

Grass group I pollen allergens (β -expansins) lack proteinase activity and do not cause wall loosening via proteolysis

Lian-Chao Li and Daniel J. Cosgrove

Department of Biology, 208 Mueller Laboratory, The Pennsylvania State University, University Park, Pennsylvania, USA

Group I grass pollen allergens make up a subgroup of the β -expansin family of cell wall loosening proteins in plants. A recent study reported that recombinant Phl p 1, the group I allergen from timothy grass pollen, was associated with papain-like proteinase activity and suggested that expansins loosen the plant cell wall via proteolysis. We tested this idea with three experimental approaches. First, we evaluated three purified native group I allergens from timothy grass, ryegrass and maize (Phl p 1, Lol p 1, Zea m 1) using five proteinase assays with a variety of substrates. The proteins had substantial wall loosening activity, but no detectable proteolytic activity. Thus we cannot confirm proteolytic activity in the pollen allergen class of β -expansins. Second, we tested the ability of proteinases to induce cell wall extension *in vitro*. Tests included cysteine proteinases, serine proteinases, aspartic proteinases, metallo

proteinases, and aggressive proteinase mixtures, none of which induced wall extension *in vitro*. Thus, wall proteins are unlikely to be important load-bearing components of the plant cell wall. Third, we tested the sensitivity of β -expansin activity and native wall extension activity to proteinase inhibitors. The results show that a wide range of proteinase inhibitors (phenylmethanesulfonyl fluoride, *N*-ethylmaleimide, iodoacetic acid, Pefabloc SC, and others) inhibited neither activity. From these three sets of results we conclude proteolysis is not a likely mechanism of plant cell wall loosening and that the pollen allergen class of β -expansins do not loosen cell walls via a proteolytic mechanism.

Keywords: expansin; grass group I pollen allergen; plant cell wall; proteinase; wall loosening.

Group I allergens from grass pollen are secreted glycoproteins of ≈ 30 kDa that are copiously released by the grass pollen grain upon hydration [1,2]. A clue to their biological function was first obtained when a sequence similarity to expansins was noted [3]. Subsequent work showed that the group I allergen from maize pollen, called Zea m 1, exhibits expansin activity specifically towards grass cell walls [4]. This discovery, combined with evidence of numerous homologs expressed in the vegetative cells of maize and rice, enlarged our definition of the expansin gene family and its functions. The group I allergens are now recognized as a subset of the β -expansin family of genes in plants [5].

Expansins are defined by their characteristic sequence and by their unique rheological effects on plant cell walls, which include rapid induction of cell wall extension and enhancement of wall stress relaxation [5,6]. These proteins, which are divided into the α and β families, are believed to be key catalysts of cell wall loosening necessary for plant cell growth, cell separation (abscission) and related processes. The limited amount of work to date indicates that the pollen allergen subclass of β -expansins are most effective on so-called Type II walls [4]. These are walls from grasses and closely allied species and they are

distinctive in composition from so-called Type I walls, which include the cell walls of all the other land plants [7]. In contrast, α -expansins have a more marked effect on Type I walls than on Type II walls. The current model for expansin action proposes that they promote a type of polymer creep or reptation of the structural polysaccharides of the plant cell wall [5]. Expansins are thought to weaken the noncovalent bonding between key wall polysaccharides, which then move in response to the mechanical stresses set up by internal cell turgor pressure or by external forces. Evidence supporting this model comes from studies of the kinetics and reversibility of the rheological effects of expansins on cell walls [8], as well as the action of expansins on artificial composites made of polysaccharides [9,10].

In contrast to this model, a recent study by Grobe *et al.* [11] concluded that group I allergens were novel C1 cysteine proteinases and proposed that wall loosening by expansin was mediated by proteinase action. This idea was largely based on the findings that recombinant Phl p 1, the group I allergen of timothy grass (*Phleum pratense*), was very unstable and was associated with proteolytic activity. This study also reported proteinase activity in native Phl p 1 after an auto-activation treatment. Further substantiating this idea, Grobe *et al.* [11] identified three sequence motifs in Phl p 1 that were claimed to be homologous to the active site motifs in the C1 family of cysteine proteinases.

Prior to this study, Pike *et al.* [12] noted an immunological cross reactivity between group I allergens and bromelain, a member of the C1 family of cysteine proteinases. They also noted a single motif (GCGSC) in common between the two proteins. Their study, however,

Correspondence to D. J. Cosgrove, 208 Mueller Laboratory, Penn State University, University Park, PA 16802. Fax: + 1 814 865 9131, Tel.: + 1 814 863 3892, E-mail: dcosgrove@psu.edu

Abbreviation: BAPNA, *N*- α -benzoyl-L-arginine-4-nitranilide.

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failed to detect proteinase activity in Lol p 1, the group I allergen from ryegrass (*Lolium pratense*) and found that the cross reaction of antibromelain antibody with Lol p 1 was due to the allergen's carbohydrate moiety, not its protein sequence.

Because of these inconsistent reports and because of the importance in resolving the mechanism of expansin action, we have critically examined the hypotheses that group I allergens are proteinases and that they cause plant wall loosening by a proteolytic mechanism. If such a mechanism were confirmed, it would also require a major revision of current models of the plant cell wall, which envision a scaffold of cellulose microfibrils that are coated with branched glucans and xylans and embedded in a gel-like pectin matrix. Specifically, the models would have to be revised to include one or more proteins as key structural and mechanical elements of the cell wall. Therefore, in this study we also test the more general notion that wall proteins are important structural determinants of wall extension activity.

MATERIALS AND METHODS

Plant materials

Ryegrass (*Lolium perenne*) and timothy grass (*Phleum pratense*) pollen were purchased from Greer Laboratories, Inc. (Lenoir, NC, USA). Maize (*Zea mays* L.) pollen was collected from plants grown in a summer field (State College, PA, USA) at the beginning of August, 1999, and stored at -80°C .

For cell wall assays, wheat coleoptile walls were used as a representative of a type II wall and cucumber hypocotyl walls were used as type I walls. Wheat (*Triticum aestivum* L., cv. Pennmore Winter) seeds were grown in moist Metro-Mix360 growing medium (Scotts-Sierra Horticultural Products Co., Marysville, OH, USA) at $27-29^{\circ}\text{C}$ in complete darkness for 3 days. Wheat coleoptiles were immediately cut, gently abraded by rubbing them between two fingers coated with a slurry of well washed carborundum (320 grit; Fisher Scientific Inc., Fair Lawn, NJ, USA), separated from primary leaves and then stored at -20°C prior to use. Cucumber (*Cucumis sativus* L. cv. Burpee Pickler) seeds were grown in darkness on wet germination paper (Kimpak K-22, Seedburo Equipment Co., Chicago, IL, USA) at $27-29^{\circ}\text{C}$ for 4 days. Cucumber hypocotyls were quickly excised from the seedlings under room light and directly frozen at -20°C .

Chemicals

Pefabloc SC, E-64 {*N*-[*N*-(1-3-thranscarboxirane-2-2-carbonyl)-*L*-Leucyl]-agmatine}, and CompleteTM EDTA-free proteinase inhibitor cocktail (a mixture of several proteinase inhibitors that inhibit a broad spectrum of serine and cysteine proteinase) were purchased from Roche Diagnostics GmbH (Sandhofer Strasse, Mannheim, Germany). Sigma Chemical Co. (St Louis, MO, USA) supplied bromelain (EC 3.4.22.32, B5144, from pineapple stem), cathepsin B (EC 3.4.22.1, C8571, from human liver), papain (EC 3.4.22.2, P4762, 2 $\times$ crystallized and lyophilized powder from papaya latex), pepsin (EC 3.4.23.1, P6887, from porcine stomach mucosa), renin (EC 3.4.23.15,

R2761, from porcine kidney), thermolysin (EC 3.4.24.27, P1512, *Bacillus thermoproteolyticus rokko*), trypsin (EC 3.4.21.4, T0134, from porcine pancreas), pepstatin A, EDTA, EGTA, cupric chloride dihydrate, phenylmethanesulfonyl fluoride, *N*-ethylmaleimide, and iodoacetic acid. Pronase (EC 3.4.24.4) and subtilisin (EC 3.4.21.62) were obtained from Fluka Chemical Corp. (Milwaukee, WI, USA). Tosyl-glycyl-prolyl-lysine-4-nitranilide (Chromozym PL) and *N*-alpha-benzoyl-L-arginine-4-nitranilide (BAPNA) were purchased from Boehringer-Mannheim Co. (Indianapolis, IN, USA). All other reagents were of the highest purity commercially available.

Proteins were quantified colorimetrically with Coomassie Plus Protein Assay Reagent (Pierce, Rockford, IL, USA) according to the manufacturer's instructions.

Rabbit polyclonal antibodies and mouse monoclonal antibodies [anti-(site D) [13]]; raised against Lol p 1 were kindly provided by D. G. Klapper (Department of Microbiology and Immunology, University of North Carolina School of Medicine, Chapel Hill, NC, USA).

Purification of β -expansins (group I allergens)

Pollen samples were extracted in 50 mM sodium acetate, pH 4.5, for 1 h at 4°C . Typically, 5 g of pollen was mixed with 20 mL of buffer. The extract was centrifuged at $15\,000\text{ g}$ at 4°C and was loaded onto a CM-Sepharose Fast Flow (Amersham Pharmacia Biotech AB, Uppsala, Sweden) column equilibrated in 20 mM sodium acetate, pH 4.5. The column was washed with the same buffer and then a 0–500 mM NaCl linear gradient with the hold at final gradient (500 mM NaCl) was applied to the column to elute the bound proteins. The fractions from CM-Sepharose column chromatography were desalted and concentrated by ultrafiltration. Active fractions (assayed by wall extension activity, described below) were pooled and loaded onto a silica-based CM-HPLC column ($4.6 \times 250\text{ mm}$, Synchropak CM300/6.5 μm , MICRA Scientific Inc., Northbrook, IL, USA). Proteins were eluted with a linear gradient of 0–550 mM NaCl in 20 mM sodium acetate, pH 4.5. Finally, the fractions containing β -expansin were further purified by two coupled high-performance gel filtration columns ($7.8 \times 300\text{ mm}$, PROTEIN PAK 125/10 μm and PROTEIN PAK 60/10 μm , Waters Co., Milford MA, USA) with 200 mM NaCl, 20 mM sodium acetate, pH 4.5 as elution buffer. N-Terminal sequencing of purified proteins was carried out at the macromolecular Core Facility of the Pennsylvania State University with an Applied Biosystems 477A Sequencer.

Purification of α -expansins

Wall proteins were extracted from maize coleoptiles and fractionated by ammonium sulfate precipitation as described for oat α -expansins [14]. After dialysis against 50 mM sodium acetate, pH 4.5, for 24 h, the proteins were loaded onto a SP-HPLC column ($7.8 \times 50\text{ mm}$, HPLC MA7S, Bio-Rad Laboratories, Richmond, CA, USA) which was washed with 20 mM sodium acetate, pH 4.5, and then a linear gradient (40 min) of 0–600 mM NaCl in the same buffer. Fractions containing α -expansin were desalted and concentrated by ultrafiltration, and then subjected to high-performance gel filtration chromatography, which

employed two coupled gel filtration columns in series as described above for β -expansin purification. Fractions containing α -expansin were identified by immunoblotting with antibody against cucumber α -expansin and by activity assays [14].

Wall extension (expansin activity) assay

Expansin activity was measured as an induction of extension in heat-inactivated wall specimens, using a constant load extensometer [15,16]. Abraded wheat coleoptiles prepared as above were boiled in distilled water for 15 s to inactivate the endogenous expansins and were secured between two clamps (with about 5 mm between the clamps) with a 20-g weight of constant tension. Extension of the wall sample was recorded at 30-s intervals. After incubation in an appropriate buffer, expansins or proteinases were added to the sample chamber (0.15 mL). In some assays cucumber hypocotyl (Type I) walls were used in place of coleoptile (Type II) walls. They were prepared from frozen cucumber hypocotyls in a manner similar to that described above for wheat coleoptile walls.

α -Expansins ($0.1 \text{ mg} \cdot \text{mL}^{-1}$) from etiolated maize coleoptiles or β -expansins ($0.15 \text{ mg} \cdot \text{mL}^{-1}$) from grass pollen were assayed in 50 mM sodium acetate, pH 4.5, 5 mM dithiothreitol. Cysteine proteinases ($0.5 \text{ mg} \cdot \text{mL}^{-1}$ papain, $1.5 \text{ mg} \cdot \text{mL}^{-1}$ bromelain, and $0.12 \text{ mg} \cdot \text{mL}^{-1}$ cathepsin B) were assayed in 50 mM Mes, pH 6.0, 5 mM dithiothreitol, 1 mM EDTA. Serine proteinases ($1.0 \text{ mg} \cdot \text{mL}^{-1}$ trypsin and $1.5 \text{ mg} \cdot \text{mL}^{-1}$ subtilisin) were assayed in 50 mM Hepes, pH 7.5, 5 mM CaCl_2 . Aspartic proteinases ($0.5 \text{ mg} \cdot \text{mL}^{-1}$ pepsin and $0.12 \text{ mg} \cdot \text{mL}^{-1}$ renin) were in 50 mM 3,3-dimethylglutaric acid, pH 3.0, and in 50 mM Mes, pH 6.0, 1 mM EDTA, respectively. Metallo cysteine proteinase ($1.0 \text{ mg} \cdot \text{mL}^{-1}$ thermolysin) was given in 50 mM Hepes, pH 7.5, 5 mM dithiothreitol, 5 mM CaCl_2 , and $1.5 \text{ mg} \cdot \text{mL}^{-1}$. Pronase was in 50 mM Hepes, pH 7.5, 5 mM CaCl_2 .

Electrophoresis and Western blot analysis

SDS/PAGE was performed in a mini-gel apparatus (Protean II; Bio-Rad Laboratories, Hercules, CA, USA) using 12% polyacrylamide gel according to the method of Laemmli [17]. Mini-gels containing proteins were stained with Coomassie Brilliant Blue R-250 in 10% acetic acid and 30% methanol. After destaining, gels were soaked in 4% glycerol and 30% methanol and wrapped with cellophane. Gels were scanned using an Epson flatbed scanner driven by the Adobe Photoshop version 5.5 and molecular weights were estimated by Kodak Digital Science 1D image analysis software (Eastman Kodak Co., Rochester, NY, USA). The protein marker used for SDS/PAGE was from Novagen Inc. (Catalog no. 69149; Madison, WI, USA).

Native PAGE was modified from Panyim and Chalkley [18] and performed on a Bio-Rad Mini Protean II system. The gel contained 10% (w/v) acrylamide, 0.27% bisacrylamide, 50 mM sodium acetate, pH 4.5. Samples were loaded in 50 mM sodium acetate pH 4.5, 10% sucrose and run in 50 mM sodium acetate (pH 4.5) at 150 V for 2 h at 4 °C. Bromophenol blue was added to each sample at a final concentration of 0.01% (w/v) to improve visibility of proteins during loading of the samples. The electrodes were

reversed at the power supply so that the net positively charged β -expansins could migrate to the bottom of the gel (the cathode). The gels were stained as described for SDS/PAGE gels. Cytochrome *c* was used as a front dye for indicating the progress of electrophoresis.

For Western blot analysis of SDS/PAGE and native PAGE gels, proteins were electrophoretically transferred on an EC140 Mini Blot Module (E-C Apparatus Corporation, Holbrook, NY, USA) to a Protran BA nitrocellulose membrane (Schleicher & Schuell, Keene, NH, USA). Transfers were carried out in a solution of 192 mM glycine, 25 mM Tris, and 20% (v/v) methanol at 25 V for 1.5 h. After electrotransfer, membranes were stained with Ponceau S solution for protein detection. For immunodetection of β -expansin proteins, the membranes were blocked with 10% horse serum in NaCl/P_i containing 0.05% Tween-20 and 5 mM sodium azide, incubated for 2 h with same solution containing mouse monoclonal antibody (antisite D) against Lol p I (1 : 200 000 dilution) or rabbit polyclonal antiserum raised against Lol p I (1 : 150 000 dilution), washed twice with NaCl/P_i or NaCl/Tris containing 0.05% Tween-20 and 5 mM sodium azide and then incubated for 1 h with goat anti-mouse IgG (whole molecule)-alkaline phosphatase conjugate (dilution of 1 : 1000; Sigma-Aldrich Co.), or goat anti-(rabbit IgG heavy and light chains)-conjugated alkaline phosphatase (dilution of 1 : 10 000; Rockland, Gilbertsville, PA, USA). The membrane blots were developed with 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (Sigma-Aldrich Co.). The prestained protein ladder for the Western blot was purchased from Gibco BRL life technologies (Rockville, MD, USA).

Auto-activation treatment of group I allergens

Auto-activation of Zea m I, Lol p I and Phl p I was performed as described by Grobe *et al.* [11], as a means of activating the presumptive proteinase activity of these β -expansins. Purified protein ($1.2 \text{ mg} \cdot \text{mL}^{-1}$) was incubated for 10 h at room temperature in activation buffer, which contained 0.1 M glycine, 10 mM CaCl_2 , 10 mM cysteine, and 5 mM dithiothreitol, pH 3.6.

Proteinase assays

(a) *Colorimetric detection of p-nitroaniline released from synthetic substrates.* Proteolytic activity was assayed by colorimetric detection of p-nitroaniline released from BAPNA and Chromozym PL. Two micrograms of auto-activated β -expansin in 75 μL of activation buffer was added to a well of a Costar flat bottom 96-well microplate (Corning) containing 25 μL of 10 mM BAPNA. The microplate was briefly vortexed, incubated for 16 h at 28 °C and read by a Series 750 Microplate Reader at 405 nm wavelength (Cambridge Technology, Inc., Watertown, MA, USA). Cleavage of Chromozym PL was assayed by a slight modification of the manufacturer's instruction. Briefly, 2 μg of auto-activated β -expansin in 50 μL of activation buffer were applied into a microplate well, then 50 μL of 100 mM Tris, 230 mM NaCl, pH 7.4, and 20 μL of 3 mM Chromozym PL in 100 mM glycine containing 0.2% Tween 20 were added. Samples were incubated and measured as in the BAPNA assay above.

(b) *SDS/PAGE analysis of BSA and ovalbumin digestion in solution.* Two micrograms of the HPLC-purified auto-activated β -expansin was added to 20 μ L of activation buffer containing 4 μ g of bovine serum albumin (BSA) and 8 μ g of ovalbumin. Protein digestion proceeded at 37 °C for 1 h and was stopped by adding 5 μ L of 5 $\times$ SDS/PAGE sample buffer and heating for 4 min at 95 °C. The samples were analyzed by SDS/PAGE. As a positive control, 0.1 μ g of papain was used in place of the β -expansin.

(c) *SDS/PAGE zymograms.* Gels containing 0.5% BSA, ovalbumin, gelatin, or casein were prepared as described by Grobe *et al.* [11]. Briefly, cysteine proteinases (papain and bromelain) and β -expansins (Lol p 1, Phl p 1, and Zea m 1) were diluted with 5 $\times$ nonreducing SDS/PAGE sample buffer, incubated for 20 min at 37 °C, and separated by SDS/PAGE at 4 °C. After electrophoresis, the gels were washed with three changes of activation buffer in 2 h, incubated in the same buffer for 22 h to allow refolding and protein digestion, then stained with Coomassie Brilliant Blue R-250. Proteinase activity appears as clear bands on a dark blue background.

(d) *Native PAGE zymograms.* Proteinase activity was also assayed on native PAGE gels containing 0.5% BSA. Papain and β -expansins were diluted with 2 $\times$ native PAGE sample buffer (20% sucrose and 0.04% bromophenol blue in 50 mM sodium acetate, pH 4.5), and separated by native PAGE, described above. The gels were washed by two changes of activation buffer in 1 h, incubated in the same buffer for 24 h, and then developed as described above for the SDS/PAGE zymogram.

(e) *Digestion in agarose gels.* Approximately 10 mL of 1.5% agarose in activation buffer supplemented with 0.1% casein or gelatin was poured into plastic Petri dishes (100 $\times$ 15 mm). Five microlitres containing 5 μ g of auto-activated Lol p 1, Phl p 1, or Zea m 1 was spotted on the surface of the gel. The gels were incubated for 24 h at 37 °C and then visualized as described above for SDS/PAGE gels. Papain was used as a positive control.

RESULTS

Purification and wall extension activity of group I grass pollen allergens

Group I allergens were extracted from grass pollen and purified by conventional cation exchange chromatography on a CM-Sepharose column, followed by HPLC on a CM-silica column and on two coupled diol-silica columns (L.-C. Li and D. J. Cosgrove, unpublished results). SDS/PAGE (Fig. 1A) shows that the final fractions for Lol p 1 and Zea m 1 contained a single major band, while Phl p 1 contained three bands corresponding to its three isoforms [11,19]. The identity of these proteins as group I allergens was confirmed by Western blot analysis using anti-(site D) monoclonal antibody (Fig. 1B) and polyclonal antibody (not shown) raised against Lol p 1. As additional confirmation, N-terminal sequencing (20 residues) of the Zea m 1 protein exactly matched the protein sequence deduced from the complete Zea m 1 cDNA sequence (not shown).

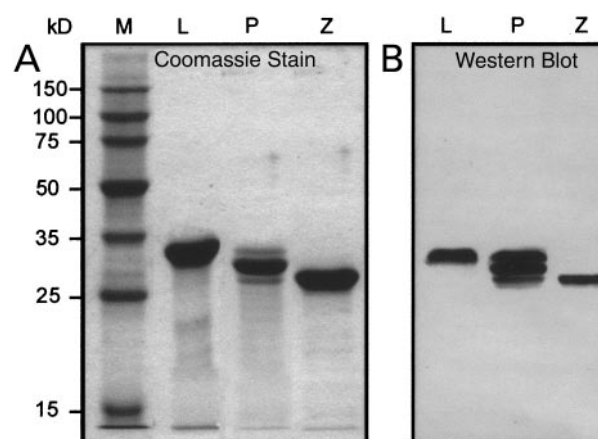


Fig. 1. SDS/PAGE and immunoblot analysis of purified Lol p 1, Phl p 1, and Zea m 1. (A) Coomassie-stained gel with 5 μ g of Lol p 1, Phl p 1 and Zea m 1 per lane. (B) Immunoblot of gel prepared as in A, probed with monoclonal antibody (antisite D) against Lol p 1. M, protein markers; L, Lol p 1; P, Phl p 1; Z, Zea m 1.

The purified proteins were shown to be functionally active in wall extension assays using wall samples from wheat coleoptiles (Fig. 2) and from maize silks, the natural substrate of Zea m 1 (data not shown). These results confirm previous results that Zea m 1 has expansin activity on grass walls [4] and extend this conclusion to include the group I allergens from timothy grass and ryegrass.

Stability of group I allergens

Grobe *et al.* [11] reported their recombinant Phl p 1 to be highly unstable, with rapid degradation into fragments. These results contrast with our experience that group I allergens are very stable. Because of concerns that their

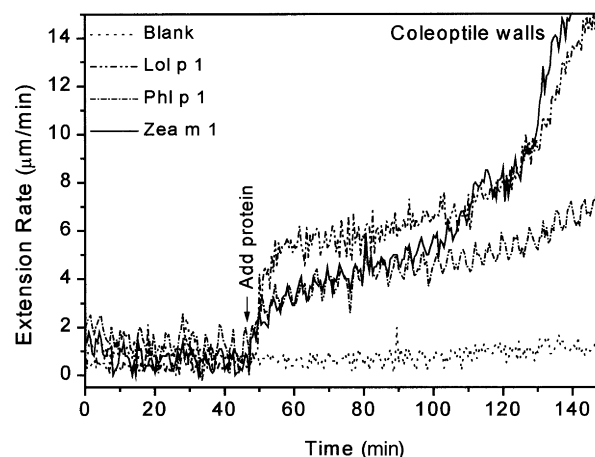


Fig. 2. Expansin activity of purified group I allergens. Heat-inactivated coleoptile walls were clamped at constant force in an extensometer. After 45 min of extension in 50 mM sodium acetate, pH 4.5, 5 mM dithiothreitol, the bathing solution was changed for the same one containing 0.15 mg·mL⁻¹ of purified Lol p 1, Phl p 1 or Zea m 1. Similar results were obtained in five independent experiments.

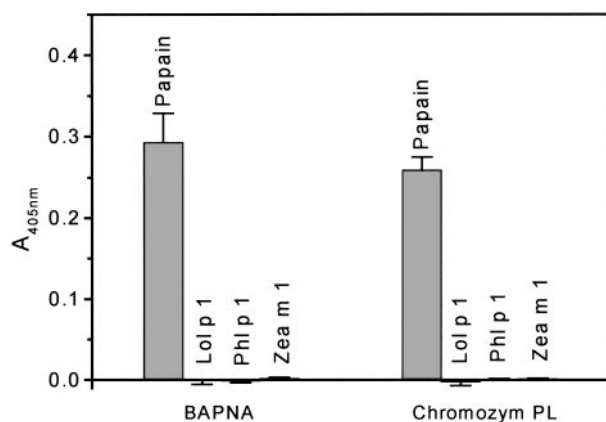


Fig. 3. Proteinase activity of group I allergens assessed by hydrolysis of BAPNA and Chromozym PL. Purified, auto-activated Lol p 1, Phl p 1 and Zea m 1 (2 μ g per well) were tested for hydrolysis of these two synthetic proteinase substrates. One μ g of papain was used as positive control. Data are means $\pm$ SD ($n = 6$).

recombinant protein may have been contaminated with a proteinase induced in *Pichia* cells used for expression, we specifically tested the stability of native Phl p 1, Lol p 1 and Zea m 1 purified as above and after an auto-activation treatment reported to enhance the proteolytic activity of Phl p 1 fractions [11]. In preliminary experiments we found that auto-activation treatment did change the wall extension activity of Zea m 1. After auto activation, Zea m 1 had 97% of wall extension activity as compared with untreated protein (SE = 15%, $n = 4$). Likewise, we did not find evidence that auto activation altered the expansin activity of Lol p 1 and Phl p 1.

We evaluated stability by assessing wall extension activity before and after incubation in 50 mM sodium acetate, 5 mM dithiothreitol, pH 4.5 at different temperatures and for varying times. For example, Zea m 1 retained

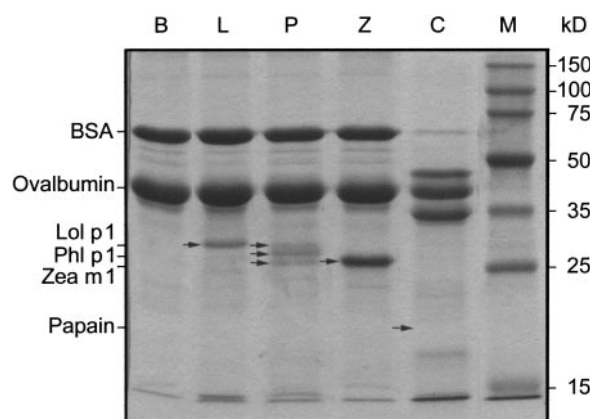


Fig. 4. Proteinase activity of purified group I allergens assessed by digestion of BSA and ovalbumin in solution. Approximately 2 μ g of purified Lol p 1, Phl p 1, Zea m 1, and 0.1 μ g papain (as positive control) were auto-activated, then incubated with 4 μ g BSA and 8 μ g ovalbumin in activation buffer at 37 $^{\circ}$ C for 1 h. B, blank (negative control); L, Lol p 1; P, Phl p 1; Z, Zea m 1; C, control (papain); M, protein markers. The positions of the group I allergens and papain are marked with arrows.

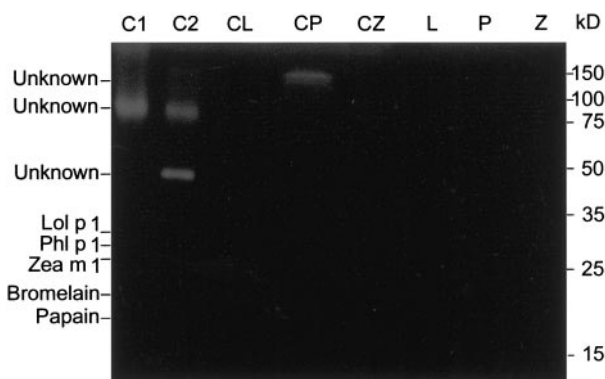


Fig. 5. Proteinase activity of group I allergens assessed by SDS/PAGE zymogram. Allergens were separated by SDS/PAGE in a gel containing 0.5% BSA. After electrophoresis, the gel was washed to allow protein refolding, incubated 22 h, then stained with Coomassie to identify renatured proteinases as cleared areas. C1: control 1 (2 μ g papain); C2: control 2 (10 μ g bromelain); CL: crude Lol p 1 (150 μ g); CP: crude Phl p 1 (150 μ g); CZ: crude Zea m 1 (150 μ g); L: purified Lol p 1 (10 μ g); P: purified Phl p 1 (10 μ g); Z: purified Zea m 1 (10 μ g). Locations of the allergens and proteinases are indicated at left.

102% of its activity when incubated at 31 $^{\circ}$ C for 27 h. Similarly, all the group I allergens maintained full expansin activity when stored at 4 $^{\circ}$ C for 1 month or at -20 $^{\circ}$ C for 9 months in 50 mM sodium acetate, 5 mM dithiothreitol, pH 4.5 (data not shown). SDS/PAGE and Western blot analysis of these samples confirmed that there were no degradation products of group I allergens after treatment in the above conditions. This stability indicates that these purified group I allergen preparations do not contain significant proteinase contamination and that the native allergens themselves do not possess substantial self-digestion activity, even after an auto-activation treatment.

Tests for proteolytic activity of group I allergens

Five different proteinase assays were employed.

(a) *Colorimetric detection of p-nitroaniline released from synthetic substrates.* BAPNA and Chromozym PL were chosen as substrates based on the report [11] that auto-activated Phl p 1 hydrolyzed them. However, we did not detect any hydrolysis of these substrates by auto-activated Lol p 1, Phl p 1, and Zea m 1, under the specific conditions used by Grobe *et al.* [11] (Fig. 3). Negative results (not shown) were also obtained when the purified allergens were first exposed to heat-inactivated coleoptile walls. This pretreatment was made to test the idea that a cell wall component might activate expansin activity *in situ*.

(b) *SDS/PAGE analysis of BSA and ovalbumin digestion in solution.* In Grobe *et al.* [11], this proteolysis experiment was carried out simultaneously with the purification of recombinant Phl p 1, resulting in a strong proteinase activity against BSA and ovalbumin. However, when we repeated the assay with native Lol p 1, Phl p 1, and Zea m 1, negative results were consistently obtained (Fig. 4). In contrast, papain at 1/20th the amount had

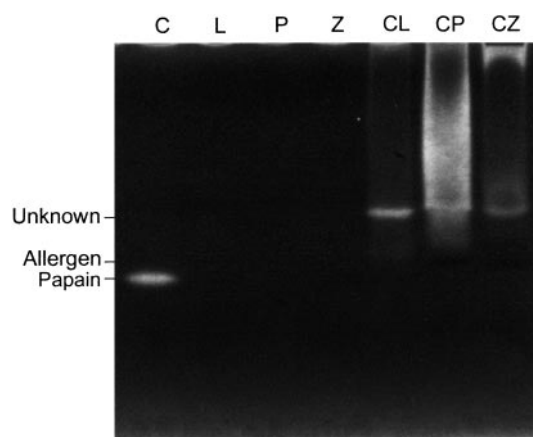


Fig. 6. Proteinase activity of group I allergens assessed by native PAGE zymogram. Allergens were separated by native PAGE in a gel containing 0.5% BSA. Gels were briefly washed three times with the activation buffer, incubated for 24 h at 37 °C, then stained with Coomassie Brilliant Blue R-250 to detect areas of clearing. The positions of the allergens were identified by a parallel immunoblot (not shown). C, control (1 µg papain); L, purified Lol p 1 (10 µg); P, purified Phl p 1 (10 µg); Z, purified Zea m 1 (10 µg); CL, crude Lol p 1 (150 µg); CP, crude Phl p 1 (150 µg); and CZ, crude Zea m 1 (150 µg).

strong proteinase activity in this assay. Moreover, papain had detectable activity when given in even smaller amounts, as little as 1/400th the amount used for testing Lol p 1, Phl p 1 and Zea m 1 (data not shown).

(c) *SDS/PAGE zymograms.* Grobe *et al.* [11] reported that a proteinase activity in the supernatant of Phl p 1-expressing *Pichia pastoris* cells could be renatured after SDS/PAGE, and they attributed this activity to Phl p 1. When we tested these conditions with purified Phl p 1, Lol p 1 and Zea m 1, we did not detect any protease activity, even after auto-activation treatment. Even papain and bromelain, used as positive controls, did not give detectable activity after SDS/PAGE (data not shown). However, when a modified protocol was adopted we did renature some proteinase activity in papain and bromelain, but still not in the purified group I allergens (Fig. 5). The proteinases renatured by this

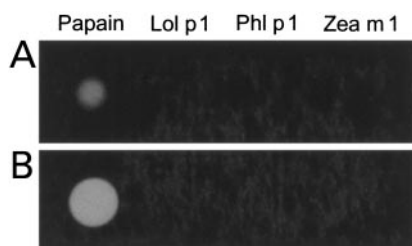


Fig. 7. Detection of proteolytic activity in agarose gel. Five micrograms of auto-activated Lol p 1, Phl p 1, and Zea m 1 were applied to 1.5% agarose plate containing 0.1% gelatin (A) or casein (B) in activation buffer. The plates were incubated for 24 h at 37 °C, then stained with Coomassie Brilliant Blue R-250. One hundred and 10 ng of papain were used as positive controls for the plates containing casein and gelatin as substrate, respectively.

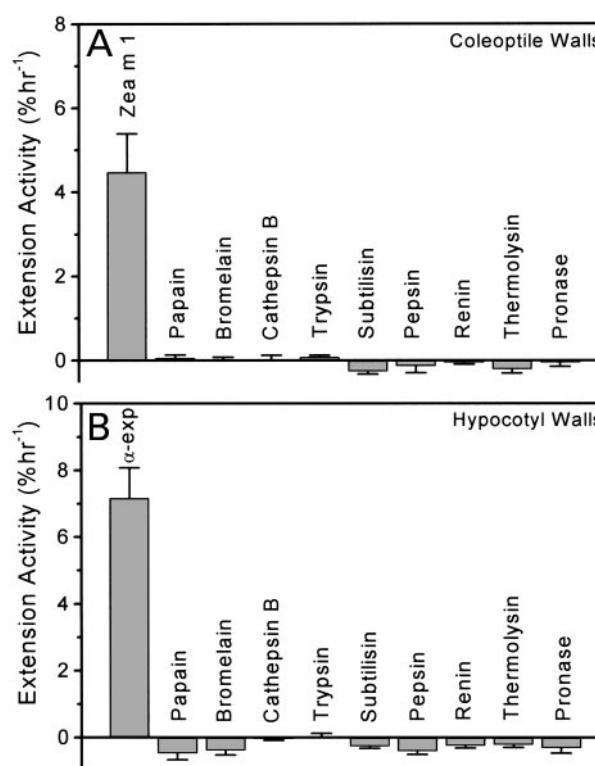


Fig. 8. Effects of proteinases on wall extension. Heat-inactivated walls were clamped in an extensometer and then proteinase was added to test for induction of wall extension. (A) Wheat coleoptile (Type II) walls were used as substrate, with 150 µg·mL⁻¹ purified Zea m 1 as positive control. (B) Cucumber hypocotyl (Type I) walls were used as substrate, with 100 µg·mL⁻¹ of α-expansin used as a positive control. Data represent means + SE of the induced extension rate, expressed as percentage increase in length per h (*n* > 5).

method migrated differently than papain and bromelain. In crude pollen extracts from timothy grass, a proteinase with molecular mass larger than 150 kDa was detected in the renatured gels. Similar results were obtained when other proteinase substrates, including ovalbumin, gelatin, and casein, were incorporated in the SDS/PAGE gels.

(d) *Native PAGE zymograms.* Because many enzymes do not renature efficiently after SDS/PAGE, we used native PAGE zymography as a more convincing test. Purified Phl p 1, Lol p 1 and Zea m 1 migrated as compact bands, but did not evince any proteinase activity (Fig. 6). Papain did show a distinctive clearing in this zymogram. Crude pollen extracts containing group I allergens showed substantial proteinase activities, but they were physically separated from the group I allergens, which were detected in parallel gels by Western blot analysis. Notably, the crude extract from timothy pollen showed strong and highly diffused proteolytic activity when compared with that from ryegrass or maize pollen.

(e) *Digestion in agarose gels.* When 5 µg of the auto-activated allergens were applied to agarose gels containing 0.1% casein (Fig. 7A) or gelatin (Fig. 7B) and incubated for 24 h at 37 °C, no proteinase activity was seen with the

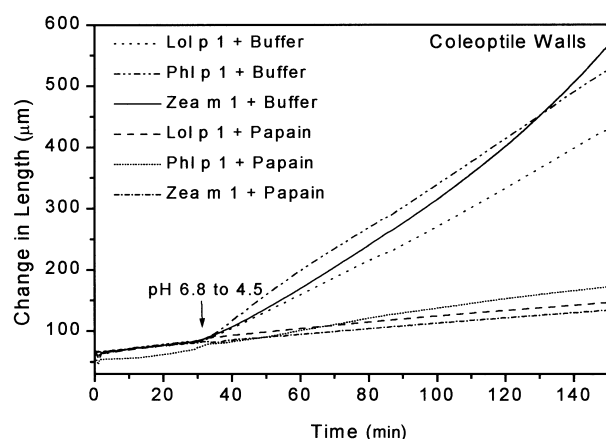


Fig. 9. Destruction of β -expansin activity by papain. Heat-inactivated wheat coleoptiles were preincubated for 1 h with $0.3 \text{ mg} \cdot \text{mL}^{-1}$ of group I allergens, then further pretreated for 1 h at room temperature with $2 \text{ mg} \cdot \text{mL}^{-1}$ of papain. The walls were then assayed for residual expansin activity by extensometer assay. At the time indicated by the arrow, the buffer was switched from the neutral buffer (50 mM Hepes, pH 6.8) to the acidic buffer in which expansins are active (50 mM sodium acetate, pH 4.5). The β -expansins used in this assay were partially purified Lol p 1 and Phl p 1 from the CM-Sepharose column, and highly purified Zea m 1 from high-performance gel filtration chromatographic columns. This experiment was performed three times with similar results.

three pure allergens. In contrast, much smaller amounts of papain gave strong activity (Fig. 7).

From these results we conclude that these group I allergens lack proteinase activity, a conclusion at odds with that reported by Grobe *et al.* [11]. Note that the validity of our conclusion does not depend on absolute purity of our protein preparations, which may contain traces of other proteins (Fig. 1). The presence of clear expansin activity in these protein preparations and the lack of proteinase activity in the same preparations demonstrates that proteinase activity is neither inherent to expansins nor required for expansin activity.

Table 1. Effects of proteinase inhibitors on β -expansin activity. Heat-inactivated wheat coleoptiles were initially extended with 50 mM sodium acetate, pH 4.5. After 30 min the bathing solution was replaced by the same buffer containing $0.3 \text{ mg} \cdot \text{mL}^{-1}$ β -expansins (partially purified Lol p 1 and Phl p 1 from the CM-Sepharose column, and highly purified Zea m 1 from high-performance gel filtration chromatographic columns) and proteinase inhibitor which was first incubated for 30 min at room temperature. Data are the means of at least four measurements.

Protease inhibitor	Residual activity (%)		
	Lol p 1	Phl p 1	Zea m 1
1.0 mM Phenylmethanesulfonyl fluoride	99.6	100.3	102.3
20 mM Pefabloc SC	75.1	82.9	94.5
10 μM <i>N</i> -ethylmaleimide	73.6	93.3	85.8
1.0 mM iodoacetic acid	93.4	92.0	74.6
5.0 mM Cu^{2+}	36.7	41.5	69.7

Effect of proteinases on wall extension

If β -expansins loosen plant cell walls by proteolysis, one might expect that some other proteinases could mimic or partially mimic the effect of β -expansins on wall extension. We tested this hypothesis using eight diverse proteinases assayed under conditions optimal for their proteolytic activity. We tested heat-inactivated walls from wheat coleoptiles (Type II walls; Fig. 8A) and cucumber hypocotyls (Type I walls; Fig. 8B), but could not detect even a trace of expansin-like wall extension activity. The eight proteinases fall into four classes with different catalytic mechanisms (cysteine proteinase: papain, bromelain, and cathepsin B; serine proteinase: trypsin and subtilisin; aspartic proteinase: pepsin and renin; metallo proteinase: thermolysin). Negative results were also obtained when an aggressive proteinase mixture (Pronase) was tested (Fig. 8). The three cysteine proteinases (papain, bromelain, and cathepsin B) were also examined at both acidic (pH 4.5) and basic (pH 8.0) conditions, with negative results (data not shown). Because the optimum pH for native wall extension is around 4.5, the bathing solutions were also

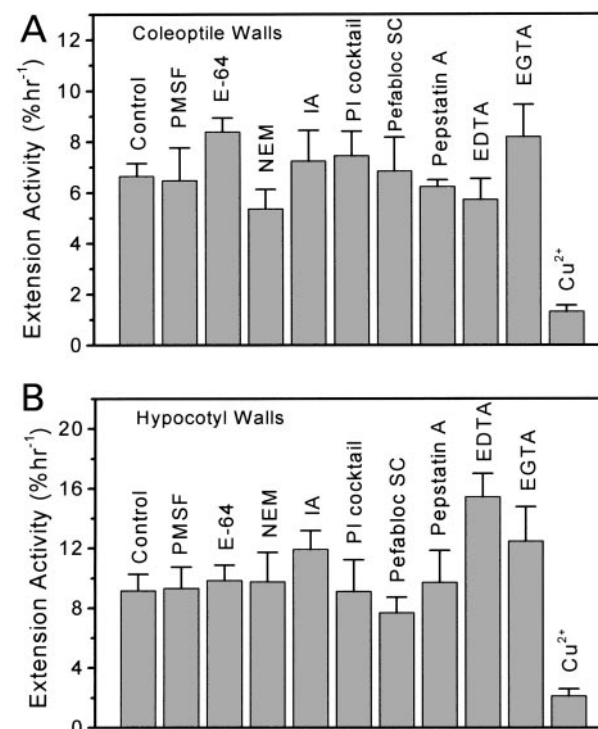


Fig. 10. Effects of proteinase inhibitors on native wall extension. Type II walls (wheat coleoptile walls) were used in A, whereas Type I walls (cucumber hypocotyls walls) were used in B. The wall specimens were initially bathed in 50 mM Hepes, pH 6.8 for about 30 min, then 50 mM sodium acetate, pH 4.5, containing the proteinase inhibitors was exchanged for the neutral buffer. Control contained no additive and represents the expansin activity endogenously present in the growing walls. The proteinase inhibitors were 2 mM phenylmethanesulfonyl fluoride (PMSF), 50 μM E-64, 10 μM *N*-ethylmaleimide (NEM), 1 mM iodoacetic acid (IA), PI (proteinase inhibitor) cocktail, 20 mM Pefabloc SC, 5 μM Pepstatin A, 10 mM EDTA, 10 mM EGTA, and 5 mM Cu^{2+} . Wall extension activity is expressed as the percentage increase in length per h. Data are means $\pm$ SE of at least five measurements.

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CsEXP1  -----DYGG-WQSGHATFYGGDASGTM--GGAC-GYGNLYSQGYGTNTVALSTALFNNGLSCGACFEMTCT
Phl p 1  IPKVPPGPNTATYGDKWLDAKSTWYKPTAAGPKDNGGAC-GYKDVKPPFSGMTGCGNTPIFKSGRGCGSCFEIKCT
Papain   IP-----EYVD-WRQK-----GAVTPVKNQSGCGSCWAFSAVVTIEGIIKIRTGNLQYSEQE-----LLDCD

CsEXP1  NDPKWCL--PGTIRVTATNFCPPNFALPNNGGWCNPPLQ--HFDMAEPAFLQIAQY-----RAGIVPVS-FRRVPC
Phl p 1  K-PEACSGEPVVVHITDDN-----EEPIAAYHFDLSGIAFGSMKKGDEQKLSAGEVEIQ-FRRVKC
Papain   RRSYGCNGG-----YPWSALQLVAQYGIHYR--NTYPYE-GVQRYCRSREKGPYAA-KTDGVRQVQPYNQAL

CsEXP1  MKKGGVRFTINGH----SYFNLVLITNVCGAGDVCSUSIKG-S-RTGWSMSRNWQCNWQSN--NYLNGQGLSFQVTL
Phl p 1  KYPEGTKVTFEVEKGSNENYAL-LVKFVAGDGDVVAVDIKEKG-KDKWIALKESWGAIWRIDTPEVLEKGP-FTVRYTT
Papain   LYSIANQPVSVVLQAAGKDFQLYRGGIFVGPCGNKVDGDAVAAGVGYGPNYILINSWGTG-----GENGYIRIKR

CsEXP1  SDGRTLTAYNLVPNSWQFGQTYEGPQF
Phl p 1  EGGTKGEAKDVIPEGWKADTAYESK
Papain   GTGNSYGVCGGL-----YTSSFYFVKK

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Fig. 11. Sequence alignment of an α -expansin, a β -expansin, and papain. Sequences were aligned by the Clustal method with PAM250 residue weight table and default penalties. Conserved residues are highlighted by bold letters. Gaps are indicated by dashes in the alignment. The three key catalytic residues and surrounding motifs identified by Grobe *et al.* [11] as critical to cysteine proteinases and common to expansins are indicated with circles and shading, respectively. Sequences: CsEXP1 (accession no. U30382[3]); Phl p 1 (accession no. Z27090[30]); papain (accession no. 231333 [31]), a family C1cysteine proteinase from papaya (*Carica papaya*). Signal peptides were removed from these protein sequences.

switched to pH 4.5 after 2 h at pH 8.0, to explore whether there was any effect on wall extension which could be displayed in acidic conditions. No change in wall-extension activity was detected (data not shown).

In another variation, we tested for the ability of papain to augment wall extension activity of β -expansins by allowing purified group I allergens to bind to heat-inactivated walls, then added papain and tested for wall extension activity after a 1-h incubation. Papain destroyed the wall extension activity of the group I allergens (Fig. 9). Similar results were found when Pronase was used to replace papain (data not shown). These results undermine the premise that wall proteins serve a key load-bearing role in the plant cell wall.

Effect of proteinase inhibitors on wall extension activities

In Grobe *et al.* [8], a variety of proteinase inhibitors, such as 1 mM phenylmethanesulfonyl fluoride, 20 mM Pefabloc SC, 10 μ M *N*-ethylmaleimide, 1 mM iodoacetic acid, and 5 mM Cu^{2+} , were reported to inhibit the proteolytic activity attributed to recombinant Phl p 1. We tested whether these proteinase inhibitors inhibited the wall extension activity of the group I allergens, as a test of the idea that β -expansins use a proteolytic mechanism for wall loosening. As shown in Table 1, the β -expansin activity was only slightly inhibited by Pefabloc SC, *N*-ethylmaleimide, and iodoacetic acid, and was not inhibited by phenylmethanesulfonyl fluoride. Cu^{2+} substantially inhibited expansin activity, but at 5 mM this metal ion interferes with many enzymatic activities and also binds strongly to acidic groups in the cell wall; therefore, we consider this result inconclusive.

We also tested a broad range of proteinase inhibitors for their ability to inhibit the endogenous extension activity of native coleoptile (Type II) and hypocotyl (Type I) walls. As shown in Fig. 10, most of the inhibitors had little or no effect when given at the start of the extension phase. We also obtained similar results when the inhibitor was directly applied in the middle of the extension period (data not shown). Cu^{2+} was a strong inhibitor, as found in the assays with the group I allergens (above), but the significance of

this result is discounted because of nonspecific effects. We conclude from these inhibitor experiments that expansin activity is unlikely to require a cysteine proteinase activity.

Sequence similarity of expansins to C1 cysteine proteinases?

Group I allergens, and expansins in general, were reported by Grobe *et al.* [11] to have significant sequence similarity to the C1 family of cysteine proteinases. Our sequence analysis undermines this conclusion. Alignments between cysteine proteinases and expansins give only $\approx 7\%$ identity (Fig. 11), and pairwise tests with the BLAST2 program [20] indicate no statistical significance to these alignments.

Grobe *et al.* [11] reported that α - and β -expansins were conserved in a 'catalytic triad' of residues and/or surrounding amino acids that are held to be important for C1 cysteine proteinases. These residues are identified as Cys25, His159 and Asn175 in the papain family [21]. The locations of these key residues and surrounding motifs are shown in Fig. 11, which is a CLUSTAL alignment between an α -expansin, a β -expansin (Phl p 1) and papain, as a representative of a C1 cysteine proteinase. It can be seen in this global alignment that the catalytic residues (C, H and N marked with dark circles) and surrounding conserved sequences (shaded boxes) identified for papain do not align well with the expansin regions identified as homologous by Grobe *et al.* [11]. The expansin Cys that aligns with papain Cys125 is not the Cys with papain-like sequence conservation in the surrounding residues. The His residues in expansin said to correspond to papain His159 do not align between α - and β -expansins (see Fig. 11). Asn175 in papain is not conserved in expansin. Our analysis dismisses the putative sequence similarity between expansins and C1 cysteine proteinases as spurious and unsubstantiated.

DISCUSSION

The growing cell wall of plants is principally composed of cellulose microfibrils embedded in a heterogeneous polysaccharide matrix [7,22,23]. Thus, the notion that cell wall loosening action by expansins may be mediated by a cryptic

proteinase activity [11] may seem unlikely at first. Nevertheless, the plant cell wall does contain structural proteins that might serve a scaffolding role in parallel with the polysaccharide network. Such ideas have been proposed in the past, albeit with little experimental support. Confirmation that expansins act via proteinase action would therefore have important implications for cell wall structure and the control of plant cell growth, as well as for the mechanism of action of expansins.

Our investigation of possible proteinase activity by the β -expansins known in the immunological literature as grass pollen group I allergens leads us to conclude that they do not loosen plant cell walls via proteolytic action. We tested purified group I allergens from three grass species, including Phl p 1, which was studied by Grobe *et al.* [11]. All three proteins possessed robust and stable wall extension activity, but we could not detect proteinase activity in the same assays (plus some additional ones) used in the study by Grobe *et al.* [11]. There was no evidence of auto-activation by the purified allergens, when assays were made of either wall extension activity or proteinase activity, and previous experience with both α - and β -expansins has not revealed any need for activation treatments to obtain wall extension activity. We suspect the proteinase activity reported for recombinant Phl p 1 by Grobe *et al.* [11] was due to trace contamination by a proteinase induced in *Pichia* upon expression of Phl p 1, whereas their native Phl p 1 may have been incompletely separated from the high proteinase activities we found in timothy grass pollen (Fig. 6). We note that Grobe *et al.* [11] did not assay their recombinant Phl p 1 for wall extension activity or attempt to assess the relationship between such activity and the proteolytic activity they detected.

Similarly, the purported sequence similarity between expansins and cysteine proteinases could not be substantiated in our analysis. This contrasts with the significant alignment (probability = 2×10^{-6} , by BLAST2) found between the Cys-rich region of expansins and the catalytic domain of family 45 glycosyl hydrolases, as reported previously [5,24]. The residues that are conserved between α - and β -expansins are also largely conserved in family 45 glycosyl hydrolases, but they are not conserved in cysteine proteinases. Finally, sequence comparisons suggest that expansins consist of two domains [5], an amino terminus homologous to family 45 glycosyl hydrolases and a carboxy terminus homologous to the Group II grass pollen allergens that fold into a compact β -sandwich [25,26]. If this is true, the residues corresponding to papain's catalytic Cys25 and His159 would fall into different domains – an unlikely situation if expansins were cysteine proteinases.

We also tested a corollary of the proposal of Grobe *et al.* [11] by testing for wall loosening activity by a variety of proteinases, but here too the results do not support a proteolytic mechanism of wall loosening. Our results thus confirm and extend previous, though more limited, studies reporting that proteolytic digestion of plant cell walls does not cause wall extension [16,27]. Moreover, the effects of the proteinase inhibitors (Fig. 10 and Table 1) support the general conclusion that plant cell wall loosening does not depend on proteinase action.

In summary, we did not find evidence of proteolytic action by group I allergens and such a mechanism does not seem to be a viable explanation for the loosening action of

expansin on the plant cell wall in light of our results. The unique rheological effects of expansins on plant cell walls are in our view most likely due to their weakening of polysaccharide–polysaccharide interactions [28]. Support for this mechanism comes from studies on the kinetics and reversibility of expansin action on cell wall rheology [8] and on the action of expansins of materials such as pure cellulosic paper [9] and artificial cellulosic composites [10], as well as the lack of hydrolytic and transglycosylation activities by expansins [8,15,29].

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