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Group I allergens of grass pollen as cell wall-loosening agents

(expansin/Lol pI allergen/*Zea mays*/plant cell enlargement/pollen–pistil interactions)

DANIEL J. COSGROVE*†, PATRICIA BEDINGER‡, AND DANIEL M. DURACHKO*

*Department of Biology, 208 Mueller Laboratory, Pennsylvania State University, University Park, PA 16802; and ‡Biology Department, Colorado State University, Fort Collins, CO 80523

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ABSTRACT **Group I allergens are the major allergens of grass pollen, but their biological function is unknown. These proteins are shown here to be structurally related to expansins, which are able to induce extension (creep) of plant cell walls. Extracts of maize pollen possess potent expansin-like activity, as measured in wall extension and wall stress-relaxation assays. This activity is selective for grass cell walls and is, at least partly, due to the action of maize group I allergens. We propose that group I allergens facilitate invasion of the pollen tube into the maternal tissues by loosening the cell walls of the grass stigma and style. Additionally, the presence of related mRNAs in vegetative tissues of rice, *Arabidopsis*, and soybean implies that allergen homologs may function to loosen walls in growing vegetative tissues as well.**

Many grasses, such rye grass, Kentucky bluegrass, and orchard grass, release prodigious quantities of wind-dispersed pollen that trigger hayfever, seasonal asthma, and related immune reactions in humans. Up to 25% of adults suffer these allergic responses as a result of inhaling pollen-laden air (1). The major and most widespread allergenic component of grass pollen are the group I allergens (2–4). These allergens are glycoproteins of about 30 kDa that are quickly and profusely released by grass pollen upon hydration; in humans they bind to IgE antibodies to initiate the allergic response. Pollen from grasses contains one or more forms of these allergens, which are named after the source species, e.g., Lol pI is from *Lolium perenne* (rye grass), Ory sI is from *Oryza sativa* (rice), etc. Although the immunological aspects of these allergens, especially Lol pI, have been extensively studied, their biological function in the plant is unknown. Nevertheless, high sequence conservation among homologs in divergent grass species implies that they serve a vital biological function (5, 6).

Recently, Shcherban *et al.* (7) noted that group I pollen allergens have a distant sequence similarity to expansins. Expansins are extracellular proteins that promote plant cell wall enlargement, evidently by disrupting noncovalent bonding between cellulose microfibrils and matrix polymers (8, 9). Here we report that the group I pollen allergens are indeed structurally and functionally related to expansins and that they and their vegetative homologs comprise a second family of expansins.

MATERIALS AND METHODS

Protein Structure Analysis. Dot plots were calculated with Antheprot (10), using the unity matrix, a window size of 15, and a similarity threshold of 10. Secondary structure predictions were made with the program PHD through its mail server (11). Hydrophobic cluster analysis used the program PCHCA (B. Boutherin, S. Lavaitte, B. Henrissat; Centre de Recherches sur les Macromolécules Végétales, Centre National de la Recherche Scientifique, Grenoble, France) to make the initial two-dimensional map and standard techniques to identify clusters (12, 13).

Protein Extraction, Purification, and Analysis. Pollen from greenhouse-grown *Zea mays* L. plants was collected daily, sifted to remove debris, and frozen at −80°C. Twenty grams of pollen was thawed, extracted at 4°C for 1 h in 80 ml of 0.125 M $NaCO_3$, and centrifuged to remove pollen debris, and the supernatant was dialyzed against 10 mM sodium acetate, pH 5.5 or 4.5. Before rheology assays, the extract was typically diluted with 4 volumes of 50 mM sodium acetate and adjusted to pH 4.5.

For purification, pollen extract was prepared as above without the dialysis step and desalted on a Bio-Gel P-10 column pre-equilibrated with 10 mM Mes, pH 6.0. The desalted fraction was brought to 60 mM NaCl, and 5 ml (typically 5–10 mg of protein per ml) was loaded onto a 2-ml CM-Sepharose column pre-equilibrated with 60 mM NaCL/10 mM Mes, pH 6.0. Protein was eluted with a pH gradient and salt steps [0–10 min: isocratic in 60 mM NaCl/10 mM Mes, pH 6.0; 10–75 min: continuous gradient to 60 mM NaCl/10 mM Hepes, pH 8.5; 75–100 min: NaCl increased in steps to 70, 90, 110, and 220 mM (in 10 mM Hepes, pH 8.5)]. Fractions were desalted on a 10- or 30-kDa Centricon microconcentrator before further testing.

Proteins were quantified colorimetrically with Coomassie Protein Assay Reagent (Pierce) and analyzed by SDS/PAGE (15% gel) and Western blotting using standard procedures (14). Gels were electroblotted onto nitrocellulose membrane and blocked with 10% horse serum in phosphate-buffered saline containing 0.05% Tween 20. To detect expansins, rabbit polyclonal antibody raised against purified cucumber "S1" expansin protein (14) was used at 1,000:1 dilution and subsequently detected using goat anti-rabbit IgG-conjugated alkaline phosphatase. Mouse monoclonal antibody against Lol pI (4) was used at 5,000:1 dilution to detect group I allergens.

Rheology Assays. Maize silks were obtained from greenhouse-grown plants; coleoptiles of wheat (*Triticum aestivum* L., cv. Pennbar) and hypocotyls of cucumber (*Cucumis sativus* L., cv. Burpee Pickler) were obtained from 4- to 5-day-old etiolated seedlings germinated in moist vermiculite (15). For creep reconstitution experiments, 1-cm segments were cut from the apical growing region, frozen at −20°C, thawed, abraded with carborundum slurry, heat-inactivated, and clamped in constant-load extensometers, as described (15). To compensate for the varying thickness of the wall specimens, 5-g weights were used to keep the silk walls under constant tension, whereas 20-g weights were used for the coleoptile and

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Data deposition: The sequences reported in this paper have been deposited in the GenBank database (accession nos. U95967 and U95968).

†To whom reprint requests should be addressed. e-mail: dcosgrove@psu.edu.

hypocotyl walls. For the stress-relaxation measurements, the walls were pretreated for 10 min in either buffer or maize pollen extract and then stored on ice before extension and stress-relaxation measurements (15). Maximal force equivalents for the stress-relaxation assays were 5 g for silks and 20 g for coleoptile and hypocotyls.

RESULTS AND DISCUSSION

When the GenBank and SwissProt Protein Sequence databases were searched (Sept. 12, 1996) using the BLAST and FASTA programs (16, 17), the only protein sequences with significant similarity to expansins were the group I pollen allergens and their homologs. Dot plots and sequence alignments show that expansins (hereafter called α-expansins) and group I allergens have short regions of conservation distributed throughout most of the protein backbone (Fig. 1*A*); these consist, notably, of five stretches of 15 amino acids with 40–53% identity (identified with the numbers 1–5 in Fig. 1*A*). The domains conserved between the specific combination of α-expansin Cs-EXP1 and the pollen allergen Lol pI (Fig. 1*A*) are also highly conserved within both groups of proteins. Likewise, both groups of proteins have hydrophobic signal peptides at the amino termini, characteristic of secreted proteins. Overall, the proteins share only 20–25% amino acid identity.

Despite this low sequence similarity, about 75% of the two proteins are predicted to have the same secondary structure, consisting mostly of loop regions (≈60%), with a small proportion of β strand (≈25%) and α-helix (≈15%) (Fig. 1*B*). These structural predictions were made with the PHD program (11, 18), using 8 aligned group I allergens to predict the allergen structure and 11 aligned α-expansin homologs to predict the structure of α-expansin. While the accuracy of this prediction method is said to be better than that of other methods (18), the important point to be made here is not that the predictions closely approximate the true structure of the proteins but rather that the two predicted patterns closely resemble each other, despite the low sequence similarity. Likewise, the structural similarity between α-expansins and group I allergens is supported by hydrophobic cluster analysis (Fig. 1*C*). This method uses a two-dimensional display of amino acids to identify spatial patterns of hydrophobic residues and other motifs that correspond to secondary structure elements and is useful for recognizing related proteins with low sequence similarity (12, 13). Hydrophobic cluster analysis indicates that α-expansins and group I allergens are structurally congruent throughout most of their protein backbones. Six conserved cysteines can be identified, suggesting a common pattern of disulfide bond formation and protein folding. An additional cysteine pair that is strictly conserved in the α-expansins (Cys-105 and Cys-119 in Cs-EXP1) is missing in the pollen allergens. From the foregoing observations and similarities, we hypothesized that group I allergens might have α-expansin-like biochemical activities.

Notwithstanding these structural similarities, α-expansins and group I allergens have notable differences in certain properties, suggesting divergent biological functions. α-Expansin proteins are found in low abundance even in rapidly growing tissues in which they are specifically expressed; they are not readily soluble in solutions of low ionic strength, are not glycosylated, and are tightly bound to cell walls (9, 19, 20). In contrast, group I allergens are found in high abundance in pollen, are highly soluble in dilute solutions, are glycosylated, and apparently do not bind tightly to the pollen wall (21, 22). These differences suggested to us that the function of the group I allergens may be to loosen the cell walls of the stigma and style to allow penetration of the pollen tube through these tissues. The grass pollen tube grows by tip growth to force its way between the tightly appressed cell walls of the stigma before entering the stylar track, where growth of the pollen tube involves further intrusive growth through and between cell walls (23). Secretion of cell wall-loosening agents with expansin-like properties would presumably aid invasion of the pollen tube into the maternal tissues.

To test this idea, we extracted protein from maize (*Zea mays*) pollen, which contains the group I allergen Zea mI (6, 24), and assayed its effects on the wall rheology of maize silks, which are the receptive stigmas and styles of the maize flower. Maize was used for these experiments because it is easy to collect large quantities of maize pollen and because the large size of the maize silk facilitates rheological assays. For these assays, silk walls were prepared so as to inactivate endogenous proteins, and they were then clamped either at constant force to measure extension behavior or at constant extension to measure stress-relaxation behavior (8, 15). Addition of the maize pollen extract induced rapid, irreversible extension (creep) of the silk walls when tested in constant-force extensometers (Fig. 2*A*). Likewise, the pollen extract enhanced stress relaxation of the silk walls over a large range of times (Fig. 2*B*). Both of these rheological effects are unique characteristics of expansin action (9, 19, 20). Moreover, these rheological effects required an acidic pH (<5.5), likewise similar to the action of expansins. These results demonstrate that maize pollen can release a potent expansin-like activity. They also give direct support to suggestions that proteins secreted by pollen may alter the walls of receptive tissues (25–27).

Despite its expansin-like activity and the limited amino acid similarity between the allergens and α-expansins, the pollen extract did not contain proteins recognized by anti-expansin antibodies (Fig. 3*B*). These antibodies recognize α-expansins of both dicot and monocot origin (14, 28, 29). Other properties also belie the possibility of a cryptic presence of an α-expansin in the pollen extract. The pollen activity was readily soluble in solutions of low ionic strength, whereas higher salt concentrations are needed to extract and maintain solubility of α-expansins. Concentrations of NaCl greater than 200 mM strongly inhibited the creep activity of the pollen extract, whereas at least 2-fold higher concentrations were required to inhibit α-expansin activity. Microcrystaline cellulose (Avicel, 10 mg/ml) depletes α-expansin solutions of creep activity by binding α-expansins and removing them from solution (9, 19), but this was not possible with the pollen activity. We conclude, therefore, that the maize pollen extract does not contain a classical α-expansin protein.

Consistent with previous work (6), the pollen extract did contain Zea mI, a group I allergen recognized by antibodies raised against the rye grass pollen allergen Lol pI (Fig. 3*C*). The pollen extract was fractionated on a CM-Sepharose column, and fractions were assayed by immunoblotting, SDS-PAGE, and wall extension assays (Fig. 3 *D–G*). Fractions testing positive for group I allergens by immunoblotting possessed significant wall extension activity, whereas fractions testing negative in the immunoblot assay lacked expansin-like wall extension activity. A fraction highly purified for Zea mI (Fig. 3 *E* and *F*) tested positive in the wall extension assay (Fig. 3*G*). Therefore, we conclude that Zea mI possesses expansin-like wall-loosening activity.

Late-eluting fractions (i.e., at 80–95 min in Fig. 3*D*) also contained isoforms of Zea mI and exhibited potent creep activity (not shown), but they also contained additional proteins. Some pollen fractions caused sudden wall breakage (unlike expansins) or acted synergistically when added to pure Zea mI fractions (data not shown); these fractions may contain pectate lyases or other wall degradative enzymes (26, 27).

Further work showed that the maize pollen extract was more effective as a wall-loosening agent with grass cell walls than with dicot cell walls. For example, the pollen extract had a marked effect on the creep (extension) and stress relaxation of

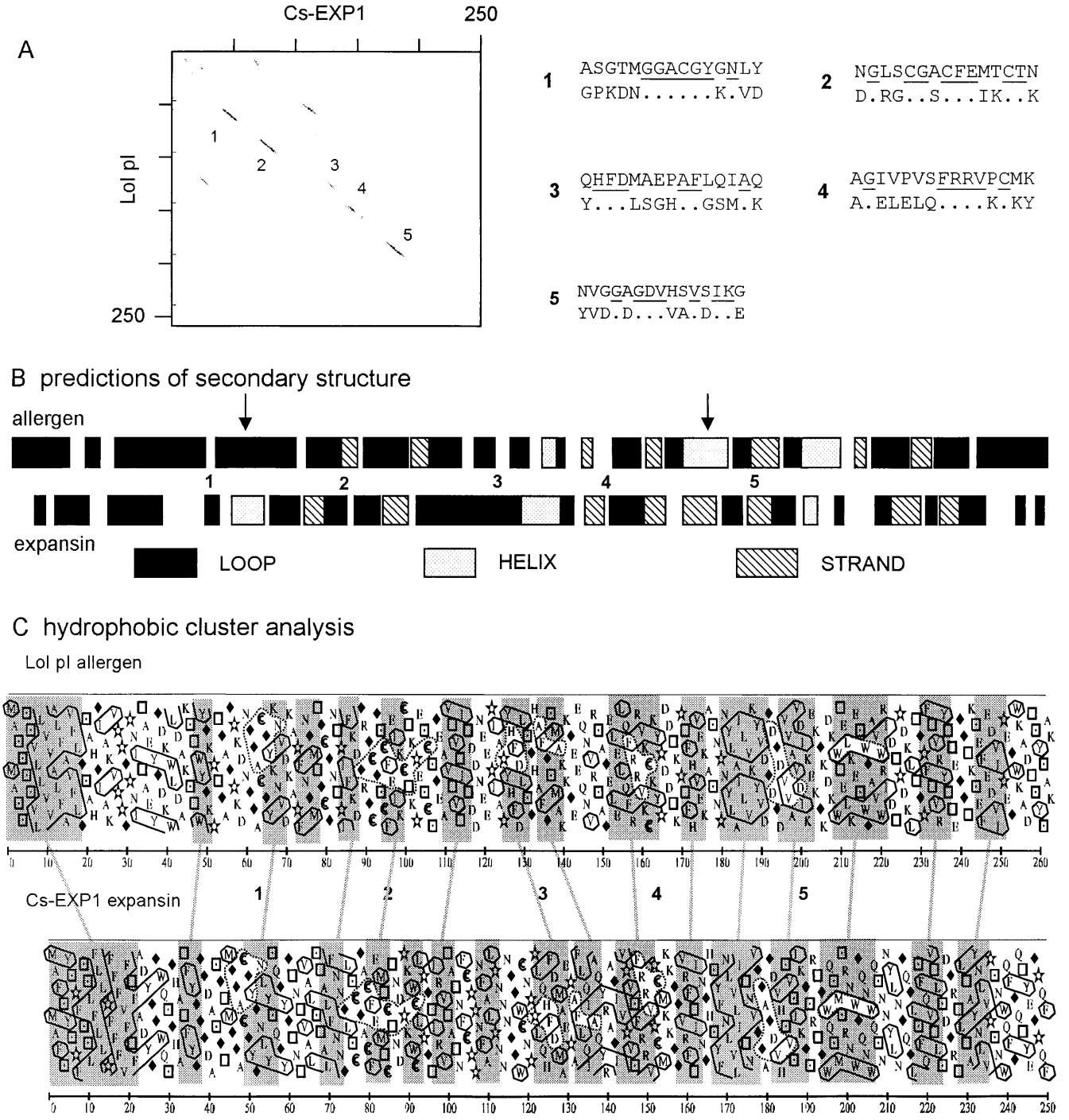

FIG. 1. Structural comparisons of expansins and group I allergens. (*A*) Dot plot of Cs-EXP1 (GenBank accession no. U30382) with Lol pI (GenBank accession no. X57678) shows limited, but distributed, sequence similarity. Alignments of the most conserved regions (regions 1–5) are shown at the right. Conserved amino acids are underlined in the upper line (Cs-EXP1) and represented by a period in the lower line (Lol pI). (*B*) Secondary structure predictions for expansins and allergens show close similarity between these two groups of proteins. Regions of the protein with a PHDsec score of <7 are shown as open blocks. Arrows mark two notable disagreements in the predicted structures. The expansin prediction was based on GenBank sequences U30460, U30476, U30477, U30478, U30479, U30480, U30381, U30382, X85187, Y07782, and U85246. The allergen prediction was based on GenBank sequences U31771, M57474, U03860, L14271, X78813, Z27084, A31060, and Z27090. Signal peptides were removed from the sequences before analysis. (*C*) Hydrophobic cluster analysis of the allergen Lol pI and expansin Cs-EXP1 indicates good concordance between the two proteins. Shaded boxes demarcate putative homologous hydrophobic clusters. Domains of high sequence similarity are outlined with dotted lines and were used as "landmarks" to identify homologous clusters. The conserved regions in *A* are also indicated in *B* and *C* (regions 1–5). The conserved cysteines are found in dotted regions 1, 2, and 4.

coleoptile walls from young grass seedlings (Fig. 2 *C* and *D*), but its rheological effects on hypocotyl walls from cucumber seedlings were small. At the same concentration that proved very effective on grass walls (i.e., at 1:4 dilution), the pollen extract had a barely detectable effect on wall creep and stress relaxation of cucumber walls (data not shown). Even at 5-fold higher concentration (i.e., undiluted pollen extract), the activity seen using cucumber walls was only about one-quarter the activity found using the 20% extract on grass walls (Fig. 2 *E* and *F*).

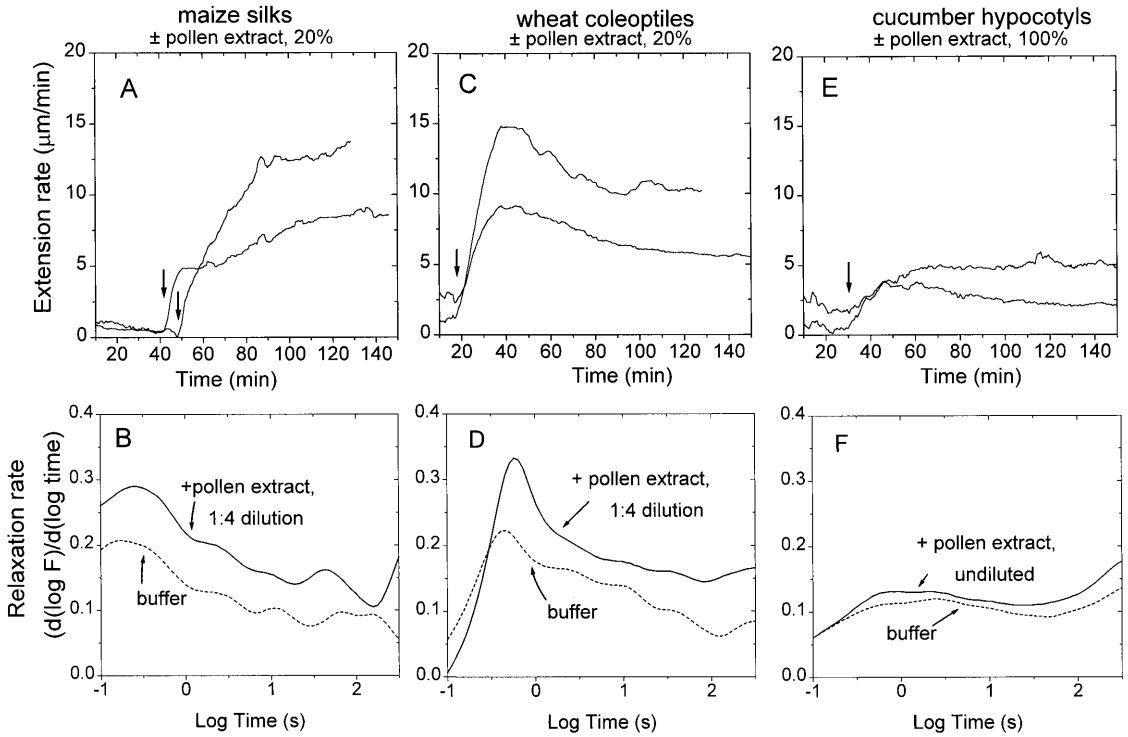

FIG. 2. Enhancement of cell wall extension (*A*, *C*, and *E*) and stress relaxation (*B*, *D*, and *F*) by maize pollen extract. *A* and *B* show rheology responses of maize silk walls to pollen extract diluted to 20% strength (1:4 dilution with 50 mM acetate buffer, pH 4.5). *C* and *D* show responses of wheat coleoptile walls to 20% pollen extract. *E* and *F* show the modest responses of cucumber hypocotyl walls to undiluted (100%) pollen extract. For the extension assays, heat-inactivated wall specimens were clamped in a constant-load extensometer in 50 mM sodium acetate buffer, pH 4.5; wall extension (creep) was detected by a position transducer attached to one of the clamps and is plotted as extension rate (9, 15). At the time indicated by the arrow, the buffer surrounding the wall specimen was exchanged for a similar one containing maize pollen extract. Extension traces show two representative results from four to eight replicates. For the stress-relaxation assays, heat-inactivated walls were preincubated in buffer ± pollen extract and then clamped in an extensometer, extended to a predetermined load, and held at constant length during the subsequent relaxation (15) either in 50 mM acetate buffer (dotted lines) or the same buffer containing maize pollen extract at the dilution indicated. The decay in stress is plotted as a relaxation spectrum (log-time derivative of stress). Each relaxation curve is the average of six to nine independent relaxation measurements.

This selectivity for grass walls complements the action of α-expansins, which appear to induce creep more effectively in dicot walls than in grass coleoptile walls (9). Even though α-expansins are found in grass coleoptiles (7), they proved more effective on dicot walls than on grass coleoptile walls, at least as assayed by reconstitution assays of wall creep (14). Similar, though less extreme, results were found in creep reconstitution assays with wall specimens from maize roots (29) and rice internodes (30). In this context, it is notable that grass walls are unusual in composition, being relatively poor in pectins and xyloglucans and rich in glucuronoarabinoxylans and $(1 \rightarrow 3)$, $(1 \rightarrow 4)$-β-D-glucans, when compared with walls of other angiosperms, including other monocots (31). It seems likely that α-expansins and Zea mI act on different components of the wall, which may differ in abundance and in their role in wall mechanics in dicots versus grasses.

Additional insight into the functional significance of the group I allergens and their homologs may be gained from analysis of the protein and DNA databases. Group I allergens have been identified in the pollen of many grass species (32) but not in pollen of species outside the grass family, including ragweed and other species that elicit potent pollen allergies. Neither have they been identified in monocots outside the grass family. We tested pollen extracts from petunia (a dicot) and lily (a monocot but not a grass) for wall extension activity, with negative results. These observations suggest that grasses may be unique in expressing high levels of these wall-loosening proteins in pollen.

An analysis of the rice and *Arabidopsis* cDNA databases shows that expression of this gene family is not limited to pollen. The rice EST (Expressed Sequence Tag) collection (Oct. 21, 1996) currently contains 18 partially sequenced cDNA entries that are close homologs to the group I pollen allergens (e.g., long stretches with 60% identity and 80% similarity at the amino acid between Lol pI and the rice EST homologs). The 18 cDNAs fall into seven distinct sequence classes, represented by GenBank accession nos. D41180, D24261, D46769, D39144, D24972, D40180, and D48180. As they are all expressed in young seedlings without flowers, these cDNAs cannot be from pollen, and so we refer to them as vegetative homologs of the group I allergens. The *Arabidopsis* EST collection (Oct. 21, 1996) currently contains at least one homolog of the pollen allergens (GenBank accession no. Z37641), which is likewise expressed in young seedlings without flowers. Additionally, *cim1*, a cytokinin-induced gene expressed in soybean cell cultures (33), is also a vegetative homolog of the group I allergens. We have sequenced a vegetative homolog of the group I allergens from the rice and *Arabidopsis* EST collections and used these sequences, together with related sequences in GenBank, to construct a phylogenetic tree for α-expansins and group I allergens (Fig. 4). The tree shows two deeply branched families, with the vegetative homologs of the group I allergens occupying a position intermediate between the group I allergens and α-expansins. Because α-expansins and group I allergens have wall-loosening activity, we postulate that the vegetative homologs of the group I allergens likewise possess expansin-like properties.

To test this idea, we attempted to identify the vegetative homologs of group I allergens by Western blotting of wall

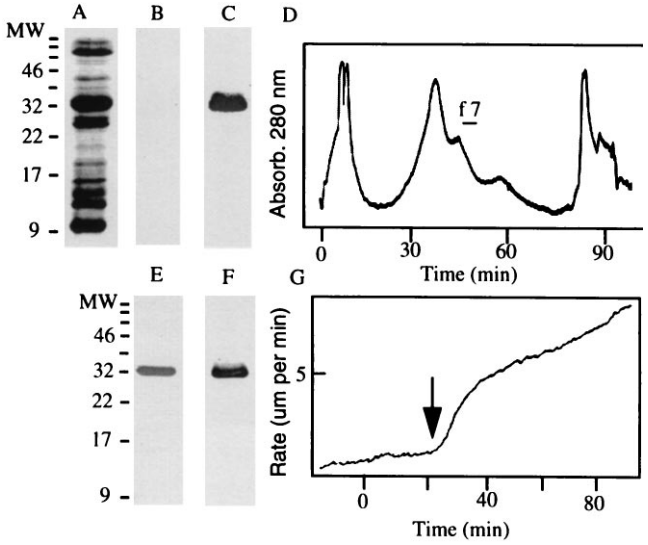

FIG. 3. Identification of Zea mI in maize pollen extracts and its association with wall extension activity. (*A*) Coomassie-stained SDS/polyacrylamide gel of total proteins eluted from maize pollen. (*B*) Western blot of total proteins eluted from maize pollen, using rabbit polyclonal antibodies against cucumber "S1" (Cs-EXP1) expansin (refs. 7 and 14). (*C*) Western blot of total proteins eluted from maize pollen, using monoclonal antibody directed against a Lol pI fragment (site D) (4). (*D*) Fractionation of maize pollen protein on CM-Sepharose. (*E*) Coomassie-stained SDS/polyacrylamide gel of CM-Sepharose fraction F7. (*F*) Western blot of fraction f7 probed with monoclonal antibody against Lol pI. (*G*) Extension curve of heat-inactivated walls of maize silks treated (arrow) with purified Zea mI (fraction f 7) brought to pH 4.5 with sodium acetate buffer.

proteins extracted from grass seedlings, using monoclonal antibody directed against Lol pI, but without success (not shown). This is consistent with previous results (24) and likely indicates that the major antigenic determinants of the group I pollen allergens are not conserved in their vegetative homologs. Our attempts to express recombinant expansins and group I allergens in *Escherichia coli* have so far failed to result in active protein, evidently because of faulty disulfide bond formation (unpublished results of M. Shieh and D.J.C.). Thus, it remains to be seen how the activity of the vegetative homologs of the allergens compares with the pollen allergens and with α-expansins.

The experimental results reported here, as well as the database observations, lead us to propose that the group I grass pollen allergens and their homologs in vegetative tissues constitute a second multigene family of expansins that function as wall-loosening agents in angiosperms. We propose that this family be referred to as β-expansins and that the original family of expansins henceforth be referred to as α-expansins. The two families of expansins exert similar biophysical effects on the wall (i.e., they induce prolonged creep and stress relaxation in a pH-dependent manner), but apparently they interact with different components of the wall. The limited sequence similarity between these two families of expansins gives obvious targets for future studies of active sites and functional domains in these proteins.

In the grasses, the group I pollen allergens represent a subset of the β-expansin family that appears to have assumed a specialized role during pollination, a role, we suggest, for wall loosening of the maternal tissues for rapid pollen tube penetration. This idea is directly supported by our results, which show that these proteins have potent rheological effects on the walls of the grass stigma and style, where they are naturally released in abundance by the grass pollen. An additional possibility is that group I allergens are involved in pollen grain germination or in loosening of the wall at the tip of the pollen tube, where surface expansion occurs.

Determination of the *in vivo* functions of β-expansins in vegetative tissues will require further work. A potential wall-loosening role for the β-expansin cim1 is consistent with induction of its expression by cytokinin (33), which stimulates cell proliferation and growth in soybean cell cultures. The large number of distinct β-expansins expressed in rice seedlings suggests that β-expansins have assumed multiple roles in grass seedling development, perhaps as agents controlling different types of cell growth, wall dissolution and separation, or other processes where wall pliancy is important.

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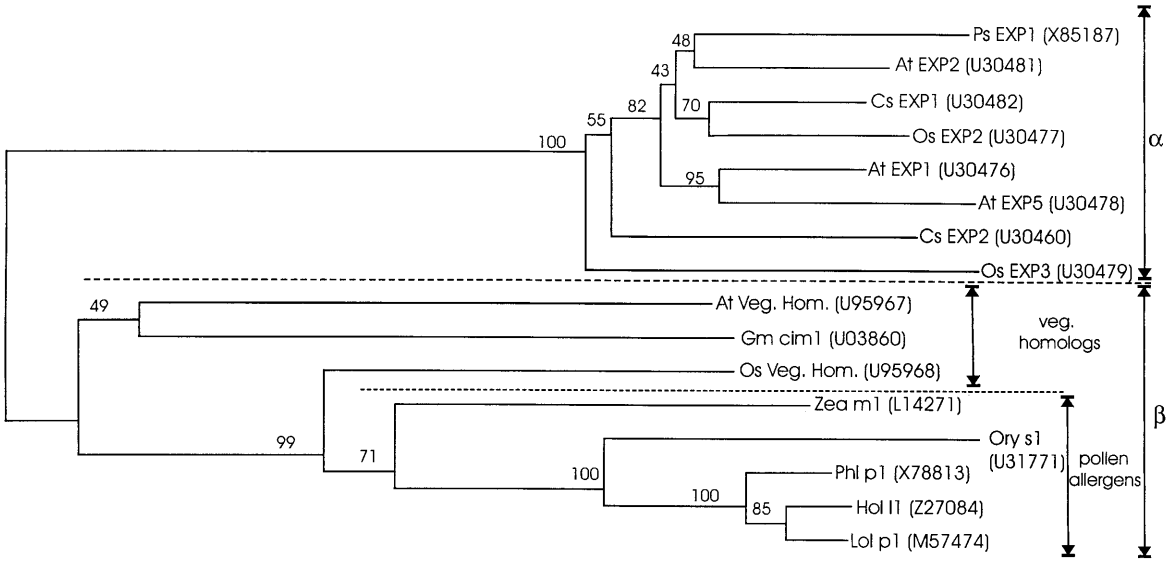

FIG. 4. Phylogenetic tree of α-expansins, group I allergens, and their vegetative homologs. Protein sequences were aligned using the CLUSTAL program with PAM250 weight table and the tree was constructed by bootstrap analysis (1,000 replications) using nearest neighboring joining of the Poisson-corrected values for amino acid differences, using the MEGA phylogenetic analysis program (S. Kumar, K. Tamura, and M. Nei, Institute for Molecular Evolutionary Genetics, Pennsylvania State University). The numbers on the tree indicate the bootstrap *P* values. GenBank accession numbers are also indicated for each sequence.

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