

Matrix solubilization and cell wall weakening by β -expansin (group-1 allergen) from maize pollen

Akira Tabuchi, Lian-Chao Li and Daniel J. Cosgrove*

Department of Biology, 208 Mueller Laboratory, Pennsylvania State University, University Park, PA 16802, USA

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*For correspondence (fax +1 814 865 9131; e-mail dcosgrove@psu.edu).

SUMMARY

Beta-expansins accumulate to high levels in grass pollen, a feature apparently unique to grasses. These proteins, which are major human allergens, facilitate pollen tube penetration of the maize stigma and style (the silk). Here we report that treatment of maize silk cell walls with purified β -expansin from maize pollen led to solubilization of wall matrix polysaccharides, dominated by feruloyated highly substituted glucuronoarabinoxylan (60%) and homogalacturonan (35%). Such action was selective for cell walls of grasses, and indicated a target preferentially found in grass cell walls, probably the highly substituted glucuronoarabinoxylan. Several tests for lytic activities by β -expansin were negative and polysaccharide solubilization had weak temperature dependence, which indicated a non-enzymatic process. Concomitant with matrix solubilization, β -expansin treatment induced creep, reduced the breaking force and increased the plastic compliance of wall specimens. From comparisons of the pH dependencies of these processes, we conclude that matrix solubilization was linked closely to changes in wall plasticity and breaking force, but not so closely coupled to cell wall creep. Because matrix solubilization and increased wall plasticity have not been found with other expansins, we infer that these novel activities are linked to the specialized role of grass pollen β -expansins in promotion of penetration of the pollen tube through the stigma and style, most likely by weakening the middle lamella.

Keywords: β -expansin, pollen, cell wall, arabinoxylan, group-1 grass pollen allergen, homogalacturonan.

INTRODUCTION

Expansins are plant cell wall-loosening proteins classified by sequence relatedness into two major groups, α -expansins (EXPA) and β -expansins (EXPB), and both encoded by multigene families in land plants (Sampedro and Cosgrove, 2005). The biological functions of α -expansins include cell enlargement, fruit softening and abscission (Cosgrove, 2000; Li *et al.*, 2003b; Belfield *et al.*, 2005; Shin *et al.*, 2005), whereas β -expansin functions are not yet well established because functional studies have been mostly limited to grass pollen (Broadwater *et al.*, 1993; Li *et al.*, 2003a; Valdivia *et al.*, 2007b, 2009) and to soybean suspension cultures, where a β -expansin transcript is regulated by cytokinin (Downes and Crowell, 1998; Downes *et al.*, 2001).

Genomic comparisons indicate that the β -expansin family has enlarged selectively during evolution of the grass lineage (Sampedro *et al.*, 2005), perhaps linked to the distinctive polymer composition of grass cell walls, which are enriched in feruloyated arabinoxylans at the expense of pectins and xyloglucan (Carpita, 1996; Gibeaut *et al.*, 2005). The arabinoxylans in grasses consist of a backbone of β -1,4-linked xylopyranosyl residues with varying degrees of

arabinosyl substitution at the C2 or C3 position and sometimes with glucuronic acid residue substitution at the xylosyl-C2 position, in which case they are often called glucuronoarabinoxylans, or GAXs. The arabinosyl residues may be esterified with phenolic acids (mostly ferulic acid), which may become oxidatively coupled to form diferulate cross-links between xylan chains (Buanafina, 2009). Beta-expansins were found to selectively loosen cell walls of grasses (Cosgrove *et al.*, 1997; Li *et al.*, 2003a) and to bind xylans preferentially over other hemicelluloses (Yennawar *et al.*, 2006), leading to the suggestion that arabinoxylans are key targets of β -expansin action in the cell walls of grasses.

Our view of β -expansin activity is based primarily, and perhaps narrowly, on an unusual subset of β -expansins that evolved specifically in the grass lineage and that are expressed abundantly in grass pollen. These proteins elicit hay fever and seasonal asthma in humans (Ball *et al.*, 2005) and consequently are known in the immunological literature as group-1 grass pollen allergens. In maize pollen they are present in several isoforms (Li *et al.*, 2003a; Petersen *et al.*,

2006) and are named 'Zea m 1' by allergen nomenclature or EXPB by expansin nomenclature. They are readily extracted from grass pollen, purified in milligram quantities, stable in solution, and form crystals suitable for X-ray diffraction analysis of protein structure (Yennawar *et al.*, 2006). Beta-expansins expressed in maize pollen are encoded by multiple genes (Valdivia *et al.*, 2007a). Genetic disruption of *ZmEXPB1* – the most abundantly expressed of these genes – impaired the pollen's ability to penetrate maize silks and to compete with wild-type pollen for reproductive success (Valdivia *et al.*, 2007b, 2009). To effect fertilization, a maize pollen grain is first captured by a multicellular trichome on the exposed surfaces of the silk. Upon germination, the emergent pollen tube pushes its way between cells of the trichome and then 'tunnels' up to 20 cm through the transmitting tract tissue to reach the ovule, whereupon the sperm cells are released (Heslop-Harrison *et al.*, 1984; Dresselhaus *et al.*, 2011). Pollen tube movement through this pathway involves deforming cell walls and forcing its way through the middle lamella of many densely-packed cells along the transmitting tract. The high expression of pollen β -expansins and their ability to cause creep of grass walls *in vitro*, combined with the results cited above, have been taken as evidence that maize pollen β -expansins function to loosen cell walls of the stigma and style.

Wall loosening by expansins involves an unusual and still enigmatic mechanism. Contrary to expectations for a 'wall-loosening enzyme', no *bona fide* lytic activity has been found for either α - or β -expansins (McQueen-Mason *et al.*, 1992, 1993; McQueen-Mason and Cosgrove, 1995; Li and Cosgrove, 2001; Yennawar *et al.*, 2006). In a comparative study of the wall-loosening actions of an α -expansin and a fungal endoglucanase, Yuan *et al.* (2001) found that α -expansin induced wall creep within seconds but did not affect wall mechanical compliances, whereas the endoglucanase exhibited a lag of many minutes before wall creep began and it greatly increased elastic and plastic compliances, which indicated substantial modification of cell wall structure, e.g. permanent breakage of cell wall cross-links. The different biophysical effects for these two wall-modifying proteins point to different biochemical mechanisms of action with distinct consequences for wall structure and biomechanical properties.

The strength and rheology (flow properties) of primary cell walls is based on a scaffold of cellulose microfibrils embedded in a matrix of complex polysaccharides that may bind to cellulose surfaces and form covalent and noncovalent networks among themselves (Cosgrove, 2005; Harris and Stone, 2008). Wall loosening by expansin is hypothesized to occur by transient dissociation of matrix-cellulose junctions, resulting in creep or reptation of polysaccharides in the cellulose-matrix network when it is stretched with sufficient force, either by turgor pressure in living cells or by clamping forces in isolated walls (McQueen-Mason and

Cosgrove, 1994; Yennawar *et al.*, 2006). Expansin action results characteristically in stress relaxation and irreversible extension (creep) of the primary cell wall.

In this study we report remarkable biochemical and biophysical changes in isolated grass cell walls treated with β -expansin from maize pollen. The results contrast greatly with the actions of α -expansins and yield new insights into the molecular means by which β -expansins promote pollen tube penetration of the maize stigma and style.

RESULTS

Pollen β -expansin (PBE) solubilizes matrix polysaccharides

We made use of a highly-purified β -expansin fraction (named 'PBE') from maize pollen to investigate its loosening effects on cell walls of the maize silk and wheat coleoptile. PBE consists of approximately 70% EXPB1 (Zea m 1d) and approximately 30% EXPB11 (Zea m 1c) (Li *et al.*, 2003a). As PBE selectively loosens grass cell walls (Li *et al.*, 2003a) and as cell walls of grasses and other so-called commelinid monocots are distinguished by their high content of arabinoxylans esterified with phenolic residues, primarily ferulic acid (Harris and Hartley, 1976; Kato and Nevins, 1984b; Carpita, 1996; Carpita *et al.*, 2001; Harris and Trethewey, 2010), our initial tests focused on this component of the cell wall. Phenolics are readily detected by their ultraviolet (UV) absorption. To test for release of wall-bound phenolics, we incubated de-proteinated cell walls from maize silks with buffer $\pm$ 250 μ g ml⁻¹ PBE at 25°C and measured 320-nm absorbance (A320) of the incubation solution after 46 h. Some A320 release was detected with buffer alone, whereas nearly four times this level was obtained with PBE treatment, in parallel with release of sugar-containing material (Figure 1a). As additional negative controls we tested bovine serum albumin (BSA) and lysozyme (Lys), both of which lack expansin activity. Lysozyme was used because, like the maize β -expansins, it is a small, basic protein. Neither of these proteins promoted A320 or saccharide release from the wall. Likewise, a crude α -expansin preparation with strong wall-extension activity (McQueen-Mason *et al.*, 1992) did not solubilize wall materials above buffer controls (Table S1). The results indicated an activity specific to β -expansin.

When coleoptile walls from maize or wheat were used instead of maize silk walls, a similar release of A320 and saccharide was detected after PBE treatment (not shown). In contrast, much smaller release of wall materials, approximately 10% of that occurring with maize silk walls (Table S2), was caused by PBE treatment of cell walls from a diverse set of non-grass species, including cucumber hypocotyls, onion bulbs, red beetroots, celery petioles and asparagus stalks. Onion is a monocot, but not a commelinid, with a wall composition similar to typical dicots (Mankarios *et al.*, 1980). The walls of celery (a dicot) contain mostly

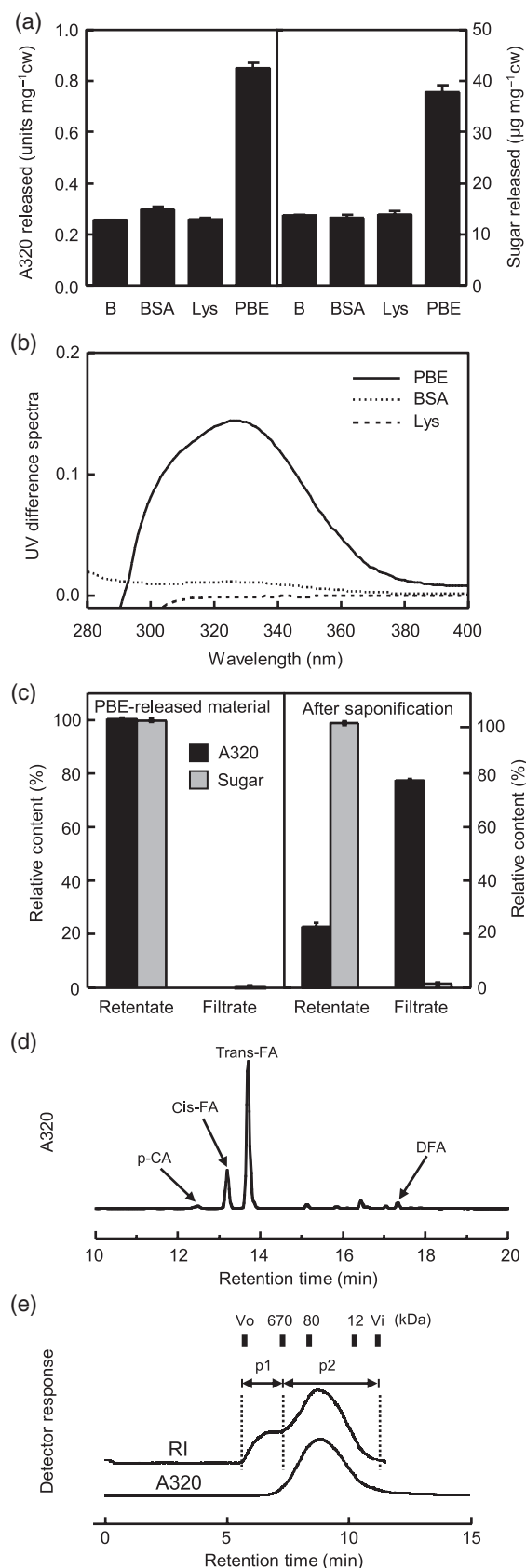


Figure 1. Release of phenolics and saccharides from maize silk walls by pollen β -expansin (PBE).

(a) Release of A320 and saccharides from walls treated with PBE (46-h incubation), compared with negative controls: buffer (B), BSA and lysozyme (Lys). Lysozyme was used because its size and pI are similar to that of pollen β -expansin.

(b) UV absorption spectrum of wall phenolic material released by PBE, compared with negative controls (BSA, Lys). Absorption spectra of corresponding solutions without cell walls were subtracted.

(c) Retention of PBE-solubilized material before (left) and after (right) saponification, using a 10-kDa filter. Values are shown for A320 and total sugars.

(d) Reversed-phase chromatography of saponified phenolics from (c). Arrows identify peaks based on authentic standards: *p*-coumaric acid (CA), *cis*- and *trans*-ferulic acids (FA), and diferulic acid (DFA).

(e) Elution profiles of PBE-solubilized wall polymers, measured by GPC with detection by refractive index (RI) and 320-nm absorbance. The numbers at the top indicate molecular mass based on dextran standards, V_i indicates the retention time for glucose, and V_o was estimated from the beginning position of elution of dextran with an average molecular mass of 2 MDa. (a) and (c) show means $\pm$ standard error (SE) of three replicates; other data are representative of at least three experiments.

pectin and cellulose and little hemicellulose (Thimm *et al.*, 2002). Walls of beetroot (a dicot) are rich in ferulic acids esterified to pectin (Oosterveld *et al.*, 1996), while walls of asparagus (another non-commelinid monocot) also contain ferulate, but the polysaccharide conjugate has not been established. These initial experiments thus uncovered a novel β -expansin activity with selectivity for grass-type cell walls, and further work was carried out to define the solubilized material.

UV spectra of the material released from maize walls showed a broad absorbance from 290 to 360 nm with maximal absorbance at approximately 325 nm (Figure 1b), consistent with ferulic acid. The phenolic material released by PBE in these assays (46 h) was equivalent to approximately 10% of total saponifiable phenolics in these walls. Both the saccharides and the phenolic material released by PBE were retained by a 10-kDa ultrafiltration membrane (Figure 1c). After saponification with 0.1 M NaOH to break ester linkages, most of the phenolics passed through the 10-kDa membrane whereas the saccharides were retained. This behavior is expected of feruloyated arabinoxylan. Analysis of the saponified material by reversed-phase high pressure liquid chromatography (HPLC) showed that 97% of the phenolics co-eluted with authentic ferulic acid (Figure 1d). Small amounts of *p*-coumaric acid and diferulic acid were also detected. We conclude that the 320-nm absorbing moiety in the solubilized material comes from ferulic acid residues esterified to larger molecules retained by the 10-kDa membrane. The rarity of diferulic acid and other multimeric forms of ferulic acid shows that the feruloyated polymer was not oxidatively cross-linked through ferulate.

For further characterization, the size of the released material was analyzed by gel permeation chromatography (GPC) with simultaneous detection by A320 and by refractive index (RI), which detects dissolved substances

nonspecifically. For these and subsequent experiments, the walls were pre-washed with buffer to reduce background; the PBE-induced release was then 5–10-fold above background. The A320 elution profile gave a broad, nearly symmetric peak with an estimated molecular mass distribution of 10–700 kDa (Figure 1e). The peak corresponded to a mass of approximately 60 kDa and matched the largest peak (p2) seen with simultaneous RI detection. The RI trace also revealed a shoulder at larger size (p1), which was absent in the A320 trace. Analysis of total sugars in individual GPC fractions gave an elution profile similar to the RI profile (not shown), which indicated that the RI signal is largely due to polysaccharide. Considering the known composition of maize cell walls, these results suggested that PBE solubilized a feruloyated arabinoxylan or glucuronoarabinoxylan (p2) as well as a second polysaccharide lacking a phenolic moiety (p1). Their physical separation on the GPC column indicates they were not covalently linked or bound to each other in the solubilized form.

We also estimated the molecular size of material in the p1 and p2 fractions with dynamic light scattering (DLS). The estimate for mass-average molecular mass for p2 was 63 kDa, using a dextran model, which is in good agreement with the 60-kDa estimate based on GPC. For p1 the mass-average estimate was 500 kDa, somewhat smaller than the GPC-based estimate (>700 kDa). The number-average DLS estimates for p1 and p2 were 27 and 7.3 kDa, respectively.

As a means to identify the major polysaccharides in p1 and p2, the solubilized material was further analyzed by digestion with specific lytic enzymes followed by GPC to detect reduction in molecular size. Digestion with pectate lyase resulted in the loss of p1, but only a slight change in p2 (Figure 2a). This result indicates that homogalacturonan is a major component of p1. The negligible change in the A320 signal shows that the feruloyated polymer is not linked to the homogalacturonan. Digestion with (1,3),(1,4)- β -glucan endo-4-glucanase caused a slight shift of the elution profile of p2 detected with RI but not with A320 (Figure 2b). This result indicates the presence of a small amount of mixed-linkage glucan in p2, but it is not linked to the feruloyated molecule. Digestion with XynC shifted p2 to much smaller sizes, in both the RI and A320 traces (Figure 2c). XynC is a xylanase requiring a pendant glucuronic acid residue to cleave the neighboring (1–4)- β -D-xylosyl linkage (Kato and Nevins, 1984a; St John *et al.*, 2006). The major rightward shift of p2 indicates it contains a large amount of xylan substituted with glucuronic acid. The fact that the peak in the RI profile shifts further than the peak in the A320 profile may be explained by the fact that smaller xylan fragments lacking ferulate are detected by RI but not A320. Another xylanase, XynM4, that hydrolyzes xylans of low substitution but cannot cut highly substituted xylans, did not change the elution profiles (Figure 2d), which indicated a high degree of substitution of the solubilized

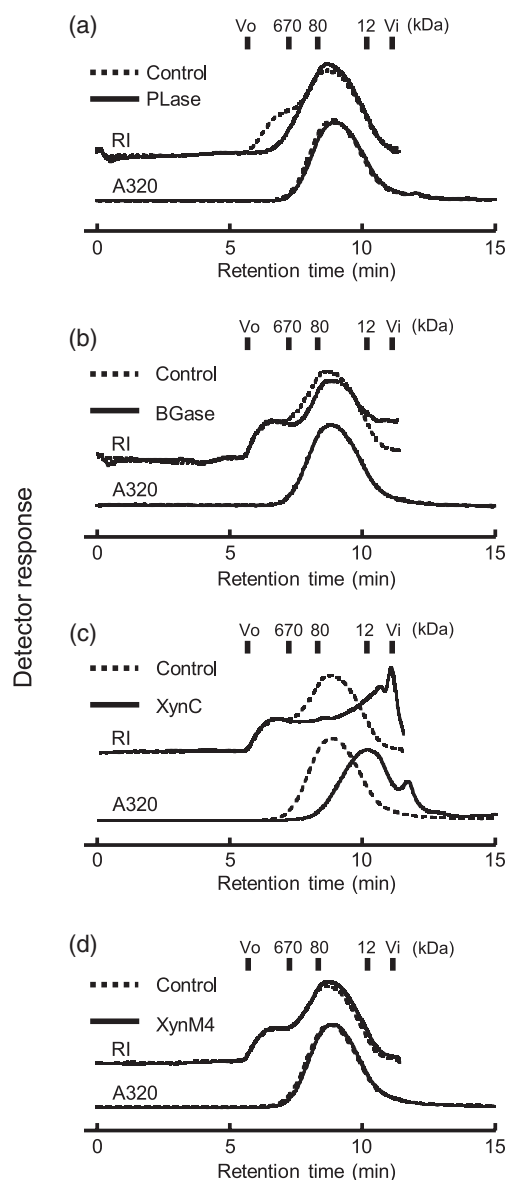


Figure 2. GPC elution profiles of PBE-released wall material digested with specific lytic enzymes.

(a) pectate lyase (PLase), (b) β -glucanase (BGase), (c) xylanase C (XynC), and (d) xylanase M4 (XynM4). Conditions as in Figure 1(e). 'Control' refers to undigested material. Data are representative of at least three replicate experiments. The numbers at the top indicate molecular mass based on dextran standards, Vi indicates the retention time for glucose, and Vo was estimated from the beginning position of elution of dextran with an average molecular mass of 2 MDa.

xylan. These results show that p1, accounting for approximately 16% of the sugars released by PBE, contains homogalacturonan whereas p2, with >80% of the sugars and most of the ferulate, contains a highly substituted feruloyated xylan with glucuronic acid substitution and a small amount of mixed-linkage glucan not linked to the xylan.

Finally, we assessed the polysaccharides in p1 and p2 by sugar composition and linkage analysis (Tables 1 and 2). The most abundant saccharide in p1 was galacturonic acid (75%), followed by arabinose (14%), xylose (9%) and traces of other sugars. This composition is consistent with the results of digestion by pectate lyase and so we conclude that p1 is largely homogalacturonan, with small amounts of arabinoxylan (approximately 15%, likely overlap from p2) and probably arabinan (approximately 5%). The low rhamnose content in p1 is notable because it indicates very little rhamnogalacturonan I (RGI), which is usually cross-linked to homogalacturonan. Low rhamnose content (0.5 Mol %) was also found in the EDTA-extracted pectic fraction of this wall material (Table S3), so the low RGI content appears to be characteristic of the pectic fraction of the maize silk wall rather than cross-link breakage by PBE.

In contrast to p1, the major monosaccharide in p2 was xylose (38%) followed by galacturonic acid (30%), arabinose (26%) and glucose (4%) (Table 1). This composition suggests the dominant polysaccharide (approximately 65%) to be a highly substituted (glucurono)arabinoxylan, with smaller amounts of homogalacturonan (30%) and mixed-linkage glucan (4%). Methylation analysis, to assess the linkage positions of neutral sugars in p2, revealed the following residues, in order of abundance: 3,4-xylosyl, t-arabinosyl, 4-glucosyl, 4-xylosyl, 3-glucosyl, 4- or 5-arabinosyl, and lesser amounts of other sugar linkages (Table 2). Together with the composition analysis and the results of enzyme digestion (particularly XynC digestion), these results indicate that the major polysaccharide in p2 is a

feruloylated, highly substituted glucuronoarabinoxylan (hsGAX) composed of linear (1–4)-linked xylopyranosyl units with arabinofuranosyl side chains at the C3 position. The degree of substitution of the xylan in p2 was estimated to be 67% by the ratio of arabinose to xylose (Table 1) and 80% by the ratio of branched xylose (3,4- and 2,3,4-linked) to total xylose (Table 2). The 67% value may be an underestimate because some of the xylose may come from a small amount of xyloglucan in p2. Glucuronic acid substitution was estimated as 1.6% of the xylosyl residues (Table 1) and feruloylation to be 1 in every 20 xylosyl residues (this estimate factors out galacturonic acid in p2 and uses 75% for the degree of arabinosyl substitution). Thus, a 60-kDa molecule (the mass-average estimate) would have approximately 230 xylosyl residues in its backbone, with approximately 12 ferulate residues and three to four glucuronate residues, on average, whereas a 7.3-kDa molecule (the number-average estimate) would have approximately 28 xylosyl residues in its backbone, with approximately 1 ferulate residue and approximately 0.5 glucuronate residue.

The presence of hsGAX in the PBE-solubilized material was further confirmed with polyclonal antibodies raised against hsGAX or against xylopentaose (xyl₅) (Suzuki *et al.*, 2000). Immunoblots showed the hsGAX epitope to be >10× more abundant in the PBE-solubilized material than the xyl₅ epitope (Figure S1a). We also used the anti-hsGAX antibody to detect the distribution of epitopes in the cross-section of maize silk tissues, to see if it specifically localized to cells in the transmitting tract. Cell walls throughout the cross-section were labeled (Figure S1b), with the strongest

Table 1 Monosaccharide composition analysis of fractions p1 and p2 of pollen β-expansin (PBE)-released material

Fraction	Sugar content (mol %)								
	Ara	Rha	Fuc	Xyl	GlcA	GalA	Man	Gal	Glc
p1	14.3	0.4	nd	8.7	nd	75.0	nd	0.5	1.1
p2	25.7	0.2	nd	38.3	0.6	29.8	nd	1.2	4.1

nd, not detected.

Table 2 Linkage analysis of neutral sugars in the p2 fraction of pollen β-expansin (PBE)-released material

Sugar residue/deduced linkage (mol %)							
Arabinose	t-Araf 21.4	2-Araf 1.0	3-Araf 0.5	4-Araf or 5-Araf 2.4	2,4-Araf or 2,5-Araf 0.4		
Xylose		3,4-Xylp 41.3	4-Xylp 9.0	t-Xylp 1.2	2,3,4-Xylp 0.8		
Galactose							
4-Galp	3-Galp 1.2	t-Galp 1.0	3,4-Galp 0.1	2,4-Galp 0.3	4,6-Galp 0.2	3,6-Galp 1.1	3,4,6-Galp 0.2
Glucose		4-Glcp 10.8	3-Glcp 4.4	t-Glcp 0.5	3,4-Glcp 0.1	6-Glcp 0.3	

labeling in the epidermis and vascular bundle, including the transmitting tract tissue, which is fused to the vascular bundle. Thus, hsGAX is indeed present in the transmitting tract, but is not unique to that tissue.

Mechanism of polysaccharide solubilization by PBE

We carried out a series of experiments to test whether the matrix polysaccharides solubilized by PBE were released by enzymatic lysis or physical dissociation. We first evaluated whether the feruloylated hsGAX is covalently or non-covalently bound to the cell wall. Physical treatments such as hot buffer and chelator extractions released a substantial amount of phenolic material from maize silk walls (Figure S2a), showing that much of the hsGAX is weakly bound to the cell wall. This is consistent with previous work characterizing hsGAX in maize coleoptile walls (Carpita, 1983, 1984, 1989). Moreover, digestions of maize silk walls with pectate lyase or (1,3),(1,4)- β -glucan endo-4-glucanase released sugars but negligible phenolic material (Figure S2c). This result is consistent with the enzyme digestions of PBE-solubilized material (Figure 2). From these results we infer that a substantial amount of feruloylated hsGAX is lightly bound to the maize silk wall and is not covalently linked to homogalacturonan or mixed-linkage glucan. However, 65% of the phenolics solubilized by hot buffer or chelator passed through a 10-kDa membrane (Figure S2b), whereas the phenolics solubilized by PBE were retained by the membrane (Figure 1c). Heat treatment of the PBE-released material did not cause the phenolics to pass through the 10-kDa membrane (not shown), so thermal instability does not explain the size difference. Evidently PBE selectively solubilizes larger feruloylated hsGAX moieties compared with the material solubilized with hot chelator or buffer.

To characterize this selectivity further, we measured the ability of PBE to solubilize polysaccharides from maize silk walls which had been pre-extracted in a series of treatments to progressively remove more tightly bound matrix polymers. Pre-extraction with hot chelator reduced the material released by PBE by 65% compared with that obtained with the unextracted wall (Figure 3). This result shows that most of the material solubilized by PBE is weakly bound to the cell wall and can be extracted with hot chelator. In other words, covalent bond breakage is not required to solubilize this material. Further extraction of the wall with weak alkali (0.1 M NaOH) further reduced the polysaccharide released by PBE to <10% of the value obtained with unextracted wall. Thus the polysaccharide solubilized by PBE is weakly bound to the cell wall. This conclusion is also supported by the moderately high background seen in initial experiments where the walls were not extensively pre-washed before treatment with PBE (see Figure 1a). These results show that a non-enzymatic mechanism of release by PBE is plausible.

On the other hand, wall digestion with xylanase XynC also released hsGAX (Figure S2c), so an enzymatic release by

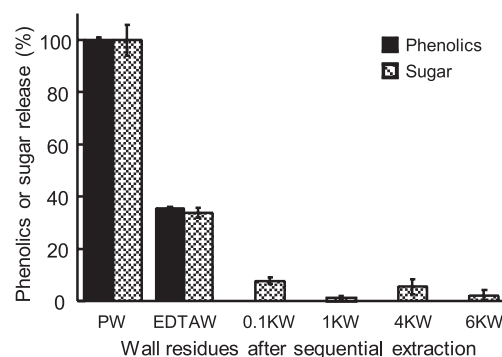


Figure 3. Release of phenolics and saccharides by PBE from maize silk walls sequentially extracted to progressively remove more tightly bound matrix polysaccharides.

PAW-washed walls (PW) sequentially extracted with EDTA, 0.1 M KOH, 1 M KOH, 4 M KOH, and 6 M KOH (designated EDTAW, 0.1KW, 1KW, 4KW, 6KW, respectively). A320 values are only shown for the first two treatments because KOH cleaves ester-linked phenolics. Means $\pm$ standard error (SE) of three replicates.

PBE must be considered. Monosaccharide analysis showed that XynC released GAX with <4% homogalacturonan (Table S4), as compared with approximately 35% homogalacturonan in the PBE-released material (combining p1 and p2 in Table 1). Thus, the two treatments solubilize homogalacturonan to a markedly different extent, suggesting different mechanisms of release.

To test for xylanase activity more directly, we assessed the ability of PBE to lytically degrade maize GAX. For these experiments we collected the high-molecular weight fraction of hsGAX from maize silk walls (i.e. the first to elute from a GPC column), incubated it with 100 $\mu\text{g ml}^{-1}$ PBE for 20 h, and assessed changes in its size distribution by GPC. If PBE possessed xylanase activity, we predicted a shift toward smaller size. We did this for feruloylated hsGAX solubilized by hot chelator and by PBE. As seen in Figure 4, prolonged incubation with PBE did not shift the hsGAX size profile obtained by either solubilization method, whereas XynC (a positive control) caused a large shift to smaller size. Thus, PBE lacked xylanase activity by this assay.

As a further test for lytic activity, we incubated insoluble arabinoxylan (from wheat flour) with PBE and assessed solubilization of saccharides and phenolics (Figure S3). PBE had negligible effect, whereas xylanases XynC and XynM4 (positive controls) caused massive release. We conclude from these experiments that PBE lacks xylanase activity with these substrates, which is consistent with previous results using dye-coupled cross-linked xylan (Yennawar *et al.*, 2006).

We also tested for pectin lytic activity by assessing changes in the size distribution of high-molecular weight pectin, extracted from maize silk walls with hot ethylenediaminetetraacetic acid (EDTA). Using the same protocol described above, we did not detect a shift in the pectin size distribution following prolonged incubation with high con-

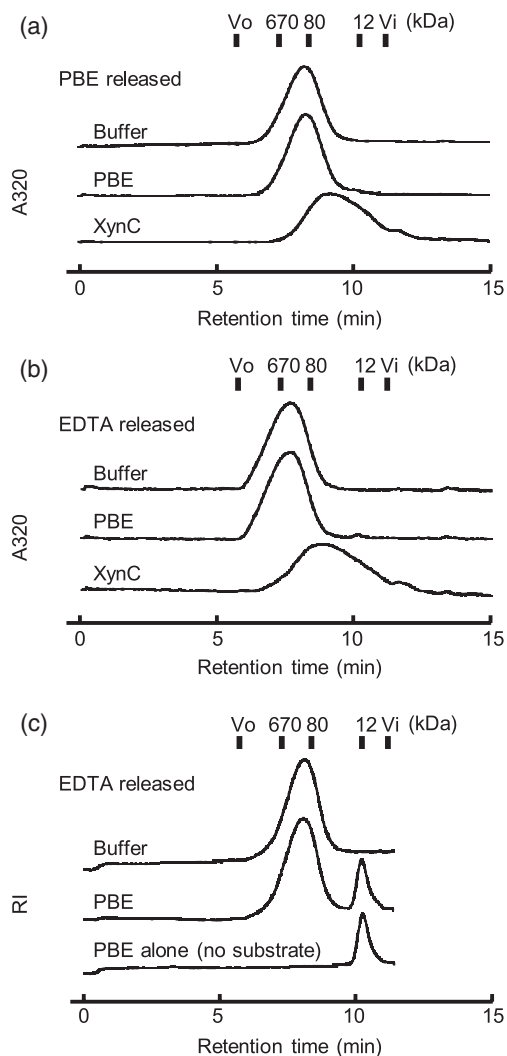


Figure 4. Tests for lytic activity by PBE, based on GPC analysis of high-molecular mass polysaccharides from maize walls. (a) A320 profiles of high-molecular mass fraction of PBE-released polysaccharide either untreated (buffer), incubated for 20 h with $100 \mu\text{g ml}^{-1}$ PBE or $5 \mu\text{g ml}^{-1}$ XynC. (b) As above except the high-molecular mass fraction of EDTA-extracted polysaccharides were used. (c) RI profile of high-molecular mass fraction of EDTA-released material treated with PBE. The majority of the material detected by RI is pectin (homogalacturonan). The numbers at the top indicate molecular mass based on dextran standards, Vi indicates the retention time for glucose, and Vo was estimated from the beginning position of elution of dextran with an average molecular mass of 2 MDa.

centration of PBE (Figure 4c). Other attempts to detect oliguronide release by capillary electrophoresis likewise proved negative, as did assays for lysis of rhamnogalacturonan I, using dye-couple RGI from Megazyme Inc (<http://www.megazyme.com>) (not shown). These results argue against pectolytic activity by PBE.

Finally, taking a more generic approach to detect enzymatic activity, we measured the time-dependence and

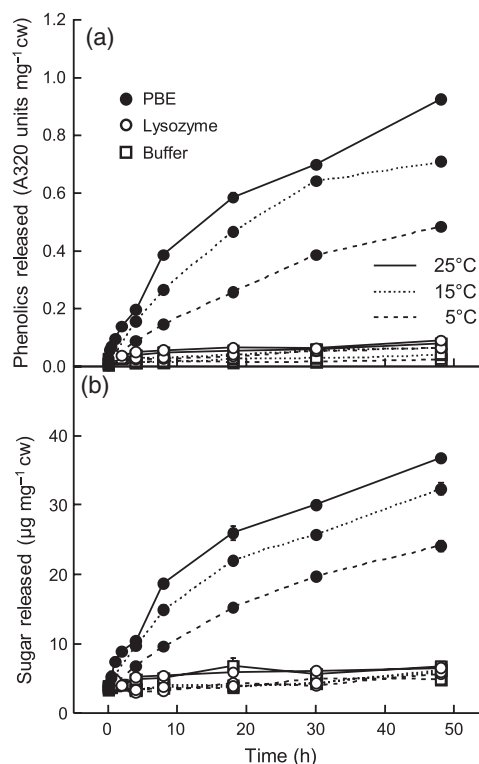


Figure 5. Time course for release of phenolics and saccharides from maize silk walls by $100 \mu\text{g ml}^{-1}$ PBE at different temperatures. Negative controls (buffer, lysozyme) are also shown. Means of three replicates; SEs are smaller than the symbols in most cases.

temperature dependence of matrix solubilization by PBE (Figure 5). At 25°C the release of saccharides and phenolics by PBE increased gradually and in parallel over a 50-h time course, with a rapid release in the first 10 h slowing to a steady rate after 20 h. Reduction of the incubation temperature by 10°C resulted in only a small decline in release and further cooling to 5°C still allowed most of the release to occur. The relative insensitivity of release to temperature argues against an enzymatically limited process, which would typically have a strong dependence on temperature. The temperature dependence is more similar to a diffusion-limited process.

To summarize this section, our tests for the most likely enzymatic activities that might solubilize hsGAX or homogalacturonan did not find evidence for a lytic mechanism, whereas the facts that hsGAX is weakly bound to the silk wall, is solubilized by hot buffer or chelator treatment, and its solubilization by PBE has weak temperature dependence are consistent with a non-enzymatic mechanism of solubilization.

PBE-induced changes in wall mechanics

We next assessed the relationship between PBE-induced solubilization of wall materials and physical changes in cell

walls, employing three biomechanical assays. The theory and interpretations of these methods are detailed elsewhere (Cosgrove, 1993) but may be summarized as follows: (i) In creep assays, wall specimens are clamped at constant force and the long-term, irreversible extension is recorded. This classic assay for expansin activity measures the slow sliding/shearing of the microfibril-matrix network. (ii) The elastic and plastic compliances of a wall specimen are measured by rapidly extending the wall specimen in two cycles. The force-extension curve in the second cycle is reversible and the slope is used to calculate the elastic compliance, while the first curve is the sum of elastic and plastic (or reversible and irreversible) extensions. The plastic compliance is calculated from the difference between the two extensions. To oversimplify a bit, the compliances depend on the density of cell wall cross-links, the 'springiness' of the cross-linking polymers, and the viscous mobility of the matrix polymers that are able to slide in response to applied force. (iii) For breaking force assays, the wall specimen is rapidly extended until it fails, and the maximum force that is generated is taken as the breaking force. This is the most *ad hoc* approach of the three methods and its interpretation requires an understanding of the mechanism of failure.

We measured these three biomechanical parameters as a function of PBE concentration, pH, and time, and in parallel measured release of wall materials to test for correlations. As PBE concentration was increased, more saccharide and phenolics were released and the rate of wall creep likewise increased (Figure 6); however, the shapes of the curves differed in detail. Compared with saccharide and phenolics release, the creep rate curve was steeper at lower concentrations and did not flatten out at higher concentrations. We note that assessment of creep rate at high PBE concentrations was complicated by higher frequencies of wall breakage, preceded by a short period of accelerated wall extension; for this reason the value at the highest concentration tested ($680 \mu\text{g ml}^{-1}$) was deemed unreliable and not calculated. Although these results show a general similarity in the concentration dependence for these processes, the differences in detail suggest that wall creep may not be closely linked to polysaccharide solubilization.

Further evidence on this point was obtained by comparing the pH dependence of the two processes. Wall creep rate gave a maximum value at pH 5.5 and decreased by 60% at pH 4.5 (Figure 7a) whereas the release of saccharide and phenolics from silk walls (Figure 7b) and from coleoptile walls (Figure S4) was nearly the same at these two pH points. Thus, while there is a general correlation between wall creep and release of wall polysaccharides, the two processes are not tightly coupled. Wall creep may not be based on polysaccharides solubilization, but it is affected by it.

In breaking force assays, PBE pretreatment greatly reduced the breaking force for coleoptile specimens, with

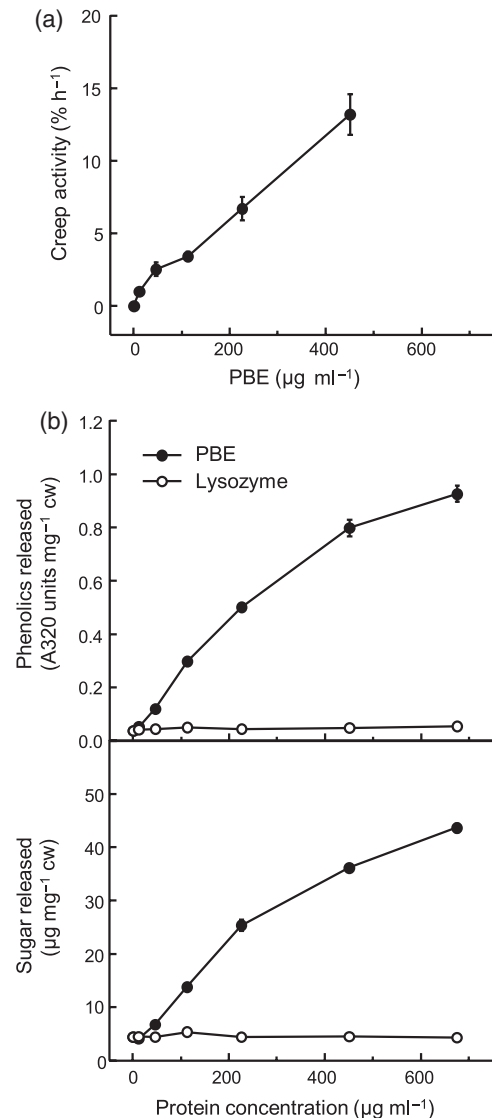


Figure 6. Wall extension and release of phenolics and saccharides as a function of PBE concentration.

(a) Creep rate of coleoptile walls versus PBE concentration. Activity at $675 \mu\text{g ml}^{-1}$ of PBE was omitted because specimen breakage occurred too early to establish a steady creep rate.

(b) Release of phenolics and saccharides from maize walls by increasing PBE concentrations (20 h incubation). Means $\pm$ standard error (SE) of three replicates.

>60% reduction in 5 h, reaching >80% reduction after 50 h (Figure 8c). This remarkable effect depended on PBE concentration (Figure 8a) and showed pH dependence (Figure 7c) very similar to the release of wall material. Comparison with the corresponding curves for matrix polysaccharide solubilization (Figure 8b,d) showed a large decrease in breaking force upon initial solubilization of the matrix, followed by diminishing mechanical effects as more of the matrix was solubilized. Negative controls, in the form of buffer or lysozyme treatments, showed no change in

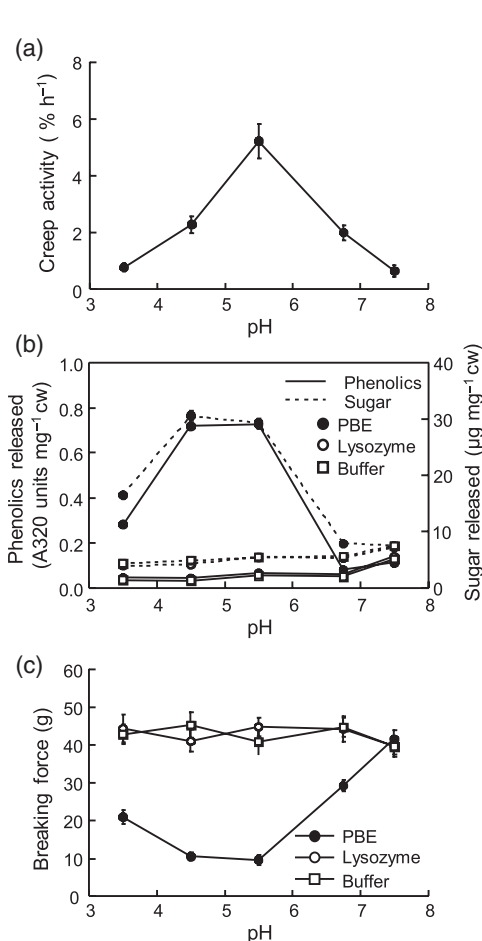


Figure 7. The pH dependence of wall properties and matrix solubilization by PBE.

(a) Wall creep rate for PBE-treated coleoptiles at different pH values. Values were measured 30 min after addition of PBE at 100 μg ml⁻¹.
 (b) Release of phenolics and total sugars from maize walls by PBE (100 μg ml⁻¹) at different pH values (20 h incubation).
 (c) Breaking force of coleoptile specimens after incubation with 100 μg ml⁻¹ PBE for 20 h, at different pH values. Means ± standard error (SE) of three replicates.

breaking force over the pH range from 3.5 to 7.5, which indicated little direct effect of pH over this range. We conclude that the marked reduction in breaking force is closely coupled to matrix solubilization by PBE. In other words, removal of the matrix polysaccharides leads to mechanical failure of the wall specimens at smaller forces. Microscopy observations to be published elsewhere indicate the mechanical failure of PBE-treated walls arises from weakening of the middle lamella.

Turning to wall compliances, PBE treatment increased the plastic compliance, with a time course that paralleled release of polysaccharides from the wall (Figure 9). Remarkably, the wall elastic modulus was not affected by PBE treatment. The increase in plastic compliance had pH dependence (Figure S5) similar to that for matrix solubilization and for

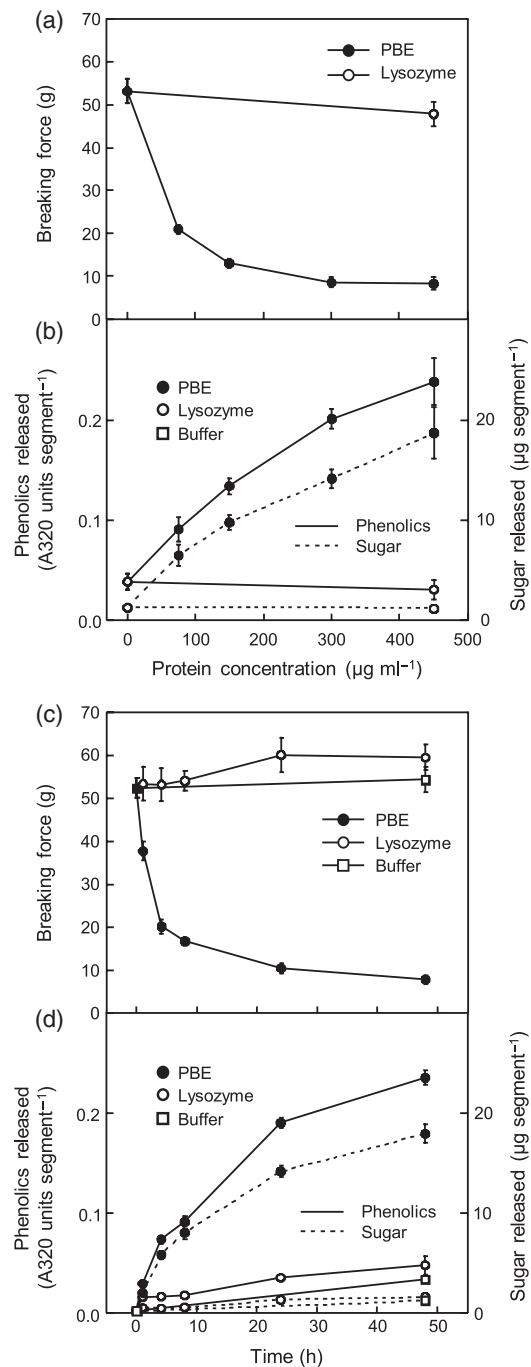


Figure 8. Correlation of breaking force with release of phenolics and saccharides as a function of PBE concentration and time.

(a, b) Coleoptile walls were treated with various amounts of PBE for 20 h at 25°C, followed by measurement of breaking force (a) or release of wall materials (b).
 (c, d) Coleoptile walls were treated with 100 μg of PBE for various times at 25°C, followed by measurement of breaking force (c) or material release (d). Buffer and lysozyme treatments were included for comparison of PBE treatment. Means ± standard error (SE) of three replicates.

breaking force, which indicated the increase in plastic compliance, like the decrease in breaking force, is linked to solubilization of the matrix polysaccharides.

We interpret the results of this section to mean that PBE affects cell wall mechanics by two mechanisms, one mechanism involves rapid induction of cell wall creep, whereas a second mechanism depends on the solubilization of matrix polysaccharides, leading to increased plastic compliance and reduced breaking force. The latter actions may be particularly relevant to the roles of PBE in pollen biology.

DISCUSSION

Grass pollen accumulates large quantities of β -expansin, estimated to comprise 4% of the pollen-extracted protein in maize (Li *et al.*, 2003a). This is vastly more expansin than has been found in rapidly growing vegetative tissues (Sampedro and Cosgrove, 2005) and it seems improbable that its sole target is the growing pollen tube wall, which is a tiny surface at the tip of the cell. On the other hand, such protein abundance would make sense if its target were cell walls of the stigma and style (Cosgrove *et al.*, 1997). Supporting this idea, pollen from maize mutants with reduced amounts of pollen β -expansin were fertile but competed poorly in fertilization assays with wild-type pollen and also exhibited morphological difficulties in entering and penetrating the silk (Valdivia *et al.*, 2007b, 2009).

Our results add biochemical and biomechanical details to the mechanism of wall loosening by pollen β -expansins and reveal a specialized activity not seen with α -expansins or with microbial expansins. PBE selectively solubilized two key matrix polysaccharides (feruloylated highly substituted glucuronoarabinoxylan and homogalacturonan) from de-proteinated maize silk walls and concomitantly weakened cell wall specimens, as measured by breaking force and plastic compliance. In previous analysis of maize leaves, feruloylated glucuronoarabinoxylan and homogalacturonan were identified as cementing substances that bind cells to one another (Ishii, 1984). Thus PBE may have a two-fold action on the cell wall, loosening the cellulose-matrix polysaccharide network, leading to cell wall creep (Li and Cosgrove, 2001; Li *et al.*, 2003a), and solubilizing an hsGAX-homogalacturonan complex in the middle lamella. The latter action might account for the increase in wall plasticity and decrease in breaking force reported here. Additionally, it may account for the high frequency of breakage of wall specimens during PBE-induced creep, noted previously (Li *et al.*, 2003a). Microscopy of PBE-loosened walls indeed confirms that the middle lamella is weakened by PBE (data to be published separately). Solubilization of matrix polysaccharides, particularly those involved in cell adhesion, may aid penetration of grass pollen tubes which must worm their way between tightly packed cells of the stigma and style (Heslop-Harrison *et al.*, 1984), and may also provide an absorbable 'biofuel' to power the pollen tube's long journey to the ovule.

These effects are particularly notable because α -expansins caused neither polysaccharide release (McQueen-Mason and Cosgrove, 1994, 1995) nor changes in wall plasticity (Yuan *et al.*, 2001) in cucumber hypocotyl walls, yet were approximately 10 times more effective at inducing cell wall creep than PBE (cf. McQueen-Mason *et al.*, 1992 with Li *et al.*, 2003a). Likewise the bacterial expansin EXLX1 from *Bacillus subtilis* did not solubilize wall polysaccharides or mechanically weaken coleoptile cell walls, though it did cause (limited) wall creep (Kerff *et al.*, 2008; Georgelis *et al.*, 2011). Thus, the novel effects of PBE reported here are not typical of other types of expansins induced by expansins.

Whether this ancillary wall activity is common to β -expansins in general or is limited to β -expansins from grass pollen is still uncertain because wall-loosening studies of β -expansins have been limited to the grass pollen class up to now. Although genes for 'vegetative' β -expansins are expressed throughout the plant (Lee and Kende, 2001; Wu *et al.*, 2001; Lin *et al.*, 2005), the corresponding proteins have not been obtained in active form, either by extraction from plant tissues or by recombinant protein expression. In vegetative tissues of rice, β -expansins bound so tightly to

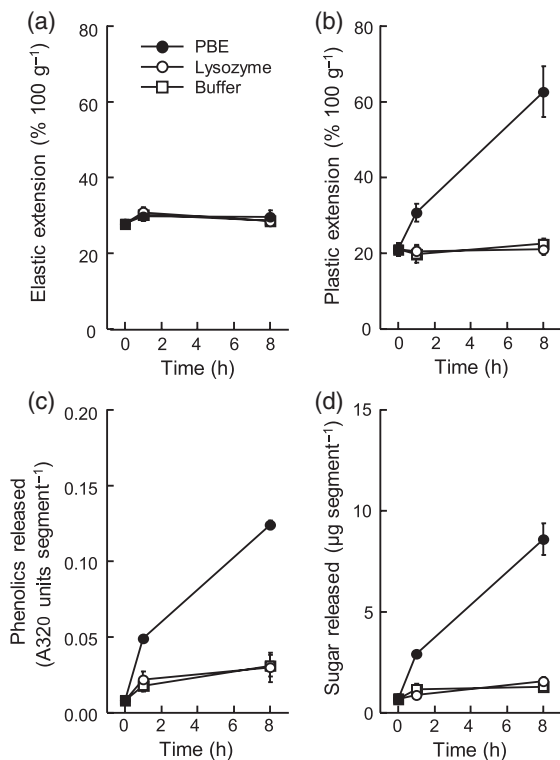


Figure 9. Comparison of times courses for changes in wall compliances with release of wall materials by PBE. At time zero coleoptile walls were incubated in buffer $\pm$ PBE (250 $\mu\text{g ml}^{-1}$) and at subsequent times they were assessed for (a) elastic compliance, (b) plastic compliance, (c) phenolics released, and (d) total saccharide released. Buffer and lysozyme controls were included for comparison. Means $\pm$ standard error (SE) of three replicates.

the cell wall that they were only solubilized under conditions that denatured the proteins (Lee and Choi, 2005). We obtained similar results in maize (L.-C. Li and D. J. Cosgrove, Penn State University, University Park, PA, USA). Thus, the properties and actions of the larger class of vegetative β -expansins may differ from that of the specialized group of β -expansins expressed so abundantly in grass pollen.

Pollen tube penetration of the stigma is also promoted by a xylanase deposited on the maize pollen coat wall (Suen and Huang, 2007). Pollen coat xylanase may work synergistically with pollen β -expansins to solubilize xylans, promoting pollen tube penetration into the stigma. It is not known, however, whether the xylanase cuts highly substituted GAX or prefers GAX with less substitution. The maize pollen xylanase is produced solely by the tapetum, collected onto the pollen coat during pollen maturation and its role in pollination is likely limited to penetration of the stigmatic surface (Bih et al., 1999; Suen et al., 2003). In contrast, pollen β -expansins are synthesized by the pollen and high transcript levels in the germinating pollen point to continued synthesis and secretion of the proteins during the journey of the pollen tube to the ovule (Broadwater et al., 1993; Valdivia et al., 2007a).

The feruloylated glucuronarabinoxylans solubilized by PBE are remarkably similar to hsGAXs characterized in detail from maize coleoptiles and proso millet (Carpita et al., 1985; Carpita and Whittern, 1986). These polymers, which may be peculiar to grass cell walls, may comprise 'the major, pore-determining interstitial material between microfibrils' in maize coleoptiles (Carpita et al., 2001). Our results indicate that hsGAX likely contributes to cell wall mechanics and to the strength of the middle lamella in grasses, although PBE-solubilized homogalacturonan may also contribute. Based on its extraction properties, Carpita et al. (2001) concluded that much of the hsGAX in maize coleoptiles was interconnected by phenolic residues. In contrast, the hsGAX solubilized by PBE from maize silk walls contained only traces of diferulate residues, which indicated negligible ferulate cross-linking. As hsGAXs (lacking ferulate) did not bind appreciably to cellulose *in vitro* (Carpita, 1983), direct tethering of cellulose microfibrils by hsGAX seems unlikely. The mechanical role of feruloylated hsGAX may be mediated through numerous weak interactions with cellulose surfaces or with other matrix polysaccharides that bind more tightly to cellulose.

In other studies (Kato and Nevins, 1984b), feruloylated GAXs were solubilized from maize cell walls by a xylanase similar to the XynC used in our study. This raises the question of whether PBE possesses xylanase activity, either as an activity inherent in β -expansin or as a contaminant from the xylanase found on the maize pollen coat (Suen and Huang, 2007). We discount both of these possibilities for multiple reasons. In developing our PBE purification protocol we specifically monitored xylanase activity from maize pollen extracts and adjusted the purification procedure to separate endogenous xylanase activity from β -expansin activity. PBE

was purified by reversed-phase chromatography using methanol in the mobile phase, which inactivated the pollen xylanase but did not inhibit β -expansin activity (A. Tabuchi, L.-C. Li, D. J. Cosgrove, Penn State University, University Park, PA, USA). Previous attempts to detect xylanase activity with purified β -expansin gave negative results (Yennawar et al., 2006). We extended this inquiry in the current study (e.g. Figure 4) using arabinoxylans from maize silk walls and from wheat flour, likewise with negative results. Thus, we discount the possibility of xylanase action by PBE.

In a similar vein, the concomitant release of homogalacturonan by PBE might suggest a pectin lytic mechanism, with arabinoxylan release being a secondary consequence of pectin lysis. However, we did not detect pectin lytic activity in PBE, nor did we find evidence of stable linkage between homogalacturonan and hsGAX (Figure 2 and Table S4), nor release of feruloylated GAX by treatment of walls with pectate lyase. Within the middle lamella or the cell wall proper, homogalacturonan and hsGAX may be weakly bound to each other by calcium bridges, as evidenced by their concomitant solubilization with calcium chelators. PBE might indirectly promote release of homogalacturonan by reducing hsGAX binding to the wall.

It is notable that the homogalacturonan solubilized by PBE contained little rhamnogalacturonan I, which is usually covalently linked to homogalacturonan (Caffall and Mohnen, 2009). Because EDTA-solubilized pectin of the maize silk wall likewise contained little rhamnose (Table S3), this seems to be a peculiarity of the maize silk wall rather than the result of PBE action. Carpita (1989) suggested that hsGAX in maize coleoptiles might be linked to rhamnogalacturonan I, but we find little evidence of such an association in the wall polysaccharides solubilized by PBE. Pertinent to this point, we note that PBE was unable to hydrolyze rhamnogalacturonan I in the form of dye-coupled cross-linked substrate.

Thus, our results do not support a lytic mechanism of release, a view further supported by the weak temperature dependence for matrix solubilization by PBE. Previous work excluded proteolytic action by PBE (Li and Cosgrove, 2001), as well as lytic activities against various other wall polymers (Yennawar et al., 2006). Our results indicate that PBE targets a wall component that is specific to, or at least enriched in, grass cell walls (cf. the selectivity shown in Table S2), the likeliest candidate being hsGAX, which together with homogalacturonan may function as a cementing substance in grass walls (Ishii, 1984). We propose that the β -expansins in grass pollen evolved this specialized activity as part of their unique function in pollen-pistil interactions in grasses. The non-enzymatic mechanism of wall loosening may be the same in all forms of expansin, but the specific targets may differ, resulting in distinct biophysical effects depending on the specific target and its role in cell wall structure. If this is true, there may be more diversity in the biological roles and biophysical effects of expansins than previously appreciated.

EXPERIMENTAL PROCEDURES

β -Expansin

Native β -expansin was extracted from maize pollen and purified by CM-Sephadex chromatography as described by Li *et al.* (2003a). Peak III was applied to a reversed-phase column (Discovery C8; Sigma-Aldrich, <http://www.sigmaaldrich.com>) pre-equilibrated with 0.1% TFA in water and eluted at 1 ml min⁻¹ with a linear gradient of 0–90% methanol (over 20 min) in the same solution. The fraction containing β -expansin (designated PBE, containing approximately 70% Zea m 1d and approximately 30% Zea m 1c) was concentrated using a 10-kDa ultrafiltration membrane (Amicon Ultra-4 or 15) with simultaneous buffer exchange to 20 mM sodium acetate, pH 5.5, and stored at 4°C. Protein concentrations were measured by Pierce BCA Protein Assay using BSA for calibration.

Wall preparation

Maize silks were collected from field-grown plants; cucumber hypocotyls were harvested from 4-day-old etiolated seedlings grown at 28°C; white onions, white asparagus stalks, red beetroot and celery were purchased from a local market. The tissues were homogenized with a Waring blender in ice-cold water; wall fragments were collected by filtration, washed thoroughly with water, de-proteinized with phenol/acetic acid/water (PAW) (Fry, 1988) and air-dried. For the experiment shown in Figure 3, PAW-washed walls were sequentially extracted as follows, using a ratio of 3 ml extractant per 50 mg of starting wall material: (a) 3× at 95°C for 15 min with 50 mM EDTA in potassium phosphate, pH 6.8; (b) 3× in 0.1 M KOH; (c) 3× in 1 M KOH; (d) 3× in 4 M KOH with 0.02% NaBH₄ and (e) 3× in 6 M KOH with 4% boric acid and 0.02% NaBH₄. Each of the KOH extractions was carried out at room temperature for a total of approximately 20 h. Extracted walls are named EDTAW, 0.1KW, 1KW, 4KW, and 6KW, respectively.

Wheat coleoptiles were harvested from 4- or 5-day-old etiolated seedlings grown at 28°C, abraded to permeabilize the cuticle and stored frozen until use (Cosgrove *et al.*, 1997).

Release of phenolics (A320) and saccharide

Except in early experiments, PAW-washed wall (1 mg) was pre-washed three times in buffer, then incubated at 25°C with agitation in 400 μ l of 20 mM sodium acetate buffer, pH 5.5, containing 1 mM dithiothreitol (DTT) and 2 mM NaN₃ $\pm$ PBE (100 μ g unless stated otherwise). After pelleting the supernatant was collected and absorbance at 320 nm was measured spectrophotometrically while total sugars were measured by the phenol-sulfuric acid method (Dubois *et al.*, 1956). For later experiments the supernatant was filtered with a 10-kDa membrane (Amicon Ultra-4 or 15, <http://www.millipore.com>). The retentate was washed with 20 mM sodium acetate, pH 5.5, three or four times prior to analysis.

Enzyme digestions

Enzyme sources were as follows: XynC, a recombinant xylanase from *Bacillus subtilis*, expressed in *E. coli* and purified by HPLC (Kato and Nevins, 1984a; St John *et al.*, 2006); recombinant pectate lyase from *Cellvibrio japonicus* (E-PLYCJ, Megazyme); recombinant (1,3),(1,4)- β -glucan endo-4-glucanase from *Magnaporthe oryzae* (MoCel12A, Takeda *et al.*, 2010), and xylanase XynM4 from *Aspergillus niger* (E-XYAN4, Megazyme).

Solubilized wall material (100 μ g) released by PBE or other treatment was incubated with 1 μ g enzyme in buffer supplemented with 2 mM NaN₃ for 24 h at 37°C. Pectate lyase was prepared in 20 mM Tris-HCl, pH 7.5, containing 1 mM CaCl₂; other enzymes

were prepared in 20 mM sodium acetate, pH 5.5. After incubation, the reaction mixture was filtered (0.45 μ m) and 10–20 μ l was analyzed by GPC (below). For some experiments insoluble arabinoxylan from wheat flour (1 mg, Megazyme) was incubated with PBE, XynC, or XynM4 as above.

Saponification

PAW walls or solubilized wall materials were saponified with 0.1 M NaOH for 20–24 h at 37°C, then neutralized with acetic acid and four volumes of methanol was added to precipitate polysaccharides (Buanafina *et al.*, 2010). After centrifugation the supernatant was dried under vacuum, resuspended with methanol followed by dilution in 20 mM sodium acetate buffer, pH 4, and applied to a reversed-phase column (Delta Pak C₁₈, 15 cm $\times$ 3.9 mm i.d., 300 Å; Waters, <http://www.waters.com>) for phenolic analysis. In other experiments the sample was filtered through a 10-kDa membrane immediately after neutralization.

GPC and DLS analysis

Polysaccharide size distribution was analyzed in 50 mM sodium acetate (pH 5.5) with a Tosoh TSK-GEL G5000PWXL column (7.8 mm i.d. $\times$ 30 cm) and a Waters Breeze HPLC with UV and RI detection, using a flow rate of 1 ml min⁻¹. Size was also estimated by DLS at 25°C using polysaccharides at 0.5 mg ml⁻¹ with a Viscotek™ model 802, using OMNISIZE software (<http://www.malvern.com>).

Glycosyl composition and linkage analyses

Glycosyl composition was measured as in Merkle and Poppe (1994) and York *et al.* (1986). For linkage analysis, the sample was permethylated, depolymerized, reduced, and acetylated; the resulting partially methylated alditol acetates were analyzed by gas chromatography-mass spectrometry (York *et al.*, 1986). These analyses were performed by the Complex Carbohydrate Research Center (Athens, GA, USA).

Wall assays

Heat-inactivated cell wall specimens from wheat coleoptiles were prepared as in Li *et al.* (2003a). For creep measurements they were clamped in a constant-force extensometer at 20-g force in 20 mM sodium acetate (pH 5.5) with 5 mM DTT, and PBE was added after 30 min. For stress/strain assays, five wall specimens were incubated in 20 mM sodium acetate, pH 5.5, 1 mM DTT with or without PBE (100 μ g in 400 μ l of reaction mixture) for various times at 25°C, then heated for 10 min at 70°C to inactivate PBE and stored on ice. Each specimen was clamped in a tensile tester with 3-mm distance between the clamps and reversibly extended in two cycles at 3 mm min⁻¹ until a force of 20 g was reached (Cosgrove, 1989). Wall compliances were calculated from the slopes of the force/extension curves (Cosgrove, 1989; Yuan *et al.*, 2001). A similar extension procedure was used for breaking force measurement, except that the walls were extended only once until they broke. The maximum force was taken as the breaking force. For pH dependence measurements, heat-inactivated wheat coleoptiles were incubated in 20 mM 3,3-dimethylglutaric acid adjusted to different pH with NaOH.

Microscopy

Greenhouse-grown maize silks were cut and fixed in 4% formaldehyde containing 50 mM sodium phosphate buffer, pH 7.2, for 1 h at room temperature and then 4°C overnight. The fixed tissues were dehydrated in an alcohol series and embedded in LR White resin (Electron Microscopy Sciences, <http://www.emsdiasum.com>).

Semi-thin sections 4 µm in thickness were cut from specimen blocks with a microtome (Sorvall MT-2, <http://www.sorvall.com>). Immunolabelling of maize silk sections with antibodies was carried out as described in Suzuki *et al.* (2000) except that goat anti-rabbit IgG linked to Alexa-488 (Invitrogen, <http://www.invitrogen.com>) was used. The sections were observed and photographed with a fluorescence microscope (Zeiss Axioplan, <http://www.zeiss.com>).

Immunoblot analysis

Wall material solubilized by PBE was spotted onto nitrocellulose membrane in a dilution series of 1, 0.1, and 0.01 µg in 0.5 µl. Birch xylan (Fluka, <http://www.sigmaaldrich.com>) in 20 mM sodium acetate buffer, pH 5.5, was used for comparison. Spotted membranes were air-dried for 30 min before blocking with 10% (v/v) horse serum in phosphate-buffered saline (PBS) containing 0.05% (v/v) Tween 20 and 5 mM Na₂CO₃, pH 7.4, incubated for 1 h with the same solution containing anti-hsGAX (1 µg ml⁻¹) or anti-xylopentose (0.1 µg ml⁻¹) rabbit polyclonal antibodies (Suzuki *et al.*, 2000), washed twice with PBS containing 0.05% (v/v) Tween 20 and 5 mM Na₂CO₃, and then incubated for 1 h with goat anti-rabbit IgG-conjugated alkaline phosphatase (Sigma, <http://www.sigmaaldrich.com>) at a dilution of 1:1000 (v/v) followed by detection with Nitro Blue Tetrazolium/5-bromo-4-chloro-3-indolyl phosphate (Sigma).

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Figure S1. Antibody detection of hsGAX.

Figure S2. Release of phenolics and saccharide from maize wall fractions by EDTA and wall-degrading enzymes.

Figure S3. Release of phenolics (a) and saccharide (b) from insoluble wheat arabinoxylan by incubation with buffer (B), lysozyme (Lys), PBE, xylanase XynM4 and XynC.

Figure S4. Release of phenolics (A320) and saccharide from wheat coleoptile walls by PBE at different pH values.

Figure S5. Plastic compliance of wheat coleoptile walls incubated for 4 h in dimethylglutarate buffer ± 250 µg ml⁻¹ PBE, as a function of pH.

Table S1. Release of phenolics and saccharides from maize silk cell walls by α-expansin.

Table S2. Comparison of PBE-induced release of saccharide and phenolics from cell walls of varying compositions and sources.

Table S3. Monosaccharide composition of EDTA-extracted material from maize silk wall.

Table S4. Monosaccharide composition of XynC-solubilized materials from maize silk wall.

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