during extraction (see Experimental), some activity of small M_r proteases in degrading casein during electrophoresis (at pH 9.0) was nevertheless present. However, it is also appropriate to note that the method lacks high specificity in that the proteases detected are not necessarily restricted to those of the cysteine protease class.

Immunological detection of cysteine endopeptidases in L. temulentum

Papain and chymopapain are cysteine endopeptidases belonging to the EC 3.4.22 group. The high degree of structural and sequence similarity amongst enzymes in this group, which have conserved amino acid sequences across the taxonomic range [28], was exploited to detect the cysteine endopeptidases of *L. temulentum*. The antibody prepared against papain reacted strongly with at least five polypeptides in juvenile tissue (Fig. 3A). Two of the antigens, a diffuse band with a M_r of more than 80 000 and another component of *ca* 100 000, were confined to J tissue; of the remaining three bands, all of M_r of *ca* 60 000, the largest was relatively unchanged in E, M and S tissue, the smallest was most abundant in E and declined thereafter, and the minor middle band was almost undetectable in M and S. In some blots, one or two fainter additional bands are visible just below the area of the rubisco large subunit in separations of extracts from E and M tissue. Figure 3B shows a western blot of protein from excised leaf tissue immunodetected with the papain antiserum. The intensity of the two major bands in the 60 000 M_r region declined with time. From day 3 onward a new antigen, M_r *ca* 30 000, appeared and was still increasing at the end of the senescence period. An antibody to chymopapain reacted less strongly than antipapain with the larger polypeptides, but had high affinity for the 30 000 component (Fig. 4B); it also detected a faint band at about 20 000 that changed in step with the 60 000 polypeptides. Prolonged incubation of a blot of J, E, M and S tissue extracts with the chymopapain antibody revealed much non-specific background staining, but also detected a band running at 30 000 in E, M and S samples (Fig. 4A). Thus, it may be that the low M_r antigen that dominates in detached tissue at advanced senescence (Figs 3B and 4B) is also present at low levels in pre-senescent tissue. As is implicit in immunoassay, it is evident that the polyclonal antibodies that were used recognize proteins with appropriate combinations of epitopes in the *Lolium* extracts and that this alone does not identify them as cysteine proteases. Equally, cysteine

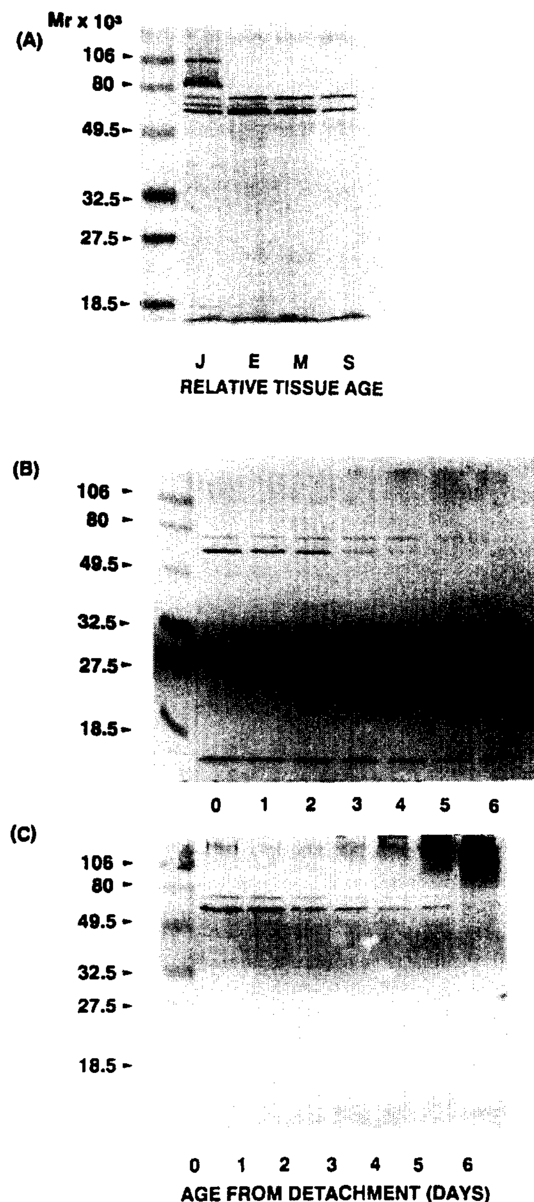

Fig. 3. Western immunoblots of proteins from *L. temulentum*. The total protein fractions from (A) juvenile, emergent, mature and senescent leaf tissues of intact plants; (B) excised leaf segments; and (C) excised leaf segments maintained on 1 mM cycloheximide, were separated by SDS–PAGE, transferred on to nitrocellulose and probed with anti-papain antiserum. M_r values shown are of pre-stained protein standards.

proteases may be present that have low cross-reactivity to papain or chymopapain. However, taken together, the data on trends in protease during development and senescence (Fig. 1), enzyme complement revealed by gel activity staining, but with the reservations in interpretation noted earlier (Fig. 2), and papain/chymopapain-like antigens (Figs 3 and 4) are consistent with the assignment of the pH 5 activity to higher M_r (> 60 000) polypeptides and pH 8 activity to the 30 000 band detected by Western blotting. Miller and Huffaker [5, 27] used activity

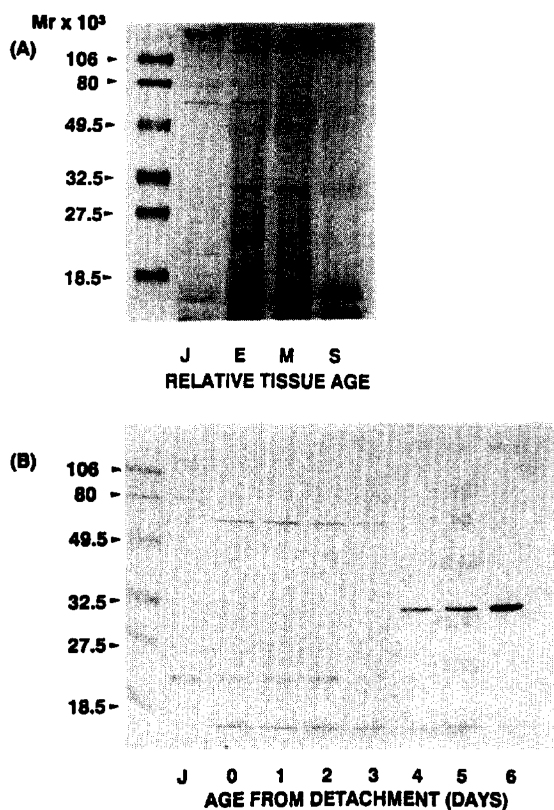

Fig. 4. Western immunoblots of proteins from *L. temulentum*. The total protein fractions from (A) juvenile, emergent, mature and senescent leaf tissues of intact plants, and (B) juvenile tissue and leaf-segments from fully expanded leaves incubated on water for the time shown, were separated by SDS–PAGE, transferred on to nitrocellulose and probed with anti-chymopapain antiserum. M_r values shown are of pre-stained protein standards.

staining after non-denaturing gel electrophoresis and electrofocusing to show that increases in barley leaf proteases in the first stages of senescence occurred without the appearance of detectable new enzymes in addition to the constitutive forms EP_1 and EP_2. Later, two other activities, EP_3 and EP_4, were observed [27]. Their gel overlay procedure used gelatin at pH 6 as the substrate, so it may be that EP_3 and EP_4 are equivalent to the pH 8 activity of *Lolium* tissue, measured away from their true pH optimum, but close enough to obtain a visible response. If this is correct, it suggests that the neutral–alkaline enzyme is distinct from the acid not only in pH optimum, but also in size, isoelectric point and perhaps subunit structure. This would make it more likely that the two groups of protease are distinct proteins and not, as suggested elsewhere [15], that the pH 8 enzyme is derived from the pH 5 enzyme by autolysis.

Inhibition of protein synthesis

We examined the response to translation inhibitors to determine how much the senescence-related quantitative and qualitative changes in endopeptidases were dependent on new protein synthesis. It is well documented that protein synthesis inhibitors delay apparent senescence [29], preventing the degradation of proteins and chlorophyll; they have also been reported to inhibit the appearance of protease activity [2, 22]. Cycloheximide, an inhibitor of 80S ribosome function, was observed to decrease the rate of protein degradation and inhibit endopeptidase activity of *L. temulentum* leaf segments during the first four days after detachment. The characteristic shift in pH optimum was also delayed in the treated segments. The 30 000 antigen was not detected in western blots of total protein extracts from cycloheximide-treated segments challenged with the papain antibody (Fig. 3C). This is consistent with the endopeptidase activities and the previous data that associate the smaller protein with the pH 8 enzyme. It was noticeable that, as the pre-existing $M_r > 60\,000$ antigens diminished, staining in a diffuse region at the top of the blot intensified (Fig. 3C). Aggregation of protease may be a response to the long-term toxic effect of the antibiotic, possibly as part of a pathological reaction invoking a crosslinking-type catabolic pathway such as the ubiquitin system.

The use of cycloheximide as a specific protein synthesis inhibitor has been questioned as it may interfere with other biochemical or physiological processes [30, 31]. An alternative protein synthesis inhibitor, MDMP [2-(4 methyl-2,6-dinitroanilino-*N*-methylpropionamide] is less open to criticism in this respect because it exists as two sterieoisomers, the D-isoform that inhibits protein synthesis and the L-form that does not [30–32]. An initial optimization of inhibitor concentration was performed using soluble protein content as an index of senescence. In segments treated with the L-isomer, protein decreased with time in a similar fashion to water controls, whereas protein loss from the D-isomer treated segments was retarded to a similar extent to that of the cycloheximide-treated tissue.

Over the range tested, retention of protein was dependent on MDMP concentration. At D-MDMP concentrations above 10 μM, tissue turgidity and integrity were severely impaired and the inhibitor failed to prevent the appearance of the alkaline endopeptidase activity in leaf segments treated for longer than four days. The optimal concentration for preserving protein content, inhibiting increase in endopeptidase activity and maintaining tissue quality was found to be 10 μM. The same concentration has been reported to give maximal retention to chlorophyll and glutamate pyruvate transaminase in detached leaves of *Festuca pratensis* [30].

Trends in proteolytic activity and soluble protein content of leaf segments incubated continuously on 10 μM MDMP or transferred from L- to D-MDMP after three days are presented in Table 1. Endopeptidase activity increased normally in the L-isomer treated segments and was completely inhibited in the D-isomer treated tissue. Because day 3 was the threshold time at which a significant alteration in endopeptidase activity and pH optimum became apparent (Fig. 1B), leaf segments were transferred from L- to D-isoform after three days to

Table 1. Endopeptidase activities of excised leaves of *Lolium temulentum* incubated on MDMP

	Treatment					
	L-MDMP Day 0–6		L-MDMP Day 0–3 D-MDMP Day 3–6		D-MDMP Day 0–6	
Days	pH 5	pH 8	pH 5	pH 8	pH 5	pH 8
0	18.4	6.0	18.4	6.0	18.4	6.0
2	41.9	7.7	41.9	7.7	11.5	8.5
4	53.8	10.3	38.9	5.1	12.0	5.1
6	57.3	123.9	14.5	4.3	10.3	6.0

The concentration of MDMP was 10 μM in each case. Activities are expressed as U g^{-1} fresh weight of leaf tissue.

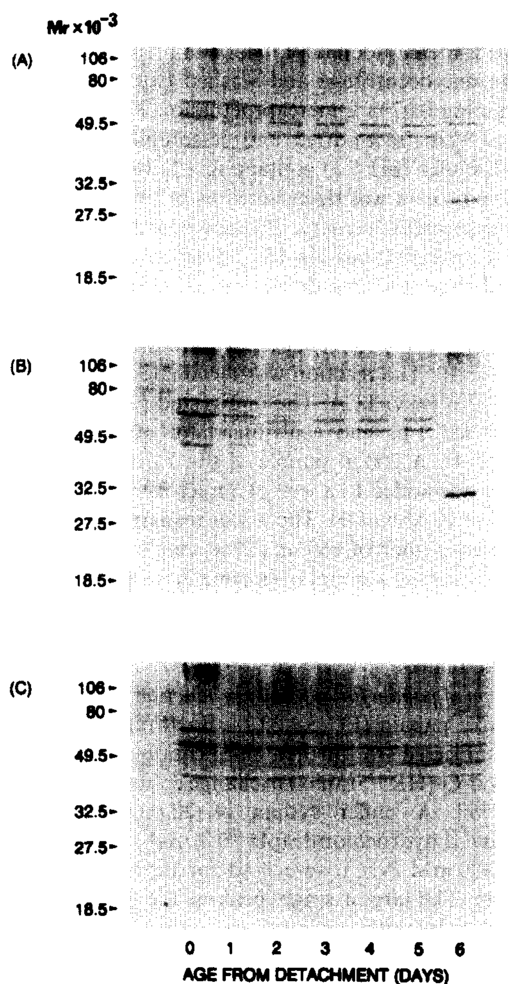

Fig. 5. Western immunoblots of proteins from leaves of *L. temulentum*. Excised leaves were incubated for the time shown on 10 μM solutions of (A) L-MDMP, (B) L-MDMP, transferred to D-MDMP after 3 days; and (C) D-MDMP. The total protein fractions were separated by SDS–PAGE, transferred on to nitrocellulose and probed with anti-papain antibody.

determine whether the changes required active protein synthesis. Tissue responded to the inhibitor applied to mid-senescence with a subsequent decline in endopeptidase activity, measured at either pH value, to levels observed in segments kept on D-MDMP for the entire incubation period (Table 1). We conclude, therefore, that changes in total endopeptidase and relative activities of the acid and alkaline forms that occur with tissue age are dependent on cytosolic protein synthesis.

Anti-papain western blots of protein from MDMP-treated tissue are presented in Fig. 5. In this experiment the minor band at M_r *ca* 45 000 is more prominent at day 0 than in the experiment of Fig. 3. Although the quantitative response of leaf proteins and protease activity to L-MDMP resembled water controls, significant differences in antigen pattern were apparent (Fig. 5A). From day 2, two bands at M_r *ca* 50 000 became visible and persisted until day 6. At day 6 the major high M_r forms of pre-senescent, M-stage tissue had been lost completely and the 30 000 component was conspicuous. Incubation with D-MDMP from day 0 essentially immobilized the antigen pattern, although a band at about M_r 30 000 had become faintly visible at day 6 (Fig. 5C). These results are consistent with the proposal that the low M_r band represents pH 8 endopeptidase and that the emergence of this activity is directly dependent on protein synthesis. The appearance of an antigen at M_r about 30 000 in day 6 extracts of segments that were transferred from L- to D-MDMP mid-way through the senescence sequence (Fig. 5B) is at odds with this interpretation, since increase in pH 8.0 activity in the tissue could not be detected (Table 1). It may be that an inactive form of the low M_r, neutral–alkaline endopeptidase is produced in the presence of D-MDMP, either because the enzyme itself is damaged in some way, or else because a cofactor necessary for its activity is missing.

Conclusions

The characteristic decrease in protein content associated with leaf senescence is accompanied by an increase

in endopeptidase activity. The phenomenon occurs during both natural and excision-accelerated leaf senescence, with a shift in the pH optimum occurring during late senescence of detached tissue. The qualitative and quantitative changes in protease activity during senescence are sensitive to treatment with translation inhibitors, indicating a requirement for active protein synthesis. The evidence from gel activity staining and western blotting indicates that the rise in acid protease between the M and S stages of leaf senescence in intact plants, and over the first three days of senescence in excised tissue (Fig. 1), occurs without modulation in enzyme complement. This is consistent with previous studies in other species (e.g. [27]).

The relationship between quantitative changes in acid protease, leaf age and protein mobilization remains enigmatic. Why, when the leaf is already equipped with high constitutive levels of acid protease, does it seem necessary to biosynthesize more of the same enzymes as the organ ages and its protein is recycled? Perhaps the answer lies with the turnover behaviour of vacuolar proteins. In the acidic environment of this organelle, the pH 5 protease is potentially at maximal activity. Casual observation of the behaviour of purified cysteine proteases *in vitro* shows that they are highly autolytic. It may be, therefore, that there is continual synthesis, transport to the vacuole and self-hydrolysis of the acid endopeptidase throughout the life of the leaf, and that the rate of autolysis decreases in older tissue, perhaps because the pH of the vacuole rises.

This still leaves unanswered the question as to whether the acid protease ever gets out of the vacuole and into the chloroplast. The evidence generally points to an intraplastidic location for proteolysis during senescence [33, 34]. Also unresolved is the issue of the origin of the pH 8 enzyme. The response to cycloheximide and MDMP is consistent with *do novo* synthesis, but the inhibitors would also be expected to stop the pH 8 activity from appearing if it is a product of autolysis by the rapidly turning-over acid enzyme. Such an indirect effect of inhibitor may reconcile the contrasting results of Table 1 and Fig. 5B, where the putative pH 8 enzyme antigen appeared without an increase in the corresponding alkaline protease activity.

We have exploited the high degree of structural similarity between cysteine proteinases to demonstrate, by means of heterologous antibodies, the immunological relatedness of pH 5 and pH 8 endopeptidases present at different stages of leaf development in *L. temulentum*. The homologies at the protein level are so close that they suggest an approach to cloning the *Lolium* endopeptidase genes, based on the exploitation of DNA-level similarities.

EXPERIMENTAL

Plant material. Plants of *L. temulentum* were grown from seed in a nutrient soln [35] under controlled environment conditions (20°, 8 hr photoperiod, 360 μmol $m^{-2} s^{-1}$ light flux). Plants were harvested 32 days after sowing when the 5th leaf was emerging and the 4th leaf had not yet fully expanded. At this state the 2nd leaf was senescent (S) and the 3rd leaf mature (M). The lamina of leaf 4 represented emergent (E) tissue and the sheath of the 4th leaf with the whole of leaf 5 was designated juvenile (J) tissue [19]. Experiments were also carried out with excised fully expanded 4th leaves, which were harvested from 35-day-old plants, cut into 1 cm segments, surface-sterilized and induced to senesce by dark incubation at 20° on sterile moist filter paper.

Prepn of protein extracts. Soluble protein extracts were prepd by homogenizing leaf tissue with 3 ml g^{-1} fr. wt of ice-cold 50 mM Tris–HCl (pH 7.5) containing 2 mM EDTA and 0.04% (v/v) 2-mercaptoethanol. The homogenate was centrifuged at 4° for 20 min at 13 000 g_{av} in a microcentrifuge. The crude protein extract was spin-desalted on a Sephadex G-25 column equilibrated with 10 mM Tris–HCl (pH 7.5) as described in ref. [36]. Proteins in aliquots of crude and spin-desalted extracts were pptd by incubating at −20° overnight with an equal vol. of 20% (w/v) TCA in Me_2CO containing 0.14% 2-mercaptoethanol. The pptd protein was pelleted in the microcentrifuge and washed twice with Me_2CO containing 0.07% 2-mercaptoethanol. The final protein pellets were freeze-dried and re-solubilized in 50 mM Na-Pi buffer (pH 7.5) containing 1% (w/v) SDS. Total soluble protein was determined as in ref. [37].

Endopeptidase activity. Proteolytic activity was determined against azocasein [38]. Each assay consisted of a 25 μl aliquot of spin-desalted protein extract incubated for 4 hr at 37° with 225 μl 50 mM Hepes/Mes buffer containing 0.4% (w/v) azocasein at the stated pH in the range 2–10. The reaction was terminated by addition of 40 μl 50% (w/v) TCA. The undigested protein was pptd on ice for 1 hr and centrifuged for 10 min at 13 000 g_{av} and 4°. A 200 μl sample of the TCA-soluble supernatant was added to a well of a microtitre plate containing 15 μl 10 M NaOH. The *A* was read at 405 nm against a reference filter of 660 nm. One unit of endopeptidase activity was the amount of enzyme that gave an *A* change of 1 at 405 nm following a 4 hr incubation at 37°. All assays were performed in duplicate with corresponding enzyme blanks.

Prepn of papain for antibody production. Commercially available papain (EC 3.4.22.2—Boehringer Mannheim) was subjected to further purification by FPLC on a Mono-Q-(HR5/5) anion exchange column according to ref. [39]. A buffer system of 20 mM 1,3-diaminopropane-dihydrochloride (pH 10.8) and a linear gradient of 0–300 mM NaCl were used for development of the column. The largest single peak in the eluate that possessed proteolytic activity was taken for immediate antiserum production. The purified papain was prepd to a final concn of 500 $\mu g\ ml^{-1}$ in buffered saline (137 mM NaCl, 2.7 mM KCl, 6.5 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 7.4). A 500 μl aliquot was emulsified with an equal vol. of Freund's complete adjuvant and injected intramuscularly into a New Zealand white rabbit. Two booster injections of the same dosage with Freund's incomplete adjuvant were administered subcutaneously

at 3 week intervals. In some experiments we also used an antibody prepn to chymopapain (EC 3.4.22.6), the kind gift of Peter Goodenough and Dean Revell of the Institute of Food Research, Reading, U.K.

Total protein extractions and western blotting. Total leaf protein for electrophoresis and immunoassay was extracted under denaturing conditions [21]. Leaf tissue was weighed and homogenized in a chilled mortar and pestle with 5 $ml\,g^{-1}$ fr. wt of ice-cold 50 mM Li-Pi (pH 7.2) containing 120 mM 2-mercaptoethanol, 1 mM Na monoiodoacetate, 1 mM PMSF and 5% (w/v) glycerol. Subsequently 20% (w/v) Li dodecyl sulphate was added to the homogenate to a final concn of 2%. The mixt. was transferred to microcentrifuge tubes, boiled for 45 sec and centrifuged at 13 000 g_{av} for 20 min. The supernatant was stored at $-20°$ until required.

Total proteins of leaf extracts were sepd by SDS–PAGE and transferred to nitrocellulose as described in ref. [40]. Each western blot was blocked with low-fat powdered milk soln and incubated with antibody. Antigens were visualized by reaction with an alkaline phosphatase-conjugated second antibody followed by colorimetric detection [41].

Substrate-containing gel electrophoresis. Crude non-denatured protein extracts were sepd by SDS–PAGE on a gel containing 130 $\mu g\,ml^{-1}$ casein; this was used in preference to, e.g. gelatin, since azocasein had been the substrate in assays of endopeptidase activity. Following electrophoresis, the sepd proteins were denatured by washing the gel in 1% (v/v) Triton X-100 soln. After overnight incubation in 50 mM Hepes/Mes buffer at pH 5 or 8, gels were stained for protein with Coomassie Blue R (0.5% (w/v) in 10% (v/v) HOAc and 45% (v/v) EtOH). Proteolytic activity appeared as clear bands against a blue background [42].

Inhibition of protein synthesis. Mature fully expanded 4th leaves were cut into 1 cm segments as described previously and incubated on H_2O, 100 mM cycloheximide or various concns of the inhibitor MDMP. Proteolytic activity was measured over a 6 day period. Total protein extracts were also prepd, sepd by SDS–PAGE, western blotted and challenged with papain antibody.

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