Protein family review

The expansin superfamily

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Summary

The expansin superfamily of plant proteins is made up of four families, designated α-expansin, β-expansin, expansin-like A and expansin-like B. α-Expansin and β-expansin proteins are known to have cell-wall loosening activity and to be involved in cell expansion and other developmental events during which cell-wall modification occurs. Proteins in these two families bind tightly to the cell wall and their activity is typically assayed by their stimulation of cell-wall extension and stress relaxation; no *bona fide* enzymatic activity has been detected for these proteins. α-Expansin proteins and some, but not all, β-expansin proteins are implicated as catalysts of 'acid growth', the enlargement of plant cells stimulated by low extracellular pH. A divergent group of β-expansin genes are expressed at high levels in the pollen of grasses but not of other plant groups. They probably function to loosen maternal cell walls during growth of the pollen tube towards the ovary. All expansins consist of two domains: domain 1 is homologous to the catalytic domain of proteins in the glycoside hydrolase family 45 (GH45); expansin domain 2 is homologous to group-2 grass pollen allergens, which are of unknown biological function. Experimental evidence suggests that expansins loosen cell walls via a nonenzymatic mechanism that induces slippage of cellulose microfibrils in the plant cell wall.

Gene organization and evolutionary history

Expansins are plant cell-wall loosening proteins involved in cell enlargement and in a variety of other developmental processes in which cell-wall modification occurs [1]. They are typically 250-275 amino acids long and are made up of two domains (domain 1 and domain 2) preceded by a signal peptide (Figure 1). On the basis of phylogenetic sequence analysis (Figure 2), four families of expansins are currently recognized in plants [2]. From the largest family to the smallest they are designated α-expansin (EXPA), β-expansin (EXPB), expansin-like A (EXLA) and expansin-like B (EXLB). α-Expansin and β-expansin proteins have been demonstrated experimentally to cause cell-wall loosening [3,4], whereas expansin-like A and expansin-like B proteins are known only from their gene sequences.

It has not been established when expansins first appeared in evolution, but the α-expansin and β-expansin families already existed by the time the vascular plants and mosses diverged (Figure 2) [5,6]. So far, the expansin-like A and expansin-like B families can be traced back only to the last ancestor of angiosperms and gymnosperms (Figure 2). More recently the expansin families have continued to grow and diversify in different plant lineages. Table 1 shows the number of genes for each family found in the available angiosperm genomes, as well as the numbers of genes estimated for the last common ancestor of eudicots (including *Arabidopsis*) and monocots (including rice). On the basis of this reconstruction, we have recently proposed a subdivision of the four expansin families of angiosperms into 17 clades (Figure 2) [7]. As shown in Table 1, the number of genes has doubled in the *Arabidopsis* lineage and more than tripled in rice since these two species diverged, approximately 150 million years ago. The main reason for this difference is the larger number of tandem duplications present in the rice genome (Figure 3). The growth of the β-expansin family in grasses is

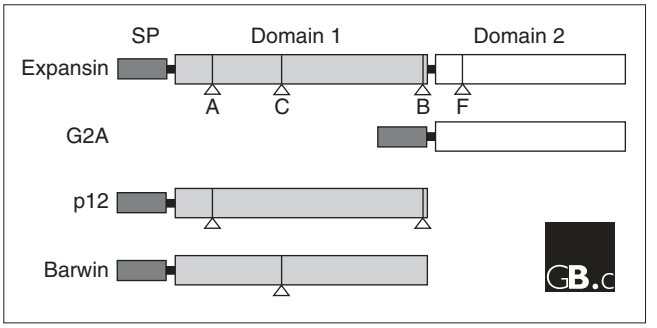

Figure 1
The domain structure of expansins and a comparison with that of distantly related single-domain plant proteins (G2A, p12 and barwin). The expansin signal peptide (SP) is 20-30 amino acids long, domain 1 is 120-135 amino acids, and domain 2 is 90-120 amino acids. Some barwin proteins have an additional chitin-binding domain after the signal peptide (not shown). The positions of the introns that are present in more than one expansin family are indicated by lettered triangles; homologous introns are present in p12 and barwin proteins. Intron letters are as in [7]. The position of intron B suggests that it could have participated in exon shuffling.

Table 1

Sizes of the four expansin families in different plants

Species	EXPA	EXPB	EXLA	EXLB
Last common ancestor	12	2	1	2
Arabidopsis	26	6	3	1
Poplar	27	2	2	4
Rice	34	19	4	1

The number of genes in each family is listed for the three plant species whose genomes have been sequenced. The number of genes in the last common ancestor of monocots and eudicots was estimated from an analysis of the rice and *Arabidopsis* genomes [7]. Numbers for poplar do not include partial gene fragments and should be taken as minimum estimates given that its genome is incompletely sequenced. EXPA, α-expansin; EXPB, β-expansin; EXLA, expansin-like A; EXLB, expansin-like B.

particularly impressive, with 18 genes in rice compared with 6 in *Arabidopsis*.

Curiously, grasses (but only grasses) also have an additional group of secreted proteins homologous only to expansin domain 2; these are known in the immunological literature as grass group-2 pollen allergens (G2As). They seem to have evolved from a truncated copy of a β-expansin gene and they share about 35-45% protein identity with their closest β-expansin relatives; their native biological function is uncertain. Although G2As evolved from a β-expansin ancestor, because of the loss of domain 1 they are considered a separate family and not part of the expansin superfamily.

Two other families of plant proteins show distant homology to expansin domain 1, but as they lack domain 2 they are not considered part of the superfamily. The closest (approximately 25-35% identity) has been variously called p12 and plant natriuretic peptide (PNP). These proteins become abundant in the xylem of blighted citrus trees [8], and they have been ascribed a signaling function [9,10]. No wall-loosening activity has been found in extracts containing p12 (D.J.C. and T. Ceccardi, unpublished observations). More distantly related (about 20-30% identity) is the barwin-like domain that defines the PR4 family of antifungal proteins [11]. Both these protein families were already present in the last ancestor of mosses and vascular plants.

Turning to non-plant organisms, various proteins with distant homology to full-length expansins or exclusively to domain 1 are found from bacteria to nematodes and mollusks [12-15]. Many of these are probably involved in the digestion of plant cell-wall material. A family of expansin-like proteins has been found in the slime mold *Dictyostelium discoideum*, where they could help to maintain the fluidity of the cellulosic cell walls in the stalk structure [16]. Recent nomenclature rules [2] recommend that only proteins with homology to both expansin domains should be designated expansins. The polyphyletic group of non-plant expansins, such as those in *Dictyostelium*, can be referred to as expansin-like family X (EXLX). The relationship of the various groups of expansin-like X proteins with the plant expansins is unclear at the moment. Their divergence could predate the origin of land plants, or they could have been acquired later through horizontal transfer of a gene from one of the plant expansin families. The same applies to proteins with homology only to domain 1, both in plants and other organisms, in that it is possible that some of them originally evolved from an expansin protein with both domains.

Characteristic structural features

Expansin proteins from different families share only 20-40% identity with each other. The degree of conservation is highest in domain 1, as shown in Figure 4. Expansin domain 1 has a distant homology to glycoside hydrolase family 45 (GH45) proteins [17], most of which are fungal β-1,4-D-endoglucanases. Proteins from this family have been crystallized and their mechanism of action determined [18]: they form a six-stranded β-barrel with a groove for substrate binding (Figure 5a). Barwin also has a similar β-barrel structure [19]. On the basis of hydrophobic cluster analysis, we expect this structural motif also to be present in expansins (Figure 6). Furthermore, expansin domain 1 shares with GH45 a number of conserved cysteines that form disulfide bridges in the fungal enzymes. It is interesting that several residues that make up the catalytic site of GH45 endoglucanases are also conserved in expansin (see Figures 4,5). Despite the presence of these conserved GH45 motifs, no hydrolytic activity has been detected for either α-expansin or β-expansin proteins.

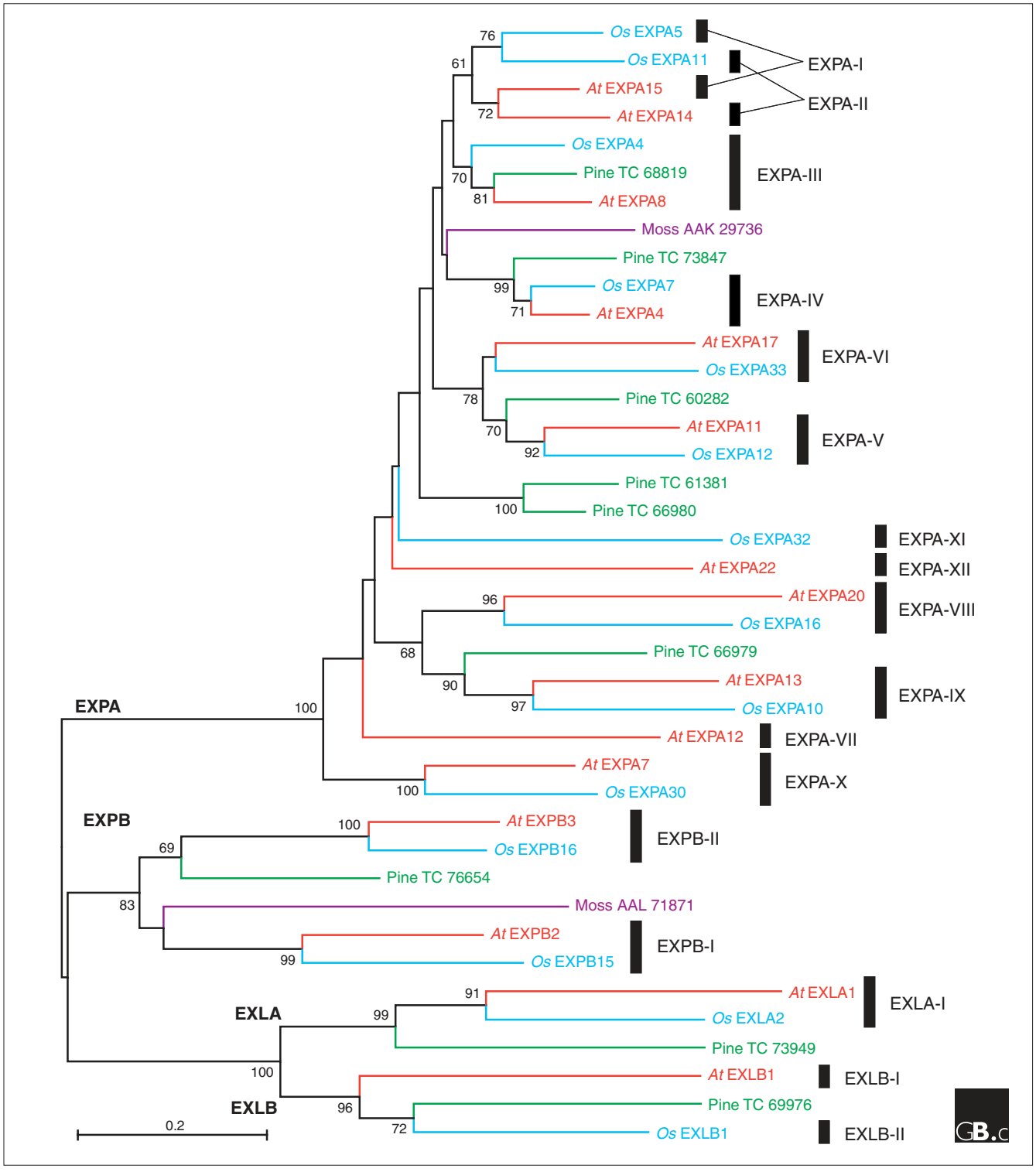

Figure 2
A phylogenetic tree of the expansin superfamily, including protein sequences from *Arabidopsis thaliana* (At), *Oryza sativa* (Os), *Pinus* species (pine) and *Physcomitrella patens* (moss). These sequences were selected to showcase expansin diversity. They were aligned with CLUSTALW (see Additional data file 1) and a neighbor-joining tree was constructed with MEGA 3. Bootstrap values above 60 are indicated next to the relevant node, and the four families are labeled at their roots. Clades, defined as all the descendants of the same ancestral gene in the last common ancestor of monocots and eudicots, are indicated by black bars to the right and given Roman numbers as in [7]. This tree does not correctly resolve clades EXPA-I and EXPA-II, possibly because of changes in amino-acid usage between *Arabidopsis* and rice expansins [7]. The numbers for pine sequences are from TIGR *Pinus* Gene Index [70]; GenBank accession numbers are shown for moss sequences. EXPA, α-expansin; EXPB, β-expansin; EXLA, expansin-like A; EXLB, expansin-like B.

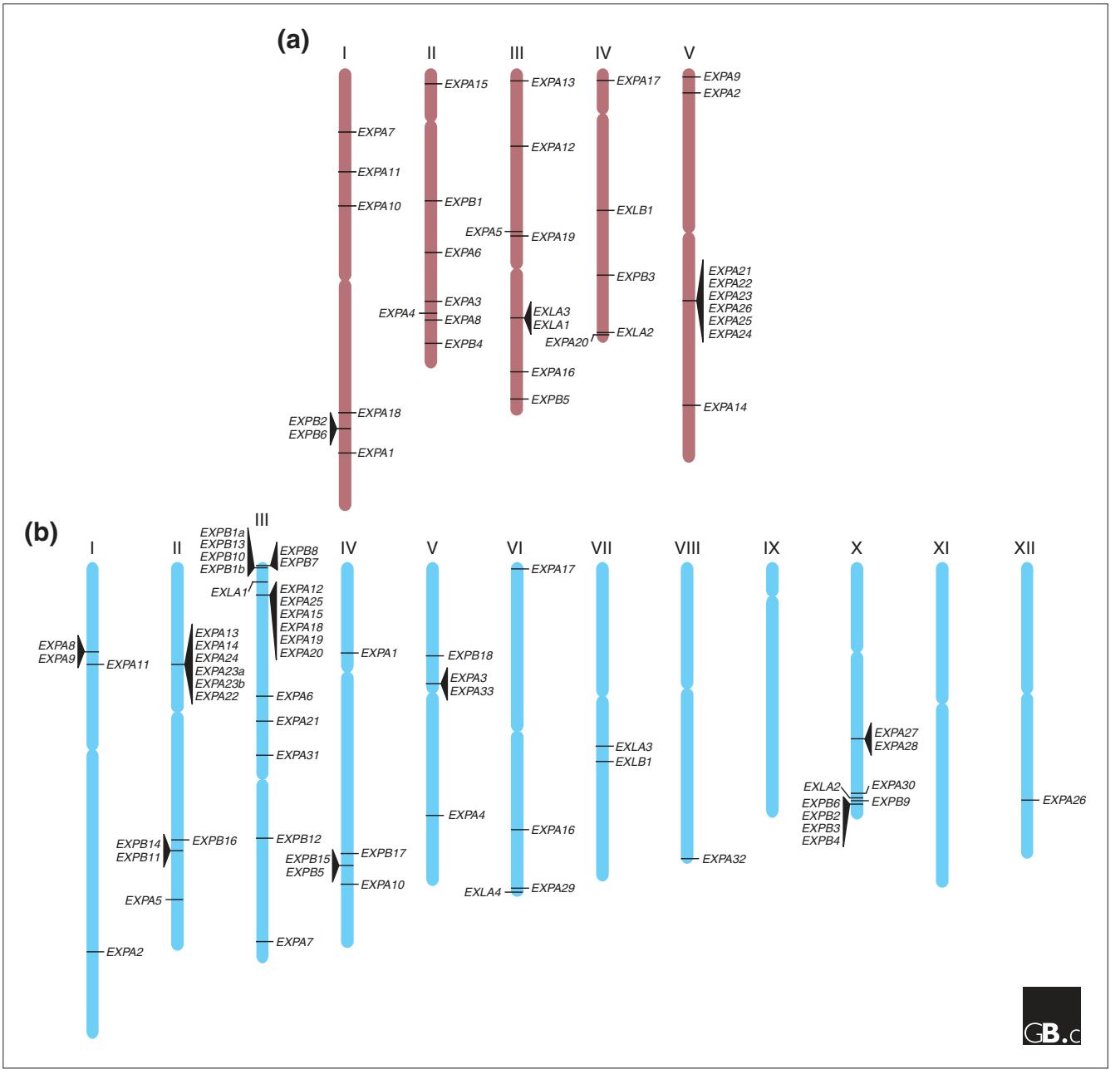

Figure 3
Genomic locations of expansin genes. **(a)** *Arabidopsis*; **(b)** rice. Genes in tandem are indicated by triangles and chromosome numbers are shown with Roman numerals. EXPA, α-expansin; EXPB, β-expansin; EXLA, expansin-like A; EXLB, expansin-like B.

α-Expansin proteins can be distinguished from other expansins by the presence of a large insertion and a nearby deletion in domain 1; these are at either side of a conserved motif that is part of the conserved GH45 active site (HFD in the single-letter amino-acid code; Figure 4). Expansin-like A and expansin-like B proteins lack the HFD motif, which suggests that their action may differ from that of other expansins. Furthermore, expansin-like A proteins have a unique conserved motif (CDRC) at the amino terminus of domain 1, and their domain 2 has an extension of approximately 17 amino acids that is not found in other expansin families (Figure 4). The functional implications of these differences among families are currently unknown.

No proteins homologous to expansin domain 2 have yet been identified except for the G2A family. The structure of a G2A protein consists of two stacked β sheets with an immunoglobulin-like fold (Figure 5b) [20]. On the basis of

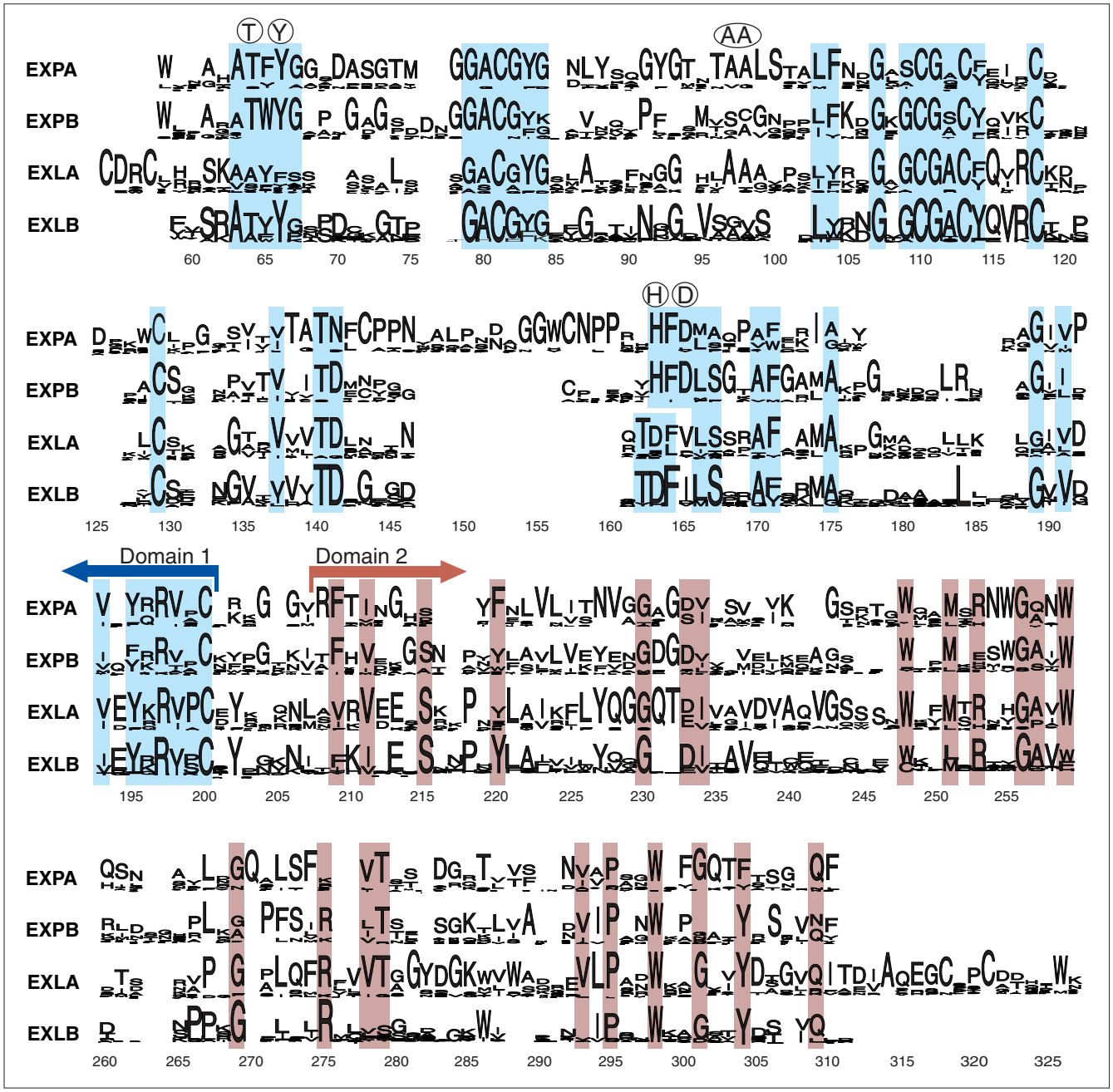

Figure 4
Sequence conservation in the expansin superfamily. Sequence logos for the four expansin families were generated with WebLogo [71] and manually aligned. The signal peptide and the poorly conserved amino terminus of the mature proteins have been removed from the alignment; because some expansins have exceptionally large signal peptides and amino-terminal extensions the alignment starts around position 60. In these sequence logos the height of the stack of amino-acid symbols at each position indicates the degree of sequence conservation, and the height of each letter within the stack indicates the frequency of the corresponding amino acid. Residues conserved between families are shaded, and the boundary between the two domains is indicated by arrows. Key residues that are part of the catalytic site of GH45 proteins and that are conserved in domain 1 of some expansin families are shown in circles above the logos. EXPA, α-expansin; EXPB, β-expansin; EXLA, expansin-like A; EXLB, expansin-like B.

this structure, some highly conserved aromatic residues present in expansin domain 2 have been hypothesized to form a binding strip for cell-wall polysaccharides [1,21], but this speculative idea has yet to be tested experimentally.

Localization and function

Expansins were first identified as wall-loosening proteins in studies of 'acid-induced growth' [3,22-24]. It was known for years that low extracellular pH (< 5.5) causes cell-wall

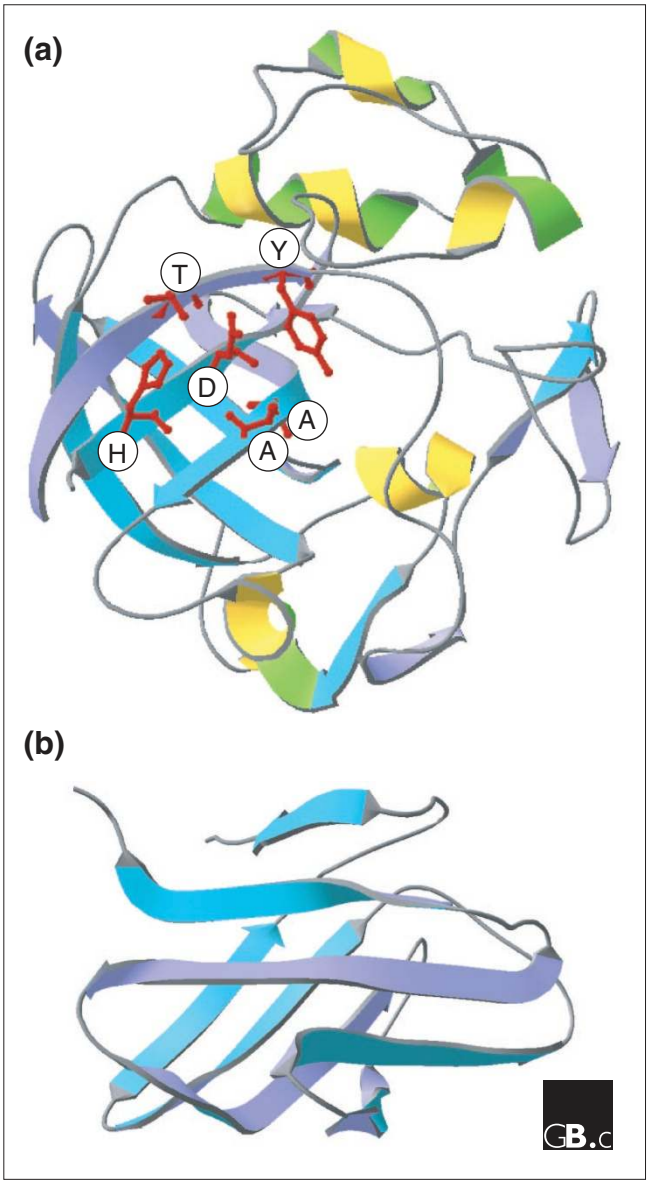

Figure 5
Structure of proteins homologous to expansin domains. **(a)** Expansin domain 1 (the catalytic domain of a GH45 endoglucanase from *Humicola insolens*; Protein Data Bank (PDB) code 2ENG). **(b)** Expansin domain 2 (a G2A protein from *Phleum pratense*; PDB 1WHO). In (a), the domain forms a β barrel; amino-acid residues that are conserved in expansins are indicated in the single-letter amino-acid code.

loosening in land plants as well as in a subset of green algae that have walls of similar structure [25]. The process is mediated in large part by wall-bound expansins with an acidic pH optimum [3]. Wall pH is normally determined by the activity of the plasma membrane H^+ ATPase, which pumps protons to the cell wall; the pH of the wall is typically about 5.5 but may go below 4.5 in some circumstances [25,26]. Acid-induced growth and expansin action are implicated in the growth responses of plants to hormones and to external stimuli such as light, drought, salt stress and submergence (anoxia) and in morphogenetic processes such as root-hair formation [27-31].

Expansin activity is usually assayed as the ability of a protein sample to induce extension of isolated cell walls (Figure 7). It may also be measured in stress-relaxation assays, in which the decay in wall stress is measured after the wall is rapidly extended and then held to a constant dimension [22]. Plant cell walls extend or relax by a process of molecular 'creep', in which the cellulose microfibrils and associated matrix polysaccharides separate from one another [32]. The energy needed to overcome the viscous resistance and entanglement of wall polymers comes from cell-wall stress, which in living plants arises from the turgor pressure within cells. Such molecular creep occurs only when the cell wall is loosened by expansins or by other factors (Figure 8); otherwise, the cellulose microfibrils are firmly held in place by matrix polysaccharides [27]. Artificial cell walls made of bacterial cellulose and xyloglucan have also been used as materials to investigate expansin action [33].

Expansin activity is most often associated with cell-wall loosening in growing cells [34]; this connection has been confirmed and extended by experiments in which expansin gene expression is manipulated in transgenic plants [35-38]. In most cases, silencing of expansin genes leads to inhibition of growth, whereas excessive ectopic expression leads to faster or abnormal growth. Localized expression of expansins is associated with the meristems and growth zones of the root and stem, as well as the formation of leaf primordia on shoot apical meristems [39] and the outgrowth of the epidermal cell walls during root-hair formation [40]. Additionally, expansins are implicated in other developmental processes during which wall loosening occurs, such as fruit softening [41-46], xylem formation [47], abscission (leaf shedding) [48], seed germination [49], penetration of pollen tubes through the stigma and style [4,50], formation of mycorrhizal associations with symbiotic fungi in root tissues [51], development of nitrogen-fixing nodules in legumes [52], development of parasitic plants [53,54], and rehydration of 'resurrection' plants, which curl up tightly when dry and expand when wet [55]. Some plants that are adapted to an aquatic environment respond to submergence with a pronounced elongation. This depends on wall acidification [56] and is correlated with activation of expansin gene expression [57-59].

In cell-fractionation studies, expansins are found bound to the cell wall, as expected from their activity [23,60,61]. With immunolocalization by light and electron microscopy, expansin proteins were localized to the cell wall [51,61,62], where they were found to be distributed throughout the thickness of the walls rather than concentrated in specific strata. There is at least one report that expansin mRNA can be found specifically at the polar ends of developing xylem cells [63]; transcript localization may be a means for ensuring

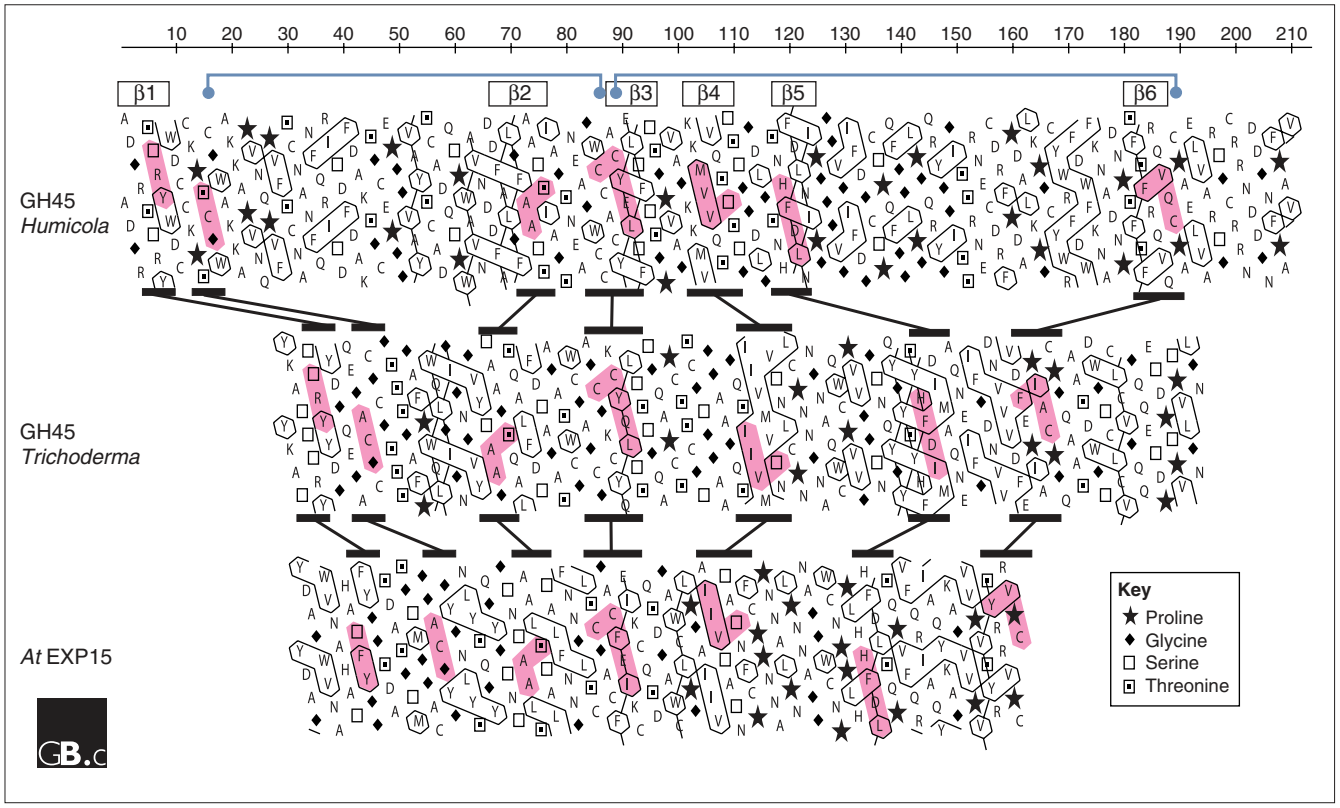

Figure 6
Aligned hydrophobic cluster analysis (HCA) plots of the catalytic domains of two GH45 proteins and domain 1 of an α-expansin protein, *Arabidopsis* EXPA15, with additional annotation based on the crystal structure of *Humicola* GH45. The GH45 sequences are from *Humicola insolens* (GenBank accession number P43316) and *Trichoderma reesei* (AAQ21385). HCA plots were constructed with DrawHCA [72]. In these plots, the amino-acid sequence of each protein is written out in duplicate in a helical representation that puts together amino-acid residues that would be next to each other in an α helix. The six β sheets that form a barrel in the GH45 from *Humicola* (see Figure 5a) are indicated by boxes above the plot. Cysteine residues involved in intramolecular bridges and conserved in expansins and GH45 proteins are shown by blue dots connected by blue lines, also above the plot. Selected conserved motifs are highlighted in pink and the differences in their relative positions between proteins are indicated by black lines between the plots. The interpretation of HCA plots is summarized in [73]. HCA uses the standard one-letter amino acid abbreviations except for four amino acids, as shown in the key. Hydrophobic residues are outlined. Clusters of hydrophobic residues are usually associated with regular secondary structures (α helices or β sheets). Zigzagging vertical lines of hydrophobic residues indicate alternating hydrophobic and non-hydrophobic residues, typical of exposed β sheets (for example, β2, β3, β5 and β6). Continuous hydrophobic clusters are more common in internal β sheets (for example, β4). Conservation of clusters and sequence motifs suggests that the core β-barrel structure with stabilizing cysteine bridges is conserved in the three proteins and that the differences are mostly in the size of the intervening loops. In *Humicola* GH45, the loops between β1 and β2 and between β5 and β6 have expanded considerably, while the other loops appear reduced in comparison with *Trichoderma* GH45. The latter appears more similar to expansin domain 1, which has an even more compact structure.

that protein production and secretion is directed to the ends of these cells. It is not clear whether this mRNA targeting is unique to expansins in xylem or whether it is a more general phenomenon. Finally, grass pollen produces and secretes specialized β-expansin proteins in copious amounts (they are known as grass pollen group-1 allergens) [4,64], but this is an unusual situation: expansins in other tissues have been found only at low concentrations.

Mechanism and regulation

All the α-expansin proteins that have been characterized so far have a pH optimum for cell-wall extension of about 4 [3,23,60]. This situation permits the cell to regulate α-expansin activity rapidly by modulating wall pH. The pH optimum of only one class of β-expansin proteins has been characterized, namely the group-1 grass pollen allergens (such as EXPB1 from maize), and it has a broad pH optimum centered at about 5.5 [65]. These pollen proteins are probably not involved in acid growth but rather in the wall loosening that is associated with invasion of maternal tissues by pollen tubes. It is expected that β-expansin proteins in somatic tissues have a pH dependence more similar to that of α-expansins, but so far β-expansin proteins in an active state have not been extracted from somatic tissues, so this expectation remains to be tested experimentally. Also, both α-expansin and β-expansin proteins are activated by reducing agents [3,4,65]; this could be biologically significant, as the cell-wall redox potential can be modulated by electron transport across the plasma membrane.

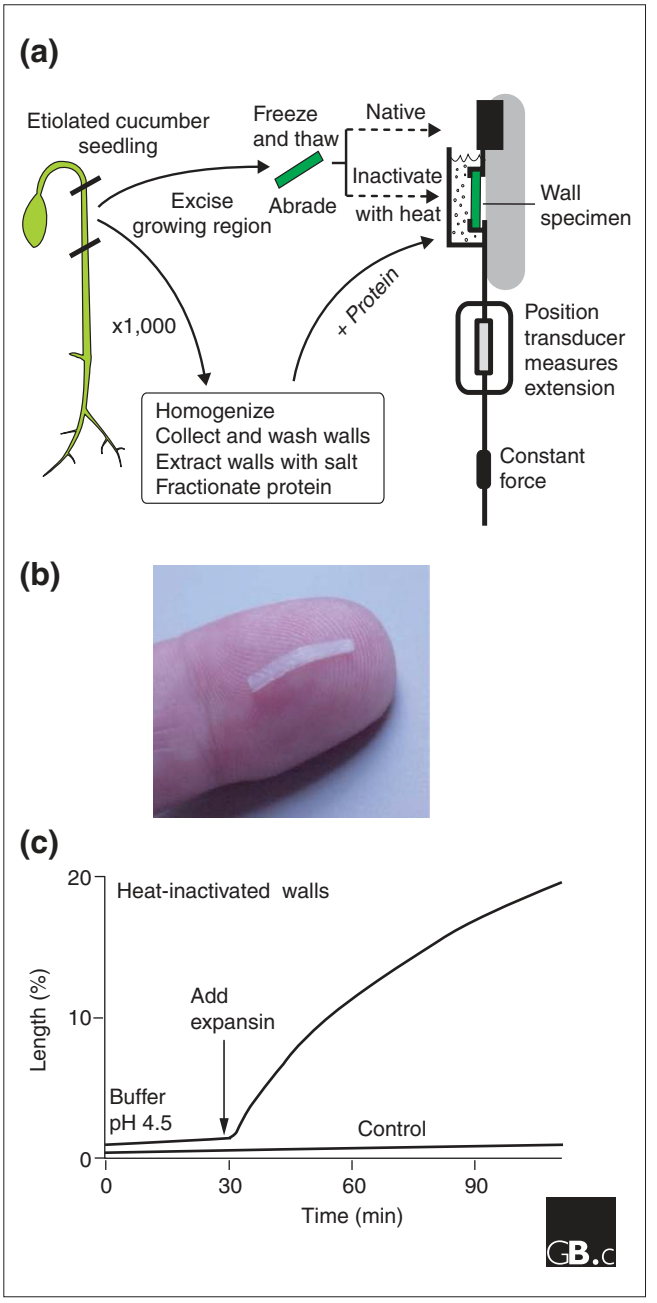

Figure 7
A common method for measuring the cell-wall extension activity of expansins. **(a)** Cell-wall specimens are excised from the growing region of a young seedling that has been grown in the dark (etiolated). The specimens are frozen and thawed in order to destroy the cells but leave the cell walls intact (the cuticle is abraded to facilitate penetration of proteins). The specimens are heat-treated to inactivate endogenous expansins and then clamped under constant tension in an extensometer. The extensometer measures the change in length of the sample, with or without the addition of exogenous expansins. Walls may be collected in parallel from other seedlings and extracted to obtain fractions with expansin activity, assayed as an increase in cell-wall length. **(b)** Photograph of a typical cell wall sample, placed on an index finger for scale, prior to clamping in the extensometer. **(c)** Time course for irreversible wall extension (creep) of heat-treated walls with and without the addition of expansin.

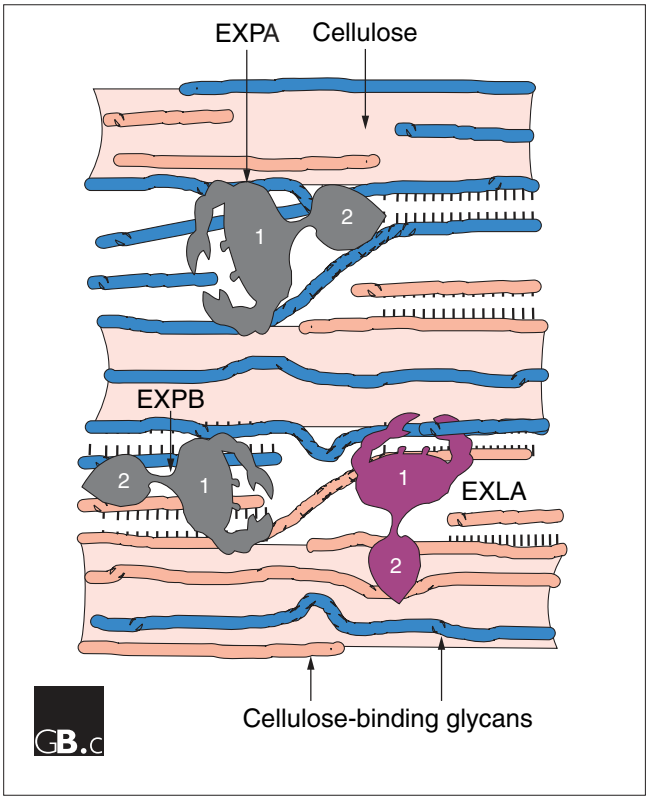

Figure 8
A simplified model of the plant cell wall and its loosening by expansins. The cell wall consists of a scaffold of cellulose microfibrils (shaded areas) to which are bound various glycans such as xyloglucan or xylan (thin strands); together these polysaccharides form a strong, flexible, load-bearing network based on hydrogen bonds (indicated by rows of short lines). Extension of the cell wall entails movement and separation of the cellulose microfibrils by a process of molecular creep. α-Expansins (EXPA) may promote such movement by inducing local dissociation and slippage of xyloglucans on the surface of the cellulose, whereas β-expansins (EXPB) work on a different glycan, perhaps xylan, for similar effect. Expansin-like A (EXLA) and expansin-like B (EXLB) proteins are predicted to be secreted to the cell wall, but their activity has not yet been established.

Expansins do not have hydrolytic activity or any of the other enzymatic activities yet assayed [64,66,67]. A report that they are proteases was later refuted [64]. Expansins also act very quickly - they induce rapid extension within seconds of addition to wall specimens, but they do not affect the plasticity or elasticity of the cell wall [68]. In contrast, cell-wall creep caused by an endoglucanase has a long lag time and is accompanied by large increases in wall plasticity and elasticity, indicative of major structural changes in the cell wall (cutting of cross-links) [68]. Thus, expansin's effects on cell walls are distinct from those expected of hydrolytic enzymes.

A nonenzymatic mechanism has been proposed for expansin action, in which expansin disrupts noncovalent bonds that tether matrix polysaccharides to the surface of cellulose

microfibrils or to each other [1,66,69]. In this model, the expansin is thought to act like a zipper that enables microfibrils to move apart from each other by ungluing the chains that stick them together. This idea is also supported by experiments in which an expansin is applied to artificial composites made of bacterial cellulose and xyloglucan [33]. Whatever their biochemical mechanism of action, expansins act in catalytic amounts to stimulate wall polymer creep without causing major covalent alterations of the cell wall [66].

Frontiers

In the published literature on expansins, gene expression has drawn the greatest amount of attention, but given the large size of the superfamily, the expression and presumptive role of many expansin genes remains unexplored. Although expression of specific expansin genes has been shown to be induced by hormones, by submergence, by drought stress, or by other stimuli, the signaling pathway has not been worked out in detail in even a single case. Major biochemical questions also remain regarding the specific wall polysaccharides on which expansins act, the differences between the action of α-expansins and β-expansins, and the molecular mechanisms underlying wall loosening. Answering these questions will require a much deeper understanding of cell-wall structure and in particular of how the cell wall is able to expand in a controlled fashion. Finally, it remains to be established whether expansin-like A and expansin-like B proteins have cell-wall loosening activity or not.

Additional data files

An alignment of the sequences used to make the phylogenetic tree in Figure 2 is available as Additional data file 1.

Acknowledgements

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