REVIEW PAPER

Relating the mechanics of the primary plant cell wall to morphogenesis

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Abstract

Regulation of the mechanical properties of the cell wall is a key parameter used by plants to control the growth behavior of individual cells and tissues. Modulation of the mechanical properties occurs through the control of the biochemical composition and the degree and nature of interlinking between cell wall polysaccharides. Preferentially oriented cellulose microfibrils restrict cellular expansive growth, but recent evidence suggests that this may not be the trigger for anisotropic growth. Instead, non-uniform softening through the modulation of pectin chemistry may be an initial step that precedes stress-induced stiffening of the wall through cellulose. Here we briefly review the major cell wall polysaccharides and their implication for plant cell wall mechanics that need to be considered in order to study the growth behavior of the primary plant cell wall.

Key words: Cellulose, mechanical feedback, morphogenesis, organogenesis, pectins, plant cell mechanics, plant cell wall, poroelasticity, wall porosity.

Introduction

The plant cell is encapsulated in a more or less stiff extracellular matrix, the cell wall. While the physical properties of mammalian cells and their interactions with their environment are largely determined by the cytoskeletal network, in plants the cell wall dominates the mechanical behavior of the cell—even in the case of young and undifferentiated cells. This is readily demonstrated by the fact that once the cell wall is removed from a plant protoplast, the rheological properties of the latter are similar to those of animal cells (Durand-Smet *et al.*, 2014). Even rapidly growing cells with thin, primary cell walls such as pollen tubes can exhibit an apparent stiffness that is orders of magnitude higher than that of animal cells (Jones *et al.*, 1999; Nguyen *et al.*, 2010; Sanati Nezhad *et al.*, 2013).

It has to be noted, however, that while plant cells are stiffer, a substantial portion of the apparent cellular stiffness in walled cells may derive from the turgor pressure that acts as a hydroskeleton (Deng *et al.*, 2011; Forouzesh *et al.*, 2013). The measurement of apparent cellular stiffness therefore does not allow for straightforward deduction of the material properties of the wall.

The differences in mechanical behavior between plant and animal cells have significant implications for the mechanics of processes such as cellular growth and morphogenesis that occur during tissue differentiation. In plant cells these processes are governed by the mechanical properties of the wall which serve as modulating parameters. While plant cell

expansion *per se* is driven by turgor pressure, the spatio-temporal control of the process relies on the mechanics of the cell wall (Geitmann and Ortega, 2009). The cell wall not only confines the growth behavior of the individual cell but also regulates the development of the entire tissue, since neighboring cells are tightly connected at their shared walls. Generally, plant cells do not detach, slide, or move against each other, and the architecture of a plant tissue is therefore much less malleable than that of an animal organ in which cells can migrate over significant distances. Exceptions exist; for example, invasively growing plant cells separate cell–cell connections to pass through the created spaces, and tissues with developmentally generated air spaces require locally controlled cell detachment. In the present review we will focus on the mechanics of the primary cell wall in early phases of plant cell morphogenesis and organogenesis when cells are tightly attached to each other. The contribution of individual cell wall components to the mechanical behavior of the overall wall material is reviewed in the context of plant morphogenetic processes.

The degree of pectin methylesterification cannot be used as a proxy to predict cell wall mechanical properties

Plant cell walls consist of a network of interconnected polymers with diverse biochemical and mechanical properties. Because of its complex structure, the cell wall has been compared with a composite material, thus underscoring the fact that different polymers play different roles in terms of mechanical behavior of the overall structure. Because of the interlinked nature, knowledge of the behavior of isolated polymers has only very limited predictive power in terms of the behavior of the complex and heterogeneous structure enveloping the living cell. Cross-links and other interactions between biopolymers render the behavior of the material both complex and also versatile. However, although considering the cell wall polymer network as a whole is crucial, general principles can be deciphered by characterizing the functionalities of its individual components.

An important group of polymers in the primary plant cell wall are pectins. These polymers have been considered to act as a gel, forming an amorphous substrate into which a structural network consisting of cellulose and hemicelluloses is embedded. The principal domains of pectin molecules are homogalacturonan (HG), rhamnogalacturonan I (RG-I), and rhamnogalacturonan II (RG-II) that can be covalently linked to each other (Ridley *et al.*, 2001; Caffall and Mohnen, 2009). Pectins are synthesized in the Golgi apparatus and transferred to the extracellular space by secretion (Mohnen, 2008).

HG pectin is a linear polymer with 1,4-linked α-D-galacturonic acid residues and is the most abundant type, representing more than 60% of pectins in the cell wall (Caffall and Mohnen, 2009). HG pectins are secreted in highly methylated form (Micheli, 2001; Wolf *et al.*, 2009). De-esterification by pectin methylesterases (PMEs) typically occurs *in muro* (in

the wall), and this process changes the mechanical properties of pectin as it enables cross-linking by positively charged calcium ions. Paradoxically, the de-esterification of pectin has been linked with both increased and decreased cell wall stiffness *in vivo* (Palin and Geitmann, 2012). In the pollen tube, the apical end of the cell displays highly methylesterified pectin which has been correlated with softer mechanics and is consistent with the physical requirements of the rapid growth behavior of this subcellular region (Zerzour *et al.*, 2009; Chebli *et al.*, 2012; Vogler *et al.*, 2013). In the *Arabidopsis thaliana* shoot apical meristem, on the other hand, decreased stiffness was found in locations displaying de-methylesterified pectins and is reported as a necessary prerequisite for organogenesis (Peaucelle *et al.*, 2011; Kierzkowski *et al.*, 2012; Wolf *et al.*, 2012; Braybrook and Peaucelle, 2013). This apparent contradiction may be explained by different actions of the respective PMEs involved. The molecular pattern of pectin demethylation can occur in a blockwise or non-blockwise manner, and parameters such as pH, initial stage of methylesterification, and cation concentration are thought to influence the mode of HG de-esterification (Osorio *et al.*, 2008). In blockwise de-esterification, PME acts continuously on galacturonic acid residues, removing the methoxyl group, resulting in a continuous region of de-esterified pectin. It is suggested that continuous demethylation of more than nine galacturonic acid residues allows strands of pectin to be linked more efficiently by Ca^{2+} bonds, leading to gelation and enhanced stiffness of the material (Willats *et al.*, 2001*a*; Wolf and Greiner, 2012). This is supported by experimental evidence, showing that the application of exogenous calcium to onion scale cell walls and to cell wall fractions of herbaceous peony inflorescence stem results in increased stiffness in the cell walls (Li *et al.*, 2012*a*; Xi *et al.*, 2015). Elevated calcium concentrations also result in the cessation of pollen tube growth—presumably because of increased cell wall rigidity—whereas removal of calcium from the medium causes pollen tubes to burst due to compromised structural integrity of the cell wall (Picton and Steer, 1983; Hepler and Winship, 2010). In non-blockwise mode demethylation, PME acts discontinuously on a pectic domain, resulting in less effective Ca^{2+} bridging over a given region. Moreover, when not bonded together via Ca^{2+} bridges, the de-esterified pectic chains might be more susceptible to degradation by polygalacturonases (PGs), which might be the process that eventually results in cell wall softening (Willats *et al.*, 2001*b*; Wakabayashi *et al.*, 2003; Arancibia and Motsenbocker, 2006). Further, the increased porosity resulting from the lack of cross-linking may facilitate the access of degrading/loosening enzymes as speculated in the case of fruit ripening (Brummell, 2006). Several studies have suggested the action of PMEs to precede that of PGs in cell wall softening during fruit ripening, although Redgwell *et al*. observed no causality between de-esterification and PG action (Redgwell *et al.*, 1990; Wakabayashi *et al.*, 2003; Arancibia and Motsenbocker, 2006). The authors speculate that rather than providing the PGs with a substrate, de-esterification may lead to cell wall loosening by decreasing the pH through the release of protons (Hall *et al.*, 1993; Bosch and Hepler, 2005). The lowered pH is thought to activate

wall loosening enzymes and agents such as expansins, resulting in increased extensibility of the cell wall and promotion of growth/creep (Suslov *et al.*, 2015). Additional studies are clearly warranted to shed light on the modes of action of PME and PG in the context of pectin de-esterification and cell wall mechanics. Finally, demethylation can also promote hydration, and therefore reduction in cell wall stiffness (Wolf and Greiner, 2012), presumably through facilitating the relative slippage and unfolding of other cell wall polysaccharides. The challenge to correlate directly the methylesterification status of pectin to the mechanics of the cell wall implies that using standard molecular probes or pectin-specific antibodies to localize the methylated or de-esterified pectin domains *per se* is not a reliable proxy for the mechanical behavior of the specific region, but that actual mechanical tests are required to this end.

Alterations in the ratio of arabinan and galactan side chains in RG-Is affect the mechanical behavior of the cell wall

RG-Is are very complex structurally heterogeneous branched glycan domains with repeating units of [→2)-α-L-Rhap-(1→4)-α-D-GalpA-(1→] in the backbone. RG-I structure is highly versatile, even in a given tissue, and is sometimes thought to act as a scaffold to which other pectic polysaccharides of HG and RG-II covalently bind, forming the pectin matrix (Vincken *et al.*, 2003). In the primary cell wall, the side chains of RG-I may contain galactan, arabinan, and arabinogalactan, while RG-I found in seed mucilage is unbranched (Caffall and Mohnen, 2009). The relative abundance of RG-I compared with HG pectin depends on the source and extraction method (Yapo, 2011). Potato tuber walls are reported to consist of 36% dry weight RG-I, while suspension-cultured sycamore walls contain only ~7% (Caffall and Mohnen, 2009). The role of RG-I in the mechanical properties of the plant cell wall is only poorly understood even if it has been implicated in cell wall extensibility and firmness. Using RG-I-specific antibodies, McCartney *et al.* (2000) observed that in pea cotyledons galactan-rich RG-I appears in the inner face of the cell wall at later stages of development, while arabinan-rich RG-I as well as HG are present throughout development. Compression tests on pea cotyledons before and after appearance of galactan-rich RG-I revealed that cotyledons with galactan-rich cell walls were twice as stiff as those without detectable galactan-rich RG-I. However, it should be noted that in this study the contribution of galactan-rich RG-I to the observed increased stiffness was not clearly distinguished from potential changes in the content of cellulose or other cell wall polysaccharides. Further, (1→4)-β-D-galactan was observed to be associated with de-esterified HG. Since (1→4)-β-D-galactans are flexible chains, whether the increased stiffness is imparted by its interaction with other wall polysaccharides or by its effect on the hydration state of the cell wall is not clear. Jones *et al.* (2003) suggested that arabinan provides the stomatal wall with flexibility required for its movement, by preventing tight packing of HG pectin. Degradation of cell wall arabinan locks the stomatal movement which can be reversed experimentally by removal of HG pectin. This phenomenon may also be explained by the effect of RG-I side chains on the hydration status of the matrix, which will be further discussed in the following sections. This is consistent with the observation that arabinan side chains extracted by enzymatic debranching of intact RG-I from potato hydrated more readily than galactan side chains (Larsen *et al.*, 2011). Ulvskov *et al.* (2005) studied the mechanical properties of potato tuber tissues under compression in two transgenic lines with truncation in either the arabinan or galactan side chains. The authors suggested that the changes in structure of RG-I can affect the hydration status of the cell wall. Further, the line with affected galactan side chains was more prone to fail under compression, was more brittle, and had a shorter relaxation time in stress relaxation experiments compared with the arabinan affected line and the wild type. The faster relaxation was speculated to be a result of depletion of a slow relaxing component of the cell wall, namely galactan. Although the pectic network has often been neglected in terms of its load-bearing capacity when compared with cellulose microfibrils, it can undeniably transmit and distribute loads through the cell wall in connections with the cellulose–hemicellulose network (McCann and Roberts, 1994) and might have higher sensitivity than the latter. Studying the behavior of the cell wall network in the epidermis of onion using two-dimensional Fourier-transform infrared spectroscopy, Wilson *et al.* (2000) showed that while pectin and cellulose–hemicellulose networks act relatively independently under mechanical stress, the pectin network is the first to sense the mechanical oscillations, rather than the more rigid cellulose fibers. A recent *in vitro* study of the interaction between cellulose from *Gluconacetobacter xylinus* and pectins with different neutral sugar contents suggests that in fact both pectin and cellulose contribute to load bearing during compression tests (Lin *et al.*, 2016). Importantly, the authors suggest that binding of pectins with a high content of neutral sugar side chains to cellulose microfibrils is stronger than that of HG to cellulose, although the bonds were reversible upon washing or mechanical compression. In stress–strain curves of compression tests, for equivalent strains, pectin–cellulose composites tolerated higher stresses compared with pure cellulose specimens, indicating a contribution of pectins to load bearing. Nevertheless, it is not clear if the contribution of the pectin in resisting compressive loads is solely due to structural reinforcement of the cellulose composite or to the formation of a denser porous matrix that slows the flow of fluid within the material towards the outside. Interestingly, the pectin–cellulose composites with higher compressive stiffness compared with pure cellulose composite released the highest amount of pectin under compression. This could be due to higher fluid pressure gradients within the specimens and resulting detachment of pectins. Further, considering that the cell wall in living plants is mainly under tension rather than other modes of mechanical deformation, it is interesting to know how tensile properties of these composites may relate to their compression behavior. A recent study seems to suggests that pectin gels from RG-I can form

hyperelastic hydrogels with nearly incompressible bulk behavior and stiffening under large deformations (Mikshina *et al.*, 2015). If the RG-I network does indeed exhibit strain stiffening, its contribution in distributing the mechanical loads to the network of cellulose and other wall polysaccharides could be critical since it might have the ability to control cell wall expansion under large pressure and strain conditions. RG-Is are a very complex class of polysaccharides with diverse functions that certainly deserve more attention in future studies as their influence on cell mechanics and growth might be more dramatic than we thought.

RG-II–borate cross-linking may be essential for polar growth

RG-II is a highly branched domain of pectin polysaccharides that constitutes <10% of primary cell walls in eudicots and is found in all vascular plants. RG-II is a highly complex structure with a HG backbone and (A–D) substituted side chains of oligosaccharides. Borate cross-links apiosyl residues in side chain A of RG-II monomers and forms apiose–borate–apiose diesters, increasing the stiffness of the wall as well as decreasing its porosity (Fleischer *et al.*, 1999; Caffall and Mohnen, 2009; Dumont *et al.*, 2014; Funakawa and Miwa, 2015). Interestingly, a recent study suggests that dimerization can occur in protoplasmic or newly secreted RG-IIs but not in those already existing in the cell wall (Chormova *et al.*, 2014). Borate cross-linking is essential for successful expansive growth of cells by providing the cell wall with proper mechanical strength. In the cell wall of *Arabidopsis* rosette leaves, >90% of RG-II content exists in dimer form. In plants of the *mur1* mutant, deficient in ʟ-fucose, a monosaccharide found in RG-II, this percentage drops to 56%. The mutant exhibits dwarfed growth, reduced rosette leaf expansion, and reduced tensile strength of the stem due to a possible truncation of side chain A in RG-II (O'Neill *et al.*, 2001; Ryden *et al.*, 2003). Watering the plants with boric acid enhances leaf expansion by promoting dimerization of RG-II (O'Neill *et al.*, 2001). The tensile modulus and tensile strength of hypocotyls of *mur1-1* grown in the presence of 2.6 mM boric acid are rescued by promoting dimerization of RG-II (Ryden *et al.*, 2003). The tensile modulus and tensile strength correspond to the slope of the elastic portion and to the failing point of the stress–strain curve in a tensile test, respectively. In pollen tubes, changing the concentration of boric acid affects the growth rate in a similar fashion to Ca^{2+} (Holdaway-Clarke *et al.*, 2003), consistent with the notion that the ion is necessary to stabilize the cell wall during cellular expansive growth. Interestingly, Koshiba *et al.* (2010) found that under boron deficiency, BY-2 cells display increased Ca^{2+} uptake. We speculate that this occurs at least partly to compensate for loss of RG-II dimerization and to control the pectin network properties including the mechanics of the network and pore size through Ca^{2+} cross-linking. Mutation in Arabidopsis *SIA2* encoding a sialyltransferase-like protein possibly involved in the transfer of Dha and Kdo (Dumont *et al.*, 2014), two disaccharides present in RG-II side chains, results in shorter, swollen or dichotomous pollen tubes and a higher frequency of bursting. As Dha and Kdo are not present in side chain A where borate bridging takes place, it seems that impairment in the assembly of other RG-II side chains can affect cell wall stability, although further studies are required to verify the impairment of RG-II structure in this situation (Holdaway-Clarke *et al.*, 2003). *bor1-1*, a high-boron-requiring *A. thaliana* mutant, shows severe defects in the expansion of rosette leaves in low boron concentration (Noguchi *et al.*, 1997). Interestingly, boron deficiency seems to impact meristems and reproductive tissues such as pollen and female gametophytes more than other somatic tissues (Chatterjee *et al.*, 2014). Increasing the boron concentration rescues the phenotype of the somatic plant body, but rescuing female sterility requires more elevated boron concentrations. *bor2-1* and *bor2-2*, the loss-of-function mutants of BOR2, a boron efflux transporter located in the epidermis of the root elongation zone, on the other hand, are compromised in root elongation under boron-deficient conditions (Miwa *et al.*, 2013). It was suggested that although the boron concentration in the root was not affected significantly compared with the wild type, the proportion of cross-linked RG-II was significantly reduced.

Although the role of RG-II dimerization for cell wall stiffness is relatively well established, the subcellular localization of RG-II in growing cells remains puzzling. In general, regions of softer cell wall are associated with pronounced cellular growth activity, whereas stiffer cell walls are present where the cell does not grow or has stopped doing so (Geitmann and Ortega, 2009). Consistent with this, in pollen tubes, highly methylesterified pectin is present mostly in the growing apex of the cell, whereas the non-growing shank displays de-esterified HG (Geitmann and Ortega, 2009; Zerzour *et al.*, 2009; Fayant *et al.*, 2010). RG-II distribution would be expected to follow the same pattern, being predominantly present in the stable, non-growing regions of the cell. However, immunolocalization showed that the opposite is the case. In pollen tubes of three plant species including *A. thaliana*, RG-II is present in all regions of the cell, both growing and non-growing (Dumont *et al.*, 2014). In wild-type *Nicotiana tabacum* pollen tubes, RG-II concentration was even found to be higher in the growing apex (Iwai *et al.*, 2006). While this is puzzling, the specificity of the antibody did not actually allow the distinction to be made regarding whether the RG-II was cross-linked by borate esters—clearly a crucial parameter for the mechanical behavior of the polymer. It has also been speculated that the HG backbone of RG-II and its side chains are resistant to fragmentation by glycanases, which maintains the integrity of the pectin network, while the framework can be enzymatically modified during cell growth (Rose, 2003).

The situation is further complicated by the fact that boron deficiency is linked to oxidative damage and cell death (Dordas and Brown, 2005). It is therefore difficult to distinguish whether RG-II dimerization is directly essential for polar growth and cell elongation or whether effects on cellular growth are mediated through the viability of the cell as a whole. Further, boron is thought to have various other functions besides cross-linking RG-II. It can act as a signaling molecule, as a stabilizer of the plasma membrane, and

is suggested to be involved in auxin metabolism (Chatterjee *et al.*, 2014). It has recently been reported that boron deficiency inhibits primary root growth in *A. thaliana* through putative auxin-, ethylene- or reactive oxygen species (ROS)-dependent pathways (Martín-Rejano *et al.*, 2011; Camacho-Cristóbal *et al.*, 2015). Auxin resistant 1 (AUX1) is reported to be involved in growth inhibition at the root tips under boron-deficient conditions (Martín-Rejano *et al.*, 2011). How compromised cell wall integrity and altered cell wall mechanical stresses due to impeded RG-II cross-linking may trigger an auxin-dependent response to inhibit further cell expansion has not been elucidated. Clearly, RG-II localization, its degree of dimerization, and its interaction with other cell wall polysaccharides merit further investigation to determine how RG-II contributes mechanically to the whole cell wall matrix in a growing cell.

Changes in pectin status alter the biphasic properties of the cell wall

The hierarchical structure and connectedness of cell wall polymers create spaces whose size affects the passage of water, ions, and macromolecules (Fig. 1A). The apoplastic water content in growing primary cell walls amounts to ~60% (w/v) (Jackman and Stanley, 1995; Pettolino *et al.*, 2012). Therefore, the mechanical behavior of the plant cell wall is influenced by how easily solutes move through spaces when the wall is deformed through tensile or compressive forces. The overall behavior can be considered to be that of a poroelastic material that is greatly influenced by the structure, size, and connectivity of its pores. In fact, the response of a poroelastic substrate to application of a load is a combination of the deformation behavior of the solid phase and the resistance of the porous structure to movement of the intrinsic fluid (similar to a sponge, Fig. 1B, C). Interestingly, even if the putatively non-linear and viscous response of the porous solid scaffold is neglected, the retarded movement of fluid through voids and cavities can exhibit a time-dependent response similar to viscoelasticity. Decoupling the relative contributions of these two behaviors in the deformation of a biological material is not a trivial task (Galli *et al.*, 2009; Strange *et al.*, 2013; Wang *et al.*, 2014). The poroelastic behavior of the plant cell wall is significantly influenced by configurational changes in cell wall polymers and their interconnections as these affect porosity. One of the parameters that influence porosity of the plant cell wall is the degree of methylesterification of HG pectin. *In vitro* compression tests on lime pectin gels with different levels and modes of de-esterification revealed a reduction in the water-holding capability of the pectin gel with the degree of de-esterification (Willats *et al.*, 2001*b*). Metal ions such as aluminum or copper are known to affect further the hydraulic conductivity of the pectic matrix as well as water uptake of the plant by saturating the polymer cavities and affecting the porosity of the composite (Blamey *et al.*, 1993; McKenna *et al.*, 2010). McKenna *et al.* used bacterial cellulose–pectin composites as plant cell wall analogs to study the changes in hydraulic conductivity of the cell wall due to ions.

They found that the hydraulic conductivity of the cell wall analogs correlates with changes observed in pectin network porosity revealed by scanning electron microscopy. Pectin methylesterification therefore not only affects the stiffness of the solid component of the wall by altering polymer cross-linking, but also influences the degree of porosity and hence the movement of liquid.

Plant organs with rapid movement requirements such as the Venus flytrap rely on rapid water exchange alongside structural instability, such as buckling, for their movement (Forterre, 2013). It is therefore plausible that the structure of the cell wall in these organs needs to possess the porosity that facilitates the movement of water at short time scales. The variety of physical requirements suggests that the short and long time scale behavior of cell walls under load can vary significantly. With respect to the role of the hydration state for the biomechanical properties of the cell wall, Köhler and Spatz (2003) suggested that the strengthening of the secondary cell wall by lignification may be mostly due to the ability of the hydrophobic cross-linked polymers to expel water from the cell wall rather than the strength of the polymer itself (Köhler and Spatz, 2002; Ulvskov *et al.*, 2005).

Changes in configuration of all types of pectin polymers can affect the porosity of the cell wall. As discussed earlier, alterations in side chains of RG-I affect the hydration state and porosity of the cell wall. De-esterification of HG pectin affects the cell wall porosity (Goldberg *et al.*, 2001). As for RG-II, adding boric acid to suspension-cultured *Chenopodium album* L. cells grown on a boron-deficient medium for more than a year caused dimerization of RG-II and a rapid decrease in wall porosity within 10 min (Fleischer *et al.*, 1999). It remains an open question whether borate cross-linking can occur in the pre-existing monomeric RG-II, a putative requirement for such a rapid change in the cell wall, or whether dimerization can occur only in cytoplasmic or newly secreted RG-II (Chormova *et al.*, 2014). Either way, RG-II dimerization and changes in cell wall porosity can occur quite rapidly, and controlling cell wall porosity can therefore have implications for the transport and incorporation of new cell wall polymers into the cell wall as well as for the access of wall-modifying enzymes and proteins to their substrate (Fleischer *et al.*, 1999). Further, poroelastic time- and strain-rate-dependent behaviors of the cell wall not only need to be scrutinized in the context of plant cell growth, but are also relevant for the interpretation of mechanical tests carried out on cell walls. These facts emphasize the need for careful evaluation of the conditions under which mechanical testing of plant specimens is performed as dehydration of the tissues can greatly alter their mechanical properties.

Cellulose, the usual suspect of cell wall mechanical anisotropy

Cellulose microfibrils have generally been considered to be the principal load-bearing components of the cell wall and the determinant of growth anisotropy (Baskin, 2005). The reason for this is the high tensile modulus of ~140 GPa that allows
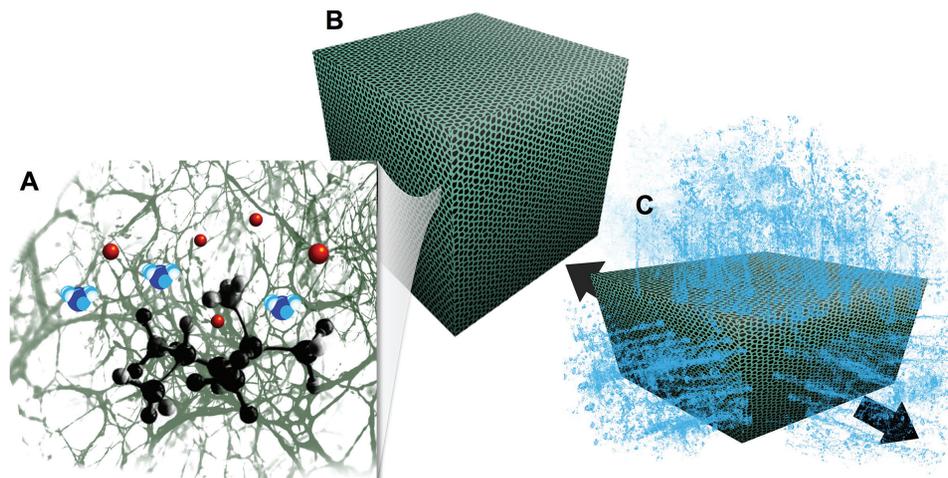
Fig. 1. Cell wall porosity affects the mechanical properties of the material. (A) Pore size of the cell wall polymers affects the passage of ions (red), water molecules (blue), and macromolecules (black). (B) Schematic representation of fluid-saturated porous material. (C) Fluid extrusion from the porous material under tensile stress.

the fibrils to impede cell expansion. Cellulose microfibrils are made up of β-1,4-glucan chains assembled into microfibrils by intermolecular hydrogen bonds between the oxygen atoms. Cellulose microfibrils are thought to behave more or less like cables. Parallel arranged cellulose microfibrils cannot withstand loading perpendicular to their long axis, but, through connections, the load is distributed among the cross-linking pectin and xyloglucan network (Dyson *et al.*, 2012). When the microfibrils in a given section of cell wall are organized to display a preferential orientation, cellular expansion under the effect of turgor pressure is reduced in that direction.

Cellulose is synthesized at grouped CESA (cellulose synthase) enzymes (rosettes), each of which can synthesize a single thread of glucan (Doblin *et al.*, 2002). Glucan chains are then packed with non-covalent hydrogen and van der Waals bonds into cellulose microfibrils (for a review, see Cosgrove, 2014). The length of the microfibrils and the shape and area of their cross-section are important in defining the type and quality of their interactions with other wall polysaccharides, such as xyloglucans, and can affect their slippage or separation during the growth of the primary wall. However, despite a large body of research on cellulose structure and interactions, surprisingly little is firmly established. Even basic characteristics such as microfibril length, number of glucan chains per cellulose microfibril, the shape of the microfibril cross-section, or the mechanism guiding CESA in the absence of a functional link to microtubules are elusive (Li *et al.*, 2012b; Cosgrove, 2014). In land plants, the diameter of cellulose microfibrils is suggested to be in the range of 2–5 nm (Thomas *et al.*, 2013; Cosgrove, 2014). Cellulose microfibrils can consist of crystalline and non-crystalline domains. In crystalline regions, glucan chains are well ordered and bonded to each other by non-covalent (hydrogen) bonds. It is suggested that the crystalline and non-crystalline regions can either be placed alternately, or the amorphous non-crystalline regions can encapsulate the crystalline part, or both (Salmén and Bergström, 2009; Burgert and Dunlop, 2011). The values reported for the longitudinal elastic modulus of cellulose I range from 25 GPa to 220 GPa, depending on certain factors such as whether or

not the intramolecular hydrogen bonding is considered in calculations (Eichhorn and Young, 2001; Diddens *et al.*, 2008; Cintrón *et al.*, 2011; Mariano *et al.*, 2014). However, most of these studies are either based on theoretical models or carried out using X-ray diffraction or Raman spectroscopy. Only a few studies have performed relatively direct mechanical tests on cellulose, for example by employing the atomic force microscopy- (AFM) assisted three-point bending test (Guhados *et al.*, 2005; Cheng and Wang, 2008; Iwamoto *et al.*, 2009). Moreover, most studies are based on the crystalline portion of cellulose, and the mechanical properties of non-crystalline or amorphous cellulose are even less well defined. Some have assumed values as low as 5 GPa for non-crystalline cellulose (Hancock *et al.*, 2000; Eichhorn and Young, 2001; Kulasinski *et al.*, 2014). The absence of well-documented mechanical parameters becomes more significant when considering that the crystalline fraction in plant cell wall cellulose content can be considerably lower compared with commonly studied cellulose from other sources such as bacteria (Mariano *et al.*, 2014). Therefore, we think that in studies correlating the orientation of cellulose microfibrils to growth anisotropy in plant cells, complementary evidence identifying cellulose crystallinity is of utmost importance.

Recent studies suggest that some microfibrils in the plant cell wall form aggregates and exist in the form of bundles with non-covalent bonding, and that it is the orientation of these bundles and not that of individual microfibrils that defines the expansion behavior of the cell wall (Anderson *et al.*, 2010; Thomas *et al.*, 2013). In higher plants, individual microfibrils of ~3 nm in thickness can form aggregates of thicker fibrils with 5–10 nm and 30–50 nm thickness in primary and secondary cell walls, respectively (Fernandes *et al.*, 2011; Li *et al.*, 2014). However, it might be that 'super bundles' of greater thickness are formed, which would explain how cellulose orientation can be resolved by common confocal microscopy that is subject to the diffraction limit of 200 nm (Fig. 2; Anderson *et al.*, 2010).

The multinet (passive reorientation) theory termed by Roelofsen and Houwink (1953) suggests that the most recently
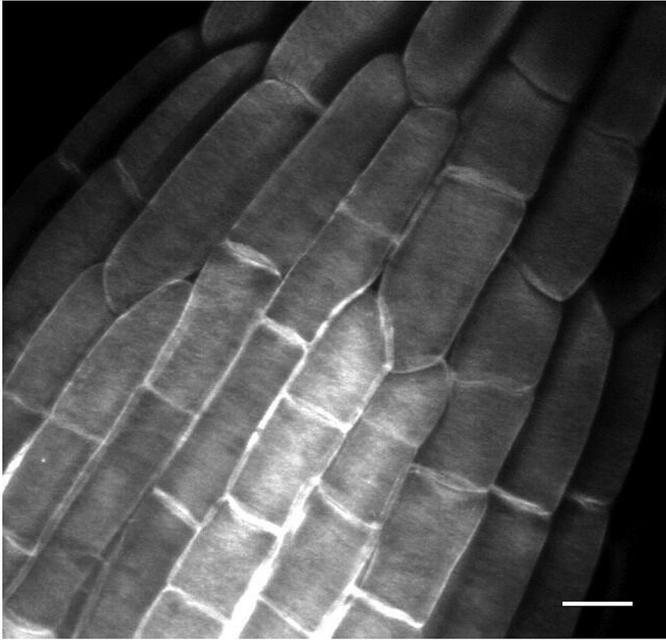
Fig. 2. Discernible arrays of cellulose are oriented perpendicular to the growth direction in the growth region of an *Arabidopsis thaliana* root. These arrays probably correspond to 'super-bundles' of microfibrils. Staining with Pontamine Fast Scarlet (S4B). Scale bar=10 μm.

added layer of cellulose microfibrils at the inner face of the cell wall is deposited in a direction perpendicular to the axis of cell growth. When thus oriented, the cellulose in the inner cell wall layer is therefore able to resist tension and is considered to be mechanically most influential, while microfibrils in the older layers are passively reoriented in the direction of growth (Richmond *et al.*, 1980; Richmond, 1991). It should be noted that this hypothesis is not necessarily incompatible with other models proposed to explain the growth of the cell wall, such as wall loosening by expansins or wall assembly by xyloglucan endotransglycosylases (XETs) (Cosgrove, 2005). Anderson *et al.* (2010) observed that in some layers of elongating root epidermal cell walls, cellulose reorients from transverse orientation in early stages to oblique orientations at later stages, in a trend similar to that previously reported for hypocotyl cell walls (Refrégier *et al.*, 2004), and consistent with the multinet theory.

Microtubules, cellulose, and growth anisotropy

The movement of rosettes to lay down cellulose microfibrils in the new cell wall layer is generally accepted to be guided by cortical microtubules through microtubule–rosette links (Crowell *et al.*, 2009). Cellulose synthase interactive protein 1 (CSI1) has been proposed as a link between microtubules and CESA (Gu *et al.*, 2010; Bringmann *et al.*, 2012; Li *et al.*, 2012b). Disruption of microtubules by drugs such as oryzalin or colchicine has been shown to adversely affect proper bundling of cellulose microfibrils and cell shape (Baskin *et al.*, 1994, 2004; Panteris and Galatis, 2005). Clearly the regulatory machinery that links internal structures to external cell

wall polymers across the plasma membrane is of fundamental importance to plant cell morphogenesis. Microtubules are also involved in targeting of CESA to the plasma membrane (Gutierrez *et al.*, 2009). Recent studies indicate that microtubules also participate in cell wall assembly by delivering vesicles containing non-cellulosic polysaccharides such as pectin; a role that was thus far assumed to rely mostly on the actin–myosin system (Oda *et al.*, 2015; Zhu *et al.*, 2015).

In anisotropically elongating cells or tissues, the orientation of the cellulose microfibrils is typically perpendicular to the main axis of growth, and this orientation has been considered to be causal for the expansion anisotropy. However, recent evidence has shown that the relationship consists of a feedback mechanism in which the mechanical status of the cell induces the deposition of cellulose in a particular direction. This is thought to be mediated by microtubules, as their orientation is believed to respond to and align with the direction of maximal stress (for a review, see Uyttewaal *et al.*, 2012). Experimental evidence to support this idea has been obtained both in single cell cultures and in tissues. Microtubules in suspended protoplasts exposed to centrifugal forces were shown to orient along the centrifugal force vector, resulting in growth of cells in the perpendicular direction (Wymer *et al.*, 1996). Similar findings were carried out in cotyledon epidermal cells where stress anisotropy due to cell shapes was linked to reorganization and alignment of microtubules in the direction of maximal stresses (Sampathkumar *et al.*, 2014b).

It is important to realize that the action of depositing cellulose microfibrils can actually maintain or even increase stresses by causing stress concentration in a particular direction and/or at a particular location. This may sound counterintuitive as it is sometimes presumed that it is the weaker walls that undergo the largest stresses. In fact aligned cellulose microfibrils, by resisting strains and limiting expansion of cells, can lead to the formation of geometrical discontinuities or curvatures which in turn result in higher stresses at specific locations. For instance, consider a cylindrical, thin-walled elastic shell, with uniform isotropic material properties (such as, for example, a Frankfurter sausage): because of the cylindrical geometry, inflation of this tube causes transverse stresses in the shank that can be computed to be twice as high as the stresses in the longitudinal direction in this region (Fig. 3A). If a spherical shell balloon has a stiffer belt-shaped band at its equator, the belt region expands less, resulting in a dumbbell shape with a narrow 'waist' region. Due to difference in deformability, this stiffer waist region is subject to the highest principal stresses (Fig. 3B). This is an example of how material discontinuities can generate geometrical changes that in turn cause local stress concentration. Since stress equals force per cross-section, the effect of geometrical discontinuities on the evolution of local stress can be offset partly or entirely if the wall becomes thicker at locations of elevated stress. However, often the addition of cellulose does not necessarily thicken the wall and thus locally elevated stresses can be expected at locations with higher density of cellulose.

Because of the effect of cellulose deposition on local stress patterns, microtubules can be considered as crucial mediators of a positive feedback mechanism as they act as enhancers of
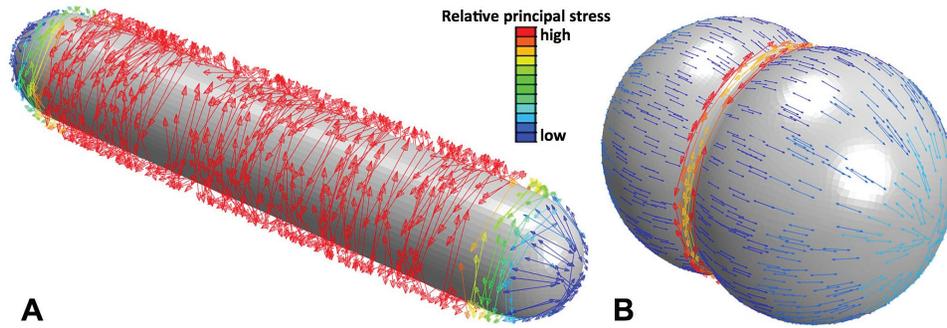
Fig. 3. Influence of geometry and material discontinuity on wall stresses: maximum principal stresses in elastic bodies in a cylindrical shell (A) and in an inflated balloon with a stiffened equatorial band resulting in a dumbbell shape (B). In (A), stress is higher in the circumferential direction than in the longitudinal direction. In (B), locally elevated stress is present in the waist region.

growth anisotropy. The microtubule-severing protein katanin is proposed to increase the ability of microtubules to self-organize in parallel arrays in response to mechanical stress. The katanin mutant *ktn1* displays impaired microtubule dynamics, resulting in a shoot apical meristem with randomly oriented microtubules, a rather bumpy meristem surface, and decreased response of meristemic cells to mechanical stress (Uyttewaal *et al.*, 2012), consistent with the notion that proper organ formation depends on this positive feedback mechanism.

Sampathkumar *et al.* (2014*a*) suggested that large-scale stresses operating at the tissue rather than the cell level act superimposed on local, subcellular stress in controlling the direction and orientation of microtubules. Further, Bozorg *et al.* (2014) showed that stress and strains in the cells are not necessarily always oriented perpendicular to each other. The authors demonstrated that stresses and strains can fall parallel in cells in a specific region of a tissue due to tissue level stresses thanks to a peculiar local shape of tissue, as in the valley between the meristem and a leaf primordium. The question is whether cellulose microfibrils orient in the direction orthogonal to the direction of maximum strain (growth) or whether they orient in the direction of maximal stress. Although these may seem difficult to distinguish, they are not of the same nature. Stress is the force that is experienced at any given point of a structure due to the application of a load (here the turgor pressure), whereas strain is the deformation that results from the application of the said load. The amplitude of local strain depends on the local stress and on the material properties determining the resistance to deformation. Strain, therefore, does not necessarily correlate with stress (Yip *et al.*, 2013). By introducing fiber reorientation in a mechanical feedback model, Bozorg *et al.* (2014) observed that simulations in which microfibrils oriented in the direction of maximum stress behaved stably and similar to a fixed anisotropy direction model, suggesting that the rule can make a robust shaping cue. However, orientation in the direction orthogonal to the maximum strain rule resulted in unstable fiber directions, and eventually the orientation of fibers became longitudinal (in the direction of maximum elongation). Based on these *in silico* simulations, the authors suggested that cellulose microfibrils orient in the direction of maximal stress (rather than strain).

Pectin: the new black?

The notion that cellulose-mediated stiffening is the result of a positive feedback mechanism raises the question of what initiates the morphogenetic process in anisotropic growth. It had long been assumed that the orientation of cellulose is both the causal and initial agent. However, this concept has recently been challenged by observations of other cell wall components that display marked inhomogeneity upon the onset of growth asymmetry and even prior to a perceivable preferential orientation of cellulose. Using AFM to map the mechanical changes in *Arabidopsis* hypocotyl epidermal cells, Peaucelle *et al.* (2015) observed that the longitudinal anticlinal walls (those that preferentially expand during hypocotyl elongation) undergo softening prior to the onset of anisotropic elongation (Fig. 4). Monitoring the orientation of microtubules and CESA tracks suggests the absence of perceivable transverse alignment of cellulose at this stage. Changes in mechanical symmetry of the cell resulting in growth symmetry breaking were attributed to softening due to selective de-esterification of pectin in longitudinal walls. The authors suggest that growth symmetry breaking is controlled at the subcellular level by changes in pectin status in selected walls of a given cell. They propose that cellulose microfibril deposition in the transverse direction follows but is not an initiator. Therefore, since changes in stiffness of pectin polymers are not as pronounced when compared with substantial stiffness of cellulose microfibrils, tools with sufficient force resolution must be used to measure the mechanical properties in growing cells, especially at early stages of growth. These studies point to a relatively novel concept—the role of changes in the pectin status as the primary morphogenetic trigger. Only once these are initiated do they lead to changes in stress distribution which in turn trigger microtubule reorganization that only in a second step leads to reinforcement by cellulose in a positive feedback loop (see the previous section). In this scenario, microtubules and cellulose microfibrils play the role of mediator and enhancer of anisotropy instead of initiating it (Fig. 4).

To corroborate further the notion of pectin as a mechanical initiator of growth anisotropy, a feedback loop has been proposed involving the phytohormone auxin and pectin de-esterification in organ formation (Peaucelle *et al.*, 2011;
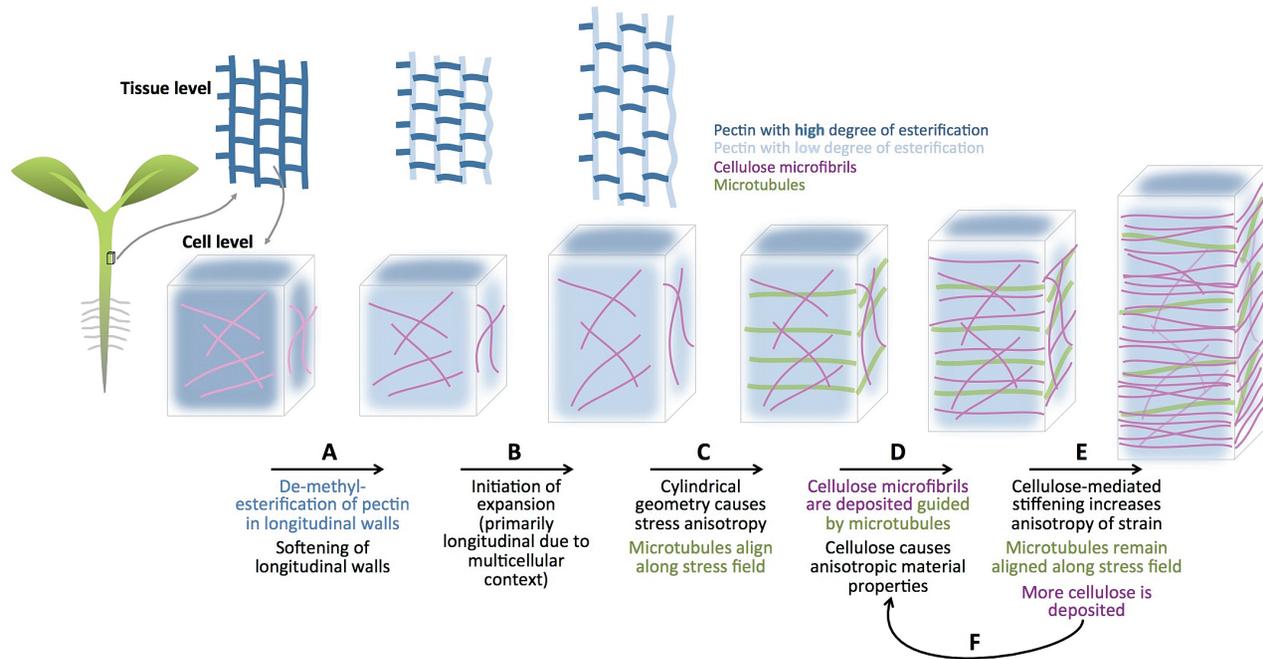
Fig. 4. General concept of a two-step mechanism achieving anisotropic plant cell growth. (A) Longitudinally oriented cell walls soften through pectin de-esterification [consistent with this, using AFM, Peaucelle *et al.* (2015) showed that longitudinal anticlinal walls in the epidermis of Arabidopsis hypocotyls become softer prior to the onset of cell expansion; immunohistochemistry suggests that this is correlated with de-esterification of pectin]. (B) The difference in mechanical properties between longitudinal and transverse walls triggers expansion of the longitudinal walls. Because of tissue geometry and tight cell–cell attachments, the expansive activity in the hypocotyl tissue occurs primarily longitudinally. (C) Due to the newly generated cylindrical geometry of the cells, the primary stress field is transverse to the growth direction. Microtubules align transversally, in the direction of maximum stress [consistent with this, Peaucelle *et al.* (2015) showed that microtubule alignment occurs only after softening of longitudinal walls]. (D) Deposition of cellulose microfibrils in transverse orientation causes material of the longitudinal walls to become anisotropically reinforced. (E) An increase in stress anisotropy maintains the transverse orientation of microtubules. (F) Continued deposition of cellulose microfibrils reinforces the cell wall anisotropy.

Braybrook and Peaucelle, 2013). Auxin-induced organogenesis is accompanied by a local decrease in stiffness of the tissue. In the *pin1* mutant defective in polar auxin transport, the shoot apical meristem grows but exhibits severe abnormalities in or the absence of the development of floral organs, resulting in needle-shaped apices of inflorescence stems (Okada *et al.*, 1991). While local application of auxin to the organ-free apex of *pin1* rescues organogenesis accompanied by a detectable decrease in stiffness at the site of the emerging leaf primordium, rescue through exogenous auxin was not possible in meristems of plants overexpressing pectin methylesterase inhibitor3 (*PMEI3oe*) (Braybrook and Peaucelle, 2013). This suggests that in the shoot apical meristem auxin-induced stiffness modulation is mediated through pectin de-esterification. Interestingly, immunolocalization of PIN1 in *PMEIoe* revealed disorganized polarity of the auxin transporter (Braybrook and Peaucelle, 2013). This is indicative of a reverse mutual influence of pectin de-esterification on auxin polarity. It is speculated that altered wall mechanics actually affect the organization of PIN1 polarity.

The enduring concept of cellulose acting as the sole mediator and initiator of growth anisotropy may have originated from or been reinforced by the presumption that other wall polysaccharides, and especially pectins, do not contribute to load bearing and determination of the mechanical properties of the cell wall, due to their relatively low elastic modulus.

However, it turns out that even in the presence of cellulose, pectins can affect the mechanical properties of the cell wall and act as distributors of mechanical load in the cellulose–hemicellulose–pectin network. Hydrated pectins might interact with and bind to cellulose microfibrils (Dick-Pérez *et al.*, 2011; Wang *et al.*, 2012). Pectin perhaps somewhat liquefies the cell wall, reducing cellulose self-association and facilitating cellulose slippage in expanding cell walls (Thimm *et al.*, 2009). Extraction of pectin is observed to be associated with increased rigidity of the cell wall (Dick-Perez *et al.*, 2012; Cosgrove, 2014), with microfibrils having a higher tendency to become denser and form aggregates (Thimm *et al.*, 2009). Pectin–xyloglucan interaction might also prevent unfolding of xyloglucan chains attached to cellulose microfibrils, resulting in increased stiffness. Abasolo *et al.* (2009) performed tensile tests on *mur1* and *qua2* mutants of *A. thaliana*. *mur1* is deficient in L-fucose, affecting RG-II dimerization and xyloglucan fucosylation, while *qua2* has 50% HG content. The results showed considerably lower tensile stiffness for these mutants compared with the wild type (Col-0) and *mur2* (affected xyloglucan fucosylation but no changes in pectin content). However, ultimate strengths (defined at the rupture point) showed no significant differences. Also, after the first cyclic loading of the tensile-testing protocol, all the hypocotyl specimens showed strain hardening, but this was much more pronounced in the case of *mur1* and *qua2* specimens.

This may indicate that unfolding of the xyloglucan network is easier in the case of a compromised pectin network showing a drop in tensile stiffness, whereas when the xyloglucan strands are unfolded, stiffness values tend to converge.

Conclusion

The emergence of feedback mechanisms regulating cell wall properties suggests that this cellular feature should be regarded as a dynamic self-regulating structure that continuously generates feedback on its mechanical properties and modifies itself to align with the requirement for plant cell growth and function. The cell wall fulfills this mechanical 'self-awareness' through modulation of its polysaccharides, cross-links, macromolecules, proteins, and ion contents. Cellulose microfibrils have conventionally been regarded as major cell-shaping components of the plant cell wall and they are known to determine growth anisotropy by restricting cellular expansion along their principal orientation. The fact that cellulose orientation is guided through links with microtubules, which in turn are sensitive to mechanical stresses in the cell wall, illustrates one possible way in which the cellular geometry and mechanical status can generate feedback onto cell wall assembly. However, this is only one of the mechanisms through which the cell wall senses its mechanical state and triggers the cell to respond accordingly. Pectins have gained increased attention in recent years. Changes in pectin polymer configuration can precede organogenesis, and these occur even prior to detectable changes in cellulose orientation. Pectins can loosen or stiffen the matrix and affect the stiffness of the cell wall directly, or indirectly through restricting the slippage of cellulose for instance by impeding the unfolding of xyloglucans. Modulation of pectins can presumably also increase the local stiffness of the matrix, triggering a positive feedback loop that in turn orchestrates a microtubule-guided cellulose reinforcement of mechanical and growth anisotropy. On the other hand, changes in pectin configuration through de-esterification, dimerization, and branching can affect the porosity of the cell wall. Cell wall porosity influences its permeability to macromolecules including enzymes that tune the expansive growth of the cell by breaking or creating cross-links between polymers. Further, changes in cell wall porosity influence how readily water can move through voids and cavities in the cell wall. Since expansive growth of plant cells is typically a relatively slow process, it is unclear how changes in poroelasticity of the cell wall affect its expansive behavior, and warrants future experimental and modeling studies. However, in measurements of the cell wall mechanics that rely on rapid and substantial deformation of the cell wall, retarded water movement within the wall could play a significant role and poroelastic properties should therefore certainly be accounted for.

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