Ara12 subtilisin-like protease from *Arabidopsis thaliana*: purification, substrate specificity and tissue localization

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A C-terminal portion of Ara12 subtilisin-like protease (residues 542–575) was expressed in *Escherichia coli* cells as a fusion protein bound to maltose binding protein. Polyclonal antisera raised against the expressed protein were used to examine the tissue specificity and subcellular localization of Ara12. The protease was found predominantly in the silique and stem of plants, but was hardly detectable in leaf and not seen in root tissue. The distribution observed using immunological techniques is different from that seen by an RNA analysis study, which demonstrated similar mRNA abundance in the stem and leaves. Using immunogold labelling, Ara12 was shown to have an extracellular localization and was found in the intercellular spaces in stem tissue. Ara12 protease was purified to homogeneity from *Arabidopsis thaliana* cell suspension cultures by anion exchange and hydrophobic interaction chromatography. Proteolytic

activity of Ara12 was inhibited by a number of serine protease inhibitors, but was almost unaffected by inhibitors of other catalytic classes of proteases. Optimal proteolytic activity was displayed under acidic conditions (pH 5.0). Ara12 activity was relatively thermostable and was stimulated in the presence of Ca²⁺ ions. Substrate specificity studies were conducted using a series of internally quenched fluorogenic peptide substrates. At the P₁ position of substrates, hydrophobic residues, such as Phe and Ala, were preferred to Arg, whilst at the P₁' position, Asp, Leu and Ala were most favoured. Possible functions of Ara12 are discussed in the light of the involvement of a number of plant subtilisin-like proteases in morphogenesis.

Key words: apoplast, pyrolysin, serine endoprotease.

INTRODUCTION

Subtilisins are serine proteases, first discovered in *Bacillus* bacteria, which rely on a well-characterized catalytic triad of residues making up a charge relay system. The activity of these extracellular proteases has been extensively studied, on account of their use as additives to detergents, which has made them the most commercially valuable group of enzymes. A large number of subtilisin-like proteases displaying homology to the subtilisins have been found in other bacteria, archae, protozoa, fungi, plants and animals [1]. The subtilisin-like proteases (herein after referred to as subtilases) are collectively grouped into clan SB of the serine proteases. Clan SB has been further divided into six different families according to the amino acid sequences of their catalytic domains [2]. Subtilases have been studied in plants and animals and have been found to belong to two of these families: the kexins and the pyrolysins.

The kexin family is composed of a group of important subtilases, including several mammalian enzymes, such as furin [3]. They function as proprotein convertases (PCs), which are proteases which convert a wide variety of inactive precursor proteins and peptides, including polypeptide hormones, growth and neurotrophic factors, receptors, adhesion molecules, certain other proteases, bacterial exotoxins and viral glycoproteins into their active counterparts [4,5]. PCs process these inactive pre-

cursors by proteolytically removing their pro-regions at sites C-terminal of mono-, dibasic or multiple basic residues [4]. Most of the mammalian subtilases are members of the kexin family, although several have been found to be pyrolysins [6,7].

The plant subtilases purified to date are members of the pyrolysin family. Pyrolysins, which can function as endoproteases or tripeptidases, have been found in bacteria, thermophilic archaeobacteria and animals, as well as in plants [2]. In animals, the pyrolysins site-1 protease (S1P) [6] and subtilisin/kexin isoenzyme-1 (SKI-1) [7] have been isolated, which exhibit specificity for cleavage C-terminal of the motif Arg/Lys-Xaa-Xaa-Leu/Thr, although cleavage at Arg-Ser-Leu-Lys↓ has also been reported [8,9]. These proteases are thought to process proteins involved in the regulation of lipid synthesis and metabolism [6].

The first subtilase to be purified from plants was cucumis, which is found in the sarcocarp of developing *Cucumis melo* fruits [10]. A number of different subtilases have been isolated from various other cucurbitaceous plants, although cucumis remains the best characterized of these proteases [11,12]. Subtilases have also been isolated from a variety of other higher plants, including *Lycopersicon esculentum* [13] and *Lilium longiflorum* [14]. There is evidence that large gene families encoding subtilases are found in tomato [15] and *Arabidopsis* [16–20]. To date, few detailed studies have been performed on the substrate

Abbreviations used: Abz, 2-aminobenzoyl; AEBF, 4-(2-aminoethyl)benzenesulphonyl fluoride; AMC, 7-amido-4-methylcoumarin; Cbz, benzyloxycarbonyl; Boc, t-butyloxycarbonyl; CLV, CLAVATA protein; DFP, di-isopropyl phosphorofluoridate; DTT, dithiothreitol; ECL, enhanced chemiluminescence; HRP, horseradish peroxidase; LRP, leucine-rich repeat protein; MALDI-TOF, matrix-assisted laser-desorption ionization–time-of-flight; MBP, maltose-binding protein; PC, proprotein convertase; RT, reverse transcriptase; SBTI, soybean trypsin inhibitor; SKI-1, subtilisin/kexin isoenzyme-1; S1P, site-1 protease; Z, 3-nitrotyrosine.

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selectivity of these proteases. With the emerging importance of *Arabidopsis* as a model plant system for molecular genetics and the complete sequencing of its genome, a number of putative subtilase-encoding genes can be identified in the *Arabidopsis* database. No definitive evidence exists showing that these code for proteins with protease activity or what their substrate selectivity is.

The function of most of these plant enzymes is currently unknown; however, evidence is emerging that a number of subtilases are involved in the modulation of plant morphology. Examples of this include the involvement of SDD1 during stomatal development [18], ALE1 during epidermal development [19], LIM9 during microsporogenesis [14], Ag12 [21] and Cg12 [22] during root nodule development, and the presumed involvement of AIR3 [16] and XSP1 [17] during lateral root formation and xylem development respectively. Two tomato subtilases appear to have a role in pathogenesis and their genes are induced in response to infection with *Pseudomonas syringae* or treatment with salicylic acid [23].

Although for a number of plant subtilases an association with a particular process in plant development or pathogenesis is suspected, the precise subcellular location, function and physiological targets of these proteases are poorly understood. Most studies on the localization of plant subtilases have been restricted to mRNA abundance and *in situ* mRNA localization. There is a clear need for immunological studies, as mRNA abundance may not reflect final protein abundance or eventual subcellular localization of the protein, which could be processed. It is still unclear to what extent plant subtilases operate in the processing and function-activation or inactivation of target proteins and peptides in a manner analogous to fungal and mammalian PCs and pyrolysins. Subtilases may cause the degradation of certain target proteins, or they may act non-specifically as tightly regulated enzymes involved in programmed cell death or nutrient scavenging, during plant development or during events following biotic or abiotic stress. The endogenous *in vivo* substrate(s) of these enzymes have largely remained elusive. Only two possible endogenous substrates of plant subtilases have been identified from *in vitro* studies. One of these is the plant hormone systemin [24] and the other is a leucine-rich repeat protein (LRP) [25]. The processing site of the LRP examined has never been definitively determined. The exact role of this protein has not been elucidated, although LRPs are known to be involved in protein-protein interactions and frequently participate in signal transduction. Thus plant subtilases may prove to have a role in signal transduction events.

In a previous study, we used a differential extraction procedure and protein sequencing to identify an array of proteins found in primary cell wall extracts and the culture medium of suspension-cultured cells from five different plant species [26]. One of the N-terminal sequences obtained from the culture medium of *Arabidopsis thaliana* cells corresponded to that of a putative subtilase, encoded by a previously described cDNA sequence [21,27]. Although the cDNA and gene sequences for this putative subtilase, previously named *ara12*, have been determined, the corresponding protein has not been isolated and no function has been established.

Arabidopsis suspension-cultured cells appear to secrete relatively large amounts of Ara12 protease into their culture medium [26]. We are interested in characterizing the activity of this enzyme, which may give us an understanding of its potential role in plant development and events such as pathogenesis. Clearly, information is required regarding the activity and substrate specificity of Ara12. It would also be of interest to determine the subcellular location of Ara12 and establish whether it is pre-

dominantly an extracellular protease in plants. This may offer an insight into the wider function of this enzyme in *Arabidopsis* and other species.

We describe here the first purification to homogeneity of a subtilase enzyme from *Arabidopsis thaliana*, the characterization of the enzyme activity and an investigation into the substrate specificity. In addition, antisera were generated against this enzyme and used to examine its tissue specificity and subcellular localization.

EXPERIMENTAL

Materials

Q Sepharose FF and phenyl-Sepharose FF resin were obtained from Amersham Biosciences. Mono Q and phenyl-Superose HPLC columns were used with a SMART system micro-separation unit (Amersham Biosciences). Dithiothreitol (DTT) was purchased from Melford Laboratories (Suffolk, U.K.). The substrates t-butyloxycarbonyl (Boc)-Leu-Arg-Arg-7-AMC.HCl and benzyloxycarbonyl (Cbz)-Gly-Gly-Leu-AMC (where AMC is amido-4-methylcoumarin) were purchased from Bachem (Bubendorf, Switzerland). Other chemicals were purchased from Sigma.

Plant material and growth conditions

Arabidopsis thaliana (Ecotype Columbia) plants were grown under a 16 h photoperiod at 24 °C. The derivation and maintenance of *Arabidopsis thaliana* cell suspension cultures has been described previously [28]. Suspension cultures were grown under a 16 h photoperiod at 24 °C and agitated on a rotary shaker at 130 rev./min. Suspension cultures were subcultured once every 7 days by aseptically transferring 10 ml of culture to 90 ml of fresh medium in 250 ml Erlenmeyer flasks.

Generation of anti-Ara12 antisera

A cDNA fragment encoding the C-terminal 216 amino acid residues was amplified from first strand cDNA prepared from total RNA from *Arabidopsis* suspension-cultured cells. Reverse transcriptase (RT)-PCR was performed for 37 cycles (94 °C/1 min; 64 °C/1 min; 74 °C/90 s) using the 5' primer 5'-ATGTCTTGCCCTCACGTTAGTGG-3' and 3' primer 5'-CTAGTC-TAGATACGACTATGTCCAGCTAATCGC-3'. Vent polymerase was used to generate blunt-ended PCR products, which were cloned into the *XmnI* site of the *malE* gene on the pMAL-c2 vector (New England Biolabs). The *malE* gene encodes the maltose-binding protein (MBP). The resulting plasmid was named pMALA12.

Polyclonal anti-Ara12 antibodies were raised in rabbits by AstraZeneca to a mixture of the fusion protein and the isolated Ara12 moiety of the fusion protein using standard protocols. A first inoculation of 200 µg of fusion protein in Freund's complete adjuvant was given. Subsequently, two further immunizations of 200 µg of fusion protein and two boosts of 40 µg of the Ara12 moiety of the fusion protein were administered subcutaneously to rabbits in Freund's incomplete adjuvant, every 4 weeks. Final antisera were obtained 22 weeks after the first inoculation.

Western blot analysis

Arabidopsis suspension-cultured cells were vacuum-filtered through two layers of Miracloth. Protein extracts were prepared by grinding fresh tissues or cells in liquid nitrogen. This material was homogenized in ice cold protein extraction buffer (20 mM

Hepes, pH 7.6, 100 mM potassium acetate). The homogenate was filtered through three layers of Miracloth. *Arabidopsis* protein extracts (13 µg per lane) were resolved by SDS/PAGE on 10% polyacrylamide gels. Proteins were blotted onto Hybond enhanced chemiluminescence (ECL) membranes (Amersham Biosciences). Blots were probed with a 1:20000 dilution of anti-Ara12 serum, followed by a 1:20000 dilution of goat anti-rabbit IgG conjugated to horseradish peroxidase (HRP), before applying ECL detection reagents (SuperSignal substrate ECL detection kit; Pierce). The blot used to examine tissue localization (Figure 8) was probed with 1:20000 anti-Ara12 followed by 1:30000 goat anti-rabbit IgG conjugate. Blots were exposed to X-ray film (X-OMAT AR, Kodak).

Purification of Ara12 protease

A volume of 6 l of 6-day-old suspension culture filtrate was diluted 2.5-fold with distilled water and the pH was adjusted to 8.5 using 10 M NaOH. A volume of 650 ml of 50% Q Sepharose FF slurry, equilibrated with 20 mM Tris/HCl, pH 8.5, (buffer A), was stirred with the filtrate. The Q Sepharose was packed into a column (2.5 cm × 120 cm), washed with buffer A, and proteins were eluted with buffer A containing 0.1 mM NaCl. Proteolytic activities of chromatographic fractions were monitored using FITC-labelled casein. Fractions 10–14 showed high activity and were pooled, mixed with an equal volume of buffer A containing 3.6 M (NH₄)₂SO₄ and applied to a phenyl-Sepharose column (0.7 cm × 15 cm) equilibrated with buffer A containing 1.8 M (NH₄)₂SO₄. Protein bound to the column was eluted with buffer A. Active fractions were pooled, mixed with 0.9 vol. of 3.6 M (NH₄)₂SO₄ and applied to a phenyl-Superose column (0.16 cm × 5 cm) equilibrated with buffer A containing 1.8 M (NH₄)₂SO₄. The column was washed with buffer A containing 1.8 M (NH₄)₂SO₄ and proteins bound to the column were eluted with a linear gradient of 1.8–0 M (NH₄)₂SO₄ in buffer A over 20 column volumes. Active fractions were pooled and desalted using a PD-10 column equilibrated with buffer A. The desalted sample was applied to a Mono Q column (0.16 cm × 5 cm) equilibrated with buffer A. After washing with the same buffer, proteins bound to the column were eluted with a linear gradient of 0–0.1 M NaCl in buffer A over 40 column volumes. The fractions showing proteolytic activity against FITC-labelled casein were pooled and stored at –80 °C until used. The N-terminal sequence of the purified protease was determined using an Applied Biosystems 477A protein sequencer.

The purified Ara12 protein was analysed by matrix-assisted laser-desorption ionization–time-of-flight (MALDI-TOF) MS using an AXIMA CFR mass spectrometer (Kratos, Manchester, U.K.). Equal volumes of purified Ara12 sample and a 50 ng/µl solution of 3,5-dimethoxy-4-hydroxycinnamic acid (sinapinic acid) in 0.05% trifluoroacetic acid/50% acetonitrile were applied to a sample plate for analysis.

Protease assays using FITC-labelled casein

Proteolytic activity was assessed according to the procedure of Twining [29] with some modifications. Assays were performed in a volume of 50 µl. Assay conditions were as follows, unless otherwise stated: 10 µl of sample was mixed with 20 µl of assay buffer (20 mM Tris/HCl, pH 7.5, 5 mM CaCl₂) and 20 µl of 0.5% (w/v) FITC-labelled casein containing 0.2% (w/v) sodium azide. Samples were incubated at 37 °C, unless otherwise stated. This assay was linear with time up to 12 h using 250 ng of purified protein. Reactions were stopped by adding 120 µl of 5% (v/v) trichloroacetic acid and mixing. Tubes were left at room

temperature for 1 h. The trichloroacetic acid-insoluble protein was sedimented at 13000 g for 5 min. Aliquots of 60 µl of the supernatant were diluted to 400 µl with 0.5 M Tris/HCl, pH 8.5 and mixed. Sample fluorescence was determined using an excitation wavelength of 485 nm and an emission wavelength of 538 nm.

Effects of additives and inhibitor studies

Relative proteolytic activities of purified Ara12 samples containing the relevant additives (CaCl₂, MgCl₂, EDTA, EGTA, DTT or inhibitors) were determined using the FITC-labelled casein assay as described above. Inhibitors were pre-incubated with 150 ng of purified Ara12 per assay for 1 h at 20 °C prior to addition of FITC-labelled casein substrate. Residual protease activity was determined at a single time point 8 h after addition of substrate to ensure that initial velocities were measured.

Synthesis of internally quenched fluorogenic substrates

Fluorogenic substrates were of the general structure Abz-(Xaa)_n-Z-Asp-OH, where the fluorescent group is 2-aminobenzoyl (Abz), the quencher Z is 3-nitrotyrosine, Xaa is any of the genetically encoded amino acids and *n* = 4–8 [30]. Peptides were synthesized on a solid support (Pega1900 resin) using an Applied Biosystems 432A Peptide Synthesizer (Perkin Elmer), as described previously [31].

Determination of enzyme activity with peptide substrates and kinetic constants

Enzymic hydrolysis of the peptide substrates was followed by observing the change in substrate fluorescence upon addition of enzyme with a Perkin Elmer luminescence spectrofluorimeter LS50, with emission at 420 nm (10 nm slit) and excitation at 320 nm (10 nm slit) at 25 °C. The substrates were dissolved in dimethylformamide at concentrations of 50–200 mM and 10 µl was added to a cuvette containing 2 ml of assay buffer (100 mM sodium acetate, pH 5.0, 5 mM CaCl₂ and 0.05% Tween 20) at 25 °C. At each of the 3 substrate concentrations (*S*₀), the initial velocity for substrate cleavage (*v*₀) was determined from the initial slope of the curve (emission versus time). The hydrolysis was allowed to proceed overnight after the addition of Ara12 and final fluorescence for total cleavage (*I*_∞ *Y*) was measured. The *k*_{cat}/*K*_m values were determined using the relationship: *v*₀ = (*k*_{cat}/*K*_m)*E*₀*S*₀, which is valid at low substrate concentrations (*S*₀ ≪ *K*_m) for systems that obey Michaelis–Menten kinetics. The cleavage site for chosen substrates was determined by amino acid sequencing of the hydrolysis products obtained after incubation of 1 µl of a concentrated solution of substrate with 1 µl of diluted enzyme in 45 µl of assay buffer for 3 h at 25 °C.

Immunocytochemistry

Arabidopsis thaliana (Ecotype Colombia) tissue embedded in resin was kindly supplied by Dr Jackie Spence (School of Biological and Biomedical Sciences, University of Durham, U.K.). Freshly excised tissue was fixed for 12 h at room temperature in 3% (w/v) paraformaldehyde, 1.25% (w/v) glutaraldehyde, 50 mM phosphate buffer, pH 7.0. Tissues were dehydrated for 1 h in a graded series of different ethanol solutions: 12.5%, 25%, 50%, 75% and 95% ethanol. Tissues were then embedded in LR White resin. The heat polymerization step was performed at 50 °C. Tissue samples were sectioned and incubated with either anti-Ara12 serum or pre-immune serum [diluted 1:100 in Tris-buffered saline supplemented with 0.05% Tween (TBST)] and then goat anti-rabbit IgG conjugated to

20 nm gold particles (diluted 1:100 in TBST). Sections were examined on a JEOL 100CX transmission electron microscope at 80 kV and images were recorded on Agfa Scientia 23D56 P3 AH EM Film (Agfa–Gevaert).

RESULTS

Generation of MBP–Ara12 fusion protein

An MBP–Ara12 C-terminal fusion protein was expressed from the pMALA12 plasmid in XL1-Blue *Escherichia coli* cells (Stratagene) and purified by affinity chromatography on an amylose column (New England Biolabs), following the manufacturer’s instructions (Figure 1). The fusion protein was resolved by SDS/PAGE and excised and eluted from the gel using an electroeluter (Biotrap; Schleicher and Schuell). The Ara12 moiety of the fusion protein was cleaved from the MBP moiety using Factor Xa protease (New England Biolabs). Expression and purification of the fusion protein and generation of the Ara12 moiety is shown in Figure 1, and these two proteins were used in the generation of anti-Ara12 antisera.

Purification of Ara12 subtilisin-like protease

The Ara12 subtilisin-like protease was purified from 6 l of *Arabidopsis thaliana* cell suspension culture, harvested 6 days

after subculture. The filtrate of these cell cultures has been shown to contain relatively large quantities of Ara12 [26]. A summary of the purification of Ara12 subtilisin-like protease is shown in

Figure 2 Purification of Ara12 protease

Chromatogram of Q Sepharose FF FPLC fractionation. Proteins were eluted with 20 mM Tris/HCl, pH 8.5, containing 0.1 mM NaCl, as shown by the dashed line, and 6 ml fractions were collected. Absorbance at 280 nm (indicated by lines with black diamonds) and proteolytic activity (indicated by grey bars) was determined using FITC-labelled casein.

Figure 1 Expression and purification of MBP–Ara12 C-terminal fusion protein

E. coli cells expressing fusion protein were lysed using a French press and centrifuged. Supernatant proteins were passed through an amylose column and eluted with 0.5 M maltose. Fusion protein in the eluate was gel-purified and digested with Factor Xa protease, releasing MBP and Ara12 moieties. Lane 1, molecular mass markers (SDS7, Sigma); lane 2, supernatant from lysed *E. coli* cells expressing fusion protein; lane 3, unbound fraction from amylose column; lane 4, gel-purified fusion protein; lane 5, fusion protein cleaved with Factor Xa protease; lane 6, gel-purified cleavage product (Ara12 C-terminal protein).

Figure 3 SDS/PAGE and Western blot analysis of purified Ara12 protease

Protein samples were resolved on 8% polyacrylamide gels. (A) SDS/PAGE of purified Ara12. Lane 1, 20 µl of 6-day-old *Arabidopsis* culture filtrate; lane 2, 20 µl of purified Ara12; lane 3, molecular mass markers (Full-Range Rainbow markers, Amersham Biosciences). Proteins were detected by Coomassie Brilliant Blue staining. (B) Western blot of purified Ara12. Lane 1, 20 µl of 6-day-old *Arabidopsis* culture filtrate protein; lane 2, 250 ng of purified Ara12. Blots were probed with 1:20 000 anti-Ara12 antiserum followed by 1:20 000 goat anti-rabbit IgG–HRP conjugate.

Table 1 Summary of the purification of Ara12 protease from *Arabidopsis* cell culture filtrate

The percentage yield and purification factor of Ara12 protease is given for each stage of the purification.

Purification stage	Protein (mg)	Total activity (units)	Specific activity (units/mg of protein)	Yield (%)	Purification (-fold)
1) Culture filtrate	390	172 000	441.0	100	—
2) Q Sepharose	7.47	25 348	3393.3	14.7	7.7
3) Phenyl-Sepharose	1.90	7797.2	4103.8	4.5	9.3
4) Phenyl-Superose	0.74	6339.7	8567.2	3.7	19.4
5) Mono-Q	0.25	5774.1	23 096.4	3.4	52.4

Figure 4 MALDI-TOF spectrometric analysis of purified Ara12 protease

Figure 5 Effect of pH on Ara12 protease activity

The substrate Abz-QPFRQAEZD and a three component buffer system of constant ionic strength were used.

Table 1. Since Ara12 was found in a very dilute form in the medium, a concentration step was required. This was effectively achieved by dilution of the sample to lower the conductivity and adjustment of the pH from 6.5 to 8.5, allowing maximum binding to a small volume of ion exchange resin, which can be easily separated from the bulk media by sedimentation. This overcame any requirement for large scale centrifugation or a precipitation step. Ara12 was purified by anion exchange chromatography and hydrophobic interaction chromatography using Q Sepharose (Figure 2), phenyl-Sepharose, phenyl-Superose and Mono Q resin. Two peaks of protease activity were eluted from the Q Sepharose column (Figure 2). Most proteolytic activity was found in fractions 10–14 and these were pooled. Fraction 18, which also showed proteolytic activity, was discarded. The phenyl-Sepharose step elution gave one peak of proteolytic activity and this sample was subsequently added to a phenyl-Superose column, where a reverse gradient was applied. Active fractions were further fractionated on a Mono Q column.

Figure 6 Effect of temperature on proteolytic activity (A) and stability (B) of Ara12 protease

(A) Proteolytic activity was measured at different temperatures using FITC-labelled casein for 1 h. (B) Ara12 was pre-incubated at various temperatures in 15 mM Tris/HCl, pH 7.5, for 10 min. Samples were placed on ice for 10 min and the remaining activity was measured using FITC-labelled casein at 37 °C for 6 h.

The purity of the protease was examined by SDS/PAGE and Western blot analysis (Figures 3A and 3B respectively), using 8 % polyacrylamide gels. In the purified sample, a single protein species of approx. 75 kDa was revealed by Coomassie Brilliant Blue staining, which was recognized by anti-Ara12 serum. No proteins were recognized by this serum in the culture filtrate (Figure 3B, lane 1) because the sample was too dilute. Immuno-recognition of a 75 kDa protein was observed after 20- and 60-fold concentration (by vol.) of the culture filtrate by ultrafiltration using a stirred filtration cell and a membrane with a 10 kDa molecular mass cut-off (results not shown). The first ten amino acid residues of the N-terminus of the purified enzyme were determined as TTRTPLFLGL, which corresponds to the predicted N-terminus of mature Ara12 subtilisin-like protease (100 % identity). Database searches have shown that no other known or putative protein (including other subtilases) contains this ten amino acid sequence in *Arabidopsis thaliana*. This confirms the identity of the purified enzyme. The molecular mass of purified mature Ara12 protease was determined as 76.1 kDa by MALDI-TOF mass spectrometric analysis (Figure 4). The predicted mass of the primary sequence of mature Ara12 protease is 67.6 kDa. Secondary modifications, such as glycosylation,

Figure 7 Effects of Ca^{2+} (A) and Mg^{2+} (B) ions on Ara12 protease activity

Protease activities were measured with FITC-labelled casein in the presence of differing concentrations of CaCl_2 or MgCl_2 and are shown as percentage activity relative to equivalent samples with no salt added.

could account for the 12 % difference between the predicted mass and the observed mass of the mature protease.

pH profile of Ara12

The pH profile of the proteolytic activity of purified Ara12 protease was examined using the peptide substrate Abz-QPFRQAEZD and is shown in Figure 5. A tri-component buffer system of constant ionic strength was used, consisting of 50 mM acetic acid, 50 mM Mes and 100 mM Tris, plus 5 mM CaCl_2 and 0.05 % Tween 20 [32]. The proteolytic activity of Ara12 protease was found to be maximal at pH 5.0.

Effect of temperature on enzyme activity

Protease activity of the purified Ara12 protein was measured at different temperatures using FITC-labelled casein. Samples containing the reaction mixture minus enzyme were pre-incubated for 10 min at a range of temperatures prior to addition of the enzyme. Activity was measured at a single time point 1 h after addition of enzyme. The relative activity observed at 60 °C was approx. twice that found at 50 °C and half of that seen at 80 °C (Figure 6A). The enzyme exhibited maximum activity around 80 °C. The thermostability of Ara12 was examined by pre-incubating the enzyme at various temperatures for 10 min.

Table 2 Effects of additives on Ara12 protease activity

Inhibitors were pre-incubated with the protease for 1 h prior to addition of substrate. Assays were performed without a pre-incubation step for EDTA, EGTA, DTT, CaCl_2 and MgCl_2 .

Additive	Concentration (mM)	Activity (%)
None		100
PMSF	0.5	84
	1.0	71
	2.0	55
	5.0	17
AEBSF	0.5	66
	1.0	56
	2.0	43
DFP	0.1	93
	0.5	77
	1.0	48
Benzamidine	1.0	105
Iodoacetamide	1.0	101
	5.0	110
Leupeptin	0.02	98
	0.1	95
Pepstatin A	1.0	96
SBTI	0.04	86
	0.4	70
DTT	1.0	206
	5.0	252
EDTA	1.0	100
	5.0	98
EGTA	1.0	103
	5.0	93
CaCl_2	5.0	146
	10.0	162
MgCl_2	5.0	113
	10.0	118

Samples were then placed on ice for a further 10 min and the remaining activity was measured using FITC-casein at 37 °C for 6 h. At the highest temperature investigated, 90 °C, Ara12 retained approx. 65 % activity (Figure 6B). The results demonstrated that, like most other plant subtilases, Ara12 is a thermostable enzyme.

Ca^{2+} -dependence

Three-dimensional structure studies have revealed that subtilases contain at least two Ca^{2+} binding sites [33], which are thought to be important in preventing thermal denaturation and autolysis. The effect of the bivalent ions CaCl_2 and MgCl_2 and the chelating agents EDTA and EGTA on the proteolytic activity of Ara12 protease was examined using FITC-labelled casein after an 8 h incubation. An apparent activation of Ara12 protease activity of 60 % was observed in the presence of 10 mM CaCl_2 above that of the no salt equivalent (Figure 7A). Magnesium ions did not cause such a large increase in activity; in the presence of 10 mM MgCl_2 , an approx. 20 % stimulation of activity was observed (Figure 7B). The presence of the chelating agents EDTA and EGTA caused only a slight decrease in protease activity at final concentrations of 10 mM (Table 2).

Effect of DTT on activity

Inclusion of the reducing agent dithiothreitol at final concentrations of 1 mM and 5 mM caused a 2- and 2.5-fold stimulation of activity respectively after an 8 h incubation (Table 2). DTT would be expected to help maintain the enzyme in a reduced state. This may account for the increase in activity observed.

Table 3 k_{cat}/K_m values for P series of substrates

↓ indicates cleavage site and n.d. indicates that the k_{cat}/K_m value could not be measured, which in our assay system equates to a value less than $0.04 \text{ min}^{-1} \cdot \mu\text{M}^{-1}$. Standard deviations of the reported values were less than 10%. Bold letters indicate the position of the varied residue within a series of substrates.

Substrate	k_{cat}/K_m ($\text{min}^{-1} \cdot \mu\text{M}^{-1}$)	Substrate	k_{cat}/K_m ($\text{min}^{-1} \cdot \mu\text{M}^{-1}$)
P₅		P₂	
Abz D FRLF AFZD	343	AbzA F AF AAZD	473
Abz V FRLF AFZD	198	AbzA F FF AAZD	465
		AbzA F SF AAZD	368
P₄		AbzA F RF AAZD	367
Abz F APF GGGZD	16.6	AbzA F DF AAZD	315
Abz L APF GGGZD	7.8	AbzA F PF AAZD	26.5
Abz D APF GGGZD	2.8		
Abz V APF GGGZD	2.5	AbzF A PF GGGZD	16.6
Abz A APF GGGZD	2.4	AbzF A AF GGGZD	1.2
Abz W APF GGGZD	1.4	AbzF A GF GGGZD	n.d.
Abz G APF GGGZD	0.60	P₁	
Abz R APF GGGZD	0.56	AbzF A PF GGGZD	16.6
		AbzF A PA GGGZD	9.2
P₃		AbzF A PD GGGZD	8.0
Abz F RPF GGGZD	50	AbzF A PW GGGZD	1.1
Abz F APF GGGZD	16.6	AbzF A PV GGGZD	0.84
Abz F GPf GGGZD	3.0	AbzF A PR GGGZD	0.61
		AbzF A PG GGGZD	0.04

Table 4 k_{cat}/K_m values for P' series of substrates

↓ indicates cleavage site, n.d. indicates that the k_{cat}/K_m value could not be measured, which in our assay system equates to a value less than $0.04 \text{ min}^{-1} \cdot \mu\text{M}^{-1}$. Standard deviations of the reported values were less than 10%. Bold letters indicate the position of the varied residue within a series of substrates.

Substrate	k_{cat}/K_m ($\text{min}^{-1} \cdot \mu\text{M}^{-1}$)	Substrate	k_{cat}/K_m ($\text{min}^{-1} \cdot \mu\text{M}^{-1}$)
P1'		P3'	
AbzFAPF D GGZD	615	AbzFAPF G G GZD	16.6
AbzFAPF L GGZD	570	AbzFAPF G G RZD	11.7
AbzFAPF A GGZD	547	AbzFAPF G G PZD	10.6
AbzFAPF F GGZD	376	AbzFAPF G G AZD	9.2
AbzFAPF R GGZD	309	AbzFAPF G G FZD	6.6
AbzFAPF E GGZD	18.3	AbzFAPF G G DZD	5.6
AbzFAPF W GGZD	17.3	AbzFAPF G G NZD	5.5
AbzFAPF G GGZD	16.6	AbzFAPF G G LZD	2.2
AbzFAPF V GGZD	15.0	AbzFAPF G G VZD	1.6
AbzFAPF N GGZD	8.4		
AbzFAPF P GGZD	n.d.		
P2'			
AbzFAPF G LGGZD	615		
AbzFAPF V GGZD	443		
AbzFAPF F GGZD	346		
AbzFAPF R GGZD	147		
AbzFAPF G AGZD	130		
AbzFAPF W GGZD	38.0		
AbzFAPF G NGZD	26.7		
AbzFAPF D GGZD	21.5		
AbzFAPF G GGZD	16.6		
AbzFAPF G PGZD	0.40		

Inhibition with protease inhibitors

The proteolytic activity of Ara12 protease was inhibited by the serine protease inhibitors 4-(2-aminoethyl)benzenesulphonyl fluoride (AEBSF), PMSF, di-isopropyl phosphorofluoridate (DFP) and soybean trypsin inhibitor (SBTI), as monitored with FITC-labelled casein after 8 h incubations (Table 2). PMSF and AEBSF inhibited Ara12 protease, such that 55% and 43% of the activity remained respectively, at final concentrations of 2 mM. Less than half of the proteolytic activity remained at a concentration of 1 mM DFP. At a final concentration of 0.4 mM SBTI, 70% of Ara12 protease activity remained. Although SBTI would not be expected to inhibit a subtilisin-like protease, SBTI is a protein and may compete for the casein substrate, particularly at high substrate concentrations. Therefore SBTI would appear to have an inhibitory effect on Ara12 at high concentrations. No inhibition was seen with 1 mM or 5 mM benzamidine or with 1 mM or 5 mM iodoacetamide, and only a minor inhibitory action was registered with 0.1 mM leupeptin and 1 mM pepstatin A.

Substrate specificity

The substrate specificity of purified Ara12 protease was analysed using internally quenched fluorescent peptide substrates. The method has been described previously [31]. The k_{cat}/K_m values for a P series of substrates are shown in Table 3 and the k_{cat}/K_m values for a P' series of substrates are shown in Table 4, using established nomenclature for protease–substrate interactions [34].

Peptide substrates based on the general formula AbzFAPF↓GGGZD (where Z is 3-nitrotyrosine) were used to investigate the substrate specificity from P₄ to P₃', altering the amino acid residue at the P₄ to P₃' position in turn. This series of substrates has been used previously to characterize the specificities of subtilisin BPN', Savinase [35] and hordolisin [36],

allowing direct comparison of their second order kinetic constants. Cleavage can be directed using these substrates, as described previously [36]. Substrates based on AbzXFRLF↓AFZD were also used to determine substrate preference at the P₅ position (bold letters indicate the position of the varied residue within a series of substrates).

Unlike hordolisin, Ara12 showed a stronger preference for AbzDFRLF↓AFZD than for AbzVFRLF↓AFZD. However, at the P₄ position, Ara12 showed a preference for large hydrophobic residues and aspartate. The following decreasing order of k_{cat}/K_m values was found: F > L > D > V = A > W > G ≥ R (Table 3), which, apart from the preference for aspartate, was very similar to the specificity of hordolisin (F > L > A ≥ V > W > G = R = D). Ara12 showed relative preferences for the P₃ substrates, based on the sequence AbzFXPF↓GGGZD, which were identical to those observed for Savinase and hordolisin. These three enzymes all display a decreasing order of k_{cat}/K_m of: R > A > G (Table 3).

At P₂, substrates based on the general formula AbzAFXF↓AAAZD were examined. k_{cat}/K_m values were determined and are listed in descending order of magnitude: A = F > S = R > D > P. For substrates based on the sequence AbzFAXF↓GGGZD, the following decreasing order of k_{cat}/K_m was observed: P > A. For the P₁ series of substrates the following decreasing order of k_{cat}/K_m values was observed: F > A > D > W > V > R > G (Table 3). This was almost identical to the preference shown by hordolisin, except that R is less preferred at P₁ by Ara12, and also matches the specificity of Savinase (F > A > G).

Preferences for substrates with different combinations of P' residues were investigated using a series of substrates

Figure 8 Tissue localization of Ara12 determined by Western blot analysis

Protein extracts of *Arabidopsis* tissues were resolved on a 10% polyacrylamide gel and blotted. Blots were probed with 1:20 000 anti-Ara12 serum, followed by 1:30 000 anti-rabbit IgG–HRP conjugate. Lanes 1–4, root, stem, silique and leaf protein extracts respectively.

based on AbzFAPF↓GGGZD (Table 4). These included AbzFAPF↓DGGZD and AbzFAPF↓GLGZD, which both showed k_{cat}/K_m values of $615 \text{ min}^{-1} \cdot \mu\text{M}^{-1}$, the highest observed for any of the substrates analysed. At P_1' , the following decreasing order of k_{cat}/K_m was observed: $D \geq L = A > F > R > E \geq W = G \geq V > N$ (Table 4). This differed somewhat from the equivalent values for hordolisin, where a preference for G and V, as well as R, A and F was seen, and a low preference for D was observed [36].

At P_2' , a preference for residues with apolar, uncharged side groups was seen, although Ara12 also showed high activity against the substrate with arginine at this position. The following decreasing order of k_{cat}/K_m was observed: $L > V > F > R > A > W > N > D > G > P$ (Table 4). This order shows more similarity to that obtained for hordolisin, and both enzymes favour substrates with leucine at this position. By contrast, both subtilisin BPN' and Savinase favoured phenylalanine residues at the P_2' position [35].

At P_3' , the following decreasing order of k_{cat}/K_m was observed: $G > R \geq P > A > F > D = N > L > V$ (Table 4), again a similar pattern to hordolisin, with a preference for G, R, P and A. Most of the substrates examined were favoured equally, with differences between the most and least favoured substrate amounting to a factor of 10, indicating that various amino acid residues were tolerated in the S_3' subsite of Ara12. Similar results were reported with this series of substrates for subtilisin BPN' and Savinase [35].

No proteolytic activity was detected using Ara12 against the subtilisin substrate Cbz-Gly-Gly-Leu-AMC or against the kexin substrate Boc-Leu-Arg-Arg-AMC.HCl (results not shown), although Ara12 may have an extended substrate specificity pocket, which would prevent cleavage of these substrates.

Tissue localization of Ara12 protease

The tissue localization of Ara12 protease in *Arabidopsis* tissues (root, stem, silique and leaf) was investigated by Western blot analysis using anti-Ara12 antisera. A single immunopositive protein of approx. 75 kDa was detected in silique and stem extracts, as shown in Figure 8. Low levels of the protease were detected in *Arabidopsis* leaf extracts, following prolonged exposure, but it was not detected in root extracts. Recognition of this single protein in silique extracts by Ara12 antisera was blocked by addition of the MBP–Ara12 fusion protein, used to raise the antibodies (results not shown).

Immunocytochemistry of Ara12 in *Arabidopsis* tissue

Polyclonal anti-Ara12 sera were used to investigate the localization of Ara12 in lateral and transverse sections of *Ara-*

Figure 9 Extracellular localization of Ara12 as shown by immunogold labelling

Transverse sections of *Arabidopsis* stem were incubated with 1:100 pre-immune serum (A) or 1:100 anti-Ara12 serum (B and C), followed by 1:100 anti-rabbit IgG conjugated to gold particles (20 nm in diameter) and viewed by electron microscopy. (A) Pre-immune control. Gold particles very rarely observed in the cytoplasm, cell wall (CW), cellular organelles and intercellular space (IS). (B and C) Anti-Ara12 serum samples. Many gold particles were observed in the intercellular spaces (as indicated by arrowheads), but very rarely in the cell wall, cytoplasm and cellular organelles. Scale bars = $1 \mu\text{m}$.

bidopsis thaliana stem tissue. Transverse sections through an *Arabidopsis* stem are shown in Figure 9. Figure 9(A) shows an electron micrograph of part of a transverse section of an *Arabidopsis* stem incubated with pre-immune serum and anti-rabbit IgG conjugated to gold particles. Gold particles were only rarely found to be associated with the cell wall, intercellular

spaces, the cytoplasm or cellular organelles, such as chloroplasts and mitochondria. Figures 9(B) and 9(C) show electron micrographs of parts of transverse sections of an *Arabidopsis* stem incubated with anti-Ara12 serum and anti-rabbit IgG conjugated to gold particles. Gold particles were found in the intercellular spaces, but were rarely found to be associated with the cell wall, the cytoplasm or cellular organelles. This localizes the Ara12 protease almost exclusively to the intercellular spaces in stem tissue of *Arabidopsis*.

DISCUSSION

We report the first purification to homogeneity of a subtilisin-like protease from *Arabidopsis thaliana*. Ara12 protease was purified from a previously characterized source: the filtrate of *Arabidopsis* cell suspension cultures [26]. Analysis of the protein using SDS/PAGE and MALDI-TOF shows that its molecular mass is higher than that predicted by DNA sequence analysis, indicative of post-translational modification. The N-terminal sequence of the purified enzyme was determined as TTRTPFLGL and this offers definitive proof that the protein is processed as predicted from its nucleotide sequence. The N-terminus of the processed protein conforms to the two threonine residues observed for other purified mature plant subtilases, such as cucumisin [11] and LIM9 [14].

Characterization of Ara12 showed that it is thermostable, like most other plant subtilases, and that it has a pH optimum around pH 5.0. Almost all previously studied plant subtilases have alkaline pH optima, however, there is a precedence for a subtilase with an acidic pH optimum. *LeSBT1*, a tomato subtilase, displays most activity between pH 4.0 and 6.0 [37]. This protease has a high degree of sequence identity to the mature Ara12 protease (62% identity and 75% similarity) and has been hypothesized to operate in the apoplast, although no direct evidence for this has been presented. Proteolytic activity of Ara12 was stimulated in the presence of Ca^{2+} ions. Three-dimensional structure studies of subtilases have revealed the presence of at least two (and possibly three) Ca^{2+} ion binding pockets in these monomeric enzymes [33]. It appears that Ca^{2+} ion binding has a stabilizing effect on subtilase activity, by reducing molecule flexibility, which prevents thermal denaturation and autolysis [1]. Inhibition studies indicated that the enzyme is inhibited by classical serine protease inhibitors, consistent with Ara12 being a serine protease. Inhibitors of cysteine, aspartic and metallo-proteases did not inhibit the proteolytic activity of Ara12 significantly.

Subtilases found in higher eukaryotes fall into two families: the kexins and the pyrolysins. These can be differentiated according to the sequence similarities of their active sites. In animals, protein processing of inactive precursors is known to occur at sites C-terminal of 1, 2, 3, 4 or 5 basic residues (Arg or Lys), which generally conform to the motif (R/K)-(X)_n-(K/R)↓, where $n = 0, 2, 4$ or 6 and X is any amino acid, except Cys [4,5]. This activity is typical of the kexins (or PCs). Mammalian pyrolysins have been shown to cleave proteins at sites C-terminal of hydrophobic residues, including Leu, Phe, Val and Met, and small amino acid residues, such as Ala, Thr and Ser [8,9]. Ara12 is a member of the pyrolysin family, according to its amino acid sequence, and internally quenched fluorogenic substrates were used to characterize its substrate specificity. Our data show that the hydrophobic residues Phe and Ala and the acidic residue Asp are favoured at the P₁ site, whilst the basic residue Arg is poorly favoured. In addition, no proteolytic activity was detected against Boc-Leu-Arg-Arg-AMC.HCl, a substrate used to monitor kexin activity. This suggests that Ara12 does not

function in kexin-like processing C-terminal of basic residues. The substrate specificity of Ara12 shows similarity to that of the homologous tomato subtilase, *LeSBT1*, which preferentially cleaves peptide bonds C-terminal of the amino acid residues Gln and Asp, using glucagon as the substrate, and the hydrophobic residue Leu using the insulin B chain [37]. Ara12 processes substrates with various residues at the P₁' position, including Asp, Leu and Ala, which were particularly favoured (Table 4). For cucumisin, residues with small side chains, such as Ser, Ala and Gly residues, are favoured in the P₁' position of the substrate [12,38]. Although specificity for substrates shown to be cleaved by mammalian pyrolysins cannot be ruled out, Arg was very poorly favoured at position P₄ of the substrates examined. Inclusion of the basic residue Arg at this position is thought to be important for substrate cleavage by S1P/SKI-1 [39]. It is still unclear whether or not Ara12 preferentially cleaves peptide bonds within a particular recognition motif, or degrades a wide variety of proteins *in vivo*. However, Ara12 was shown to degrade a series of extracted extracellular proteins over a period of 18 h (results not shown) and therefore, to effect its role correctly, it is presumably vital that its expression is under tight spatial and temporal control.

Western analysis has shown that Ara12 protein is found predominantly in silique tissue and also, but to a lesser extent, in stem tissue. Leaf tissue appeared to contain little immunopositive protein and none was detected in root tissue. Our results are largely in accordance with Northern analysis conducted previously [21,27]. However, whilst *ara12* transcript levels were found to be low in stem tissue in one previous study [21], we found relatively large amounts of Ara12 protein in the stem. This is consistent with another previous RNA study, which found significant expression of *ara12* in the stem [27]. Immunocytochemical studies using anti-Ara12 sera directly show that Ara12 is secreted to an apoplastic location in *Arabidopsis* stems. Furthermore, the protein appears to reside in the intercellular spaces. The presence of Ara12 at relatively high levels in tissues undergoing rapid cell expansion growth in the stem and silique may highlight an involvement in enabling or directing morphogenesis, or in catabolism, making nutrients available to developing tissues. Acidic conditions, of around pH 5.0, in the apoplast favour optimal activity of Ara12 protease. During auxin-induced cell elongation in rapidly growing tissue, the apoplastic pH lies between 4.5 and 5.0, whereas in the absence of auxin the pH is between 5.5 and 6.5 [40]. At sites of root hair initiation, a localized apoplastic pH of ≤ 5.0 is observed, which falls temporarily to 4.5 during the initial stage of root initiation, before rising to a level of pH 6.0, which is seen in the rest of the apoplast [41]. In addition, elevated extracellular Ca^{2+} levels observed in tissues undergoing elongation growth [42] would help to stabilize Ara12 protease, increasing its potential for hydrolysis.

The exact target substrate(s) of Ara12 has not been identified; however, with direct evidence for localization to the apoplast and its presence in rapidly dividing cells, it is tempting to speculate that it is in some way intimately involved in cell wall metabolism or the processing of precursor proteins, such as polypeptide hormones, several of which have been discovered recently [43,44]. It seems that plant subtilases have roles analogous to mammalian subtilases in processing important precursor molecules. In plants, systemin, a polypeptide hormone involved in signalling in response to physical damage, is processed from prosystemin by an undetermined proteolytic activity [45]. Interestingly, there is a possible pyrolysin cleavage site (REDL↓) at the N-terminal presumptive processing site bordering systemin. Further processing of a labelled systemin analogue has been shown to occur *in vitro*, by kexin-like activity of a 50 kDa plasma membrane

protein (SBP50), which binds this analogue [24]. Most plant polypeptide hormones studied to date are involved in the control of morphogenetic events, for example, CLAVATA (CLV) 3 participates in adjusting the balance between meristem cell proliferation and differentiation [46]. Evidence is now emerging for the involvement of plant subtilases in morphogenesis [18,19]. CLV3 and most systemins contain motifs potentially recognized by both kexins and pyrolysins isolated from animals, indicating that they may be processed by homologous plant proteases. Thus, Ara12 may be involved in signal transduction pathways during processes such as development and homeostasis, much as subtilases clearly are in animals.

Alternatively, Ara12 may be involved in cleavage of certain proteins in the apoplast related to cell wall structure or metabolism. It has previously been suggested that a subtilase, AIR3, may participate in lateral root formation by cleaving structural proteins, such as extensins, in the cell wall in *Arabidopsis* [16]. Ara12 could potentially cleave extensins or arabinogalactan proteins, which have both been implicated in processes in plant development [47]. Other possible specific targets of Ara12 include enzymes involved in modifying the extracellular matrix, proteins associated with the cytoskeleton or adhesion molecules.

We have evidence for the existence of a large gene family of *Arabidopsis* subtilases from Southern analysis, database searches and RT-PCR results [20], resembling that reported for tomato [15]. The generation and study of mutant *Arabidopsis* plants lacking normal Ara12 (or other subtilase) activity may allow further progress to be made in establishing *in vivo* function. It would also be of interest to see if ectopic expression of these proteases could result in the generation of plants with unusual morphologies, and what regulates normal expression. Further work is required to establish the identity of the physiological substrate(s) of Ara12, especially amongst silique and stem proteins found in the apoplast, and the intercellular space in particular.

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