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ORIGINAL ARTICLE

The two main endoproteases present in dark-induced senescent wheat leaves are distinct subtilisin-like proteases

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Abstract We have previously reported the occurrence of two serine endoproteases (referred to as P1 and P2) in dark-induced senescent wheat (*Triticum aestivum* L.) leaves. P1 enzyme was already purified and identified as a subtilisin-like serine endoprotease (Roberts et al. in Physiol Plant 118:483–490, 2003). In this paper, we demonstrate by Western blot analysis of extracts obtained from dark-induced senescent leaves that an antiserum raised against P1 was able to recognise a second protein band of 78 kDa which corresponded to P2 activity. This result suggested that both enzymes must be structurally related. Therefore, we purified and characterised P2 activity. According to its biochemical and physical properties (inhibition by chymostatin and PMSF, broad pH range of activity, thermostability and ability to hydrolyse Suc-AAPF-pNA) P2 was classified as a serine protease with chymotrypsin-like activity. In addition, P2 was identified by mass spectrometry as a subtilisin-like protease distinct from P1. Western blot analysis demonstrated that P1 appeared in extracts from non-detached dark-induced senescent leaves but was undetectable in leaves senescing after nitrogen (N) deprivation. In contrast, P2 was already present in non-senescent leaves and showed increased levels in leaves senescing after N starvation or incubation in darkness. P1 signal was detected at late stages of ethephon or methyl jasmonate-induced senescence but was undetectable in senescent leaves from plants treated with abscisic acid. None of the three hormones have any effect on P2 protein levels. These results indicate that despite their biochemical and structural similarities, both enzymes are probably involved in different physiological roles.

Keywords Leaf senescence · Proteolysis · Subtilisin-like serine protease · Wheat

Abbreviations

ABA	Abscisic acid
Ethephon	2-Chloroethylphosphonic acid
MALDI-TOF	Matrix-assisted laser desorption ionisation-time of flight
MJ	Methyl jasmonate
MS/MS	Tandem mass spectrometry
N	Nitrogen
PMF	Peptide mass fingerprint
PMSF	Phenylmethanesulphonyl fluoride
Rubisco	D-Ribulose-1,5-bisphosphate carboxylase-oxygenase (EC: 4.1.1.39)
RLS	Rubisco large subunit
Suc-AAPF-pNA	*N*-succinyl-alanine-alanine-proline-phenylalanine-*p*nitroanilide

Introduction

Proteolysis participates in almost all aspects of plant development ranging from germination (Müntz 1996) and tissue differentiation (Ye and Varner 1996) to programmed cell death (PCD) (Beers et al. 2000) and

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senescence (Huffaker 1990). Most of the studies of senescence-associated proteolysis have put emphasis on their crucial role in massive protein degradation that contributes to nitrogen (N) remobilisation from senescent leaves to new developing and reproductive organs. However, recent knowledge indicates that many processing proteases are connected with highly specific functions and substrates revealing the importance of protein degradation as a regulatory component of several processes occurring during plant development (Schaller 2004; Schwechheimer and Schwager 2004).

In the last years, the use of techniques such as subtractive hybridisation and differential screening allowed the identification in several plant species of numerous genes which are increasingly expressed during the senescence process (Buchanan-Wollaston et al. 2003; Gepstein et al. 2003). As expected, an important fraction of these genes are predicted to encode different types of proteases, mainly cysteine proteases. More recently, the sequence analysis of ESTs collections obtained from cDNA libraries prepared from senescent leaves of *Arabidopsis thaliana* (Guo et al. 2004) and *Populus tremula* (Bhalerao et al. 2003; Andersson et al. 2004) revealed that mRNAs coding for cysteine proteases are among the most abundant transcripts found in senescent leaves.

A recent publication by Antão and Malcata (2005) provides a comprehensive review on plant serine proteases including a list of the so far isolated enzymes and the current knowledge about their functions. Although serine proteases have been related to a great variety of biological functions, only a few of them could be associated with plant senescence. The induction of serine proteases has been demonstrated in peroxisomes from senescent pea leaves (Distefano et al. 1997), in post-harvest senescent parsley leaves (Jiang et al. 1999) and in broccoli florets (Wang et al. 2004) mainly by in-gel protease activity assays combined with the use of protease inhibitors.

We have recently shown that upon fractionation of extracts from senescent wheat leaves by DEAE-Sepharose column chromatography, two peaks of endoproteolytic activity could be detected (Roberts et al. 2003). One of them (referred to as P1) appeared in the flow-through while the second one (referred to as P2) eluted at 150 mM NaCl. P1 was purified to near homogeneity and characterised. It was identified as a subtilisin-like serine protease by biochemical and partial sequence analysis (Roberts et al. 2003). In this paper, we report the purification and characterisation of P2 and demonstrate that it also belongs to the subtilisin-like serine proteases family. We also show that both enzymatic activities are differentially expressed depending on the senescence-inducing stimuli.

Materials and methods

Plant material and growth conditions

Wheat seeds (*Triticum aestivum* cv. Pro INTA Isla Verde obtained from INTA Marcos Juárez, Córdoba, Argentina) were sown on vermiculite and watered daily with nutrient solution (Hoagland and Arnon 1950) containing 10 mM KNO_3. Plants were maintained in a growth chamber under a photoperiod of 16 h light/8 h darkness at 23°C and with an irradiance of 350 μmol photons m^{-2} s^{-1}. Fifteen days after sowing, plants were subjected to one of the following treatments. For dark-induced senescence in excised leaves, the third leaf of each plant was detached, placed in plastic boxes with distilled water and incubated in continuous darkness or maintained under the aforementioned photoperiod. Samples were taken at 24, 48, 72 and 96 h after detaching. Dark-induced senescence in intact plants was attained by covering the third leaf with an aluminium foil cap. For N deprivation-induced senescence, pots were thoroughly rinsed with distilled water and from then on, watered daily with nutrient solution lacking KNO_3. For ethephon (2-chloroethylphosphonic acid) treatment, plants were sprayed daily with 1 mM ethephon and 0.005% (v/v) Tween 20. Methyl jasmonate (100 μM) or abscisic acid (50 μM) was applied daily dissolved in the nutrient solution. Samples of the third leaf were taken every 2 days. For all treatments, three replicates of leaf samples were collected, weighed, frozen in liquid nitrogen and stored at −50°C until processing. In all cases, the first sample (time 0 h) corresponded to the third leaf of 15-day-old plants frozen immediately after detaching. Control treatments consisted of plants maintained under the initial growth conditions (aforementioned light/dark cycles and 10 mM KNO_3).

Extracts preparation

Frozen leaves were ground with mortar and pestle in the presence of liquid nitrogen and the powder obtained extracted with 50 mM Tris–HCl buffer, pH 7.5 containing 1% (w/v) polyvinylpolypyrrolidone using 2 ml buffer g^{-1} fresh weight (FW). The homogenate was filtered through cheesecloth, centrifuged at 100,000×*g* for 1 h and the supernatant (hereafter named S100 fraction) saved for enzymatic assays.

DEAE-Sepharose column chromatography of S100 fractions from senescent and non-senescent leaves

S100 fractions from leaves incubated in darkness for 72 h and from leaves just detached (0 h) were desalted

by filtering through a Sephadex G-25 column equilibrated with 50 mM Tris–HCl buffer, pH 7.5 (buffer A). Each sample, containing approximately 20 mg protein, was fractionated on a DEAE-Sepharose column (1×5 cm) equilibrated in buffer A. After washing with 20 ml of buffer A, the column was developed at a flow rate of 75 cm h^{-1} with 80 ml of a linear gradient from 0 to 500 mM NaCl in buffer A. Fractions of 1 ml were collected and analysed for Suc-AAPF-pNA (*N*-succinyl-alanine-alanine-proline-phenylalanine-*p*nitroanilide) hydrolytic activity and immunoreaction with a polyclonal antiserum raised in mouse against purified P1 (anti-P1 antiserum).

Measurement of enzymatic activity

Chymotrypsin-like activity was assayed with the synthetic chromogenic substrate Suc-AAPF-pNA. The reaction mixture contained, in a final volume of 200 µl, 50 mM sodium phosphate buffer, pH 8.5, 5 mM substrate and 50 µl of each fraction. Samples were incubated at 37°C for 45 min and the reaction was stopped by adding 500 µl of 2 M acetic acid and 300 µl of reaction buffer. The *p*-nitroaniline liberated was monitored at 405 nm. A control sample without substrate was routinely included. The enzymatic activity was expressed as moles of *p*-nitroaniline liberated per hour. Calculations are based on a molar absorption coefficient of 9,500 l mol^{-1} cm^{-1} for *p*-nitroaniline at 405 nm.

P2 partial purification and properties

All steps were performed at 4°C. S100 fraction was prepared as described above from 2.45 kg FW (24 g protein) of leaves detached from 15-day-old plants and incubated in continuous darkness for 72 h. The sample was concentrated by ammonium sulphate precipitation to 80% saturation, resuspended in buffer A and desalted by gel filtration on Sephadex G-25. The first purification step consisted of a DEAE-Sepharose chromatography similar to that mentioned above except that the size of the column was 2.5×38.5 cm and the elution volume was scaled up accordingly. Four cycles of 6 g of protein were performed. Fractions containing the activity peak named P2 were collected and pooled and proteins were fractionated by ammonium sulphate precipitation. P2 activity was recovered in the fraction corresponding to 50–80% saturation. The pellet was resuspended in buffer A, desalted through a Sephadex G-25 column (2.5×10 cm) equilibrated in 50 mM Tris–HCl buffer, pH 8.5 (buffer B), and applied on to a Q-Sepharose column (1×6 cm) equilibrated in the same buffer. After washing with 20 ml of buffer B the column was developed at a flow rate of 75 cm h^{-1} with 110 ml of a linear gradient from 0 to 500 mM NaCl in buffer B. Fractions of 1.1 ml were collected and analysed for Suc-AAPF-pNA hydrolytic activity and immunoreaction to anti-P1 antiserum. Fractions containing the peptidasic activity peak were pooled, diluted tenfold with buffer B and concentrated by filtering through a small Q-Sepharose column (0.5×1 cm) equilibrated in the same buffer. Bound proteins were eluted with 500 mM NaCl in buffer B. This fraction (0.5 ml) was loaded onto a Sephacryl S-300-HR column (1×41 cm) equilibrated and developed with buffer A containing 150 mM NaCl. Fractions of 0.7 ml were collected at a flow rate of 2.3 cm h^{-1}. Active fractions were pooled and used to study the properties of the enzyme.

The effect of pH on P2 activity was assessed in the range 4–13 using the following buffers: 50 mM citrate–NaOH (pH 4, 5, 5.5 and 6.5), 50 mM potassium phosphate (6, 6.5, 7, 7.5 and 8), 50 mM Tris–HCl (pH 7, 8 and 8.5), 50 mM CAPS–NaOH (pH 9, 10, 10.5 and 11) and 50 mM KCl–NaOH (pH 12 and 13). Aliquots of 40 µl of enzymatic preparation were incubated for 10 min at 37°C.

Dependence of the enzymatic activity on temperature was determined assaying Suc-AAPF-pNA hydrolysis in the range 0–80°C for 10 min. P2 thermal stability was determined by pre-incubating the enzyme at different temperatures (0–80°C) for 10 min and measuring residual activity at 37°C for 30 min.

Effect of different protease inhibitors on P2 activity was tested by pre-incubating the enzymatic preparation (20 µl) for 30 min at 37°C in the presence of: 10 µM *trans*-epoxysuccinyl-L-leucylamido(4-guanidino)-butane (E-64), 10 µM fluorescein mercuric acetate (FMA), 5 mM *o*-phenanthroline, 1.45 µM pepstatin, 45 µM leupeptin, 1 mM EDTA, 5 mM *N*-ethylmaleimide (NEM), 100 µM tosyl phenylalanyl chloromethyl ketone (TPCK), 1 mM phenylmethanesulphonyl fluoride (PMSF) and 10 or 50 µM chymostatin. A control sample without inhibitors was included. After the pre-incubation period, the reaction was initiated by adding the chromogenic substrate, and residual activity was determined after 35 min incubation at 37°C.

Polyacrylamide gel electrophoresis and immunoblotting

Samples of leaf extracts were fractionated by 10 and 15% SDS-PAGE according to Laemmli (1970) for Western blots analysis and for Rubisco detection, respectively. Rubisco large subunit (RLS) was visualised by CBB R-250 staining. For Western blots, electrophoresed proteins were transferred to polyviny-

lidene difluoride (PVDF) membranes (Immobilon, Millipore) essentially as described by Towbin et al. (1979). Immunodetection of P1 and P2 was achieved with anti-P1 antiserum. Bound antibodies were detected with alkaline phosphatase-conjugated anti-mouse IgG and NBT/BCIP solution (Pierce Biotechnology, Inc., Rockford, IL, USA). A control sample (C) of 0.2 μg of purified P1 was included in all blots.

Samples of purified P1 and partially purified P2 were submitted to IEF-SDS-PAGE according to O'Farrell (1975). Isoelectric focusing in the first dimension was in the pH range 3–10 and electrophoresis in the second dimension was in a 10% SDS gel. Proteins were stained with CBB R-250.

Mass spectrometry analysis

After performing the bidimensional electrophoresis, P1 and P2 spots were excised, digested in-gel with trypsin and submitted to MALDI-TOF mass spectrometry analysis (Proteomics Unit, Autonomous University of Madrid, Spain). Three peptide peaks from P2 spectrum (1,044.5, 1,320.6 and 1,393.7 Da) were selected and subjected to tandem mass spectrometry (MS/MS) fragmentation. Peptide mass fingerprint (PMF) and MS/MS data were used to search in the nr database at NCBI with Mascot program (http://www.matrixscience.com). Sequence homology was determined using the BLAST program (http://www.ncbi.nlm.nih.gov/BLAST) for searching in the nr database at NCBI and sequence alignment was created with the ClustalW program (http://www.ebi.ac.uk/clustalw).

Results

Changes in P1 levels in dark-induced senescent leaves

The presence of P1 protein in extracts from dark-induced senescent and non-senescent detached leaves was assessed by Western blot analysis using an antiserum raised against purified P1. As can be seen in Fig. 1a, an immunoreactive band of 59 kDa was detected in extracts from senescent leaves. This band corresponded to P1 as shown by a control sample of the purified protease (lane C). As expected, the 59 kDa band appeared only after 48 h of incubation in darkness and its signal increased until 96 h. Surprisingly, a second band of apparent molecular mass of 78 kDa was revealed by the antiserum. This signal was already present in non-senescent leaves (Fig. 1a, 0 h) and increased along the incubation period (Fig. 1a). This band did not increase in detached leaves incubated under the light/darkness cycle used for plant growth (Fig. 1b) while P1 band was undetectable, except for a weak signal appearing at the last sampling day (96 h).

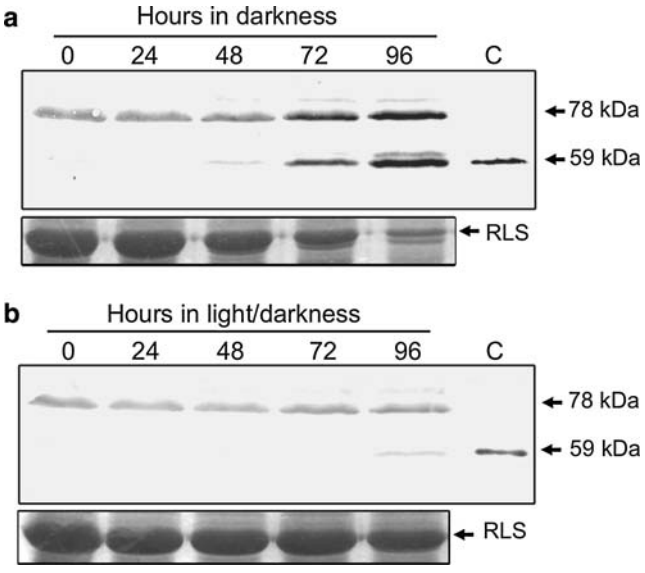

Fig. 1 Immunorecognition of P1 in extracts from dark-induced senescent leaves. S100 fractions were prepared as described in Materials and methods from detached leaves after 24, 48, 72 and 96 h of incubation in continuous darkness (**a**, *upper panel*) or under the light/darkness cycle used for plant growth (**b**, *upper panel*). Samples corresponding to 7.5 mg FW of each extract were separated by 10% SDS-PAGE, transferred to a PVDF membrane and blotted with anti-P1 antiserum. *Lane C* corresponds to a control sample of 0.2 μg of purified P1. *Arrows* indicate the position of immunoreactive bands and their molecular masses. *Lower panels* in **a** and **b** show Rubisco large subunit (RLS) stained with CBB R-250 after 15% SDS-PAGE of the same extracts (3 mg FW). Data shown are representative of four independent experiments with three replicates

The decay in RLS content was used as a control marker of the senescence progress. An active RLS hydrolysis was observed as a consequence of the dark treatment reaching 85% degradation after 96 h (Fig. 1a, lower panel). In contrast, only 33% of RLS initial content was hydrolysed in control leaves at the same time (Fig. 1b, lower panel).

Identification of the 78 kDa immunoreactive band as P2 activity

The expression pattern of the 78 kDa band shown in Fig. 1 strongly resembled the P2 proteolytic activity profile previously detected on DEAE-Sepharose columns (Roberts et al. 2003). To clarify this point, fractions from a DEAE-Sepharose column chromatography of extracts prepared from dark-induced senescent (72 h) and non-senescent (0 h) leaves were analysed by Western blot using the anti-P1 antiserum (Fig. 2). Also, the Suc-AAPF-pNA hydrolytic activity

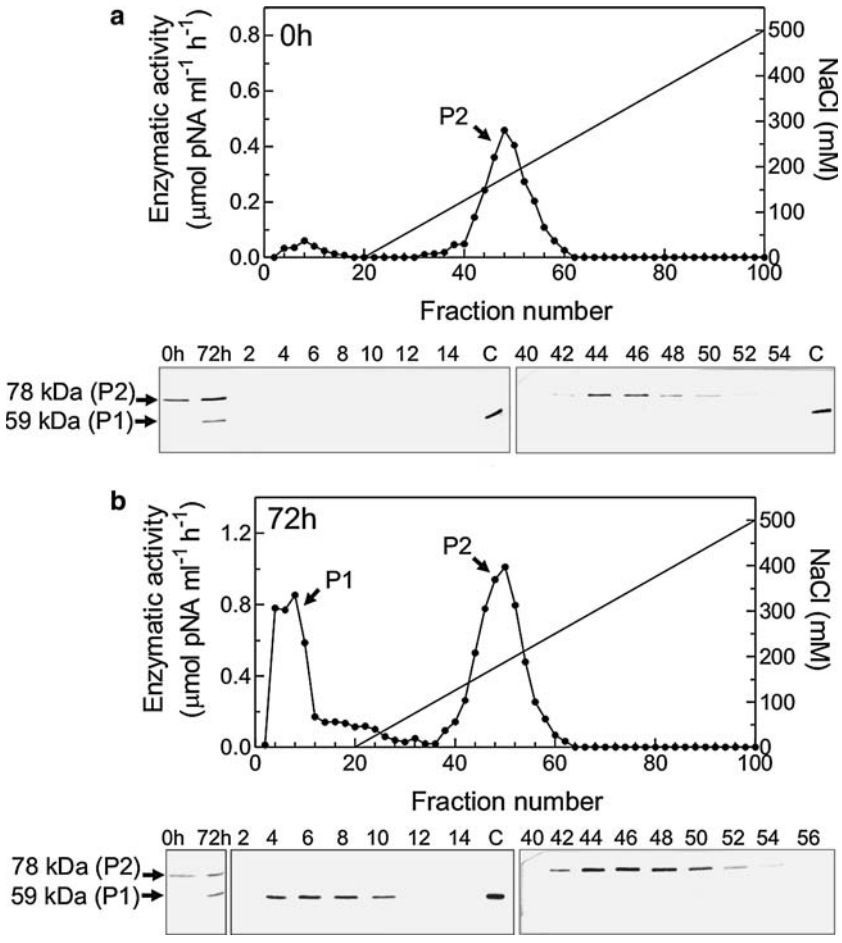

Fig. 2 DEAE-Sepharose column chromatography of S100 fractions prepared from non-senescent (**a**) and dark-induced senescent (**b**) wheat leaves. *Upper panels* in **a** and **b** show the chromatographic profile of Suc-AAPF-pNA hydrolytic activity. *Arrows* indicate the activity peaks designated P1 and P2. *Lower panels* in **a** and **b** show immunoreactive bands of the corresponding fractions (15 µl). Samples corresponding to 3.5 mg FW of whole extracts from non-senescent (0 h) and senescent leaves (72 h) were included as controls. *Arrows* indicate the position of the immunoreactive bands. *Lane C* corresponds to a control sample of 0.2 µg of purified P1. *Numbers above the lanes* indicate the fraction number of the column. Data shown are representative of five independent experiments

was assessed in column fractions. As can be seen, in fractions of extracts from senescent and non-senescent leaves the profile of the immunoreactive 78 kDa band perfectly matched P2 enzymatic activity peak. The senescence-associated increase in P2 activity was reflected in a higher intensity of the immunoreactive 78 kDa band in fractions of the 72 h treated leaves (Fig. 2b). These observations allow us to undoubtedly assign the 78 kDa band to P2 activity.

As expected, the 59 kDa band corresponding to P1 appeared only in the flow-through from senescent leaves extract (Fig. 2b), also following the enzymatic activity profile.

Partial purification of P2 and biochemical properties

The fact that both proteases were active against the same synthetic substrate, showed higher activity levels in dark-induced senescent leaves and reacted with anti-P1 antiserum raised the question on the possible biochemical/structural relationship between them. Therefore, in order to study some of the P2 biochemical properties in comparison with those of the previously characterised P1, we addressed the purification of P2 activity starting from the DEAE-Sepharose step. Column fractions containing P2 activity were pooled and submitted to the purification steps described under Materials and methods. Figure 3 shows the Suc-AAPF-pNA hydrolytic activity profile of the last purification step (Sephacryl S-300 column chromatography). As can be seen, only one symmetric peak was obtained which coincided with the fractions reacting with anti-P1. A native molecular mass of 115 kDa was estimated for P2 activity according to Andrews (1965).

The effect of several protease inhibitors on P2 peptidasic activity is shown in Fig. 4a. Among the different inhibitors tested, only PMSF and chymostatin were able to inhibit P2 activity. Inhibition by PMSF but not by E-64 suggests that P2 is a serine protease. The chymostatin inhibitory effect together with P2 ability to hydrolyse Suc-AAPF-pNA clearly indicate the chymotrypsin-like character of the enzyme.

When P2 peptidasic activity was assayed at different pHs, maximum activity was obtained in the range 10–11 (Fig. 4b). However, the enzyme was active over a

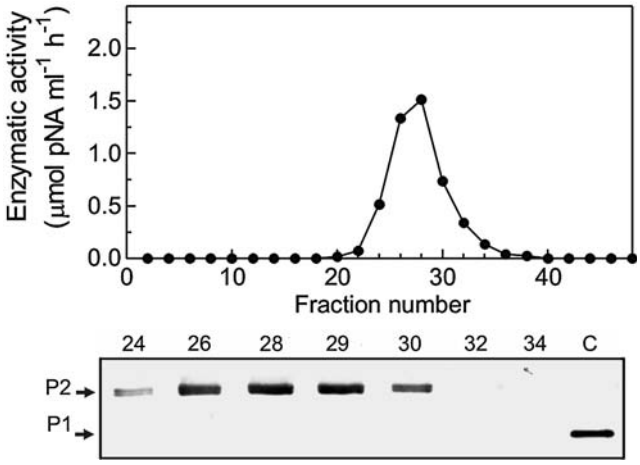

Fig. 3 Sephacryl S-300 column chromatography of P2 activity. *Upper panel* shows the chromatographic profile of P2 activity measured with Suc-AAPF-pNA as described in Materials and methods. *Lower panel* shows Western blot analysis of the column fractions (15 µl) revealed with anti-P1 antiserum. *Arrows* indicate the position of P1 and P2. *Lane C* corresponds to a control sample of 0.2 µg of purified P1. *Numbers above the lanes* correspond to fraction numbers of the column. Data shown are representative of five independent experiments

broad pH range maintaining a significant residual activity at acidic and extreme alkaline pHs.

The effect of temperature on P2 activity was determined by incubating the complete reaction mixture at different temperatures from 0 to 80°C for 10 min. As can be seen in Fig. 4c, the enzymatic activity increased with temperature reaching a maximum at 50°C and still retaining about 70% of its activity at 70°C. Analysis of the thermal stability of protease P2 showed that the enzymatic activity was not affected by pre-incubation for 10 min up to 40°C (Fig. 4d) but decreased drastically at higher temperatures.

Taken together, the results showed above indicate that the biochemical and physical properties of P2 were closely similar to those previously reported for P1 (Roberts et al. 2003).

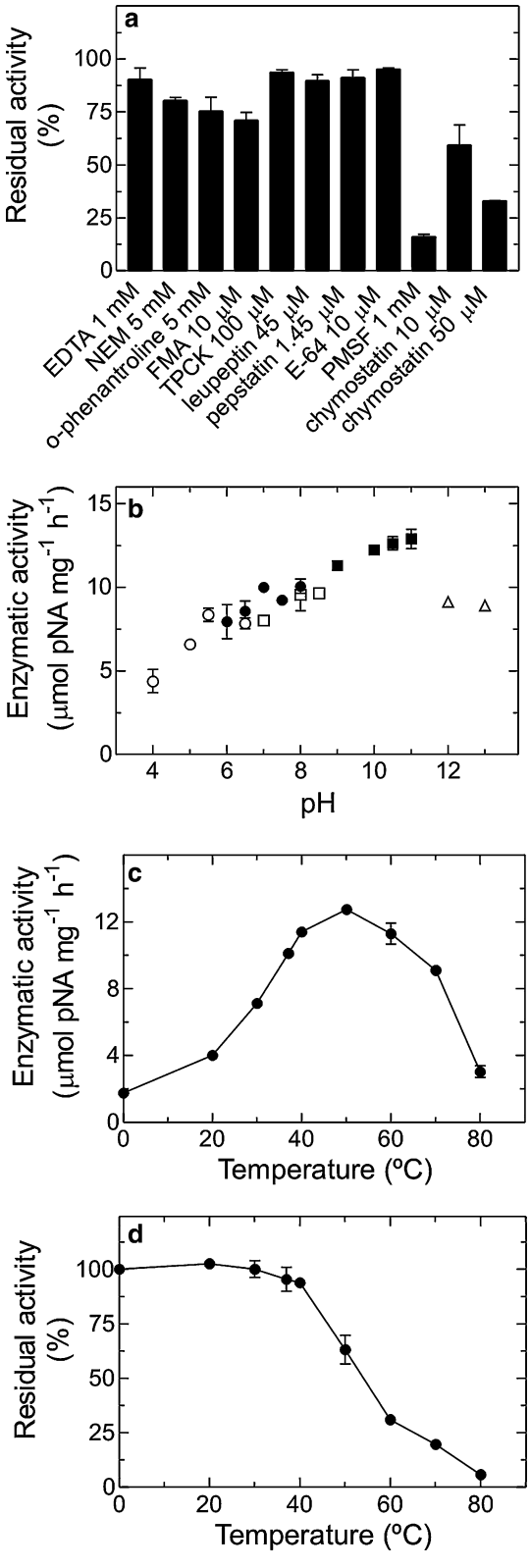

Fig. 4 Biochemical properties of P2. **a** Effect of several protease inhibitors on P2 activity was determined after a pre-incubation period of 30 min in the presence of different inhibitors as described in Materials and methods. **b** pH dependence of the enzyme was determined in the range 4–13 as described in Materials and methods. *Symbols* represent buffers: citrate–NaOH (*open circles*), potassium phosphate (*closed circles*), Tris–HCl (*open squares*), CAPS–NaOH (*closed squares*) and KCl–NaOH (*open triangles*). **c** Effect of temperature on P2 activity was assessed incubating the reaction mix for 10 min at different temperatures in the range 0–80°C. **d** Thermal stability of P2 was determined by pre-incubating the enzyme for 10 min at different temperatures in the range 0–80°C and subsequent analysis of residual activity at 37°C. Values are means ± SD from three independent experiments

Mass spectrometry analysis of P1 and P2

The similarities found between P1 and P2 pointed to a potential structural relationship between these prote-

ases. The consistent appearance of P1 when high amounts of P2 were accumulated, together with the knowledge that several plant subtilisins are generated by pre-protein processing, suggested that P1 could be derived from P2 by proteolytic cleavage. To investigate this possibility we performed PMFs from purified P1 and P2. Purified samples of P1 and P2 were submitted to bidimensional gel electrophoresis as described in Materials and methods. The corresponding spots were excised and subjected to in-gel tryptic digestion and subsequent MALDI-TOF mass spectrometry analysis. Comparison of PMFs obtained clearly showed that P1 and P2 do not share any tryptic peptide (see Electronic Supplementary Material, Fig. S1).

A few isoelectric variants of P2 differing in their abundance were visualised in the 2D gel by CBB R-250 staining as well as by Western blot analysis (data not shown). The two most abundant spots were selected to perform the MALDI-TOF analysis. The identity of their peptide maps allowed us to corroborate that both spots corresponded to isoforms of the same protein, even though the nature of the modifications involved was unknown.

PMF data were used to search in the protein database nr at NCBI with Mascot program but neither P1 nor P2 retrieved significant matches. In order to obtain additional information on the identity of P2, three peptides from the peptide maps were selected and sequenced by MS/MS fragmentation. The MS/MS data of the peak corresponding to a mass of 1,393.68 Da matched with a putative subtilisin-like proteinase from *Oryza sativa* (gi: 50915254). This result was confirmed using the peptide sequence obtained (DLATGTESTPFVR) to search for similarity with known sequences on the nr database at NCBI using the BLAST program. Besides the expected rice putative subtilisin-like proteinase already mentioned, three other hits corresponding to plant subtilisin-like proteases were found. These sequences corresponded to a subtilisin-like protease (gi: 757534) and to the deduced amino acid sequence of a putative gene coding for Ara12 (gi: 23296832) from *A. thaliana* and to STB1 from *Lycopersicon esculentum* (gi: 1771160), all of them exhibiting 72% identity to the P2 fragment (8 out of 11 identical amino acids). The sequence alignment of these subtilases together with P2 fragment is shown in Fig. 5. We also included the previously reported 17 amino acids fragment of protease P1 (Roberts et al. 2003) which is also present in the sequences homologues to P2 peptide. Moreover, the putative rice subtilisin from which P2 was identified showed the highest homology to P1 in an independent search (82% identity, 14 out of 17 amino acids).

Changes in P1 and P2 protein levels in senescent leaves induced by darkness or N deprivation in intact plants

To investigate if the results obtained using detached dark-induced senescent leaves are maintained in a system closer resembling physiological conditions, we studied P1 and P2 expression in non-detached leaves submitted to darkness and in leaves from plants stressed by N deprivation.

Senescence was induced by darkness in non-detached leaves by covering the third leaf of 15-day-old plants with aluminium foil as described in Materials and methods. Samples of the darkened leaf were taken every 2 days and the presence of P1 and/or P2 was assessed by Western blot analysis of leaf extracts (Fig. 6a). As expected, only P2 was detected in non-

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P1      ---APEIFKPDVTAPGVDIL-----------------------------------
Rice    NYRAPEILKPDVIAPGVNILAAWTGESAPTDLDIDPRRVEFNIISGTSMSCPHVSG   562
At      NSITPNILKPDLIAPGVNILAAWTGAAGPTGLASDSRRVEFNIISGTSMSCPHVSG   539
Ara12   NSITPNILKPDLIAPGVNILAAWTGAAGPTGLASDSRRVEFNIISGTSMSCPHVSG   550
SBT1    NPITPDVLKPDLIAPGVNILAGWTGKVGPTGLQEDTRNVGFNIISGTSMSCPHVSG   550

P2      -----------------------------------DLATGTESTPFVR-------
Rice    LAALLRQAQPDWSPAAIKSALMTTAYNVDNSSAVIKDLATGTESTPFVRGAGHVDP   618
At      LAALLKSVHPEWSPAAIRSALMTTAYKTYKDGKPLLDIATGKPSTPFDHGAGHVSP   595
Ara12   LAALLKSVHPEWSPAAIRSALMTTAYKTYKDGKPLLDIATGKPSTPFDHGAGHVSP   606
SBT1    LAALLKAAHPEWSPAAIRSALMTTSYSTYKNGKTIEDVATGMSSTPFDYGAGHVNP   606
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Fig. 5 Sequence alignment of the 13 amino acids fragment of P2 obtained by MS/MS and other plant subtilisin-like proteases. Sequence similarities were determined with the BLAST program in the nr database at NCBI and the alignment was created with the ClustalW program. The previously sequenced 17 amino acids fragment of P1 was included in the alignment, labelled with a *frame*. Amino acids identical to P1 or P2 sequence are *boxed in black* and those strongly similar (E=D=N=Q and V=L=I=M) are *boxed in grey*. Accession numbers are as follows: Rice (gi: 50915254, putative subtilisin-like proteinase from *Oryza sativa*), At (gi: 757534, subtilisin-like protease from *Arabidopsis thaliana*), Ara 12 (gi: 23296832, putative subtilisin serine protease ARA12 from *A. thaliana*) and SBT1 (gi: 1771160, subtilisin-like protease from *Lycopersicon esculentum*). *Numbers* refer to the amino acid position in the full-length sequences

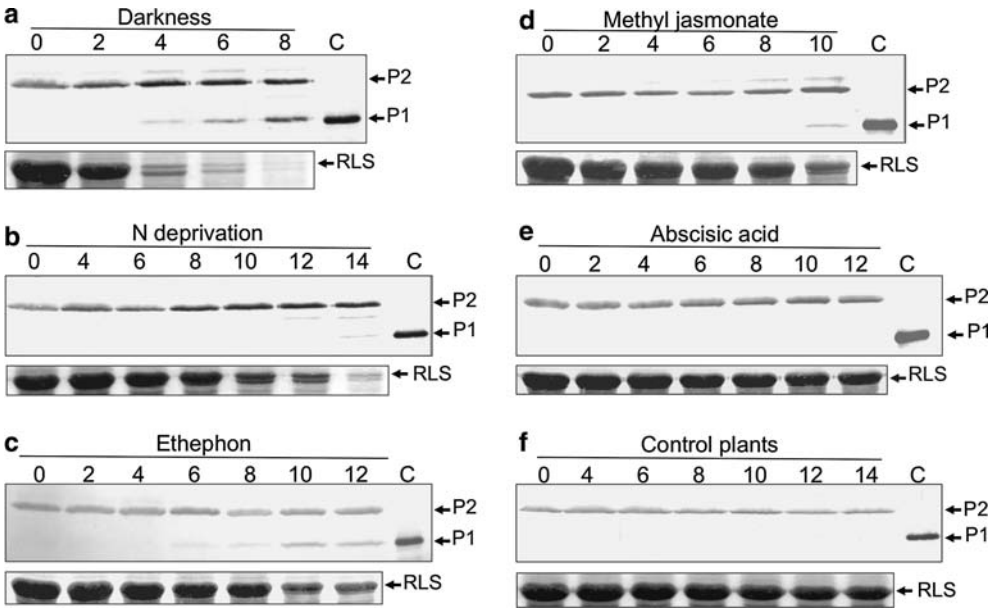

Fig. 6 Immunorecognition of P1 and P2 in extracts from senescent leaves induced by darkness, N deprivation or phytohormones application in whole plants (*upper panels*). S100 fractions were prepared from the third leaf of wheat plants incubated in darkness without detaching (**a**), submitted to N deprivation (**b**), sprayed daily with 1 mM ethephon (**c**), watered daily with 100 μM methyl jasmonate (**d**) or 50 μM abscisic acid (**e**), and from control plants maintained under their normal light/darkness cycle, with N supply and no hormone addition (**f**). Samples corresponding to 7.5 mg FW of each extract were processed for Western blot as described in the legend to Fig. 1. *Lane C* corresponds to a control sample of 0.2 μg of purified P1. *Arrows* indicate the position of P1 and P2. *Lower panels* in **a**–**f** show RLS stained with CBB R-250 after 15% SDS-PAGE of the same extracts (3 mg FW). Data shown are representative of at least two independent experiments with three replicates

senescent leaves (day 0). Moreover, levels of both proteases increased with increasing time in darkness.

Senescence at the whole plant level was achieved by N starvation in 15-day-old plants. Samples of the third leaf were taken every 2 days from the beginning of the N deprivation treatment (day 0) to complete yellowing (day 14). Analysis of leaf extracts from N starved plants with anti-P1 antiserum revealed that only P2 levels increased under these conditions (Fig. 6b). A faint P1 signal could be detected at the last sampling day when RLS levels turned almost undetectable. In control plants grown under their normal light/darkness cycle and maintained with proper N supply (Fig. 6f) P2 levels did not change and P1 was undetectable.

Effect of plant growth regulators on P1 and P2 levels

The effect of three known senescence-inducing plant growth regulators on P1 and P2 protein levels was also studied by Western blot analysis. Ethephon, an ethylene-realising compound, was applied to 15-day-old plants by spraying while methyl jasmonate (MJ) and abscisic acid (ABA) were added to the nutrient solution. In all three cases, the third leaf was sampled since the beginning of the treatment up to abscission or complete deterioration. As shown in Fig. 6c, ethephon application produced a modest increase in P1 levels compared to darkness (Fig. 6 a). However, ethephon was the strongest inducer. In fact, plants watered with MJ showed a weak P1 signal at the last sampling day (Fig. 6d) while no changes at all in P1 status was observed in ABA-treated plants (Fig. 6e). Additionally, none of the three hormones had any effect on P2 protein levels. Lower panels in Fig. 6 show the progress on RLS loss, included as a marker of the protein degradation process.

Discussion

The study of the biochemical properties of P2 revealed several similarities with P1, summarised here in Table 1. They are both serine proteases as inferred from their inhibition pattern. Moreover, as P2 activity is inhibited by chymostatin and it is able to hydrolyse Suc-AAPF-pNA, a typical substrate for chymotrypsin, its activity can be predicted to be of the chymotrypsin-like type as previously demonstrated for P1 (Roberts et al. 2003).

As the vast majority of known plant subtilisins, P1 and P2 are active over a broad pH range with an optimum at alkaline pH and have a relatively high thermal stability.

Table 1 Comparison of P1 and P2 main characteristics

P1	P2
Serine-protease with chymotrypsin-like activity belonging to the subtilisin-like proteases group	Serine-protease with chymotrypsin-like activity belonging to the subtilisin-like proteases group
Optimum pH at 8–10	Optimum pH at 10–11
Thermal stability up to 50°C	Thermal stability up to 40°C
59 kDa in SDS-PAGE and 110 kDa in non-denaturing conditions	78 kDa in SDS-PAGE and 115 kDa in non-denaturing conditions
Only present in senescent leaves	Present in senescent and non-senescent leaves
Appearance by incubation in darkness in detached and non-detached leaves. Absent in N starved plants	Increased levels by incubation in darkness in detached and non-detached leaves. Also increased by N starvation
Appearance by ethephon and methyl jasmonate application. Absent in ABA-treated plants	Unchanged levels by ethephon, methyl jasmonate or ABA application
Ref: Roberts et al. (2003)	Ref: this work

The fact that an antiserum raised against P1 can also react with P2 strongly indicates that they must share some structural features.

The majority of subtilisin-like serine proteases (subtilases) are synthesised as prepro-enzymes and converted to their active form by limited proteolysis. In plants, this has been demonstrated for Cucumisin (Yamagata et al. 1994), SBT1 (Janzik et al. 2000) and hordolisin (Terp et al. 2000). Many other plant subtilases are predicted from their nucleotide sequence to be synthesised as zymogens and subsequently matured by processing (Tornero et al. 1996; Beilinson et al. 2002; Berger and Altmann 2000; Yamagata et al. 2000). At least for Cucumisin, a total processing is not necessary for activation of the enzyme since the 67 kDa native and the 54 kDa autolysed forms are both active (Yamagata et al. 1994).

Physical similarities between P1 and P2 together with the observation that P1 appearance correlates with an increase of P2 levels raised the possibility that the 59 kDa band corresponding to P1 protease could have derived from the 78 kDa band assigned to P2 by proteolytic cleavage. To verify this hypothesis, we compared the PMFs obtained by MALDI-TOF searching for common peptides. From the results obtained it can be concluded that P1 and P2 are two different serine proteases.

The analysis of the MS/MS spectrum of one peptide (1,393.68 Da) derived from P2 demonstrated that it also belongs to the subtilisin-like proteases group, in agreement with its biochemical characteristics. Interestingly, the deduced amino acid sequence of a putative subtilisin-like protease from rice, which allowed us to classify P2, also contained a region highly homologous to P1 fragment previously sequenced (Roberts et al. 2003). At that time, the most similar sequence found for P1 fragment corresponded to a subtilase from *A. thaliana* (NP_568890) presenting 76% identity. An updated search revealed that the rice sequence shares an 82% identity with P1 fragment. An alignment of several plant subtilases sequences showed that the P1 peptide is localised in a region more conserved than that corresponding to the P2 fragment (data not shown).

Two of the subtilisin-like proteases exhibiting homology with the P2 fragment have been thoroughly studied and are well characterised, Ara 12 from *A. thaliana* (Hamilton et al. 2003) and SBT1 from *L. esculentum* (Janzik et al. 2000). Ara 12 is an extracellular enzyme localised in the intercellular spaces of stem tissue. The subtilase SBT1 is also presumed to be apoplastic as its active form operates at acidic pH.

According to their function, two main groups have been recognised for subtilases, those acting as degradative enzymes involved in general protein turnover and those associated with highly specific regulatory processes (Rautengarten et al. 2005). In the last few years more evidences appeared associating subtilases with regulatory and signalling functions in morphogenesis and development (Berger and Altmann 2000; Tanaka et al. 2001). Recently, two serine proteases with caspase-like enzymatic activity have been described in *Avena sativa* leaves undergoing PCD induced by a fungal toxin (Coffeen and Wolpert 2004). They presented sequence homology with subtilisin-like proteases and constitutive expression, being translocated to the extracellular fluid once PCD has been induced. Concomitant Rubisco degradation is observed but the evidence points to an indirect action of these enzymes, possibly due to their participation in signalling cascades leading to Rubisco degradation by other proteases.

Despite the similarities between P1 and P2, they exhibited different expression patterns according to the stress conditions promoting senescence. Although both enzymes showed increased levels in leaves incubated in

darkness, P2 was already present in non-senescent leaves while P1 only appeared once the senescence process had already begun. More interestingly, P1 was not detected in senescent leaves from plants deprived of N supply while P2 showed an early and persistent increase until the last stages of senescence triggered by N starvation. The increase of P2 levels and activity in response to such different stimuli as darkness and N deprivation suggests that it could be implicated in the most general aspects of leaf senescence. In contrast, appearance of P1 seems to be a response limited to dark-induced senescence, which proceeds at a faster rate than N starvation-induced senescence.

Three different plant hormones are known to influence the senescence process: ethylene, methyl jasmonate and abscisic acid. Ethylene has been largely recognised as a senescence regulator (Aharoni and Lieberman 1979; Weaver et al. 1998; Klee 2004). Although ethylene cannot induce senescence until leaves reach a defined age, it can modulate the timing of leaf senescence (Grbić and Bleecker 1995; Jing et al. 2005). Here, ethephon application to young wheat plants resulted in active protein degradation as shown by decreasing RLS levels (Fig. 6c, lower panel) but it was less effective than darkness to induce senescence and protein remobilisation (Fig. 6a, lower panel).

Although it has been demonstrated for various plant species that exogenous MJ application promotes typical symptoms of senescence (Beltrano et al. 1998; Rossato et al. 2002), under our experimental conditions P2 levels did not change and a weak P1 signal appeared only after 10 days of treatment despite the progressive RLS hydrolysis observed.

Abscisic acid has also been connected with induction of plant senescence. He et al. (2005) reported that endogenous ABA levels were lower in leaves of an earlier senescent maize cultivar compared to another with retarded leaf senescence. Major markers of leaf senescence, protein degradation and chlorophyll loss, occurred when ABA was exogenously applied to leaves of *A. thaliana* (Weaver et al. 1998) and wheat plants (Yang et al. 2003). Here, ABA was moderately effective in promoting protein degradation, since about 60% of initial protein content remained in the third leaf after 12 days of treatment (data not shown). Even when leaves were visibly damaged they still retained high amounts of RLS (Fig. 6e, lower panel) indicating that no efficient protein remobilisation was achieved in ABA-treated plants. In addition, P1 and P2 subtilase levels did not change in response to ABA application.

It can be concluded that although the three growth regulators were able to trigger a senescence-mimic response, their effect on P1 and/or P2 levels was different. In fact, P1 levels increased only in those treatments where RLS was thoroughly hydrolysed, suggesting some role for this enzyme in the latest stages of leaf proteolysis. The in vivo functional roles of P1 and P2 during senescence as well as their subcellular localisation remain to be elucidated.

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