

# Expansive growth of plant cell walls

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**Abstract** The enlargement of plant cell walls is a key determinant of plant morphogenesis. Current models of the cell wall are reviewed with respect to their ability to account for the mechanism of cell wall enlargement. The concept of primary and secondary wall loosening agents is presented, and the possible roles of expansins, xyloglucan endotransglycosylase, endo-1,4- $\beta$ -D-glucanase, and wall synthesis in the process of cell wall enlargement are reviewed and critically evaluated. Experimental results indicate that cell wall enlargement may be regulated at many levels. © 2000 éditions scientifiques et médicales Elsevier SAS

**Cell wall growth / endo-1,4- $\beta$ -D-glucanase / expansin / xyloglucan endotransglycosylase**

**EG, endo-1,4- $\beta$ -D-glucanase / XET, xyloglucan endotransglycosylase**

## 1. INTRODUCTION AND OVERVIEW OF CELL WALL GROWTH

Some of the largest organisms on Earth are plants and this achievement is based largely on the growth and mechanics of the plant cell wall. To grow, plant cells must physically expand their restraining cell walls, while at the same time preserving the wall's mechanical integrity in the face of high turgor pressures. This is a delicate process that may be considered from many points of view. In this article, I review some of the molecular processes thought to modulate cell wall enlargement, but first I wish to put this issue in the broader context of spatial, temporal and evolutionary control of cell wall growth.

Spatial patterns of wall expansion can vary considerably within a cell and between different cell types. We recognize cell types to a large extent on the basis of their size and shape, which are generated by highly controlled and cell-specific patterns of enlargement and division. Wall expansion may be highly localized, as in tip-growing cells such as root hairs and pollen tubes, or it may be very diffuse, as in potato tuber parenchyma. Between these two extremes one finds a wealth of intermediate patterns where diffuse expansion is limited to certain walls or where there are gradients in expansion rate along cell walls. This wealth of spatial growth patterns gives rise to the large

menagerie of cell shapes and sizes illustrated in any textbook on plant anatomy. Our understanding of the spatial control of wall growth at the cellular and molecular levels is still very limited. Presumably, the spatial patterning is largely regulated by localized secretion of structural materials and catalysts that influence the assembly, cross linking, turnover, and slippage of wall polymers, but the details of these processes and their localization are incompletely known and may be complex [18, 37,66]. Even for the apparently simple case of diffuse cell wall growth, many different molecular modifications of the cell wall have been invoked to explain regulation of cell growth [9, 104, 105].

Cell wall enlargement also has a temporal aspect, which may be conveniently divided into two time scales. First, there is the large scale that encompasses the entire growth phase of the cell. If we consider a cell in the typical stem or root, the growth phase begins with relatively slow meristematic growth, continues through rapid expansion and vacuolation of the cell as it is displaced through the 'zone of cell elongation', and ends with rigidification of the cell wall and the cessation of growth (*figure 1A*). Meristematic cells enlarge slowly, typically at a rate that matches their rate of cytokinesis, with the result that average cell size changes little over time [44]. As cells

are displaced into more rapidly expanding regions of the plant, they cease dividing and begin vacuolation, which provides a relatively inexpensive form of volume expansion. It is sometimes stated that meristematic cell growth differs qualitatively from expansive cell growth, yet it is likely that the same mechanisms of wall enlargement are involved in these two growth periods. The switch to a vacuolated mode of cell growth is accompanied by higher expression of numerous genes, including those encoding for abundant membrane proteins such as aquaporins [117] and those encoding for wall synthesis and wall metabolizing enzymes [85, 99]. In the final growth phase, the cell wall becomes more rigid and may become thicker as wall deposition exceeds the thinning effect of expansion. During this large-scale time period, which may last from many hours to days, wall structure and composition may change in subtle and not-so-subtle ways, as a result of (a) expansion itself, (b) changes in the deposition of wall materials, and (c) action by wall modifying enzymes. Numerous studies have documented changes in the physical and biochemical properties of walls as they progress through this large-scale growth period, which overlaps the early phases of cell differentiation (for reviews see [9, 92, 114]). The cessation of growth in the last phase probably involves multiple simultaneous processes that reduce wall loosening and promote cross linking of the cell wall [18, 19].

The second time scale is the short one, in the range of seconds to hours. With sensitive growth measuring methods, it is evident that cell wall expansion does not progress at a smooth even rate; instead, it often exhibits dynamic fluctuations (*figure 1 B*), sometimes cyclic, at other times highly irregular and complex [23, 50, 59, 75]. The underlying molecular basis for these rapid fluctuations in wall expansion is not well established, but may be based on ephemeral changes in the wall environment (such as pH, reactive oxygen species, and redox potential) that are regulated by the cell via transport across the plasma membrane. These fast changes are superimposed on the more gradual trends based on the age and position of the cell in the growth zone (*figure 1 C*). It seems likely that distinct processes regulate cell growth at these two time scales.

As a last point for this introduction, I wish to note that diffuse growth and tip growth are only two of the many possible solutions to the general problem of cell wall expansion. Other groups of walled organisms have invented other solutions. For example, diverse groups of green algae have evolved alternative cell wall structures and diverse mechanisms of cell wall

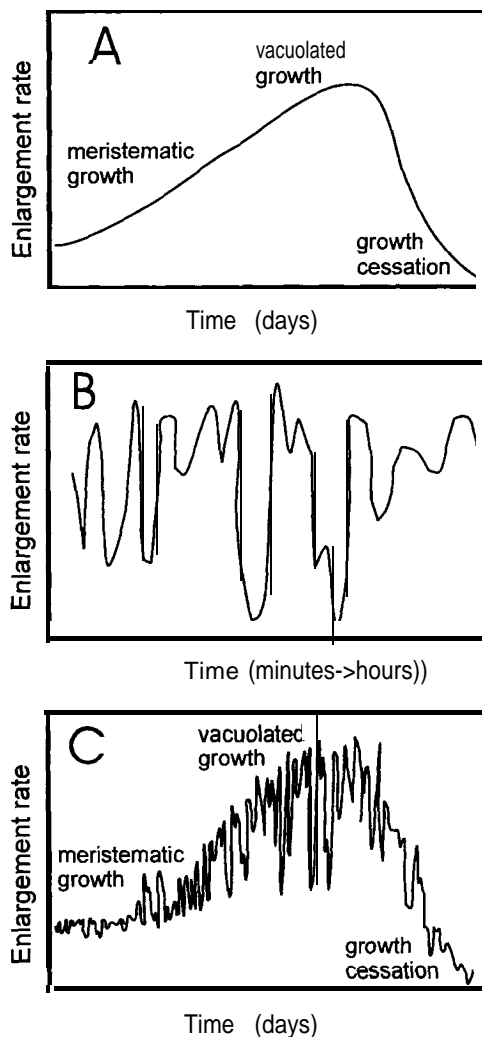
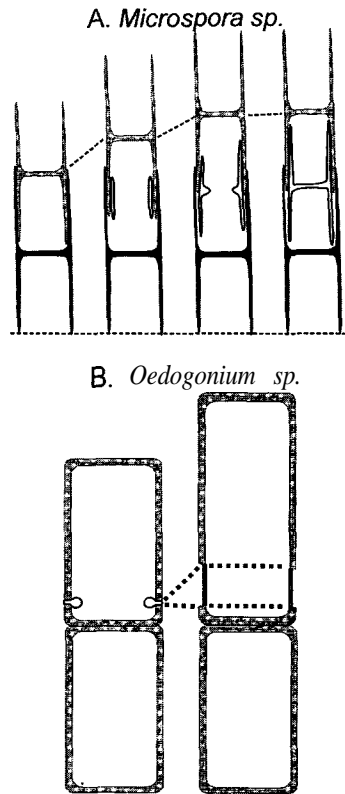


Figure 1. Two time scales on which plant cell wall growth may be analyzed. A, Large time scale (many hours or days), in which cells graduate from meristematic growth to rapid expansion accompanied by vacuolation, followed by cessation of enlargement. B, Short time scale (minutes to hours), in which growth may reversibly fluctuate. C, Summation of the long-term trends with the short-term fluctuations.

enlargement [86]. Some, such as *Microspora* sp., use a system of telescoping walls, in which surface area is generated by the sliding of two pre-formed, nested wall cylinders (*figure 2 A*). This process is very different from that used by land plants (Embryophyta) which generate new surface area by expansion (stretching/slippage) of the wall material itself. Another green alga, *Oedogonium* sp., secretes a ring of pliant wall material inside the tough wall, near one end of its cylindrical cell (*figure 2 B*). It then ruptures the



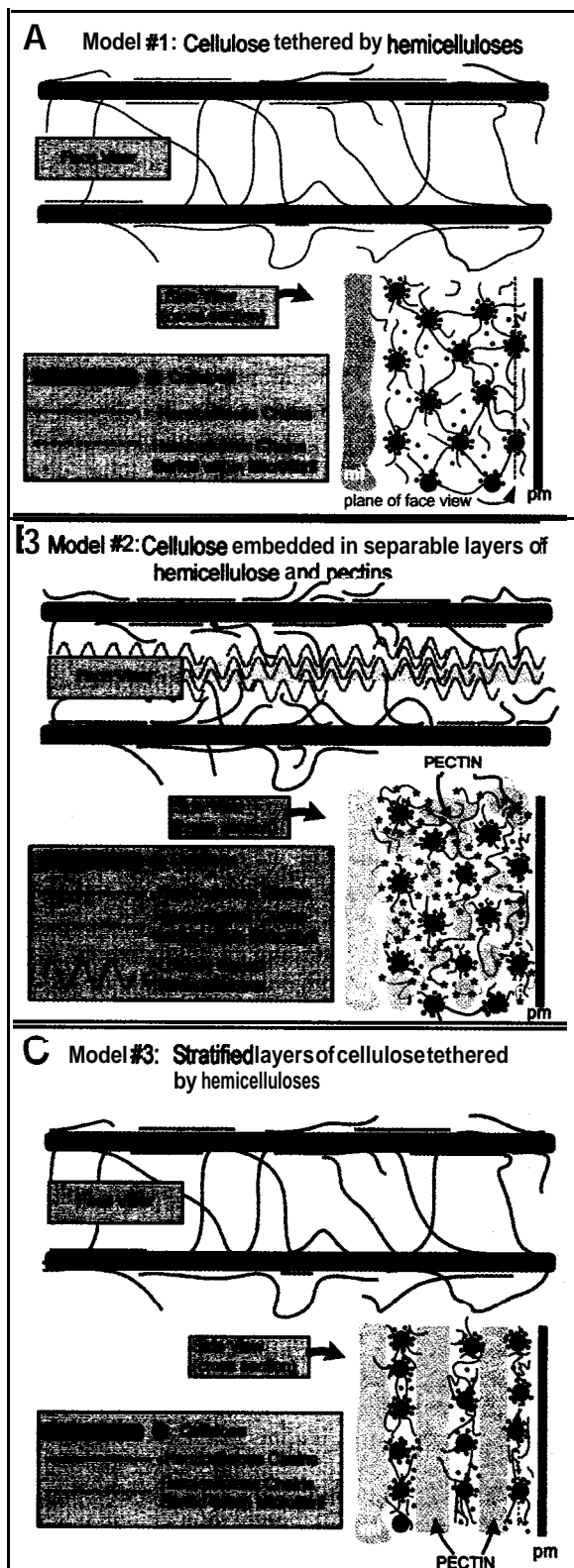
**Figure 2.** Alternative mechanisms of cell wall enlargement. **A**, *Microspora* sp. walls consist of nested cylinders which can slide outwards from each other. A hypothetical time series is shown, in which the gray half (upper half of cell at left) gradually slides outward (upward) while a new wall cylinder is formed inside and the cell eventually divides (right-most figure). **B**, *Oedogonium* sp. walls enlarge only in a localized ring where the old wall fractures and a plug of previously deposited wall material spreads out to generate the wall surface (indicated by the dotted lines). Redrawn after Pickett-Heaps [86].

wall underneath the ring of material, which stretches out to form new wall surface area. These are wildly different schemes for wall growth. They may work well for organisms configured as single files of cells, but it is hard to imagine such schemes operating in organisms with multicellular, parenchymatous tissues in which cells are attached on all sides to neighboring cells which must communicate and exchange materials with each other for growth, transport and other intricate processes. The process of diffuse cell growth can be seen as a key prerequisite to the evolution of the complex forms seen in land plants.

For the remainder of this article, I will consider recent ideas about how cell wall expansion (diffuse growth) is achieved and regulated in land plants.

## 2. STRUCTURE OF THE GROWING WALL

An adequate understanding of cell wall expansion demands a detailed model of wall structure and how such structure accommodates surface expansion and incorporates newly secreted polymers into the expanding load-bearing network. Existing models of the cell wall of land plants fall short of this standard. Current views of the architecture of the growing cell wall are synthetic pictures based largely on wall microscopy, in vitro dissection of walls by selective extraction and structural analyses, and spectroscopic methods. Each of these methods yields restricted types of information (e.g. about spatial configurations, or biochemical structures, or polymer conformation and mobility). Wall models are attempts to synthesize these diverse data into a consistent picture. Microscopic methods have informed us about the overall appearance and morphology of the wall, but they lack sufficient resolving power to discern the physical interactions and lateral associations between the individual polymers that hold the cell wall together. Analysis of extracted polymers can give important clues on the structure and association of wall components, but these methods are hampered by the possibility of degradation artifacts and significant structural rearrangement during the extraction procedures [105, 107]. Spectroscopic methods hold much potential for identifying bulk polymer conformation and interactions within the intact wall [33, 36, 67, 116], but presently are limited by technical issues (see also the review by Jarvis and McCann in this issue [54]). The striking images of extracted primary walls from deep-etched specimens [68-70] confirm the view of the primary cell wall as a multilamellate cellulosic network embedded in a matrix that influences the spacing and lateral association of the cellulose microfibrils. These images of extracted walls were interpreted by the authors as supportive of an idealized wall model that I call the 'sticky network' model (figure 3 A). In this model, cellulose microfibrils are pictured as rods linked to each other by long xyloglucan chains that bind to the microfibril surface or perhaps become physically entrapped in the microfibril during its formation [39, 47]. The xyloglucan is hypothesized in this model to provide mechanical strength and physical integrity to the weave of cellulose microfibrils, while at the same time, it coats the microfibrils to keep them from laterally associating into large bundles. The importance of hemicelluloses such as xyloglucan and xylans for the physical structure of the wall also draws support from earlier microscopic studies [92-94, 114]



and viscoelastic studies [13, 89] of walls, as well as from studies of the cellulose-binding properties of these polymers [48, 77, 115] and the effects that hemicelluloses have on microfibril formation and aggregation *in vitro* [2, 118].

In this simplified model of the growing wall, xyloglucan is inevitably seen as the polysaccharide of key importance in controlling wall expansion. (In grasses, which have a so-called 'Type II' cell wall, xyloglucans and pectins are present, but reduced in abundance; mixed-link glucan and glucuronoarabinoxylan are thought to be used in structurally analogous roles as are xyloglucan and pectin in the 'Type-I' walls found in dicots and other species outside the grass/commelinid group [8, 9]). To extend such a network, the xyloglucan 'tethers' must either be cut (perhaps with insertion of additional xyloglucan, as happens during an endotransglycosylation) or the xyloglucans must be stripped off or must slide along the surface of the cellulose microfibril. Although these ideas have been discussed, supported and accepted by many authors [9, 41, 42, 80, 91, 95], it should be noted that there is only scanty evidence for direct tethering of microfibrils by individual xyloglucan chains, as envisioned in this model. This detail of the model is based largely on the possibility of such direct tethering, which is plausible because xyloglucan chains have a propensity to stick to cellulose surfaces and because they have sufficient length that they might extend between microfibrils. Additionally, microscopic images of primary walls show what appear to be cross links (presumed to be xyloglucan on thin evidence) between microfibrils [69, 70]. These images were obtained after extraction of pectin and some hemicellulosic materials with *trans*-1,2-diaminocyclohexane-*N,N,N',N'*-tetra-acetic acid,  $\text{Na}_2\text{CO}_3$  and 1 M KOH, causing substantial changes in cell wall appearance. It is not certain, therefore, whether these cross links are artifacts of wall preparation or indeed represent endogenous cross links that can be visualized only after

**Figure 3.** Alternative models of the plant cell wall. For each model, sections through two different planes of the wall are shown. pm, Plasma membrane; ml, middle lamella. A, Model #1, the sticky network model, in which cellulose microfibrils are directly tethered to one another by xyloglucans that bind non-covalently to the surface of the cellulose. Pectins are not shown, but are assumed to fill the space not occupied by cellulose and hemicellulose. B, Model #2, the multi-coat model, in which cellulose is embedded in layers of hemicelluloses and pectins fill the interstices. C, Model #3, a stratified hybrid model, in which pectin is largely restricted to the space between distinct layers or lamellae of cellulose microfibrils which are connected laterally by xyloglucans. The physical connection between lamellae is made only through the pectin.

removing most of the matrix polymers. Independent NMR methods detect xyloglucans of varying mobilities in the wall, and it is thought that the immobile xyloglucans might be those most tightly adhering to the cellulose surface, whereas the more mobile fraction may be xyloglucans between microfibrils [33]. Assuming these cross links are not artifacts, it is unclear how strong they might be or whether they are present in sufficient density to control the cohesiveness, strength and growth properties of walls. These cross links are assumed to be crucial in the sticky network model, but this point remains hypothetical. Studies of wall viscoelasticity would seem to require a more complex picture of wall structure [14, 65, 89].

Veytsman and Cosgrove [113] recently attempted to analyze this simple sticky network model from the perspective of polymer thermodynamics. Their aim was to predict the extension properties of a reversibly-tethered network in which cellulose microfibrils were glued together by glucan 'strands', as envisioned in the sticky network model. Using parameters taken from the cell wall literature, they found that such an ideal network should exhibit a distinct yield threshold (that is, a minimum wall stress or turgor pressure above which point the wall begins to extend, or yield, irreversibly), and the yield threshold was predicted to be in the range actually measured in growing plant cells (that is, equivalent to approximately 0.1–0.3 MPa of turgor pressure). Additionally, with this thermodynamic model, they were able to explore the effect of wall synthesis (adding more cellulose or glucan strands) on the predicted extension properties of the polymer network. Their model indicates that addition of either cellulose or hemicellulose should strengthen the wall and raise the yield threshold. In other words, addition of hemicellulosic polymers should retard the extension rate, but increase the amount of extension the wall could undergo before losing its mechanical integrity. Thus, this model does not support the oft stated idea that secretion of matrix polymers will make the wall extend faster. Quite the contrary. Only under very restricted conditions would hemicellulose secretion have the effect of lowering the yield threshold and enhancing wall extension rate: specifically, only if the newly secreted glucans were very short and thus not long enough to form cross links between microfibrils, or if they were in vast excess of cellulose, in which case the hemicellulose strands would be linked to only one cellulose microfibril and would not provide effective cross linking [113]. Changes in hemicellulose length, e.g. by the action of endoglucanases or transglycosylases, were predicted to affect wall extension in

the intuitively obvious way (shorter hemicelluloses lower the yield threshold and accelerate enlargement).

If this sticky network model were fundamentally correct, one would reasonably expect that endoglucanases that hydrolyzed xyloglucan would induce extension of cell walls that were clamped in tension. However, direct tests of this prediction have failed to detect significant wall extension when such enzymes were tested on cucumber hypocotyl walls [22]. Cell walls treated with such enzymes became mechanically weaker and eventually broke, but they did not display significant creep (time-dependent wall extension) prior to mechanical failure. To me this result presents a serious challenge to the sticky network model. Because this wall extension assay works well for expansins (see following section), the technical feasibility of inducing prolonged wall extension *in vitro* by simple protein preparations is not in doubt [11,62,73].

A slightly different model of the wall (figure 3 B) gets around this problem and in my view is probably closer to reality. This 'multi-coat' model was most recently proposed by Talbott and Ray [105] on the basis of wall extraction data and draws circumstantial support from measurements of polymer mobility as assayed by NMR spectroscopy [46, 116]. It envisions cellulose microfibrils coated with a layer of xyloglucan and then embedded in layers of successively less tightly bound hemicellulosic polymers and finally pectins, which fill the interstices between the cellulose/hemicellulose fibrils. Note that in this model, there are no tethers directly linking neighboring microfibrils together. Instead, microfibrils are held together only by the cohesive forces between successive layers of laterally associated matrix polymers. This model predicts that xyloglucan chain cleavage would not necessarily induce wall extension (at least until the xyloglucan were highly degraded), and in this regard the model is consistent with the results of wall extension assays in which walls were digested with lytic enzymes [22]. In this model, xyloglucans do not necessarily hold the key to wall expansion. Pectins or the intermediate hemicelluloses are also candidates, in some respects more likely candidates because of their spatial locations. These polymers have higher molecular mobility, as assayed by NMR methods [33,46], and so they are plausible determinants of wall extension. They might also act like grease, lubricating the slippage of cellulose microfibrils. This is not to say that they are the major control point for wall extension, but rather that their mobility and space-filling attributes might ease the slippage of microfibrils that were freed to move.

There is also a hybrid of these two models, suggested by Ha et al. [46]. In this 'stratified wall model', cellulose is proposed to be directly cross linked by xyloglucan, as in the sticky network model, except that this cross linking operates only within a single layer or lamella of microfibrils. Pectins are hypothesized to be situated in a distinct zone between individual microfibril lamellae and to control the spacing and interactions between lamellae, as well as the total wall thickness, but to have less influence on the tensile strength of the wall (*figure 3 C*).

Although these three models have much in common, they differ in the details of the spatial location and types of associations between the major wall components. These points are still a matter of educated guess work and inference, rather than direct measurement. Unfortunately, these are crucial details for a definitive understanding of how the cell wall enlarges.

### 3. WALL LOOSENING AGENTS

Auxin, light and other agents can modulate cell wall growth rates in living plants very rapidly, within minutes or even seconds. This time is too short to entail substantial change in the composition of the cell wall, and so dynamic changes in the bonding between wall components have been the focus of attention in this field. In particular, the concept of a wall loosening agent has often been invoked to account for such growth modulations. Recent analyses suggest that we must distinguish between primary and secondary wall loosening agents [21]. I define a primary wall loosening agent as a substance that induces stress relaxation of the cell wall, resulting in secondary water absorption by the cell and expansion of the wall [19]. The substance could be an enzyme that acts to cleave a structural polymer or to break apart associations between wall polysaccharides or it could be a wall polymer with the ability to insert itself in the wall by competitive binding and exchange with load-bearing components of the wall. In either case, this definition requires that stress relaxation and expansion of the cell wall result from the action of the wall loosening agent. This activity is most readily tested by extensometer assays for induction of wall extension of inactivated cell walls [22, 73]. A secondary wall loosening agent does not itself cause the crucial relaxation and expansion of the wall, but acts as a synergist to make the wall more sensitive to the action of primary agents. This might be achieved, for example, by hydrolytic enzymes that partially cleaved the matrix polysaccha-

rides, thereby reducing the matrix viscosity. Of the potential wall loosening agents discussed below, expansin is most clearly a primary wall loosening agent, whereas the wall lytic enzymes may not meet this criterion, but may act as secondary wall loosening agents.

#### 3.1. Expansin

These proteins were first identified as mediators of the 'acid growth' response. Acid growth refers to the enhanced expansion of living cells or isolated cell walls by acidic pH, a phenomenon that is common to most, if not all, groups of land plants. Acid growth has had its share of controversy with regard to how auxin stimulates growth [1,30,38,60,64,90]. For years this controversy overshadowed a more fundamental question, namely, what is the molecular nature of this pH-dependent wall expansion? An early hypothesis proposed that acid growth arose from a direct inhibition, by acidic pH, of hydrogen bonding between xyloglucan and cellulose, but binding studies disproved this idea [112]. The loss of acid growth after protein denaturation treatments suggested that it was a protein-mediated process [16] and this was directly confirmed when it was shown that heat-inactivated cucumber hypocotyl walls could be restored to nearly full competency for acid growth by the addition of purified wall protein fractions [73]. This basic conclusion has since been confirmed with wall proteins from other materials, including tomato leaves [56], oat coleoptiles [62], rice internodes [11], soybean hypocotyls [61] and tobacco cell cultures [63]. Green algae such as *Valonia* and *Nitella* sp. also exhibit acid-induced wall extension, but it does not appear to be a protein-mediated response [103, 108]. Hence, this particular mechanism of wall enlargement may be restricted to land plants.

The proteins responsible for acid-induced wall extension were named expansins and growing tissues were found to contain the highest expansin activity [12, 24, 62]. However, the protein is not an abundant one even in rapidly growing tissues and we estimate that it is present only in minor amounts in the cucumber seedling (perhaps one part protein to  $\approx 5\ 000$  parts wall on a dry weight basis). Most of what we know about expansin biochemical activities comes from studies of two expansins purified from cucumber hypocotyls [71-73]. These proteins bind strongly to cell walls, apparently to the non-crystalline regions of cellulose microfibrils [72]. They do not exhibit hydrolytic activity against cell wall components, and indeed we have not been able to detect any enzymatic activity

in expansin preparations. Nevertheless, they act catalytically, not stoichiometrically, to induce wall expansion *in vitro*. They also have been shown to be capable of stimulating cell expansion in living cells when applied exogenously to *Arabidopsis thaliana* hypocotyls and cucumber root hairs [76], tomato meristems [34, 35] and tobacco cell cultures [63]. These results are important because they show that endogenous expansin activity is rate limiting for the enlargement of these cells, and thus expansins may serve as a potential control point for cell growth.

The cloning of the first expansins [97] revealed them to be a novel class of wall protein without strong sequence similarity to other known wall proteins. We now distinguish two families of expansins, called  $\alpha$ -expansins and  $\beta$ -expansins.  $\alpha$ -Expansins comprise the first-discovered family of expansins and have been studied in greatest detail.  $\beta$ -Expansins were more recently recognized when it was found that *Zea m*, a member of a group of grass pollen allergens, has significant expansin activity [25]. A number of related sequences that are present in GenBank have now been classified as  $\beta$ -expansins on the basis of sequence homology to *Zea m* and are presumed to have related wall loosening functions [20, 25]. However, this assumption about activity needs to be tested further because the  $\beta$ -expansin family is relatively divergent in sequence, at least as compared with the  $\alpha$ -expansins. The recent report of successful expression of active recombinant  $\alpha$ - and  $\beta$ -expansins in the Bacculo virus/insect cell culture system should provide a useful means of testing the activity of specific expansin gene products [98].

Between the  $\alpha$ - and  $\beta$ -families there is relatively little sequence conservation (figure 4 A), consisting most notably of a series of conserved cysteines in the N-terminal half of the protein, a HFD (histidine, phenylalanine, aspartate) motif and an FRRV (phenylalanine, arginine, arginine, valine) motif in the middle of the protein, and a series of tryptophans (W) near the carboxy-terminus (figure 4).

Despite the low sequence identity, the two groups of proteins have similar rheological effects on cell walls (induction of creep and stress relaxation; [25]). However, they appear to have different substrate preferences. The  $\alpha$ -expansins were found to work well on a range of dicot and monocot cell walls (or Type I walls, in the terminology of McQueen-Mason et al. [73]), but to have relatively low effectiveness on grass cell walls (or Type II walls). In contrast, *Zea m*, the only

$\beta$ -expansin tested to date, has strong selectivity for grass walls and only marginal effect on dicot walls [25].

It is a curious fact that  $\alpha$ -expansins possess greater action when tested on dicot walls than on grass walls, even when the  $\alpha$ -expansin is from grass walls [11, 62]. This is perhaps understandable if we consider that the grass wall contains unusual hemicelluloses (mixed-link glucan and glucuronoarabinoxylan), in addition to the usual xyloglucan, pectin and other matrix polymers found in dicots [8]. I think it likely that in the course of evolution of the grass (Type II) cell wall, when these new matrix polymers partially displaced xyloglucan and pectin and assumed some of their structural roles, the  $\beta$ -expansins co-evolved for wall loosening action on these new matrix polysaccharides.

$\alpha$ -Expansins make up a large multigene family, with at least 22 members in *A. thaliana* (see the expansin web site, <http://www.bio.psu.edu/expansins/>, for up-to-date listings of known expansin genes in *A. thaliana*, rice, tomato and other species).  $\alpha$ -Expansin sequences are now known from many flowering plants, including numerous dicots and grasses [20,63]. In addition,  $\alpha$ -expansins have been cloned from a gymnosperm (pine [52]), a fern (*Marsilea quadrifolia* [57]), and even from a liverwort (*Marchantia* sp.; R. Carey and D.J. Cosgrove, unpubl. data). Attempts to find  $\alpha$ -expansins in the green alga *Chara* sp. have not proven successful (Y. Wu and D.J. Cosgrove, unpubl. data). This failure could signify that expansins evolved later in the evolutionary line leading to land plants (Embryophyta); alternatively, the failure could be due to technical limitations of the methods. The high sequence similarity between  $\alpha$ -expansins from these divergent taxa suggests that the biochemical function of these proteins is highly conserved. The reason for the large numbers of different  $\alpha$ -expansin gene members, e.g. in *A. thaliana*, may have to do with the need for selective control of cell wall loosening in different cell types. Preliminary evidence indicates that these genes are indeed expressed in highly selective and distinct patterns ([27] and unpubl. data).

$\beta$ -Expansins also comprise a multigene family. In *Arabidopsis*, we have identified only three genes that clearly fall into the  $\beta$ -expansin family. With over 68 % of the *Arabidopsis* genome sequenced as of this writing, we can be confident that the  $\beta$ -expansin family is much smaller than the  $\alpha$ -expansin family in *Arabidopsis*. In contrast, in rice and maize,  $\beta$ -expansins appear to be slightly more numerous than  $\alpha$ -expansins, at least judging from extensive analyses of cDNA libraries (Y. Wu and D.J. Cosgrove, in prep.).

A: Alignment of  $\alpha$ - and  $\beta$ -expansin proteins

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-----DY--GGWQSGHATIFYGGGDASGTM CsEXP1
MNLTEYSHILFSLSLCTLNFLY-----SINSDDNGG WERGHATIFYGGADASGTM AtEXP2
MASSSS--VLLVVALFAVFLGSAHGIKVPPGPNITAEYGDK WLDAKSTWYQKPTGAGPK LOL_PI
MAGASAKVVALLSVLATY-GFAAGVV-----YTND WLPKAKATWYQPNAGGPD OsEXPb

--GGACGYGNLVSQGYGTNTVALSTALENNGLSCGACFEMTCTNDPKWCLPGTIRVTAITN CsEXP1
--GGACGYGNLHVSQGYGLQTAALSTALENNSGQKCGACFELTCEDDPEWCIPGSIIVSATIN AtEXP2
DNGGACGYKNVDKAPFNGMTGCGNTPIEKDGRGCGSCFEIKCTKPES--CSGEAVTITTD LOL_PI
DNGGACGFKNTNQYPFMSMTSCGNEPLEQDKGCGACYQIRCTNNPS--CSGQPRTVIITD OsEXPb

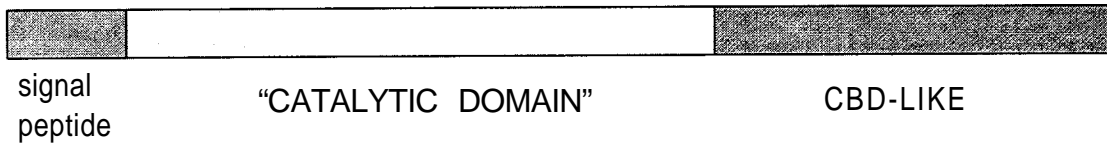
FCPPNFALPNNNGGWCNPPLQHFDMAEPAFLQIA-----QYR-AGIVPVVSFRRVPCMKK CsEXP1
FCPPNFALANDNGGWCNPPLKHFDLAEPAFLQIA-----QYR-AGIVPVAFRRVPCCEKG AtEXP2
--DNEEPIAPY-----HFDLSGHAFGSMAKKGEEQNVRSAGELELQFRRVKCKYP LOL_PI
--MNYYPVARY-----HFDLSGTAFGAMARPGLNDQLRHAGIIDIQFRRVPC-YH OsEXPb

GG--VRFITIN--GHSYFNLVLIITNVGGAGDVSVSIK-----GSRTG-WQSMSRNWQNW CsEXP1
GG--IRFITIN--GNPYFDLVLIITNVGGAGDIRAVSLK-----GSKTDQWQSMSRNWQNW AtEXP2
DDTKPTFHVEKASNPNYLAIIIVKYVDGDCDVAVDIKE-----KGDKWTELKESWGA VW LOL_PI
RGLYVNFHVEAGSNPVYLAVLVEFANKDGTTVVQLDVMSLPSGKPTRVWTPMRRS WGSITW OsEXPb

--QSNNYLNGQGLSFQVTLSDGRTLTAYNLVPSNWQFGQTYEG-PQF CsEXP1
--QSNTYLNRGQSLSFQVTDSDGRTVVSVDVVEHDWQFGQTFEG-GQF AtEXP2
RIDTPDKLITG-PFTVRYTTEGGTKSEFEDVIEGKADTSYSAK LOL_PI
RLDANHRLQCPKSLRMVSESGQTVIAHQVIEANWRANTNYGSKVQFR OsEXPb

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## B: Probable domains of the expansin protein



## C: Limited conservation between expansin and family-45 glycosyl hydrolase

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NtEXP5      GGGDASGTMGGACGYGNLVSQGYGTNTAALSTALENN-----GLTCCACCYELTCNNDGQ
cons.       G G +SG      G GN      G TAA S ALF+      G CC CY+LT      GQ
Tr_EG5     GCGSSSGAFPWQLGIGN-----GVYTAAGSQALFDTAGASWCGAGCCKCYQLTST--GQ

NtEXP5      ----SCLQG-----SIIVTATNFCPPNPSLPNNGGWCNPPL-----Q-----HFD-LAQ
cons.       SC G      SIIV TN C PN      N N WC P+      Q      HFD +AQ
Tr_EG5     APCSSCSTGGAAGQSIIIVMTNLCPN----NGNAQWC-PPVGGTNQYGYSYHFDLMAQ

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**Figure 4.** Structure of expansin proteins. A. Alignment of  $\alpha$ - and  $\beta$ -expansins reveals limited sequence conservation. The top two sequences (CsEXP1, AtEXP2) are  $\alpha$ -expansins from cucumber and Arabidopsis; the bottom two sequences (Lol pl, OsEXPb) are  $\beta$ -expansins. Conserved residues are enclosed in boxes. B. Schematic diagram of the putative domain structure of expansins, with a signal peptide, a central domain that resembles family-45 glycosyl hydrolases, and a carboxy-terminus with resemblance to cellulose-binding domains. C. Alignment of the 'catalytic domains' of an expansin (top line, NtEXP5) with a family-45 glycosyl hydrolase (endoglucanase-5 from *Trichoderma reesei*).

This fact supports the idea that  $\beta$ -expansins have duplicated and diverged in unique ways in the grasses, and perhaps have assumed some of the functions of  $\alpha$ -expansins.

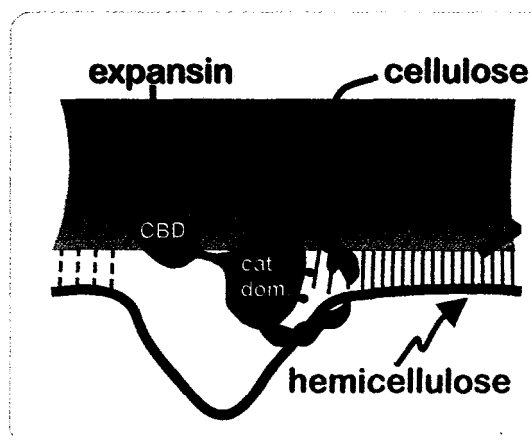
The amino acid residues conserved between  $\alpha$ - and  $\beta$ -expansins (figure 4 A) presumably reveal the parts of the protein that serve essential structural and catalytic

roles for expansin activity. We currently recognize three probable domains in the expansin protein (figure 4 B). The first  $\approx 22$  amino acids encodes for a classical signal peptide that directs the protein into the secretory pathway and is cleaved off to form the mature protein. A putative binding domain ( $\sim 10$  kDa) at the carboxy-terminus of the protein contains a series

of conserved tryptophans (W) with spacing that resembles that of cellulose-binding domains of cellulases. The 1.5kDa piece of the polypeptide between the signal peptide and the putative binding domain has distant, but significant, sequence similarity to the catalytic domain of family-45 glycosyl hydrolases [19]. Within this putative catalytic domain of the expansin protein (*figure 4 C*), the conserved cysteines are likely to function in disulfide bridge formation. The 'HFD' motif and a second conserved aspartate (D) residue near the carboxy-terminus make up the key residues for the catalytic site of the family-45 hydrolase, and these residues can be found in corresponding places in both  $\alpha$ - and  $\beta$ -expansins (D.J. Cosgrove, in prep.).

This sequence similarity with the catalytic domain of family-45 enzymes is highly suggestive that these domains from these two groups of proteins are structurally and evolutionarily related. It also gives a strong hint that expansins might have glycosyl hydrolase activity; however, renewed attempts to detect such activity continue to support the conclusion that expansin-induced wall extension is not based on wall hydrolytic activity (L.-C. Li and D.J. Cosgrove, in prep.). Similarly, transglycosylation activity has not been detected in expansin preparations [74]. A recent study reported that recombinantly produced Phl p 1, the grass pollen allergen from *Phleum* sp., was highly unstable and was associated with very high protease activity [45]. Phl p 1 is a homolog of Zea m1 and falls into the  $\beta$ -expansin family. Grobe et al. [45] proposed that Phl p 1 is a cysteine protease and that proteolytic activity may account for the cell wall loosening effect of these pollen allergens and perhaps expansins in general. Opposing this hypothesis, we have not been able to detect any proteolytic activity associated with active Zea m1 proteins (L.-C. Li and D.J. Cosgrove, in prep.). Moreover, wall digestion with cysteine proteases and other more aggressive protease preparations did not cause cell wall extension or in any way mimic the effects of expansin [16]. Thus, this provocative idea merits critical testing before it is accepted.

Our current working model for  $\alpha$ -expansin action is diagrammed in *figure 5*. In this model, the binding domain anchors expansin to the surface of the cellulose microfibril and restricts mobility. This anchoring may be important to prevent expansins secreted by one cell from influencing the growth of neighboring cells. Expansin anchoring could also provide a way for cells to selectively expand certain walls, while leaving others in an inextensible state. It is possible that tightly bound expansins may still move along the surface of



**Figure 5.** Schematic diagram of how expansin might induce cell wall enlargement by breaking the non-covalent bonding (short bridges) between cellulose (large rod) and hemicellulose (curved line). The presumptive binding domain ('CBD', sketched as a tail) is hypothesized to anchor expansin to the cellulose surface, while the putative catalytic domain ('cat dom.') would be able to interact with hemicellulose at the microfibril surface or in the matrix between microfibrils. The dotted arrow indicates the direction of expansin motion, which would be driven by release of mechanical strain energy in the wall polymers. The hemicellulose bonding to the cellulose is reversible (short bridges, broken lines) and results in an inchworm-like motion of the hemicellulose and a stress relaxation of the wall.

the microfibril in a type of two-dimensional diffusion, as was recently shown for a cellulase [55]. Such localization could even provide a plausible mechanism for the idea that specific microfibril layers control the directionality of wall expansion, a concept suggested years ago [92], but not generally embraced by the cell wall community. On the other hand, less avid binding to the wall may enable some expansins to be more mobile and to have a more widespread loosening effect. This appears to be the case with Zea m1 and related grass pollen allergens that are secreted by grass pollen as it germinates and as the pollen tube penetrates between cells of the stigma and pollen transmission track [25].

In this working model of expansin action, the putative catalytic domain is likely to function by disrupting the non-covalent bonding between wall polysaccharides, either at the surface of the cellulose microfibril or more distantly in the matrix between microfibrils. This idea, that expansin locally dissolves polysaccharide associations, is supported by studies of the effect of expansins on the mechanical strength of paper [71] and by the reversibility and unusual time dependence of expansin action [72]. Moreover,

although expansins do not themselves show hydrolytic activity against cellulose, they are able to synergistically enhance the digestion of cellulose by microbial cellulases [26]. We interpret this effect as an indication that expansins are able to help pry glucans off the surface of cellulose microfibrils, thereby making them more accessible to attack by hydrolases. In the growing cell wall, this activity is presumably directed to the release of hemicelluloses from the surface of cellulose, or perhaps to weakening the association between two hemicelluloses. In a wall under tension, this could generate a kind of polymer creep, in which the short segments of a hemicellulose are released from the cellulose surface, move, and then re-bond to the surface. This hypothetical action would cause hemicelluloses to move 'relative to the cellulose in an inchworm fashion (in some fields this is called reptation). Such a mechanism could account for the linear, long-term creep induction by expansins, as opposed to the breakage induced by wall hydrolases [22].

### 3.2. Xyloglucan endotransglycosylase (XET) and endo-1,4- $\beta$ -D-glucanase (EG)

Both of these enzymes have been proposed to act as cell wall loosening agents by cleaving the xyloglucans that hypothetically knit cellulose microfibrils together. There is substantial evidence that xyloglucan chains undergo significant modification after their secretion into the wall, and that these two classes of enzymes are likely to be of prime importance in these modifications [40, 51, 110].

XET (also called EXGT, for endo xyloglucan transferase, by Nishitani [79]) is an enzyme that cleaves xyloglucan mid-chain, forming a covalently bonded xyloglucan-enzyme intermediate that can then transfer the xyloglucan to the non-reducing end of another xyloglucan chain [101, 102]. The result of this molecular grafting reaction is a new hybrid xyloglucan plus a fragment of the original chain. XET activities were first characterized independently by several groups using enzyme preparations from different plant sources [31, 32, 81, 82, 100] and cloning of the first XET cDNAs soon followed [28, 84]. Today we know that XETs and closely related sequences make up a large multigene family (> twenty in *Arabidopsis* [7]), whose specific functions are the object of intense current study [3]. Recombinant protein expression of XET in insect cultures has proven successful [6] and can now be used to explore the biochemical functions of XET-related proteins.

One of the attractive aspects of XET activity is that it could, in theory, simultaneously solve the problem

of how to loosen the wall and how to incorporate newly deposited polymers into the wall to maintain its strength. Consequently, some of the first XET studies proposed that this enzyme might catalyze turgor-driven cell wall expansion [43, 82]. This concept gained circumstantial support by the finding that growing cells in various plant systems expressed relatively high XET activity [49, 87, 88, 119]. However, direct tests of wall extension failed to find induction of wall extension *in vitro* by XET preparations [74]. Moreover, some cells, e.g. those below the growth zone of the pea epicotyl, may continue to express high levels of XET after cell elongation has ceased [43], thus breaking the correlation between expansion and XET activity. At present, there is no direct evidence that XET has a primary wall loosening function, but perhaps the correct tests have not yet been done.

The second proposed function of XET, namely ligation of newly synthesized xyloglucans into the existing wall, has gained in support in recent years [7, 80]. Newly synthesized xyloglucan chains appear to be of much shorter length than the bulk of xyloglucans extracted from the cell wall [106]. This difference implies that xyloglucans are grafted together within the wall, perhaps weaving the xyloglucans between microfibrils and making for a tighter, less extensible cell wall. As evidence that such grafting indeed occurs *in vivo*, density labeling was used to follow the fate of newly synthesized xyloglucans [111]. The results showed that hybrid molecules were indeed formed, presumably by the action of endogenous XET. Older studies likewise indicate that xyloglucans can undergo significant, reversible up shifts in molecular size after they are deposited to the cell wall, but how this might be regulated and how it might be related to wall expansion is far from clear ([106] and references cited therein).

Like XET, endo-1,4- $\beta$ -D-glucanase (EG) may cleave xyloglucans, but addition of water results in hydrolysis rather than transglycosylation. EG action clearly ought to weaken cell walls, but it has not been demonstrated that such hydrolysis can cause wall extension (as opposed to breakage). On the other hand, treatment of cell walls *in vitro* with fungal EGs makes the walls more susceptible to expansin-induced wall extension [22]. Thus, it would appear that EGs are strong potential candidates as secondary wall loosening agents.

Recent work in this area is based largely on the cloning of EGs, which make up a multigene family in plants (> twelve genes in *Arabidopsis* [29]). Most

plant EGs that have been cloned to date fall into the group of family 9 endo- $\beta$ -1,4-glucanases. A preliminary report of the cloning of a maize coleoptile EG indicates that this enzyme belongs to a novel class of endoglucanases [109]. Unlike their microbial counterparts, plant EGs typically lack a cellulose-binding domain [5]. However, recent studies have described plant EGs with membrane anchors that appear to localize the enzymes to the Golgi and plasma membranes [4, 78]. Unfortunately, there has been relatively little detailed characterization of the activity and substrate specificity of plant EGs, and heterologous expression of active recombinant EGs from plant sources has yet to be reported. This is a major gap that needs to be filled by future work.

Stimulation of EG activity has long been implicated in auxin-induced growth, particularly in grass coleoptile. However, induction of EG gene expression by auxin takes many hours [10], whereas auxin-induced growth begins within 15 min. In the tomato hypocotyl, auxin-induction of EG occurs principally in the cortex rather than in the epidermal and sub-epidermal layers, which have the thickest walls and may impose greater restraint on hypocotyl elongation than does the cortex [10]. Furthermore, auxin induction of EG occurs equally well in the basal region of the tomato hypocotyl, which is not stimulated to grow by auxin. These observations do not lend themselves to a simple explanation of the relationship between auxin induction of growth and EG genes. Cooperative and synergistic activities between EG, XET and expansins have been hypothesized [95, 96], although specific examples of such interaction *in vivo* have yet to be documented.

Genetic evidence supporting a role for EGs in plant morphogenesis comes from the recent discovery that an *Arabidopsis* mutant with stunted growth has a genetic lesion in an EG with a membrane anchor domain [78] (see also the review by Höfte in this issue). The precise function of this EG and how its mutation leads to the growth phenotype is not yet clear, however. Since the enzyme appears to be associated with the plasma membrane, it probably does not have access to the majority of the cell wall, and so it probably does not function as a wall loosening enzyme. A role in the processing of newly synthesized cellulose microfibrils has been proposed for a membrane-anchored EG in *Acetobacter xylinum* [58], but further work is needed to establish the actual biochemical activity of the related plant protein.

### 3.3. Other wall enzymes

Two other recent reports of potential synergists or wall loosening enzymes are notable. Inouhe and Nevins [53] report on a protein, which they call an 'acidic wall protein', that can stimulate the activity of exo- and endoglucanase activities in maize coleoptile walls. They speculate that the acidic wall protein may mobilize glucanases bound to the cell wall and that these glucanases can act as wall loosening agents. It might also be that the acidic wall protein renders the wall polymers more accessible to enzymatic attack, as hypothesized for expansins. In another study, Okamoto and Okamoto [83] report indirect evidence, based on differential sensitivity to heat inactivation, that the yield threshold and extensibility of cowpea hypocotyl walls are controlled by two distinct wall enzymes. We await further characterization and evaluation of these intriguing wall activities.

### 3.4. Wall polysaccharides as loosening agents

Wall synthesis is sometimes viewed as an essential and sufficient cause of cell wall expansion, but it is not certain that it is either. Of course, the synthesis and deposition of wall polymers usually accompanies cell wall enlargement, with the result that cell wall thickness remains more or less constant as the wall expands. However, this general correlation between expansion and synthesis is not universal [93], and wall synthesis is mechanistically separable from expansion. The clearest example of this separation is prolonged extension (without synthesis) of isolated cell walls clamped in an extensometer in acidic buffers [15, 16]. Such walls can extend for many hours or even days and in rare cases, the wall can double in length before it breaks. Of course, breakage does not normally occur *in vivo*, and this is presumably because the progressive thinning of the wall during expansion is countered by the incorporation of newly secreted wall substances, which strengthens the wall.

The foregoing remarks indicate that wall expansion can occur without wall synthesis, at least in the short term. But can the secretion of wall polymers directly result in cell wall expansion, as sometimes assumed [106]? Under some theoretical scenarios, this seems possible, although it has never been demonstrated experimentally and our attempts to induce extension in clamped wall specimens by the addition of solubilized polymers have not yielded a positive result (unpubl. data). This failure could be because of technical limitations or because the concept is faulty. However, let us consider the conditions which would

enable newly secreted wall materials to stimulate cell wall extension. For this to occur, they would have to induce stress relaxation in the wall [17]. In the sticky network model of the wall (figure 3 A), this would mean that they would have to compete off the load-bearing xyloglucans that are bound to the microfibril surface. Thermodynamic analysis of this situation indicates that in most cases such action would actually result in a stronger, not looser, wall [113]. If the newly synthesized polymers were too short to form cross links with neighboring microfibrils, they might have a net loosening effect. This idea might be tested experimentally by addition of high concentrations of short xyloglucan chains to walls clamped in an extensometer.

It is intriguing in this regard that auxin treatment results in the synthesis of shorter xyloglucan chains in pea epicotyls [106]. The length of the newly synthesized hemicelluloses could have a critical influence on whether such materials acted as wall strengthening agents or loosening agents [113]. This change in length might be achieved very simply by speeding up or slowing down the delivery of materials via the Golgi system. Shorter transit times would allow less time for lengthening the polymers: the result being a higher flux of shorter polymers to the wall. If the microfibrils became coated with only short xyloglucan segments, this could be as dangerous as indiscriminately activating a wall hydrolase. A loss in wall coherence could be mitigated if XET acted to ligate the short xyloglucan fragments together, thus strengthening the wall after the stress relaxation had been achieved.

In the case of the multi-coat model of the cell wall (figure 3 B), where all of the matrix polymers potentially contribute to wall cohesiveness by their lateral associations, it is easier to imagine a scenario whereby newly secreted matrix polymers would be spontaneously drawn into association with the existing matrix polymers and, in so doing, would relieve the tensile stress borne by the lateral association of polymers. The practical feasibility of testing this concept by addition of matrix polymers to clamped wall specimens seems low.

#### 4. CONCLUSION

The challenge for growing cell walls is to selectively release the matrix that holds microfibrils in place, and to do this without creating an aneurysm. Expansins are able to accomplish this feat, but the

precise biochemical mechanism of its action is still speculative. Cellular control of wall enlargement could be exerted at several levels. For example, rapid and reversible responses could be controlled by changing wall pH, which would activate or inactivate expansins in the wall. Cells could secrete additional expansin, or enhance wall hydrolytic activities. Changes in the size and type of hemicelluloses could influence the strength of the hemicellulose-cellulose bonding, and thereby modify the effectiveness of expansins and other wall loosening agents. The daunting complexity of the wall's structure and the variety of wall modifying enzymes present us with much grist for the years to come.

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