Loosening of plant cell walls by expansins

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Plant cell walls are the starting materials for many commercial products, from lumber, paper and textiles to thickeners, films and explosives. The cell wall is secreted by each cell in the plant body, forming a thin fibreglass-like network with remarkable strength and flexibility. During growth, plant cells secrete a protein called expansin, which unlocks the network of wall polysaccharides, permitting turgor-driven cell enlargement. Germinating grass pollen also secretes an unusual expansin that loosens maternal cell walls to aid penetration of the stigma by the pollen tube. Expansin's action has puzzling implications for plant cell-wall structure. The recent explosion of gene sequences and expression data has given new hints of additional biological functions for expansins.

For plants, the cell wall is an important structure that determines cell shape, glues cells together, provides essential mechanical strength and rigidity, and acts as a critical barrier against pathogens. Secreted by growing cells, the primary wall is a polymeric network of crystalline cellulose microfibrils embedded in a hydrophilic matrix of hemicelluloses and pectins[1] (Box 1). Unlike the bacterial wall, which may form one giant, covalently linked macromolecule, the polysaccharides of the growing plant cell wall are mostly separate long-chained polymers that form a cohesive network through non-covalent lateral associations and physical entanglements.

The growing wall possesses a remarkable combination of strength and pliancy, enabling it to withstand the large mechanical forces that arise from cell turgor pressure, while at the same time permitting a controlled polymer 'creep' that distends the wall and creates space for the enlarging protoplast. With a tensile modulus greater than $10^{11}\,\mathrm{N\,m^{-2}}$, cellulose microfibrils themselves are effectively inextensible. Wall expansion occurs by slippage or rearrangement of the matrix polymers that coat the microfibrils and hold them in place. Until recently this was thought to occur primarily by hydrolysis of matrix polysaccharides, but the discovery of expansins has uncovered another mechanism of wall enlargement.

Discovery of expansins

Plant cells originate mainly in small pockets of dividing cells, called meristems, that are located at the growing points of shoots, roots and

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Box 1

Structure and biogenesis of the primary cell wall in plants

All land plants make a primary cell wall that is remarkably similar in structure to, but distinct from, that of most algae, fungi, bacteria and other groups with cell walls. The left-hand panel of the Figure shows the microfibrillar nature of the wall as a 'relief map' of the cell-wall surface after extraction of most of the matrix polysaccharides (image from ref. 4, used with permission). The right-hand panel is a diagram of cell-wall assembly. Cellulose microfibrils are synthesized by large complexes in the plasma membrane. Newly secreted cellulose then associates with matrix glycans (hemicelluloses and pectins) which are synthesized in the Golgi apparatus and delivered to the wall by

secretory vesicles. The cellulose microfibril is a thin ribbon, about 5 nm in width and many micrometers in length. It consists of an ordered array of many parallel chains of an unbranched glucose polymer (1,4-β-glucan). Hemicelluloses typically are branched polysaccharides characterized by a strong tendency to bind to cellulose, whereas pectins are generally acidic polysaccharides with a strong tendency to form ionic gels. A small amount of structural protein is also found on the wall, but its function is uncertain.\

Golgi apparatus

Cellulose synthase complex

plasma membrane

Cellulose

Matrix glycans

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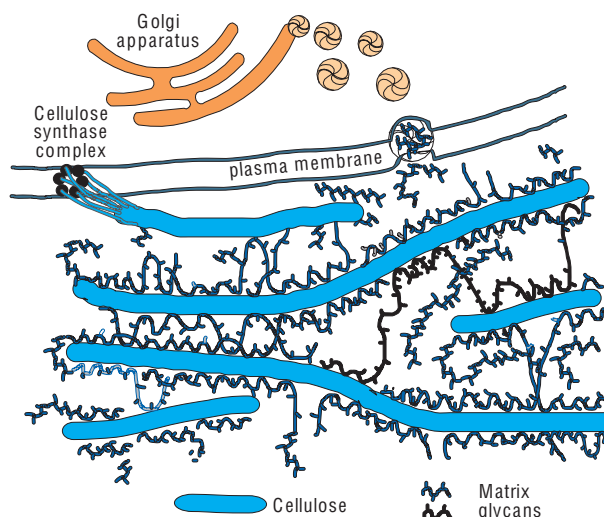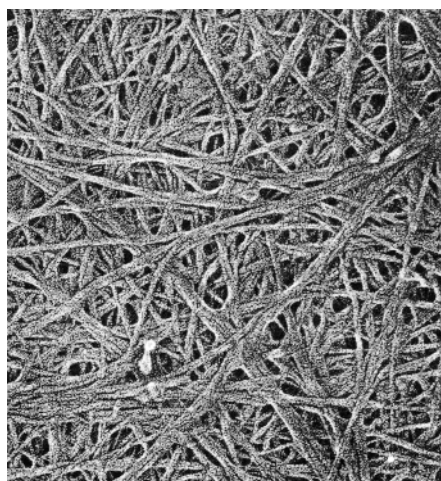
other organs. Meristematic cells are small (~5 µm) and densely packed with cytoplasm. When cells are displaced from the meristem, they typically undergo a prolonged phase of enlargement and differentiation during which cell volume greatly increases. To take an extreme example, a water-conducting cell of the xylem may be a million times larger in volume than its meristematic initial. Such cell enlargement is achieved economically by filling the cell with a large central vacuole containing water and solutes. The physical control of this process resides in the ability of the cell wall to undergo turgor-driven expansion[8].

Growing plant cell walls typically extend faster at low pH, a phenomenon referred to as 'acid growth'[9]. This is readily seen in isolated walls clamped at constant tension in an extensometer (Fig. 1a). At neutral pH the walls soon stop extending but they rapidly extend when pH is lowered (Fig. 1b). Acid-induced extension is not merely a physical property of wall polysaccharides, but requires active wall proteins[10]. A key breakthrough[11] came with the discovery that wall proteins may be added back to denatured cell walls to restore their ability to extend (Fig. 1c). The active proteins were of a new protein class, subsequently named expansin[12]. Their action is referred to as 'wall loosening', a general term that does not specify the exact mechanism for induction of cell wall extension.

Expansins, proteins with relative molecular mass of about 26,000, were isolated first from young cucumber seedlings and subsequently from other plant tissues, where their activity was associated with cell growth. Expansins are also implicated in the drought responses of maize seedlings, where maintenance of root growth involves increased expansin activity in the growing region[15]. Recent work indicates that drought increases the expression of expansin genes in a spatial and temporal pattern that closely matches the changes in expansin protein activity (Y. Wu, R. E. Sharp and D.J.C., manuscript in preparation). It may thus be possible to engineer enhanced drought tolerance into crop plants by manipulating expansin gene expression.

Other studies have shown close correlations between expansin gene expression and growth. For example, deep-water rice responds to submergence with rapid stem elongation, which is preceded by large increases in expansin messenger RNA[16]. The growth hormone gibberellin has similar effects on stem elongation and expansin expression. In the wetland plant *Rumex palustris*, submergence stimulates elongation and expansin expression, but this stimulation is not found in a related species that lacks the submergence growth response[17]. Auxin-induced root formation in pine cuttings is accompanied by a 100-fold increase in expansin mRNA[18]. Finally, in the shoot apical meristem, which produces leaf primordia in a carefully orchestrated spatial and temporal pattern, an expansin gene is expressed at the site of future primordium emergence, preceding the first morphological signs of primordium outgrowth[19]. These results confirm the connection between expansin gene expression and growth induction.

Consistent with these gene-expression results, other studies found that cells grew faster upon treatment with expansin proteins[20,21]. Moreover, when expansin-loaded microbeads were applied locally to shoot apical meristems at the sites of incipient leaf primordia, the primordia emerged prematurely[22]. The resulting out-of-sequence leaf emergence caused the spiral pattern of primordium initiation (phyllotaxy) on the meristem dome to reverse[23].

The concept that expansins are key endogenous regulators of plant cell enlargement is now supported by four lines of evidence. First, expansin proteins induce stress relaxation and extension of isolated cell walls in a pH-dependent manner[11,24]. No other proteins have been found with this activity[7,25]. Second, application of expansins to living cells stimulates cell enlargement. This shows that expansins can function under the normal conditions found in the cell walls of living cells and that they are present in sub-saturating amounts in these tissues. Third, expansin genes are expressed at the right time and in the right place to function in growth control,. And finally, reduction of expansin gene expression by antisense methods inhibits growth[32]. Thus, the concept of expansins as catalysts of cell-wall enlargement is well supported.

Allergens, β-expansins and grass cell walls

When the first expansin complementary DNAs were sequenced[33], BLAST searches of GenBank revealed a distant sequence similarity to a group of grass pollen allergens called group-1 allergens[34]. These pollen proteins were identified by immunologists in the mid-1980s as the main causative agents of hay fever and seasonal asthma induced by grass pollen[35]. Assays of the group-1 allergen from maize
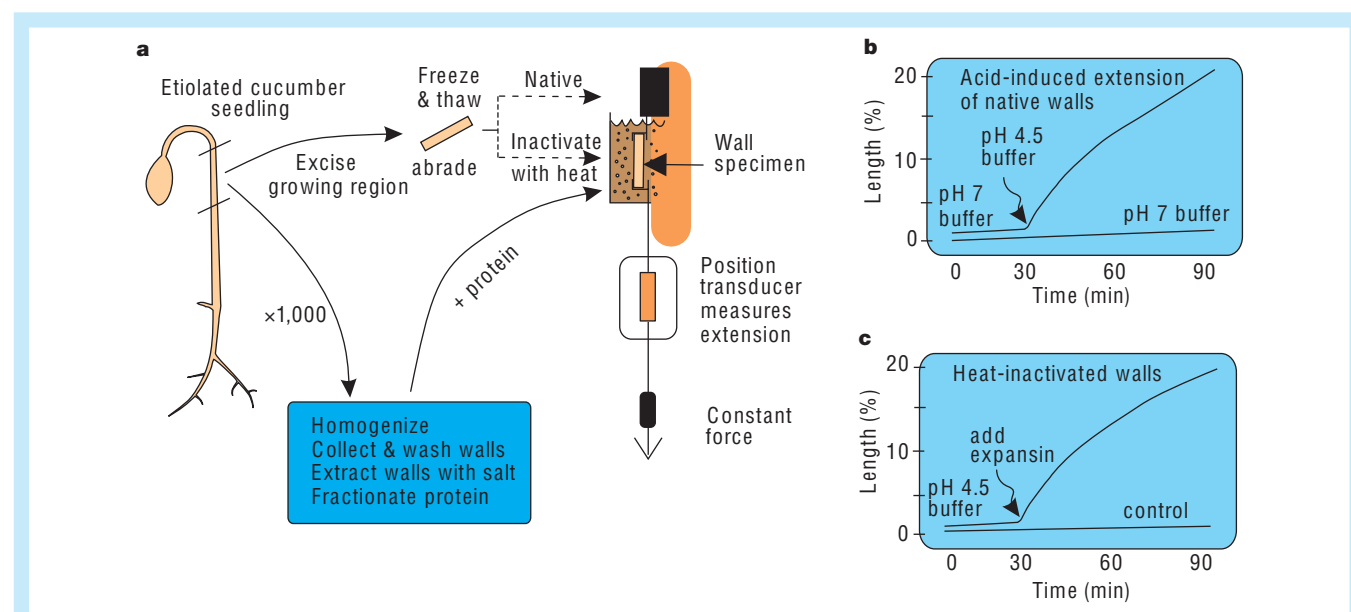

Figure 1 Diagram of extensometer assays. **a**, The growing hypocotyl of a seedling is cut and frozen to kill the cells. The wall specimen is either directly clamped in a constant-force extensometer ('native walls') or first inactivated with a brief heat treatment before being clamped in the extensometer ('heat-inactivated walls'). Expansin protein is prepared by extraction from native walls, followed by fractionation and addition to the wall. **b**, Native walls extend very little at neutral pH, but rapidly extend in acidic pH. **c**, Heat-inactivated walls lack acid-induced extension, which can be restored by addition of expansin to the walls.

pollen (called Zea m1) showed it to have wall-loosening activity characteristic of expansin[36], but with an intriguing twist: the allergen induced extension only in grass cell walls and was not effective on walls from dicotyledons. This activity has since been confirmed for group-1 allergens from other grasses (L.-C. Li and D.J.C., unpublished data). This discovery has several implications.

First, it appears that group-1 allergens have a wall-loosening role, aiding penetration of the pollen tube through the stigma and style by softening the maternal cell walls. This function has not been proven, but is indicated by the unusually high abundance and solubility of these proteins[36]. These properties would be anomalous if the group-1 allergens were targeted for action on the growing pollen-tube wall, which consists of a very small area at the tip of the pollen tube. However, they make sense if the target were the many walls between which the pollen tube must push on its way to the ovary[37]. Indeed, experimental measurements[36] show that maize style walls are readily loosened by Zea m1.

Second, it appears that grasses are the only flowering plants to have discovered the 'trick' of using a pollen-secreted expansin to assist pollen-tube penetration. This inference is based on the finding that pollen extracts from other plant groups lack expansin-like loosening activity[36]. Moreover, BLAST searches of GenBank show homologous pollen allergens only in grasses. If grasses have discovered this method of invading plant tissues, however, can fungi and bacteria be far behind? These organisms might possess novel expansins.

A third inference is that the group-1 allergens act on polymers specific to grass walls, which differ from the typical plant cell wall in several regards. In particular, grasses have reduced the usual matrix polysaccharides (xyloglucan and pectin) and substituted other polysaccharides, most notably branched xylans and mixed-linked glucan[38]. These may be the natural targets of the group-1 allergens.

Fourth, if we recognize group-1 allergens as a new group of expansins, then a number of closely related GenBank entries become immediate suspects as wall-loosening proteins. We have named this group the β-expansin family, to distinguish it from the original group of expansins, which are now called α-expansins. The β-expansin family comprises the group-1 allergens and related genes expressed in vegetative tissues (Fig. 2), including *CIM1*, a cytokinin-induced

message identified in soybean cell cultures[39,40]. The sequences of β-expansins are highly represented in the rice and maize expressed sequence tag (EST) databases, where at last count 150 maize EST sequences could be classified as β-expansins encoded by at least 19 different genes (Y. Wu and D.J.C., unpublished data). In comparison, only a single *Arabidopsis thaliana* EST is considered to be a β-expansin. If *Arabidopsis* is representative of other dicotyledons, this comparison implies an exceptional diversity and abundance of β-expansin genes in grasses such as maize and rice. We suspect that the evolutionary radiation of β-expansin genes in grasses is linked to the evolutionary divergence of the grass cell wall[7].

Finally, because the two families of expansins have similar wall-loosening activity, we infer that the key structural and catalytic residues are to be found in the very limited conservation (~20% identity) between α- and β-expansins. Scrutiny of these regions leads to some new guesses about how these proteins might loosen the cell wall, as discussed below.

A role in fruit softening

During ripening of many fruits, wall polysaccharides are hydrolysed by a suite of digestive enzymes whose genes are specifically expressed during the final stages of fruit development[41]. For many years these polysaccharide hydrolases were thought to cause softening of fruit[42]. These ideas were cast into doubt, however, when plants were genetically engineered to alter enzyme activity and polysaccharide breakdown in the fruit, but fruit softening occurred more or less normally.

Following these studies, an α-expansin gene *Le-EXP1* was found to be expressed specifically in the later stages of tomato fruit ripening and its expression was also stimulated by the ripening hormone ethylene[46]. These observations suggested that expansin may function in fruit softening. To test this idea, expression of the *Le-EXP1* gene was reduced by antisense methods; the resulting fruit indeed proved to be firmer throughout ripening[47]. Similarly, overexpression of *Le-EXP1* resulted in softer fruit throughout ripening. These textural effects were relatively small, however, and fruit softening still occurred; it was simply delayed or accelerated. Thus, while *Le-EXP1* expression is involved in tomato fruit softening, it is not the sole determinant of this process.

In tomato plants where expression of the *Le-EXP1* gene was driven with a strong constitutive promoter (CaMV 35S), the fruits were smaller and more rubbery but vegetative growth was normal[47]. The lack of effect on vegetative growth indicates either that the *Le-EXP1* protein does not have a cell-growth function or that the plants were able to compensate for ectopic expression of *Le-EXP1* in vegetative tissues.

In other fruits, such as strawberry and cantaloupe, α-expansin mRNAs are also expressed in late stages of ripening[46,48] and so this may be a common feature of fruit softening[41]. Wall protein extracts from several kinds of ripening fruit possess significant expansin activity[49], although such activity was not detected in wall proteins from tomato fruit. This is another hint that *Le-EXP1* may lack wall-extension activity. However, it is also possible that much of the extracted protein was inactivated either during extraction or while still within the cell wall. There is precedent for expansin inactivation in growing cucumber seedlings, where expansins are secreted in an active state but become inactivated after the growing cells are displaced from the growth zone. The molecular basis for this inactivation is unclear, but it may relate to thiol oxidation[11,50].

More genes, more biological roles

Thanks to the plant genome projects, we now have a nearly complete inventory of the genes in *Arabidopsis* and an extensive, but still incomplete, inventory of expressed genes in rice, maize and other plants. This has provided us with some surprises about expansins. As mentioned above, we have learned that the β-expansin gene family is large and abundantly expressed in grasses (rice and maize), whereas in *Arabidopsis* this gene family is much smaller and not highly
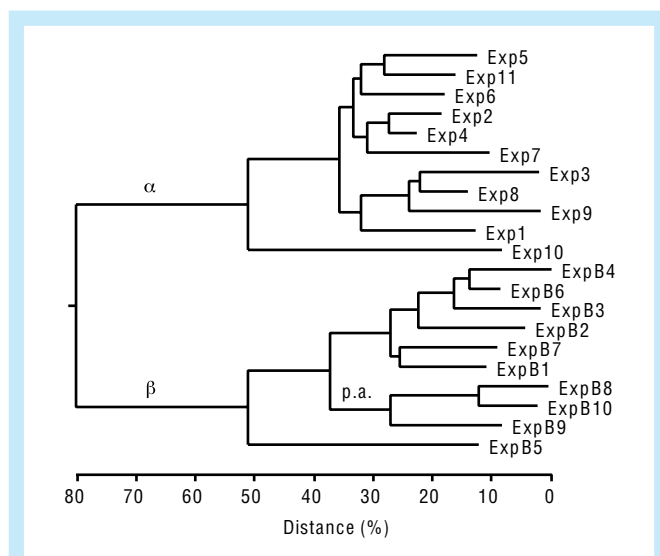

Figure 2 Phylogenetic tree of rice α- and β-expansins. The α- and β-expansins share only about 20% amino-acid identity. Rice appears to have three group-1 pollen allergens (p.a.) that form a distinct subclass of β-expansins. The other β-expansins are expressed in tissues other than pollen and so are referred to as 'vegetative' β-expansins.

expressed. In comparison, *Arabidopsis* has at least 24 α-expansin genes (see the expansin web site at www.bio.psu.edu/expansins/ for an up-to-date listing).

There is evidence that many of these genes have cell-specific expression[51], with each cell type expressing a distinct subset of expansins during growth; for example, trichomes, guard cells, root hairs and xylem each express a distinct set of expansins (ref. 51 and unpublished data). In some tissues, such as the xylem, at least three different expansin genes are expressed, indicating that there may be functional overlap and genetic redundancy. Expansin mRNAs in *Zinnia* xylem are localized to the ends of the cells, where they may function for targeted secretion of expansin to specific walls[52].

The wealth of GenBank data also lets us evaluate the evolutionary distribution of expansins. So far, they are restricted to land plants (Embryophyta) and are not reported in other kingdoms. In particular, the completed model genomes for bacteria, fungi, insects and nematodes *Escherichia coli*, *Saccharomyces cerevisiae*, *Drosophila melanogaster* and *Caenorhabiditis elegans* lack anything that resembles expansin. Because these organisms make a very different kind of cell wall or extracellular matrix compared with that of plants, this finding is consistent with expansin's unique function. However, one might expect to find expansin-like proteins in organisms that invade plant tissues or digest cell walls, such as termites, nematodes that form root cysts, plant parasites and pathogenic fungi. In this regard, it is noteworthy that the digestive track of garden snails *Helix pomatia* contains expansin-like proteins[25]. These presumably help digestion of plant tissues in the snail's diet. That this is the only documented case of expansin-like proteins in organisms other than plants probably reflects our lack of comprehensive information.

Characteristics of wall loosening by expansin

Expansin's unique physical effects on plant cell walls include rapid induction of wall extension and stimulation of stress relaxation[11]. Expansins do not progressively weaken the cell wall, nor do they cause a lasting change in wall structure[53] (except, of course, that the wall is longer and thinner after it extends). No ligands or cofactors have been identified as necessary for expansin action, although thiol reductants help to maintain stable activity.

Normally, expansin is a very minor component of the cell wall[24]; for instance, in cucumber seedlings expansin is found at roughly one part protein to 5,000 parts cell wall (on a dry mass basis) and induces wall extension when added in amounts as low as 1:10,000. Binding and activity both saturate at a protein-to-wall ratio of about 1:1,000.

From the architecture of the plant cell wall, one might expect expansin's loosening action to result from hydrolysis of the matrix polymers that hold the cellulose microfibrils in place. However,

attempts to identify hydrolytic activity by expansin against the major wall components have failed[11,24]. Attempts to mimic expansin's effects with wall hydrolases have also failed[25]; such enzymes can reduce the mechanical strength of the cell wall, but do not induce extension and stress relaxation, at least not until the wall is degraded to the point of mechanical failure.

A recent study[54] proposed that group-1 allergens are cysteine proteases, based on proteolytic activity associated with recombinant protein. Spurred by this report, we have examined Zea m1 and other group-1 allergens for proteolytic activity, but have not detected such activity (L.-C. Li and D.J.C., manuscript in preparation). We also tested a broad range of proteases[10], but failed to detect significant wall-extension activity. Thus, proteolysis does not appear to be a probable mechanism of expansin action.

Model of expansin action

In contrast to these conventional enzymatic theories of wall loosening, we have proposed an alternative mechanism, in which expansins weaken the non-covalent binding between wall polysaccharides, thereby allowing turgor-driven polymer creep[55] (Fig. 3). In this scheme, expansin would make use of the mechanical strain energy in the wall to catalyse an inchworm-like movement, or reptation, of the wall polymers. Expansin movement may be confined to lateral diffusion along the surface of the cellulose microfibril, as been observed for other polysaccharide-binding proteins[56]. Such contained diffusion would enable expansin to search the microfibril surface, locally loosening its attachment to the matrix, and allowing chain movement and stress relaxation.

This model of expansin action is consistent with the lack of progressive weakening of the cell wall by expansin and also fits the biophysical characteristics of cell-wall extension, the rate of which is dependent on the wall stress in excess of a minimum yield threshold[57]. Other support for this mechanism comes from the finding that expansin weakens pure cellulose paper, whose strength derives from non-covalent binding between cellulose fibres[55], and artificial composites composed of cellulose alone or cellulose and xyloglucan[58]. Finally, although expansin lacks hydrolytic activity by itself, it does enhance the hydrolysis of crystalline cellulose by cellulases[59]. This synergistic action may indicate that expansin makes glucans on the surface of the microfibril more accessible to enzymatic attack by cellulases.

Sequence analysis of expansins

Although the protein structure for expansin has not yet been solved, sequence analysis hints at a two-domain structure (Fig. 4a). The carboxy terminus is up to 50% identical to a small protein produced by grass pollen, known as a group-2 grass pollen allergen. The biologi-
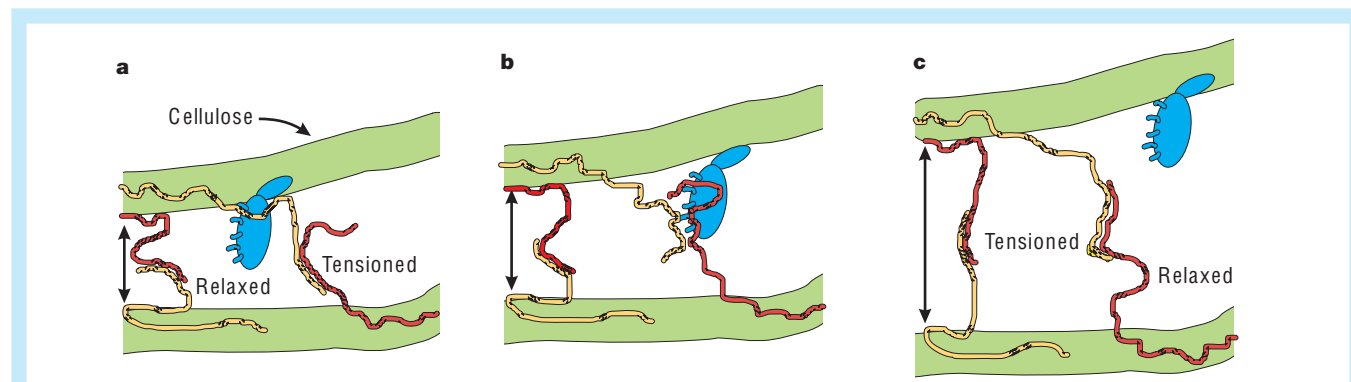

Figure 3 A model of expansin's wall-loosening action. Cellulose microfibrils are connected to each other by glycans (thin yellow and red strands) that can stick to the microfibril surface and to each other. The expansin protein (blue) is hypothesized to disrupt the bonding of the glycans to the microfibril surface (**a**) or to each other (**b**). Under the mechanical stress arising from turgor, expansin action results in a displacement of the wall polymers (**c**) and slippage in the points of polymer adhesion (compare **b** and **c**).

cal function of group-2 allergens is unknown, but a potential function in wall loosening is suggested by its homology to expansin and by the fact that it is secreted to the cell wall by grass pollen.

Recently the structure of a group-2 allergen was solved[60,61] and we have used this structure to model the C terminus of the β-expansin Lol p1 (). On the protein surface are a number of aromatic residues that could function in polysaccharide binding, for example, by ring stacking as found for other carbohydrate-binding proteins[62]. However, the surface does not closely resemble the flat binding surface reported for cellulose-binding domains[63], which preferentially bind to the crystalline surface of cellulose.

Expansins also have limited sequence similarity to the catalytic domain of a small family of endoglucanases (family 45)[64]. The sequence identify is only around 30%, but it includes a series of cysteines (Fig. 4c), suggesting a conserved protein fold[50]. In addition, the His-Phe-Asp (HFD) motif found in the enzyme's catalytic site[65] is also well conserved in most expansins. This conservation led us to re-examine expansins for endoglucanase activity, using more sensitive methods, but the results continue to support a nonhydrolytic mechanism of action.

The resemblance of expansin to family-45 endoglucanases remains enigmatic. Perhaps expansins act by a transglycosylation mechanism, in which the backbone of a polysaccharide is cut and ligated to the end of another similar polymer[66]. Tests for transglycosylation of xyloglucan (the major hemicellulose of most growing cell walls) by α-expansins have given negative results[67]. Moreover, xyloglucan endotransglycosylase lacks wall-extension activity. Also weighing against this idea is that family-45 endoglucanases are inverting enzymes, which do not form the covalent intermediates needed for transglycosylation[65].

Implications for wall structure

Another enigma concerns the model of the plant cell wall (Box 1). One would reasonably expect such a structure to extend under the action of hydrolases or transglycosylases that act on matrix polysaccharides, but this has not proven to be the case.

Similarly, the immediate induction of wall extension by expansin (starting within seconds and reaching a maximum rate within a couple of minutes after addition) and the miniscule amounts of expansin needed for wall extension suggest that there may be very few 'sticky' spots or entanglements at any moment where expansins act to promote polysaccharide slippage. This inference is supported by NMR studies indicating that expansin does not increase the general mobility of wall polysaccharides[53]. Thus wall enlargement may be controlled by action at relatively rare sites that link microfibrils together. The nature of these linkage sites remains a mystery.

Finally, the two-domain model of expansin structure suggests that the loosening action of expansin may be a separate function from its binding to the wall. Binding could serve to anchor the protein and to prevent its diffusion to neighbouring cells, where it could interfere with cell-specific control of cell enlargement. Such tight binding, however, would not necessarily prevent protein diffusion along the surface of the microfibril; hence, expansin's action could be localized by its wall-binding properties, which may vary among different kinds of expansin.

Perspectives

This class of protein appears to have evolved specifically in the land plant lineage, with biological functions in cell growth and in other situations where the movement, adhesion and enzymatic accessibility of wall polysaccharides are important. Expansins offer potential applications for bioengineering of cell walls, either to manipulate the growth and texture of plants or to modify the structure and physical properties of cell walls used in commercial products such as wood, textiles and polymers.

Although the plant cell wall is composed of polymers characteristic of plants, analogous structures based on different polymers but similar physical principles are found in fungi, arthropods and other organisms. Perhaps they too have evolved proteins functionally analogous to expansin. □
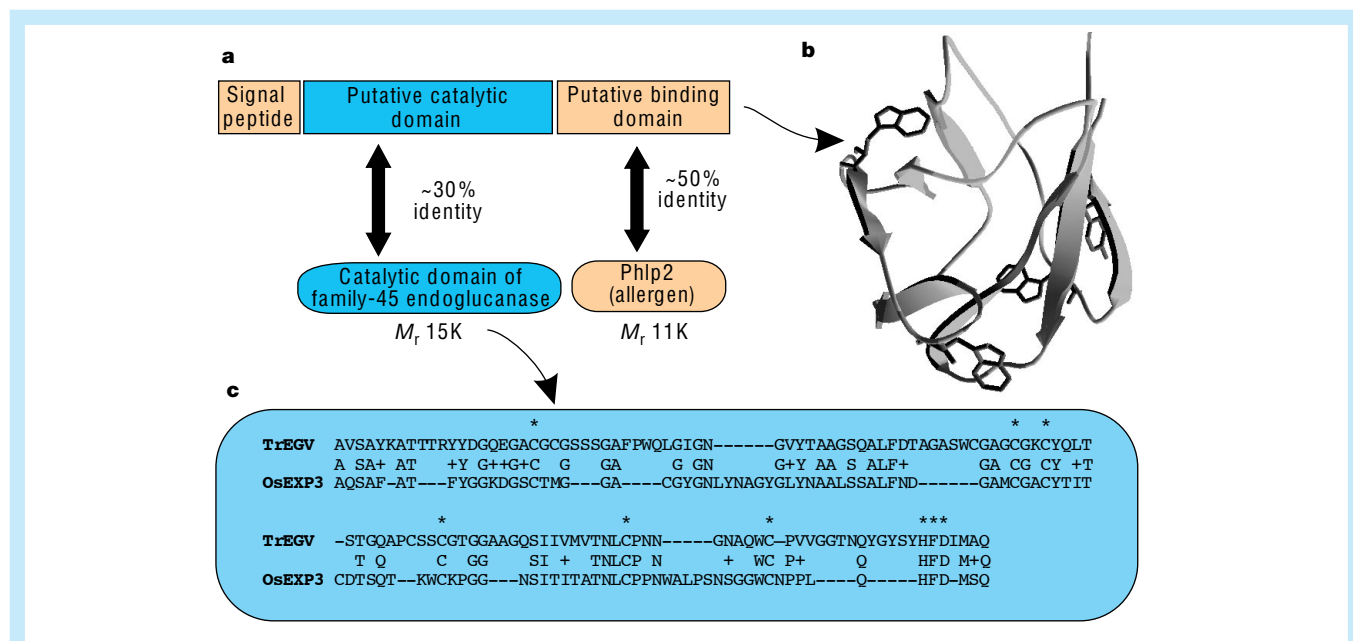

Figure 4 Predicted structure of expansin protein. **a**, Block diagram of putative expansin protein domains, mapping the carboxy terminus to grass pollen group-2 allergens and the amino terminus to the catalytic domain of family-45 endoglucanase. **b**, The structure of the C terminus of Lol p1 (a group-1 pollen allergen and β-expansin), modelled by SWISS-MODEL[68] using the structure of Phl p2 as a template. Conserved surface aromatic residues (tryptophan, tyrosine) with a possible role in polysaccharide binding are displayed. **c**, Sequence alignment of the catalytic domain of a family-45 endoglucanase (EGV from *Trichoderma reesii* to a rice α-expansin (Os-EXP3). The endoglucanase also has a linker region and a cellulose-binding domain (not shown) which are not homologous with expansin sequences.

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