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3 **Plant expansins: diversity and interactions with plant cell walls**
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Abstract

Expansins were discovered two decades ago as cell wall proteins that mediate acid-induced growth by catalyzing loosening of plant cell walls without lysis of wall polymers. In the interim our understanding of expansins has gotten more complex through bioinformatic analysis of expansin distribution and evolution, as well as through expression analysis, dissection of the upstream transcription factors regulating expression, and identification of additional classes of expansin by sequence and structural similarities. Molecular analyses of expansins from bacteria have identified residues essential for wall loosening activity and clarified the bifunctional nature of expansin binding to complex cell walls. Transgenic modulation of expansin expression modifies growth and stress physiology of plants, but not always in predictable and even understandable ways.

Introduction

Plant cells grow in volume by a coordinated process in which the primary cell wall, which confines and shapes the cell, is selectively loosened, resulting in wall stress relaxation and consequential water uptake and cell enlargement [1]. An understanding of the molecular nature of cell wall loosening inevitably depends on our concepts of primary cell wall structure. Although the major structural components of growing cell walls are well known, how these components are connected to one another to make a physically strong yet extensible primary wall is an active area of current research and changing paradigms [2]. Growing cell walls in plants consist of one or more layers of cellulose microfibrils embedded in a hydrophilic matrix. Microfibrils make direct contact with one another and may also be linked together by noncovalent interactions with matrix polymers (**Figure 1A**). The matrix polymers include pectins and xyloglucans in most land plants, except in grasses and related species where arabinoxylans predominate. One of the novel ideas to emerge from recent work is that cell wall loosening during growth does not occur at all cellulose:matrix interfaces but is limited to selective sites where cellulose microfibrils make close contact with one another. These sites appear to be the targets of plant expansins, the focus of this review.

Like the proverbial blind men encountering an elephant for the first time, when we identified the first group of expansins (now called α -expansins), we glimpsed one facet of these cell wall-loosening proteins with their enigmatic effect on cell wall rheology [3,4]. Unlike any other plant protein characterized to date, α -expansins rapidly induce creep and stress relaxation of primary cell walls in a pH-dependent manner, yet without the lytic activity previously expected of a 'wall loosening' enzyme. Expansins provided an unorthodox solution to the mystery of how cell wall acidification increases wall extensibility and induces plant cell enlargement, e.g. in the early phases of auxin-induced growth. In the intervening years this view has solidified and deepened, but we've also come to appreciate the wider diversity of expansin genes, expansins' roles and the continuing molecular mystery of their remarkable action on plant cell walls.

The last major review of expansin was in 2005 [5]. This review focuses on emerging themes in the expansin field since then, with insights from recent structure-function analyses (**Figure 1B**), updated perspectives on new expansin-like sequences, and the lessons learned and questions

raised by transgenic manipulation of expansin expression. The potential application of microbial expansins in the biofuel field was reviewed recently [6] and is not detailed here.

Defining, refining and delimiting the expansin concept

The earliest definition of expansin was based on the wall-loosening activity of what we now call α -expansin. This was superseded by a sequence-based definition [7], later confirmed and extended by structural information [8,9]. Early bioinformatic studies identified the multigene, multi-family character of expansin sequences in plant genomes and documented the presence of distantly-related sequences in microbes [10,11]. The astonishing growth of sequence databases, combined with recent advances in expansin structure and phylogeny, as well as the discovery of related proteins in bacteria and fungi, leads to the current overview diagrammed in **Figure 2**.

Canonical plant expansins are torpedo-shaped proteins (~5 nm long and 3 nm wide) consisting of two domains (**Figure 1B**). The N-terminal domain is a six-stranded double-psi beta-barrel (DPBB) which is packed tightly next to a C-terminal domain having a β -sandwich fold. An open surface suitable for polysaccharide binding spans the two domains; recent data demonstrate that binding is largely determined by the β -sandwich domain, which is also classified as a family-63 carbohydrate binding module (CBM63) [12]. The DPBB domain is structurally related to family-45 glycosyl hydrolase (GH45), so this domain has sometimes been described as GH45-like. Despite this structural similarity, expansins lack the β 1,4-glucanase activity of GH45 enzymes, which in turn lack the wall-extension activity of expansins [8].

Plant expansins are classified by sequence-based phylogeny into two major families named EXPA (α -expansins) and EXPB (β -expansins). EXPAs were identified as mediators of acid-induced wall loosening while EXPBs include a subset of well-studied proteins known as group-1 grass pollen allergens as well as a larger set of proteins about which we know very little [13]. The group-1 allergens facilitate pollen tube invasion of the stigma by dissolving the middle lamella, which in grasses contains hemicellulose (arabinoxylans) and pectin (homogalacturonan). Expansins also include two smaller families named expansin-like A and B (EXLA;EXLB). Phylogenetic analysis shows these proteins to constitute separate and well-resolved groups, but their biological functions are uncertain. Because they have signal peptides directing them to the cell wall and because their structure is predicted to be the same as other expansins, they probably target the cell wall for modification.

This system of family nomenclature, which was originally based on the Arabidopsis and rice genomes, has proved robust for classification of expansins in numerous additional plant genomes (**Table 1**). Microsynteny between expansin-containing segments in the Arabidopsis and rice genomes helped define orthologous groups that originated from 12 ancestral *EXPA* genes, 2 ancestral *EXPB* genes and 1-3 ancestral *EXLA/EXLB* genes [10]. This analysis, which is applicable to the expansin family in other angiosperm genomes such as soybean [14], maize [15], poplar [16], grape [17], Chinese cabbage [18], and apple [19], showed that some clades have remained relatively constant in gene number since the last common ancestor of rice and Arabidopsis, whereas a few clades have expanded, the most striking example being EXPA clade V and EXPB clade-I; the latter has greatly and specifically proliferated in grasses (**Figure 3**),

giving rise to the pollen-allergen subclass of β -expansins as well as other grass-specific EXPBs [13]. Expansion of specific clades in grasses is associated with the distinctive cell wall composition of grasses compared with eudicots and most monocot groups, but the details of this connection still need to be worked out.

In more distantly related organisms, we find multi-gene *EXPA* and *EXPB* families in *Selaginella* (a lycopod) and *Physcomitrella* (a moss), but their orthology with the established angiosperm clades is harder to pinpoint because shared microsynteny is too weak [20,21]. Their common ancestor with angiosperm expansins may predate the diversification of the expansin genes represented by these clades. These genomes also lack *EXLA* and *EXLB* genes (Table I).

In *Micrasterias* (a Streptophyte green alga) four expansin-related genes were characterized [22]. Two genes had the same domain structure as plant expansins, one had an additional DPBB domain whereas another contained only the DPBB domain. Phylogenetic analysis suggested that the *Micrasterias* expansins may be sister group to the *EXPA* family in land plants. Ectopic expression of a *Micrasterias* expansin led to abnormal morphogenesis, as one might expect if the protein has wall-loosening activity. Expansin-related sequences are also found in other green algae such as *Spirogyra*, *Nitella* and *Penium*, so it seems likely that expansin-mediated wall loosening evolved long before the origin of land plants. Wall-loosening activity of algal expansins has not yet been documented, but is needed to confirm this hypothesis.

The *Micrasterias* DPBB protein named MdEXP4 does not conform to the two-domain structure of canonical expansins [22]. This raises a difficulty that increases as more, diverse genomes are sequenced: what to call proteins that either do not conform to the two-domain structure of expansin or are so distant in sequence that it is unclear whether they have the same structure or function as an expansin? We first faced this situation soon after identifying *EXPA* cDNAs [23]; BLAST analysis revealed a *Bacillus subtilis* protein named YOAJ that shared limited sequence similarity with some expansin sequences, but lacked the conserved motifs common to plant expansins. Was this a bacterial expansin? Evidence for homology became clear when the crystal structure of YOAJ was solved, showing it to be congruent with plant expansins [9]. Activity assays then showed that YOAJ could indeed loosen plant cell walls, confirming this protein (renamed EXLX1) as the first documented instance of a bacterial expansin.

A more difficult example is that of a protein named swollenin (SWO), from the fungus *Trichoderma* [24]. It contains 493 aa, which is much larger than the ~250 aa of canonical expansin. The SWO C-terminus is distantly related to plant expansin, but the alignment contains many insertions and deletions [25]. In limited tests, SWO did not induce creep of plant cell walls as do plant expansins [8]. And unlike plant expansins, SWO has clear β 1,4-glucanase activity, although with unusual kinetics [24]. Should SWO be considered an expansin? Probably not, as its wall-loosening activity, its β 1,4-glucanase activity and very likely its 3D structure differ from expansin. Moreover, a phylogenetic analysis indicates that SWO is well separated from plant expansins and several classes of GH45 enzymes [26]. A 3D structure for SWO would help to clarify the distinctions between more typical GH45 enzymes and expansins.

With further delving into the sequence databases it becomes clear that proteins with an expansin-like DPBB fold are very diverse [27] and have undergone considerable evolution [25],

particularly in fungi. In addition to expansins and GH45 enzymes, they include a variety of other enzymes and proteins with no known enzymatic activity. Plant genomes contain one or more genes for one-domain DPBB proteins, variously named citrus blight p12 or plant natriuretic peptide or kiwellin (Figure 2). There is no evidence that they have wall-loosening activity (tests of p12 and kiwellin in my lab proved negative), and they are not classified as expansins, although they are more closely related in sequence to plant expansins than are some of the nonplant DPBB sequences that have been called expansin-like.

In fungi, proteins with low sequence relatedness to the expansin DPBB domain are particularly numerous and have been clustered into several distinctive groups that include SWO and GH45 [26]; in some reports such proteins have been named 'loosenin' [28] or EXPN (for N-terminus of expansin) [29]. Another fungal protein that has been called 'cerato-platanin' has structural similarity to expansin's DPBB domain and is reported to disrupt cellulose fibers without evidence of lytic activity [30]. In other studies, proteins with only the DPBB domain have been simply called expansins or expansin-like, e.g. [31,32], despite clear differences from canonical expansins. Such breaks from accepted nomenclature leads to avoidable confusion. *Caveat lector*. The lesson is that plants and fungi have single-domain DPBB proteins that share partial sequence relatedness and structural features with expansin, but their functions are likely distinct from that of expansin. It appears that the DPBB domain in some cases has β 1,4-glucanase activity, yet in other cases the hydrolytic activity is lacking while an ability to disrupt cellulose fibers is evident. In no case has a single-domain DPBB protein been shown to cause plant cell wall creep. Here it is worth reiterating that, while GH45 enzymes have a DPBB fold and degrade β 1,4-glucans in plant cell walls, they do not induce cell wall creep [8].

Two recent reports suggest that mushroom stipes have a spatial pattern of elongation and wall extensibility characteristics that resemble etiolated hypocotyls [33,34]. Attempts to isolate proteins with wall extension activity from stipes were not successful. Nevertheless, the mushroom stipe might provide suitable material to test some of the fungal DPBB proteins mentioned above for fungal wall-loosening activity. A protein from snail gastric contents was isolated that stimulated elongation of stipe cell walls without hydrolytic activity [33], i.e. resembling expansin action. Partial amino acid sequences of the protein did not reveal a match to expansin or indeed to any other protein in public sequence databases.

Expansin structure, binding to cell walls and mechanism of wall loosening

The most detailed structure-function information comes from recent studies of bacterial expansins, which in at least some cases facilitate bacterial colonization of plant tissues [6]. Heterologous expression of plant expansins in active form has been problematic, but bacterial homologs are readily expressed in active form in *E. coli* [35]. Unlike plant α -expansins, which seem to precipitate at concentrations much above 10 μ g/mL, the bacterial proteins can be concentrated to high levels, facilitating crystallography, NMR and other analyses. The expansin from *Bacillus subtilis*, named BsEXLX1 (=YOAJ), has proved particularly informative. Expression of individual BsEXLX1 domains showed that the β -sandwich (CBM63) domain alone determined protein binding to whole cell walls and to pure cellulose, whereas wall-loosening activity required both domains to be linked together [36]. The DPBB domain showed negligible binding to cell walls or cellulose and did not induce wall extension even at concentrations 26X higher

than values of full-length protein that caused wall extension. Site-directed mutagenesis enabled the function of specific residues to be studied. Binding to cellulose is mediated predominately by three conserved aromatic residues on the 'belly side' of the β -sandwich domain, whereas binding to pectin is determined by non-conserved basic residues on the 'backside' of the domain (**Figure 1B**). With an isoelectric point of ~ 9 , BsEXLX1 predominantly bound to pectin in whole cell walls via nonspecific electrostatic interactions. Mutagenesis of three basic residues to glutamine reduced binding to pectin, but increased wall-loosening activity, showing that binding to pectin diverted the protein from its site of wall loosening. Innovative solid-state NMR spin diffusion studies further documented BsEXLX1 interactions with pectin and cellulose in complex cell walls [37]. In an 'RKK' mutant lacking three basic residues on the back side of the β -sandwich, BsEXLX1 interactions with cellulose were increased, as was wall loosening activity, thus confirming the significance of cellulose binding for wall-loosening activity. It is noteworthy that the NMR shift of the cellulose bound by BsEXLX1 was different from that of bulk cellulose, indicating BsEXLX1 preferentially targets a distinctive structural form of cellulose of low abundance in the cell wall. These results complement binding experiments showing that BsEXLX1 bound to a selective region of cellulose [12]. They also jibe with independent experiments showing that plant cell wall extensibility is controlled at limited sites, dubbed biomechanical hotspots, where cellulose microfibrils come into close contact with each other [2,38].

Cellulose-expansin interactions were confirmed and extended with crystal-based structures of BsEXLX1 in complex with cellohexaose, a cellulose oligosaccharide with six glucose residues (**Figure 1B**). The crystal complex revealed a unique dimeric structure in which a single cellohexaose was sandwiched between the β -sandwich domain of two proteins [12]. The six glucose residues made CH- π bonds with alternate aromatic residues on the two protein surfaces. Glucose rings have two surfaces, one with two axial CH groups, the other with three axial CH groups. Glucose interactions with the aromatic residues were always via the more hydrophobic face populated with three CH groups. Binding isotherms combined with calorimetry led to the conclusion that BsEXLX1-cellulose interactions were predominantly driven by entropy changes. This most likely involved the release of structured water from the cellulose and protein surfaces.

Additional studies have confirmed that other bacterial expansins have the ability to bind cellulose and cell walls [39-43]. The binding determinants generally appear to be similar to the situation described above for BsEXLX1, except for the expansin of *Pectobacterium carotovorum*, which has an acidic pI (~ 4.8); as a result, this protein binds to cellulose but not pectin [43].

In contrast to the wealth of new data on cell wall binding by bacterial expansins, there appears to be only a single detailed study of the binding activity of α -expansin [44]. The results indicate that the protein binds to noncrystalline regions of cellulose with a maximum ratio of $\sim 1:1,000$ (dry mass ratio, protein:cellulose) at saturation and with a K_d of $\sim 0.1 \mu\text{M}$ (this is recalculated from the data in the article). This binding is tighter than that of BsEXLX1 and other bacterial expansins ($K_d \sim 1-3 \mu\text{M}$). The basis for tighter binding is unknown, but it may contribute to the greater wall-loosening activity of α -expansins compared with bacterial expansins. Other results showed that most of the endogenous α -expansin remained tightly bound to hypocotyl walls

after extraction with detergent, 1 M NaCl and 1 M urea and was only extracted under conditions that denatured the protein [44]. Thus the standard protocol for α -expansin extraction from plant cell walls by use of 1 M NaCl removes only a small fraction of the protein in the wall, most likely protein that is ionically bound to pectins.

In a more recent report the CBM63 domain of a strawberry EXPA was expressed in *E. coli* and its binding to various polysaccharides was characterized [45]. The binding constant to cellulose was 1,000X less than that of native cucumber EXPA and the binding curve showed no sign of saturation, unlike the results described above for native EXPA. In our experience *E. coli* expression of plant expansin domains does not produce properly folded protein; the anomalous binding behavior of this recombinant protein suggests a misfolded protein. In another study, transgenic expression of this expansin CBM63 fragment in *Arabidopsis* led to pleiotropic effects on growth and cell wall properties, including composition, mechanics, metabolism and resistance to the necrotrophic fungus *Botrytis cinerea*, effects that are interesting, puzzling, and not well understood mechanistically [46].

Tight binding also seems to be the case for EXPB proteins endogenously bound to rice cell walls, where expansin could be extracted only under denaturing conditions [47]. Clearly, our understanding of the binding of plant expansins to cell walls and its mobility within the cell wall needs deeper study.

Concerning the mechanism of wall loosening by expansin, all the expansins examined to date, whether from plants or bacteria, lack detectable lytic activity. Paper weakening or cellulose fiber disruption has been reported for several bacterial expansins (reviewed in [35]) as well as for EXPA [48]. This result, in combination with the lack of lytic activity, has been taken as evidence that expansins may disrupt noncovalent binding between cellulose microfibrils. When such results were first obtained [48], the common model of the cell wall held that cellulose microfibrils did not make direct contact with each other, but were tethered together by xyloglucans. This model is no longer tenable [2,49], and alternative models have been proposed in which microfibrils make closer, limited contacts, potentially glued together by lateral adhesion with a monolayer of xyloglucan. Such contact regions may be the limited sites targeted by α -expansin [44,48] and by bacterial expansin [37].

If expansins disrupt noncovalent bonding of polysaccharides, they might make surface glucans of cellulose more accessible to enzymatic attack by cellulases. Experimental results on this point, however, are ambiguous. When cellulases were added in miniscule amounts to cellulose, the limited hydrolysis of cellulose was indeed enhanced appreciably by addition of plant or bacterial expansins (reviewed in [35]). However, inert proteins such as bovine serum albumen produced the same or nearly the same degree of synergy, which likely arises from nonspecific effects, such as blocking sites of nonproductive binding of the cellulase. In some cases there may be more to the synergism than this explanation, but further work is needed to demonstrate it decisively.

Turning to plant β -expansins, a remarkable action of ZmEXPB1, the β -expansin from maize pollen, was recently discovered: quite unlike α -expansin and bacterial expansins, ZmEXPB1 solubilizes arabinoxylans and homogalacturonan from grass walls [50]. This action is specific to

grass cell walls, which have evolved a distinctive composition and a selectivity for loosening by this pollen subclass of β -expansins [13]. Despite the abundant release of matrix components, no evidence for lytic activity could be obtained in extensive experiments [50]. This solubilizing action may not be common to the larger class of β -expansins that are found in eudicots as well as monocots, but may be a special attribute of the pollen allergen β -expansins that are unique to grasses (**Figure 3**).

Although the various forms of expansins consistently lack demonstrable wall lytic activity, negative results cannot prove that expansins lack any form of enzymatic activity. A recent case in point is the discovery of lytic polysaccharide mono-oxygenases (LPMOs), some of which were considered nonenzymatic cellulose binding modules until recently [51,52]. LPMOs contain copper, they produce lytic oligosaccharide fragments, and their activity is reduced by copper chelators. None of these attributes applies to expansins, which clearly are not LPMOs. Do expansins have some other cryptic enzymatic activity? The identification of specific residues in the DPBB domain of BsEXLX1 that are essential for wall extension activity [36] is suggestive of an enzymatic site, but hardly conclusive. Pages could be written for and against this idea, but the fact remains that every direct test for enzymatic activity has so far proved negative and so alternative mechanisms of wall loosening, however anathema to enzymologists, have to be considered. As described above, one such alternative proposes that expansins disrupt noncovalent bonding of cell wall polysaccharides to each other, enabling turgor-driven creep of cell wall polymers [48]; this remains a viable working hypothesis with implications about cell wall structure [2].

Developmental expression

With so many expansin genes in plant genomes (See Table 1), it comes as no surprise that different expansin genes exhibit different expression patterns, varying by cell and organ specificity and by external conditions and response to plant hormones. Recent examples include the diverse expression of EXPB genes in soybean, particularly during *Rhizobium* infection and mycorrhiza associations [53], the expression of expansin genes in the wheat peduncle with modulation by Rht alleles for dwarfism, the involvement of multiple expansin genes in Arabidopsis leaf growth [54], and the complex regulation of expansin gene expression in grape during berry ripening and during phylloxera-induced gall formation [17,55]. Building on earlier studies (reviewed in [56]), recent work has also documented expression of specific expansin genes in response to various hormones [57-60]. Wherever cells are growing or modifying their cell walls, one or more expansin genes are usually involved.

Root-specific development

A notable set of developmental studies has documented expansin involvement in root hair formation and elongation [61]. Partial silencing of AtEXPA7 – a gene previously identified as root-hair specific [62] – resulted in a short root hair phenotype in Arabidopsis [63]. A root hair mutant in rice was mapped to a point mutation in OsEXPA17 [64]. The mutation could be partially complemented with OsEXPA30 and AtEXPA7 under control of the CAMV 35S promoter. These three genes - plus AtEXPA18 - are root-hair specific and are members of EXPA Clade-X [10]; the new results support the view that the expression and function of at least some

expansin genes are highly conserved throughout angiosperm evolution and that the clade system of classification may yield valuable hints about specific expansin gene functions. An informative experiment in this series with the OsEXPA27 mutant would have been to test for complementation with genes from other expansin clades, making use of a root-hair specific promoter. This would test if root hair growth specifically requires the Clade-X proteins or whether any EXPA could do the job. The differences between diffuse growth of most cells in the plant body versus tip growth of root hairs might require specific features of Clade-X expansins for interactions with cell wall components of root hairs.

In the case of grasses a specialized group of β -expansins are additionally involved in root hair growth: *OsEXPB15* in rice and *HvEXPB1* in barley [65]. The genes in this phylogenetic group are specific to grasses and are part of the impressive radiation of EXPB genes that is grass specific [13]. Expression of *HvEXPB1* was previously shown to be lacking in a root-hair deficient mutant of barley [66]. The results imply that root hair elongation in grasses requires coordinate action by both α - and β -expansins, perhaps another consequence of the change in cell wall polymer composition during evolution of the grass lineage.

Other recent studies of root development in Arabidopsis have shown that α -expansins are involved in the emergence of lateral root primordia, both in the primordium itself and in the cortical cell layers covering the pre-emergent primordia; this involves *AtEXPA4*, *14*, *17*, which may promote cell separation [67,68]. One might expect a parallel set of EXPB genes to be involved in lateral root formation in grasses, but this has yet to be investigated. One notable aspect of this work is that the transcription factor LBD18/ASL20 was shown to bind directly to the promoter of *AtEXPA14* and to activate its expression. LBD18 is downstream of the TIR1 auxin receptor and the ARF7 and ARF 19 transcription factors, and so it seems that auxin induction of lateral roots may provide an example (the first?) of a full explication of the signal transduction network from initial hormone receptor to the activation the cellular mechanisms that loosen cell walls and stimulate cell growth and morphogenesis. Additional cases of upstream control of expansin gene expression by other specific transcription factors have been documented [69-73].

In other root-related work, studies of mycorrhizae reported an EXPA epitope concentrated in arbuscule branches within tobacco roots [74] and an epitope of 'EXPN' (=a fungal DPBB protein) in fungal (*Laccaria bicolor*) cell walls within ectomycorrhizal mantles in poplar roots [29]. These studies build on previous work indicating that plant expansins are specifically recruited during cell wall remodeling of roots during mycorrhiza symbiosis [75]. Another less symbiotic association with roots is made by nematodes which have their own expansin-like proteins (at least the DPBB domain) [76] that are reported to suppress plant defenses [77]. Plant expansins are also involved in root remodeling by nematodes [78]. Thus expansins have emerged as a central player in numerous aspects of root development, extending the early work on shoot growth and development.

Stress responses and genetic manipulation of expansin gene expression

Many plants respond to water deficits by increasing their root/shoot ratio, an adaptive change in plant form that is achieved in part by maintaining root growth and reducing shoot growth.

Changes in expansin expression facilitate this growth adaption in maize where water deficits enhance expression of selected expansin genes within the growth zone of roots [79]. In leaves a reverse response is observed: when maize seedlings were treated with 100 mM NaCl, leaf elongation was inhibited and ZmEXPB6 protein concomitantly vanished from leaf cell walls [80]. Leaf growth could be partially restored by exogenous application of recombinant ZmEXPB6 to leaf surfaces, an effect seen only in NaCl-treated plants, indicating a specific action of NaCl on ZmEXPB6 abundance. Whether these maize responses are representative of other species is uncertain, but there is some reason to believe so [81].

Genetic manipulation of expansin gene expression has become a common approach in this field. Numerous studies have reported that transgenic expression of expansin genes in tobacco and Arabidopsis, under control of the constitutive CAMV 35S promoter, leads to various changes in growth and enhanced resistance to biotic and abiotic stresses (**Table 2**). Changes in growth and development can be rationalized from the wall-loosening action of expansins, but enhanced resistance to various oxidative stresses and pathogens remains unexplained. One wonders whether ANY expansin, including bacterial expansins, might cause such effects and whether these effects are specific to expansin. If so, are the responses due to the action of expansins on the cell wall or are they mediated by some other mechanism? Perhaps the mis-regulation of expansin is sensed as a disturbance in cell wall integrity, invoking pre-programmed defense responses [82]. A deeper understanding of the general applicability and mechanistic bases for these effects on stress tolerance is needed if we are to go beyond this genetic version of ‘spray and pray’ trials and deploy the transgenic approach for improving crop responses to stress.

An interesting series of related experiments involved overexpression of the *OsEXPB8* gene in rice, reportedly improving growth and root system architecture [83]. *OsEXPB8* overexpression in rice suspension cells resulted in a 6X reduction in cell wall stiffness, measured in whole cells by atomic force microscopy, with a concomitant increase in cell growth rate. In the opposite direction, silencing of *OsEXPB8*, as well as *OxEXPA8*, increased wall stiffness and inhibited cell growth [84,85]. The apparent stiffness values were ~1,000X less than the values obtained by other measurement techniques with comparable plant cells [86]. The reason for this large difference in wall stiffness is unclear. The expansin-dependent changes in rice cell wall stiffness resemble the action of pollen protein ZmEXPB1 on (grass) coleoptile cell walls [50] but contrast with the application of EXPA to (dicot) hypocotyl cell walls, which induced cell wall creep but did not change wall stiffness [87]. These comparisons reinforce the notion that the cell walls of grasses and dicots differ in their responses to expansins.

Questions remaining

While our understanding of expansin interactions with the plant cell wall have deepened considerably in detail, the long-standing question of its loosening action remains enigmatic. This is partly due to the complexity of the plant cell wall and our rudimentary understanding of how cell wall polymers are assembled to make a load-bearing yet extensible primary cell wall. Solving the mystery of expansin’s loosening actions will likely reveal important features of wall structure. How ectopic expression of expansins results in enhanced tolerance to a variety of stress is perplexing and merits deeper exploration. Finally, a search for expansin-like sequences

in the increasing number of fungal genomes reveals a huge diversity of distantly related proteins whose functions and biology have yet to be explored.

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Table 1. Minimum gene numbers reported for the four expansin families of selected plant genomes and for the polyphyletic EXLX group in selected microbial genomes. Some of the gene counts may be underestimates due to incomplete genome annotations at the time of analysis (marked *).

Species [ref#]	EXPA	EXPB	EXLA	EXLB	total
Angiosperms:					
<i>Arabidopsis thaliana</i> [10]	26	6	3	1	36
Poplar [16]*	27	3	2	4	36
Grape [17]	20	4	1	4	29
Soybean [14]	49	9	2	15	75
<i>Medicago truncatula</i> [14]*	16	1		1	18
<i>Phaseolus vulgaris</i> [14]*	25	6		5	36
Apple [19]*	34	1	2	4	41
Chinese cabbage [18]*	39	9	2	3	53
Rice [10]	33	18	4	1	56
Maize [15]*	36	48	4		88
Nonflowering Plants:					
<i>Selaginella moellendorffii</i> [20]	15	2			17
<i>Physcomitrella patens</i> [20,21]	28	7			34
Algae					
<i>Micrasterias denticulata</i> [22] *					4 "EXP"
Bacteria & fungi: [25]					
<i>Bacillus subtilis</i>					1
<i>Clavibacter michiganensis</i>					2
<i>Ralstonia solanacearum</i>					1
<i>Xanthomonas campestris</i>					1
<i>Aspergillus niger</i>					1
<i>Sclerotinia sclerotiorum</i>					1
<i>Magnaporthe oryzae</i>					1

750 **Table 2:** Selected examples of studies reporting effects of ectopic expression of expansins on
751 stress physiology and morphogenesis

752

Gene (source)	Transgenic plant	Effects	Reference
TaEXPB23 (wheat)	tobacco	Tolerance of drought, oxidative stress, low P	[88-91]
PpEXPA1 (<i>Poa pratensis</i>)	tobacco	Tolerance of heat, oxidative stress	[92]
GmEXPB2 (soybean)	soybean	Improved leaf growth, P efficiency	[93]
AtEXPA1 (Arabidopsis)	Arabidopsis	Stomatal functions	[94]
GhEXPA1/GhRDI1 (cotton)	Arabidopsis, cotton	Larger seeds, more fruits, higher fiber yield	[95]

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Figure Legends

Figure Legends

Figure 1: (A) Atomic force micrograph of the innermost surface of the epidermal cell wall of onion scale, showing a multi-layered structure made up of dispersed cellulose microfibrils that are aligned in short regions (indicated with arrows). The wall was never dried and was imaged under water under condition where the matrix polysaccharides are too flexible for clear imaging. The smooth, nonfibrillar regions are thought to be semi-rigid matrix polysaccharides covering cellulose microfibrils. Image courtesy of Tian Zhang, based on [96]. **(B)** Structure of BsEXLX1 in complex with cellohexaose. Cellohexaose (green) is sandwiched between the CBM63 domain of two EXLX1 proteins (red, blue). The surface responsible for binding to cellulose is populated with a linear arrangement of three aromatic residues (two tryptophans and one tyrosine, shown in red and blue above and below the cellohexaose). Binding to pectin is determined by nonconserved basic residues on the reverse side of the CBM63 domain, indicated by arrow. Image based on [12].

Figure 2: Distribution of expansins and related proteins. The top panel shows the structure of expansin (middle) to be a fusion of an N-terminal DPBB domain and a C-terminal β -sandwich (CBM63). The next three panels show the names and modular structure of the major proteins related to expansin or the individual domains in plant, bacterial and fungal genomes. The group-2 pollen allergens are specific to grasses. In bacterial and fungal genomes additional binding modules or family-5 glycosyl hydrolases (GH5) are often linked to expansin.

Figure 3: Phylogenetic structure of EXPB Clade I, demarcated with a broken line. This clade consists of a 'conservative' branch (lower region), populated by EXPBs from eudicots and monocots, including grasses, and a 'divergent' (upper region) found only in grasses and closely related species. One set of these divergent sequences includes the group-1 grass pollen allergens (indicated with red arrow), expressed only in grass pollen and closely related species. The other genes in the figure are only shown here for phylogenetic rooting and are more fully explained in [13]. The figure was produced by Javier Sampedro and published in a different form in [13].

Cosgrove Figure 1

Cosgrove: Figure 2

Cosgrove Figure 3: