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An oat coleoptile wall protein that induces wall extension in vitro and that is antigenically related to a similar protein from cucumber hypocotyls

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Abstract. Plant cell walls expand considerably during cell enlargement, but the biochemical reactions leading to wall expansion are unknown. McQueen-Mason et al. (1992, Plant Cell **4**, 1425) recently identified two proteins from cucumber (*Cucumis sativus* L.) that induced extension in walls isolated from dicotyledons, but were relatively ineffective on grass coleoptile walls. Here we report the identification and partial characterization of an oat (*Avena sativa* L.) coleoptile wall protein with similar properties. The oat protein has an apparent molecular mass of 29 kDa as revealed by sodium dodecyl sulfate-polyacrylamide gel eletrophoresis. Activity was optimal between pH 4.5 and 5.0, which makes it a suitable candidate for "acid growth" responses of plant cell walls. The oat protein induced extension in walls from oat coleoptiles, cucumber hypocotyls and pea (*Pisum sativum* L.) epicotyls and was specifically recognized by an antibody raised against the 29-kDa wall-extension-inducing protein from cucumber hypocotyls. Contrary to the situation in cucumber walls, the acid-extension response in heat-inactivated oat walls was only partially restored by oat or cucumber wall-extension proteins. Our results show that an antigenically conserved protein in the walls of cucumber and oat seedlings is able to mediate a form of acid-induced wall extension. This implies that dicotyledons and grasses share a common biochemical mechanism for at least part of acid-induced wall extensions, despite the significant differences in wall composition between these two classes of plants.

Key words: Acid growth – *Avena* (coleoptile growth) – Cell wall extension – Expansin – Protein (cell wall)

Abbreviations: ConA = concanavalin A; CM = carboxymethyl; DEAE = diethylaminoethyl; DTT = dithiothreitol; Ex29 = 29-kDa expansin

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Introduction

Numerous studies have shown that the primary walls of many plants, including dicotyledons, monocotyledons, ferns and algae, extend when they are acidified via exogenous buffers (see reviews in Rayle and Cleland 1977; Taiz 1984) or via the action of an H^+-ATPase in the plasma membrane (Hager et al. 1991). These observations provide substantial support of the acid-growth hypothesis (Rayle and Cleland 1992). Although the significance of acid growth for auxin action continues to be debated (Schopfer 1989; Luthen et al. 1990; Rayle and Cleland 1992), there is little doubt that wall acidification can induce wall extension. However, the biochemical mechanism of such wall extension is not understood. Wall acidification has been postulated to activate cell-wall glycosidases or other hydrolases that break glycosyl bonds between wall structural polymers and allow the wall to yield to turgor-generated wall stress (Johnson et al. 1974; Fry 1989). Although this hypothesis is attractive and is supported by indirect evidence (Fry 1989; Inouhe and Nevins 1991), it remains speculative because it has not been shown directly that such wall hydrolytic enzymes can catalyze extension of isolated walls.

Recently, two proteins from cucumber walls were identified with the ability to induce extension of isolated plant walls (McQueen-Mason et al. 1992). In this report we refer to these proteins as *expansins*, to designate the class of wall-associated proteins that mediate the acid-induced extension of isolated walls. Cucumber expansins induced extension of walls from several di- and monocotyledonous species, but had little effect when assayed with coleoptile walls from maize and barley. Grass coleoptiles have been a favored object of growth studies since Darwin's analysis of phototropism and have been instrumental in many discoveries about plant growth, including the discovery of auxin. The wall composition of grass coleoptiles is notably different from that of dicotyledons and other monocotyledons (Bacic et al. 1988; Carpita and Gibeaut 1993); nevertheless, coleoptile walls,

like dicotyledonous walls, do exhibit a strong acid-induced extension in vitro and in vivo (Rayle and Cleland 1972). These observations led us to postulate that a wall protein, with functions analogous to the cucumber expansins, might mediate the endogenous acid-induced extension of coleoptile walls. Therefore, in this study we attempted to identify oat coleoptile proteins that could induce wall extension, using the wall extraction and reconstitution approach that proved successful with cucumber walls (McQueen-Mason et al. 1992).

Materials and methods

Plant material. All seedlings were grown in moist vermiculite in complete darkness at 27°C. Except as noted, coleoptiles were from seedlings of 4-d-old oat (*Avena sativa* L. cv. Olge, from Carolina Biological Supply, Burlington, N.C., USA). Cucumber hypocotyl walls were from 4-d-old seedlings of cucumber (*Cucumis sativus* L. cv. Burpee Pickler, from A.W. Burpee, Westminster, Pa., USA). Pea epicotyls were from the third internode (counting from the base) of 6-d-old seedlings of pea (*Pisum sativum* L. cv. Alaska, from A.W. Burpee).

Seedlings were quickly harvested under room lights. For wall-extension assays, the apical 2 cm region of the growing stem or coleoptile was excised, sealed in aluminum foil and frozen at −20°C prior to use. Coleoptiles were separated from primary leaves. Cuticles were abraded by rubbing the coleoptiles or stems between two fingers coated with a slurry of carborundum (320 grit, well washed prior to use; Fisher Scientific, Fair Lawn, N.J., USA). For oat coleoptiles, the cuticle was generally abraded prior to freezing, whereas for cucumber and pea stems the frozen segment was quickly abraded. In some instances as noted, coleoptile cuticles were removed by stripping the epidermis from the tissue with fine forceps and the remaining coleoptile cylinder was bisected longitudinally prior to freezing. Tissues were thawed, pressed under weight for 5–10 min to remove tissue fluids and clamped in an extensometer (5 mm between the clamps, corresponding to the apical 3–8 mm of the stem or coleoptile), as described previously (Cosgrove 1989).

Protein preparation. For oat protein extraction, oat seedlings were rapidly cut under room lights and placed in ice water. The apical 2.5 cm (± 0.5 cm) of each coleoptile was then cut, separated from the primary leaf, and placed on ice while the other coleoptiles were harvested. About 500 coleoptiles were homogenized in 200 mL of 10 mM sodium phosphate, pH 6.0. In some instances the coleoptiles were collected in lots of 100–200 and frozen (−20°C) for 1–3 d prior to homogenization. The homogenate was filtered through a nylon screen (70 μm mesh), and the cell walls were collected and washed four times by resuspending in the homogenization buffer (300 mL) followed by filtration. Ionically-bound proteins were extracted for at least 1 h at 4°C with 50 mL of 1 M NaCl containing 20 mM Hepes (pH 6.8), 2 mM EDTA and 3 mM sodium metabisulfite. Wall fragments were removed by filtration or centrifugation and the wall proteins in the supernatant were precipitated with ammonium sulfate (0.4 g added to each mL). Precipitated proteins were dissolved in 1.5 mL of water and desalted on an Econo-Pac 10DG desalting column (Bio-Rad Laboratories, Richmond, Calif., USA) which was equilibrated with 20 mM Tris-HCl, pH 8.0, containing 100 mM NaCl. Without NaCl the active proteins tended to bind to the desalting column, resulting in a lower recovery. Protein solution from the desalting column was centrifuged in a microcentrifuge for 3 min to remove precipitates. Proteins were then loaded onto a diethylaminoethyl (DEAE)-column (Sephadex A-25; Sigma) equilibrated with 20 mM Tris-HCl, pH 8.0, containing 100 mM NaCl at 25°C. The proteins bound to the DEAE-column were eluted by 1 M NaCl in 20 mM Tris-HCl buffer, pH 8.0.

A 1-mL concanavalin A (ConA) column (Sigma) was equilibrated with 200 mM NaCl containing 1 mM each of Mg^{+2}, Ca^{+2}, and Mn^{+2}. Proteins from the DEAE-column were passed through this column. After the column was washed extensively with the same solution, the bound proteins were eluted with the same solution containing 0.5 M α-methyl mannoside. Extension activity was associated with the fractions that did not bind to the column.

Active fractions were further separated by HPLC using a carboxymethyl (CM) cation-exchange column (4.6 id., 250 mm long, CM300/6.5 μm; ISCO, Lincoln, Nebr., USA) equilibrated with 10 mM Mes, pH 5.5. Before the protein sample (1 mL) was loaded on the column, the sample buffer was exchanged for 10 mM Mes, pH 5.5, by use of a 30-kDa Centricon microconcentrator (Amicon, Berverly, Mass., USA). Proteins were eluted from the CM column at a flow rate of 1 mL/min with a gradient of 0 to 0%, 0 to 4%, 4 to 6% and 6 to100% of 1.0 M NaCl in 10 mM Mes, pH 5.5, from 0 to 5 min, 5 to 10 min, 10 to 30 min and 30 to 50 min, respectively, and detected by absorbance at 280 nm.

Soluble protoplasmic proteins were obtained by homogenizing oat coleoptiles in 10 mM sodium phosphate buffer (in some cases, 10 mM Hepes, pH 6.8) and centrifuging at 4°C at 26 000·*g* for 10 min to remove particulate matter.

Active cucumber expansin fractions were prepared by NaCl extraction of cucumber hypocotyl walls, as described in detail by McQueen-Mason et al. (1992). For most experiments reported here, the C3 protein fraction was used (i.e. the protein in the NaCl extract was precipitated with ammonium sulfate, resolubilized and fractionated on a C3 column, per McQueen-Mason at el. (1992)). In some cases the proteins in the ammonium sulfate pellet were desalted and partically purified on a DEAE-Sephadex column, as described above.

Proteins were quantified colorimetrically using the Coomassie Protein Assay Reagent (Pierce, Rockford, Ill., USA). For SDS-PAGE (Laemmli 1970), proteins were separated on a 14% polyacrylamide gel or a 4–20% gradient polyacrylamide gel (Bio-Rad Ready Gel). For Western analysis, proteins were electrophoretically transferred to a nitrocellulose membrane in a solution of 192 mM glycine, 25 mM Tris, 20% methanol (v/v) at 10 V/cm for 3 h or in some cases 16 h. After the membrane was blocked with 3% bovine serum albumin in phosphate-buffered saline containing 0.05% Tween-20 (PBST), it was incubated for 2 h in PBST containing antiserum (1:3000 dilution). The membrane was washed four times with PBST and then incubated for 1 h with goat anti-rabbit IgG-conjugated alkaline phosphatase (Sigma; dilution of 1:4000) in PBST. The Western blot was developed using bromochloroindolyl phosphate/nitro blue tetrazolium and the reaction was stopped with 10 mM EDTA.

Antibodies. A wall protein with molecular weight of 29 kDa (now called cucumber expansin-29 or Ex29) was previously isolated from cucumber hypocotyls and shown to induce extension in isolated walls (McQueen-Mason et al. 1992). Antiserum with specific recognition of this protein was raised in a female New Zealand White rabbit by subcutaneous injections of cucumber Ex29 with Freund's adjuvants (Harlow and Lane 1988). Serum dilutions in the range of 2000:1 to 4000:1 proved optimal for Western analyses of cucumber and oat proteins.

Results

Figure 1 shows an example of acid-induced extension of oat coleoptile walls. When native walls were clamped under constant tension at neutral pH, the extension rate was low 30 min after application of the load and could be greatly increased by replacing the neutral buffer with a buffer of pH 4.5. The wall extension rate decreased continuously with time after the change to acid buffer (Fig. 1B). Addition of 10 mM dithiothreitol (DTT), a sulfhydryl reducing agent, increased the extension rate

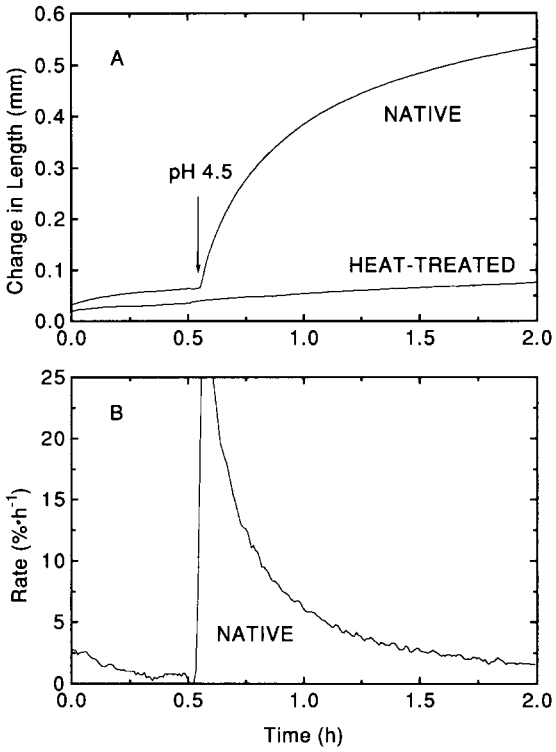

Fig. 1A,B. Acid-induced extension in native and heat-treated oat coleoptile walls. **A** When switched from a neutral buffer (50 mM Hepes, pH 6.8) to an acidic buffer (50 mM sodium acetate, pH 4.5), native walls extended at a high rate which fell continuously with time, whereas heat-treated walls lacked an appreciable response. The native and heat-treated (15 s in boiling water) walls were prepared and mounted on an extensometer (5 mm between two clamps) with 20 g of tension as described by Cosgrove (1989) and McQueen-Mason et al. (1992). **B** A plot of extension rate for the native walls shown in **A** demonstrates that the rate decreases continuously. Similar results were obtained in ten repetitions

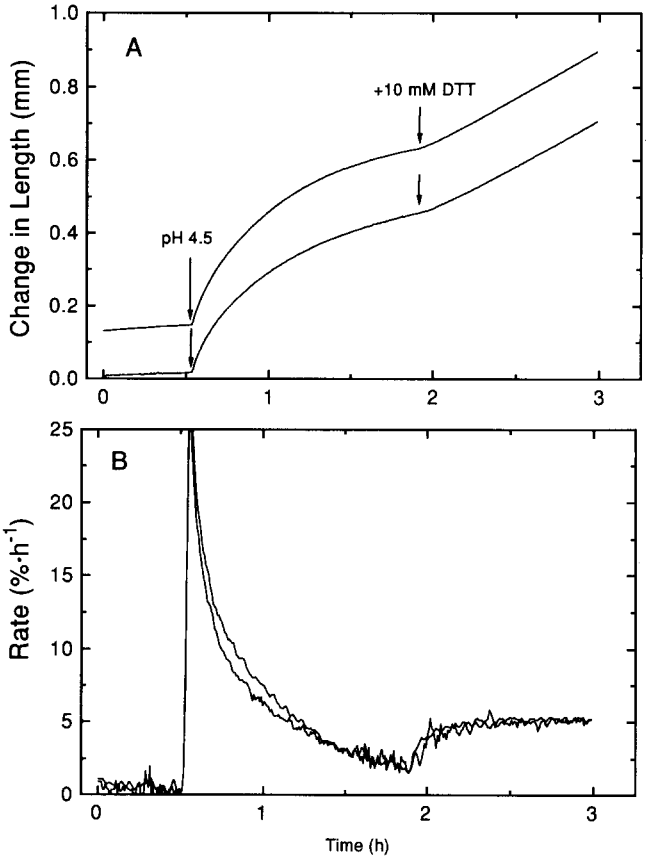

Fig. 2A,B. Effect of DTT addition to acid-induced extension of oat coleoptile walls. Native walls were prepared as in Fig. 1 and the buffer was exchanged for the same buffer containing 10 mM DTT at approximately 80 min after start of acid-induced extension (**A**). The plot of extension rate (**B**) shows that the rate is stable at about 5% per h after addition of DTT. Similar results were obtained in six independent trials

and stabilized it (Fig. 2). This effect is similar to that previously found with cucumber walls (Cosgrove 1989). Inclusion of DTT throughout the extension period resulted in a simpler decay to a constant extension rate (Fig. 3). Pretreatment of the coleoptile wall with boiling water for 15 s to inactivate wall proteins eliminated the acid-induced extension (Fig. 1A), suggesting that extension of these walls may be due to a protein-mediated reaction. These observations are consistent with previous reports of acid-induced extension in oat and cucumber walls (Tepfer and Cleland 1979; Cleland et al. 1987; Cosgrove 1989).

To identify the hypothetical proteins responsible for wall extension, we used 1 M NaCl to extract proteins from cell walls of etiolated oat coleoptiles. Proteins in this crude extract were precipitated with ammonium sulfate, desalted, and fractionated on a DEAE-Sephadex anion-exchange column. The unbound proteins passing through this column possessed the ability to induce extension in heat-inactivated walls from oat coleoptiles (Fig. 4A). Moreover, this active fraction also induced extension of heat-inactivated cucumber hypocotyl walls and pea epicotyl walls (Fig. 4A). This result was surprising because earlier results led us to expect poor cross-reactivity between extension proteins and walls from dicotyledons and grass coleoptiles (McQueen-Mason et al. 1992).

To pursue this point further, we tested the ability of cucumber expansins to induce extension of oat coleoptile walls. As shown in Fig. 4B, they indeed induced extension in oat coleoptile walls, but with less effectiveness than when assayed with cucumber walls. This last point is quantified in Fig. 5A, which compares responsiveness of cucumber and oat walls to a partially purified cucumber expansin fraction. The maximal response of cucumber walls was about three times higher than that of oat walls. To obtain the same extension response in oat walls as was elicited in cucumber walls by 2 $\mu g \cdot mL^{-1}$ of cucumber protein, ten times as much protein was required. Figure 5B shows that cucumber walls were similarly more sensitive to the active oat extract than were oat walls. The protein-induced extension was stabilized by inclusion of 1–10 mM DTT in the incubation buffer (data not shown). We also confirmed that cucumber expansins caused very little extension of barley coleoptiles (data not shown), as reported previously (McQueen-Mason et al. 1992). Thus,

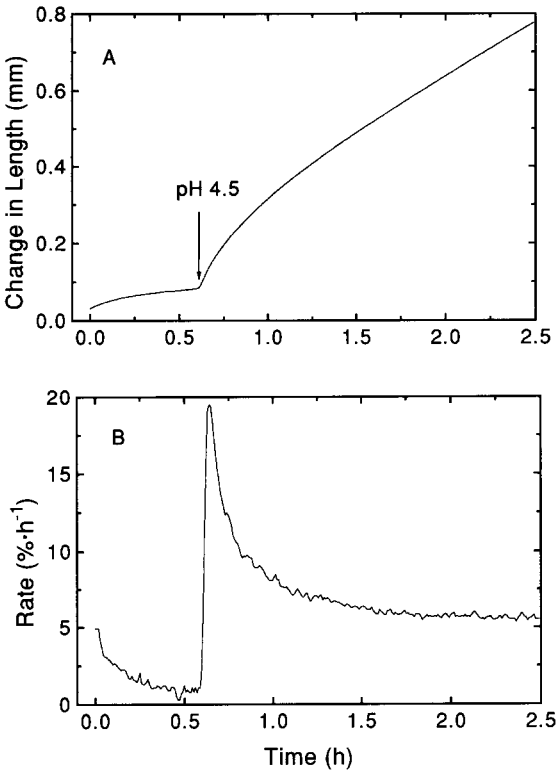

Fig. 3A,B. Acid-induced extension in native oat coleoptile walls in the continuous presence of DTT. **A** Walls were prepared as in Fig. 1 and incubated in 50 mM Hepes, pH 6.8, with 2 mM DTT; after about 35 min the buffer was switched to 50 mM sodium acetate, pH 4.5, with 2 mM DTT. **B** The plot of extension rate shows that the decay in extension rate after 1 h is greatly diminished (compare with Fig. 1). This experiment was repeated four times with similar results

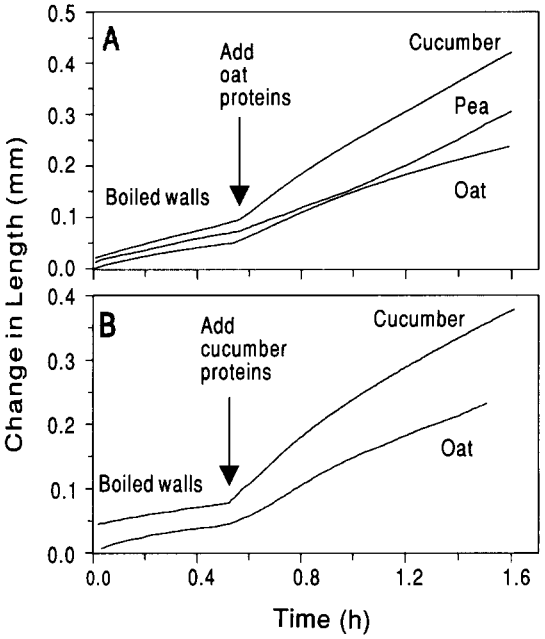

Fig. 4A,B. Extension induced in oat, cucumber and pea walls by proteins extracted from oat coleoptiles walls (**A**) and cucumber walls (**B**). Coleoptile wall proteins and cucumber wall proteins were extracted with 1 M NaCl, precipitated with ammonium sulfate, desalted and partially purified on a DEAE-Sephadex column. Heat-inactivated cell walls from oat coleoptiles, cucumber hypocotyls or pea epicotyls were clamped at 20 g tension in 50 mM sodium acetate, pH 4.5. After about 0.5 h, the solution was replaced with 0.4 mL of the same buffer containing about 10 µg proteins partially purified from oat or cucumber cell walls by DEAE-anion-exchange chromatography. The data represent a typical experiment from at least four replicates

we conclude that oat coleoptile walls are substantially more responsive in these reconstitution assays than barley coleoptile walls (McQueen-Mason et al. 1992), but less responsive than cucumber walls.

Despite the quantitative differences, these results show that oat and cucumber proteins can induce qualitatively similar extension responses in oat and cucumber walls. We thus infer that a similar biochemical mechanism is involved in acid-induced wall expansion in grasses as in dicotyledons.

One might expect that extraction of frozen/thawed coleoptiles with 1 M NaCl might remove most or all of the acid-extension response. Figure 6 shows that overnight extraction of coleoptiles indeed diminished their acid-extension response. This loss could be due to extraction of the expansins; alternatively, the walls or their proteins may have been modified in some other way so that they lost the acid-extension response. Cucumber walls were previously found to lose their native extension response when they were pre-incubated in neutral pH (Cosgrove 1989). Further work will be needed to differentiate between these and other possible explanations.

To assay the pH dependence of the extractable expansin activity from oats, we used cucumber hypocotyl walls as the "substrate" for measuring the extension activity of oat proteins. Cucumber walls were used because they were easier to prepare, broke less often, had lower baseline extension rates, and proved to be a more sensitive substrate for extension assays than did the walls from oat coleoptiles. The active oat fractions from the DEAE-column had a pH optimum between 4.5 and 5.0 (Fig. 7). At pH 3.5 or 5.5, the activity was reduced by about 50%. In contrast, cucumber expansins displayed a broader pH optimum, with high activity maintained at pH 3.5 (McQueen-Mason et al. 1992).

When the active proteins from the DEAE-column were passed through a ConA column, the majority of the extension activity did not bind to the lectin column, suggesting that the activity was not associated with glucosyl- or mannosyl-glycoproteins. When the active fractions which passed through the ConA column were further fractionated by HPLC on a carboxymethyl cation-exchange column, extension activity was eluted as a single major peak at about 15 min (Fig. 8). The activity of this protein fraction had an acid optimum similar to that shown in Fig. 7 (data not shown). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis revealed that a protein with molecular mass of about 29 kDa was associated with this active fraction (Fig. 9). We designate this protein as oat expansin-29 (oat Ex29).

A summary of the purification steps for the oat Ex29 is shown in Table 1. This protein was purified sequential-

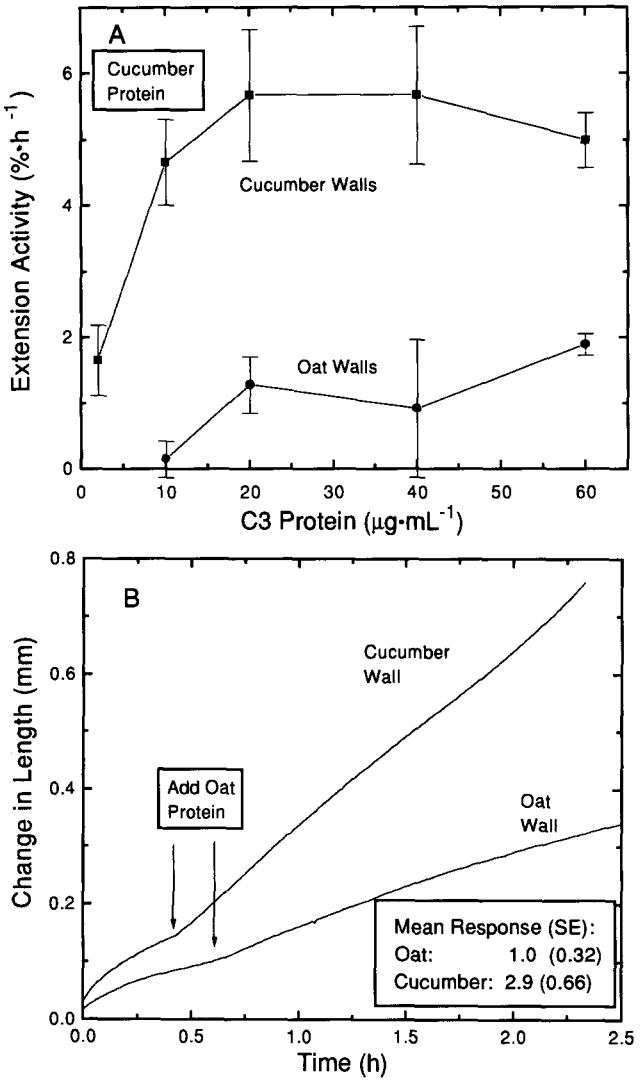

Fig. 5A,B. Comparative responsiveness of oat and cucumber walls to exogenously added oat or cucumber proteins. **A** Concentration dependence of cucumber and oat walls to exogenous cucumber protein. Cucumber walls were frozen, thawed, abraded, heat-inactivated and loaded in the extensometer, as in Fig. 1. Oat coleoptiles were treated similarly, except that the epidermis was stripped with fine forceps and the coleoptile bisected longitudinally prior to freezing. The walls extended for approximately 30 min in 50 mM sodium acetate, pH 4.5, before the buffer was exchanged for the same one containing various concentrations of partially purified cucumber expansins (the active fraction from the C3 HPLC separation; see McQueen-Mason et al. 1992). Extension activity is calculated as the increase in extension rate in the first 15–20 min after addition of C3 protein and is expressed as % increase in length per h. The average extension rate prior to addition of protein in these experiments was 2% per h for oat walls and 1.7% per h for cucumber walls. **B** Response of oat and cucumber walls to added oat protein. Heat-inactivated oat and cucumber walls were prepared as in **A** and extended in 50 mM sodium acetate, pH 4.5, for about 30 min prior to exchange of buffer for the same one containing 73 μg of crude oat coleoptile protein (ammonium sulfate precipitate) per sample holder (about 400 μL). The extension curves shown illustrate two examples with responses close to the mean (2.8%·h^{-1} increase for the cucumber wall and 1.1%·h^{-1} increase for the oat wall). The mean responses ($n=4$) of oat and cucumber walls to added oat protein are shown in the *inset* and are calculated as the increase in extension rate, expressed as % per h. The baseline extension rates prior to addition of protein averaged 2.4% per h (oats) and 1.1% per h (cucumber)

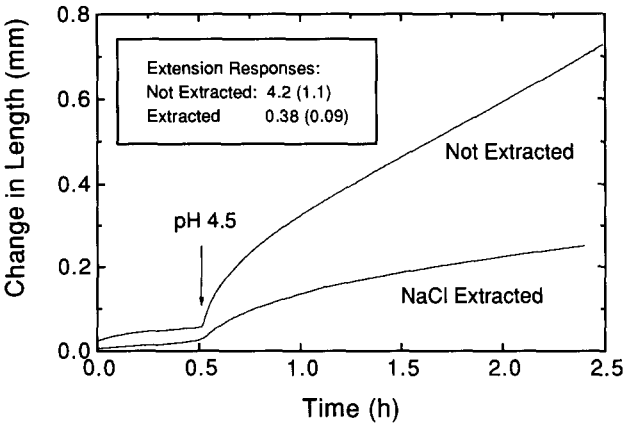

Fig. 6. Effect of 1 M NaCl extraction on acid-induced extension of oat coleoptile walls. Coleoptiles were abraded prior to freezing, then either directly clamped in the extensometer or extracted for 16 h in 1 M NaCl containing 10 mM Hepes, pH 6.8, 3 mM EDTA and 2 mM sodium bisulfite prior to measurement. Walls were rinsed briefly in water, clamped at 20 g tension, then incubated in 50 mM Hepes, pH 6.8, with 2 mM DTT. At about 30 min the buffer was exchanged for 50 mM sodium acetate, pH 4.5, with 2 mM DTT. The curves shown are representative of eight trials for each treatment. The *inset* shows the average extension response (SE, $n=8$) of each treatment. Response is calculated as the rate at 1.5 to 2 h minus the rate prior to pH 4.5 buffer, and is expressed as % increase in length per h

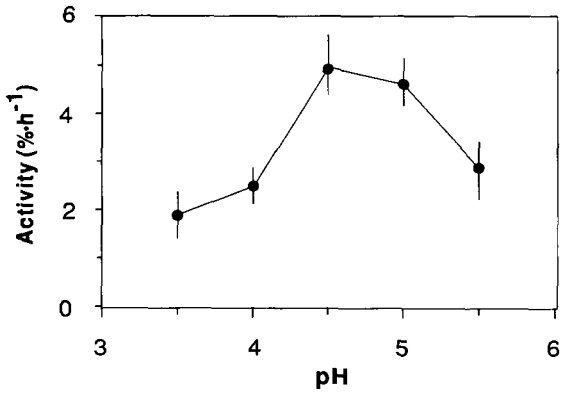

Fig. 7. pH dependence of the extension activity reconstituted by oat wall proteins. Heat-inactivated walls from cucumber hypocotyls were clamped in an extensometer as in Fig. 1 and placed in 50 mM sodium acetate buffers at pH 3.5, 4.0, 4.5, 5.0 or 5.5. After 20 min, the solutions were replaced with 0.4 mL of the corresponding buffer containing 10 μg oat-coleoptile wall proteins partially purified by DEAE-chromatography. The extension activity was calculated by subtracting the baseline rate without proteins from the linear rate (5–30 min) after the addition of the proteins, and expressed as % increase in length per h above the baseline rate. The data represent the means ± SE ($n=5$ to 8). The average baseline rate prior to addition of protein ranged between 2.52 and 2.96 μm·min^{-1} (or 3.0–3.55% per h) for all pH groups

ly by ammonium sulfate precipitation, DEAE-chromatography, ConA affinity chromatography and CM cation-exchange chromatography. If the extension activity in ammonium-sulfate-precipitated proteins is taken as 100%, a purification of 51-fold with 69% yield was obtained after two steps of ion-exchange chromatography.

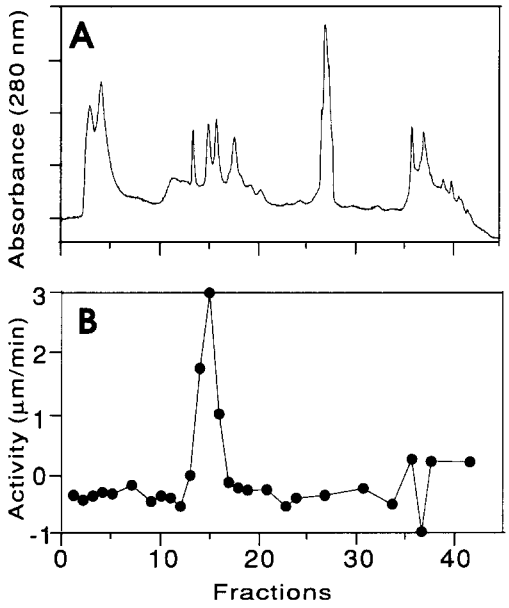

Fig. 8A,B. Purification of oat expansin by HPLC on a carboxymethyl (CM) cation-exchange column. **A** Elution of proteins (absorbance at 280 nm) from a CM-column. Proteins were solubilized from the cell walls of etiolated coleoptiles by 1 M NaCl and then sequentially fractionated by ammonium sulfate precipitation, DEAE-chromatography, and ConA chromatography, prior to CM-HPLC. **B** Wall extension activity of HPLC fractions. The extension activity was assayed by addition of fraction samples (equal volumes, typically 20 μL) to sample cuvettes containing 400 μL of 50 mM sodium acetate buffer, pH 4.5. Activity is expressed as increase in extension rate after addition of protein to a 5-mm, heat-inactivated, abraded oat coleoptile. A single peak of activity eluted at about 15 min or 3.5% of 1 M NaCl. Similar results were obtained in six independent trials

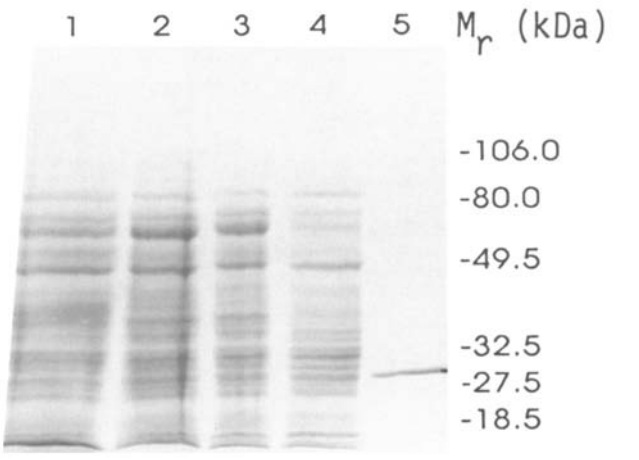

Fig. 9. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of proteins from different purification steps. Active protein fractions were separated on an SDS-PAGE gradient gel (4–20%) and stained with Coomassie Blue R-250. *Lane 1*, 1 M NaCl extraction (20 μg); *lane 2*, ammonium sulfate precipitate (20 μg); *lane 3*, active DEAE-fraction (10 μg); *lane 4*, proteins passing through ConA column (5 μg); *lane 5*, active fraction from CM-HPLC (0.3 μg). The protein with apparent molecular mass of about 29 kDa was designated as oat expansin (oat Ex29). Similar results were obtained in five trials, except in some of these an additional protein band at about 35 kDa appeared in lane 5

Because ConA affinity chromatography did not markedly purify Ex29, this step was often omitted without appreciable effect on the purification.

To examine whether expansin activity was also present in the protoplasm of oat coleoptiles, we fractionated the soluble protoplasmic proteins (all proteins in the coleoptile homogenate not bound to the wall) by ammonium sulfate precipitation and DEAE-column chromatography. Little or no activity was detected in any fractions (data not shown), suggesting that the responsible protein was bound to the cell walls of oat coleoptiles.

Because oat Ex29 was capable of catalyzing extension of walls from cucumber, we wished to test whether it is immunologically related to the 29-kDa cucumber expansin (McQueen-Mason et al. 1992). Figure 10 shows that the oat Ex29 was specifically recognized by an antibody raised against cucumber Ex29. Pre-immune serum under the same conditions did not label Ex29 (not shown). Little signal was detected in the soluble protoplasmic fraction of oat coleoptiles, which is consistent with our other evidence that the Ex29 is a wall-bound protein. When equal amounts of crude and purifed wall protein were assayed by Western analysis, the purified protein gave a much greater signal (Fig. 10). These results demonstrate that the extension-inducing activity co-purifies with the protein that is antigenically related to cucumber Ex29.

Discussion

Our results show that oat coleoptile walls possess a protein that can mediate acid-induced extension of grass

Table 1. Purification of oat Ex29 from etiolated oat coleoptiles. Activity was assayed as described in Fig. 4 and expressed as the initial increase in the extension rate of isolated cucumber cell walls upon addition of the protein fraction (e.g. 5 to 30 min after protein addition). Total activity was calculated by dividing the activity (measured in one to three extension assays) by the fraction of protein used for each assay. Specific activity was calculated by dividing the total activity by the total protein

Purification step	Total protein (μg)	Total activity units (μm/min)	Specific activity (units/mg protein)	Purification (fold)	Yield (%)
$(NH_4)_2SO_4$ precipitation	444	123	278	1	100
DEAE	190	104	546	2	84
CM-HPLC	6	85	14278	51	69

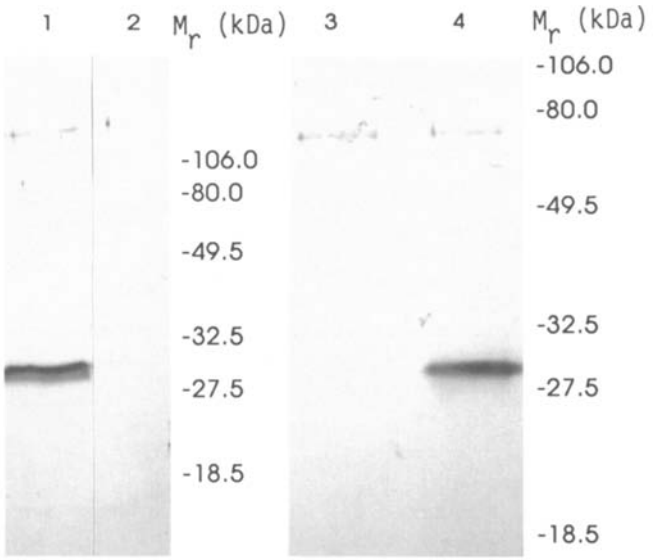

Fig. 10. Western analysis of oat coleoptile proteins probed with antiserum against cucumber Ex29. Comparison of lane 1 (crude wall protein) with lane 2 (crude protoplasmic protein) shows that the antiserum recognizes an Ex29-like protein (*arrow*) specifically bound to the coleoptile cell wall. Comparison of lane 3 (crude wall protein) with lane 4 (purified protein with extension activity) shows that the Ex29-like protein co-purifies with the extension activity. Methods: *Lane 1* was loaded with 15 μg of crude wall protein (ammonium sulfate precipitate of 1 M NaCl extract). *Lane 2* had 15 μg of the soluble protoplasmic protein fraction. These samples were separated on the same 4–20% gradient SDS polyacrylamide gel, blotted onto nitrocellulose, and probed with rabbit antiserum against cucumber Ex29. *Lane 3* was loaded with 0.2 μg of crude wall protein. *Lane 4* had 0.2 μg of active oat wall-extension protein purified sequentially by DEAE-Sephadex and CM-HPLC. These samples (3–4) were separated on the same 14% SDS polyacrylamide gel, blotted onto nitrocellulose, and probed with antiserum. This assay, with minor variations, was carried out four times with similar results

coleoptile walls and dicotyledonous walls. This protein resembles the cucumber 29-kDa expansin of McQueen-Mason et al. (1992) in that it induces extension at acid pH but not at neutral pH, its activity is stabilized by dithiothreitol, it has a similar size as judged by SDS-PAGE, and it is specifically recognized by an antiserum raised against the cucumber protein. Because of these similarities between the oat and cucumber expansins, we propose that expansins are evolutionarily conserved proteins that underlie at least part of the acid-extension response common to the walls of many plant species.

The similarity between oat and cucumber expansins surprised us because McQueen-Mason et al. (1992) found that cucumber expansins caused little extension of maize coleoptile walls and no extension of barley coleoptile walls. We confirmed that barley walls were unresponsive to cucumber expansins, but our positive results with oat coleoptiles show that this insensitivity is not a general property of grass coleoptiles. We do not know why barley walls failed to extend in the presence of cucumber or oat expansins.

The similarity between oat and cucumber expansins is also surprising because the matrix components of the wall are believed to be important for wall loosening and extension, yet these components are quite different for grass walls and dicotyledons (Bacic et al. 1988; Carpita and Gibeaut 1993). The major matrix polysaccharides of the coleoptile wall are (1->3, 1->4)-β-D-glucans and arabinoxylans whereas dicotyledons contain principally xyloglucans and pectins. Dicotyledonous walls contain hydroxyproline-rich glycoproteins of the extensin family whereas grass walls contain much lower amounts of such proteins (Cassab and Varner 1988). It may be that expansins interact directly with wall components common to both types of wall (e.g. cellulose or a minor matrix component) or that they can act on different glycans with similar functions, e.g. xyloglucans and (1->3, 1->4)-β-D-glucans.

Although oat Ex29 can induce an acid-dependent extension in coleoptile walls, there are notable differences between the native and reconstituted acid-extension responses in coleoptiles. First, the acid response of native walls includes a large, but transient, burst in extension, which is mostly decayed away by 60–90 min. This burst is largely lacking in Ex29-reconstituted extensions, which resemble more closely the steady extensions which outlast the transient (e.g. Figs. 1, 3). Second, the pH dependence of reconstituted extension (Fig. 7) does not exactly match the pH dependence reported for acid-extension responses of isolated coleoptile walls (Rayle and Cleland 1972; Tepfer and Cleland 1979; Cleland et al. 1987). The reconstituted extensions displayed a maximum at pH 4.5 and fell off at lower pH values, whereas acid extensions of native coleoptile walls did not fall off at lower pH values. Third, the maximum extension rate inducible with exogenous Ex29 was substantially less than acid-extension responses of native coleoptile walls (i.e. about $2\% \cdot h^{-1}$ for reconstituted extensions versus $4–5\% \cdot h^{-1}$ for the stable component of native wall extensions or $20–30\% \cdot h^{-1}$ for the immediate response of native walls).

These differences between the reconstituted and native acid-extension responses of coleoptile walls could indicate that oat coleoptiles possess additional acid-extension processes, other than the one mediated by Ex29. On the other hand, these differences might also result from inadequacies in our reconstitution methods. For example, heat inactivation of the coleoptile wall by treatment with boiling water may modify the wall's structure so that it is not as susceptible to Ex29 action. There may also be differences due to poor accessibility of exogenous Ex29 to its site of action in the wall, or with need for ancillary wall enzymes that are inactivated by heat treatment. Hence, we believe that, at this stage, caution is warranted in interpreting the differences between native and reconstituted extensions.

From the characteristics of Ex29-induced extension of coleoptile walls and the above considerations, we propose that oat Ex29 is responsible for at least part of the long-term (> 1 h) acid-induced extension responses of oat coleoptiles. This view is strengthened by the findings that reconstituted wall extension and endogenous wall extension exhibit similar sensitivities to biochemical activators and inhibitors (McQueen-Mason et al. 1992). In cucumber, exogenous expansins can restore extension rates as

high or higher than the long-term extension rates in native walls exposed to acid pH. Although we believe there to be good reasons for thinking that expansins mediate the long-term acid-induced extension of isolated walls, their role in the growth of living tissues has not been directly addressed. This assessment will require experiments in which the action of expansins are specifically inhibited or enhanced. Further studies of the biochemical action of expansins and the genes that encode them may provide the tools for this assessment.

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