

Cell Wall, Cytoskeleton, and Cell Expansion in Higher Plants

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ABSTRACT To accommodate two seemingly contradictory biological roles in plant physiology, providing both the rigid structural support of plant cells and the adjustable elasticity needed for cell expansion, the composition of the plant cell wall has evolved to become an intricate network of cellulosic, hemicellulosic, and pectic polysaccharides and protein. Due to its complexity, many aspects of the cell wall influence plant cell expansion, and many new and insightful observations and technologies are forthcoming. The biosynthesis of cell wall polymers and the roles of the variety of proteins involved in polysaccharide synthesis continue to be characterized. The interactions within the cell wall polymer network and the modification of these interactions provide insight into how the plant cell wall provides its dual function. The complex cell wall architecture is controlled and organized in part by the dynamic intracellular cytoskeleton and by diverse trafficking pathways of the cell wall polymers and cell wall-related machinery. Meanwhile, the cell wall is continually influenced by hormonal and integrity sensing stimuli that are perceived by the cell. These many processes cooperate to construct, maintain, and manipulate the intricate plant cell wall—an essential structure for the sustaining of the plant stature, growth, and life.

Key words: cell wall; cellulose; hemicellulose; pectin; biosynthesis; cellulose synthase; microtubules; actin; cytoskeleton; trafficking; hormonal regulation; cell wall integrity sensing.

INTRODUCTION

By forming a physical barrier that surrounds every plant cell, the cell wall is inherently involved in regulating cell expansion. Tremendous effort has been put into the study of cell wall structure and formation over the past two decades not only due to interest in the biological perplexities of the cell wall function in plants, but also due to the dependence of society on the utilization of cell wall material by the timber, pulp, paper, and cellulosic biofuel industries. Many outstanding reviews cover the recent progress in the rapidly expanding field of plant cell walls (Cosgrove, 2005; Saxena and Brown, 2005; Somerville, 2006; Joshi and Mansfield, 2007; Mohnen, 2008; Guerriero et al., 2010; Scheller and Ulvskov, 2010; Fujita et al., 2012; Li and Gu, 2012; Wolf et al., 2012a; Li et al., 2014). As the major load-bearing cell wall polymer, cellulose will be the central focal point in a majority of our discussion on how several properties of the biosynthesis and organization of the plant cell wall influence cell expansion. We will discuss how several aspects relate to cell expansion, including: the interaction network between cellulose microfibrils and other wall polymers, the relationship between microtubules and cell wall architecture, the influence of hormones and cell wall integrity sensing in cell wall regulation, the role of the cytoskeleton and trafficking in cell wall maintenance, and the

key players between cellulose synthase complexes and microtubules. The authors apologize to many colleagues whose work could not be cited because of space limitations.

THE INTERACTION BETWEEN CELLULOSE AND NON-CELLULOSIC WALL POLYMERS

The plant cell wall is composed of cellulose, non-cellulosic wall polysaccharide polymers such as hemicellulose and pectin, and a small amount of protein. The architecture of the cell wall is required not only to be strong and rigid to provide the structural support for the plant, but also to be forgiving in a controlled way to allow for anisotropic cell expansion. Therefore, knowledge of cell wall architecture is fundamental for understanding how cells control growth through cell wall synthesis and cell wall remodeling. Unlike cellulose, which is

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synthesized at the plasma membrane (PM) by PM-resident cellulose synthase (CESA) complexes (CSCs), non-cellulosic polysaccharides are assembled within the Golgi, secreted into the apoplast by fusion of Golgi-derived vesicles with the PM, and associated with newly synthesized cellulose microfibrils in the cell wall (Driouich et al., 1993). Because cellulose and non-cellulosic polymers are synthesized at different locations, non-cellulosic polymers might not directly influence the biosynthesis of cellulose, but rather the mechanical properties of cellulose microfibrils and the assembly of the cell wall matrix.

Xyloglucan (XyG) is the most abundant hemicellulose in dicot primary walls. XyG is thought to form hydrogen bonds along the lengths of cellulose microfibrils and to play a large role in forming the crosslinks between cellulose microfibrils. Historically, there have been two proposed mechanisms by which XyG acts in crosslinking cellulose microfibrils, either by XyG chains directly connecting two microfibrils or by XyG chains indirectly connecting two microfibrils through an intermediate network of pectin polysaccharides (Keegstra et al., 1973; Fry, 1989; Hayashi, 1989).

Indirect linkages between XyG and cellulose microfibrils were first proposed by Keegstra and his colleagues (1973). In this early cell wall model from studies on sycamore cells, XyG was proposed to be hydrogen-bonded to the surface of cellulose microfibrils and covalently attached to a pectin crosslinking network that was in turn covalently attached to other XyG chains that were hydrogen-bonded to the surface of other cellulose microfibrils. Alternatively, XyG has been proposed to tether cellulose microfibrils by acting as the sole crosslink by binding to the surface of two different microfibrils or by being intercalated within multiple microfibrils during the crystallization of cellulose microfibrils (Fry, 1989; Hayashi, 1989). This XyG/cellulose network is imbedded within a pectin matrix. This 'tethered network model' is widely cited and supported by *in vitro* binding data, by microscopic visualization, and by enzyme digestion experiments (Hayashi and MacLachlan, 1984; Hayashi et al., 1987; McCann et al., 1990; Pauly et al., 1999; Yuan et al., 2001). In one supporting study, expansins induced creep more effectively when supplemented with a fungal endoglucanase that digests XyG (Yuan et al., 2001). In a transmission electron microscopy (TEM) study, cell wall polymers were observed to crosslink cellulose microfibrils. These polymers were proposed to be XyG because alkali treatment abolished detectable crosslinks and endoglucanase treatment reduced observable crosslinks (McCann et al., 1990; Yuan et al., 2001). The identities of the polysaccharide crosslinks that have been visualized are inferred in a subtractive manner by analyzing which treatment abolishes crosslinks. Direct immunological labeling of XyG might aid in determining the identity of the crosslinks between cellulose microfibrils.

Recent findings challenge the tethered network model. The primary walls of many dicot plants including celery, potato, and carrot and of monocot grasses have such a small

amount of xyloglucan that it is not enough to coat cellulose microfibrils in their entirety (Stevens and Selvendran, 1984; Thimm et al., 2002; Zykwiniska et al., 2005). Also, NMR studies have detected limited XyG–cellulose interactions in *Arabidopsis* primary walls (Dick-Perez et al., 2011). Moreover, the *xxt1xxt2* mutant, which lacked detectable XyG, displayed a relatively mild phenotype, suggesting that the load-bearing function might not only be assumed by XyG–cellulose interactions, but also by interactions between other wall polymers and cellulose (Cavalier et al., 2008; Park and Cosgrove, 2012b). In the absence of XyG, pectins and arabinoxylan assume a larger share of the mechanical load of the wall (Zykwiniska et al., 2005; Dick-Perez et al., 2011). Recent studies suggest that up to one-third of the total XyG in the wall of suspension-cultured rose cells is covalently attached to acidic pectins (Thompson and Fry, 2000). This is consistent with the idea that load-bearing function is not exclusively dependent on cellulose–XyG tethering, but also on the covalent binding between pectin and hemicelluloses (Pauly et al., 1999; Thompson and Fry, 2000). Recent experiments using cell wall-degrading enzymes revealed that cell wall loosening requires enzymes with dual specificities to cut both XyG and cellulose (Park and Cosgrove, 2012a). A revised primary cell wall model was proposed in which load-bearing XyG is located in limited regions of tight contact between cellulose microfibrils. This revised model is conceptually different from the tethered network model such that, rather than spanning 20–40 nm of space between adjacent cellulose microfibrils, load-bearing XyG acts as an adhesive layer to connect two adjacent cellulose microfibrils that are bundled together at 'biomechanical hotspots' (Figure 1A).

With ever-improving models of the cell wall matrix interactions, a better understanding of cell wall loosening mechanisms, which allow turgor-driven cell expansion to occur, continues to develop. Potential *in muro* cell wall loosening mechanisms involving expansins, xyloglucan endotransglucosylase/hydrolases, endoglucanases (cellulases), hydroxyl radicals, and pectin modification have been reviewed elsewhere (Cosgrove, 2005; Peaucelle et al., 2012; Wolf and Greiner, 2012). Recently, experiments using sensitivity-enhanced solid-state nuclear magnetic resonance (ssNMR) spectroscopy have provided a putative mode of action for expansins in cell wall loosening (Wang et al., 2013b). In this study, ¹³C- and ¹⁵N-labeled bacterial expansin was shown to selectively bind to cellulose in XyG-enriched areas within ¹³C-labeled cell wall extracts from *Arabidopsis*. The binding of expansins is believed to weaken the interactions between the polysaccharides to induce creep in the cell wall. Interestingly, this model of expansin-mediated loosening is consistent with the recently proposed 'biomechanical hotspot' model of cellulose–XyG interaction (Park and Cosgrove, 2012a; Wang et al., 2013b). A more complete understanding of the biosynthesis of cell wall polymers and the network of interactions between cell wall polymers will continue to cause refinements of the

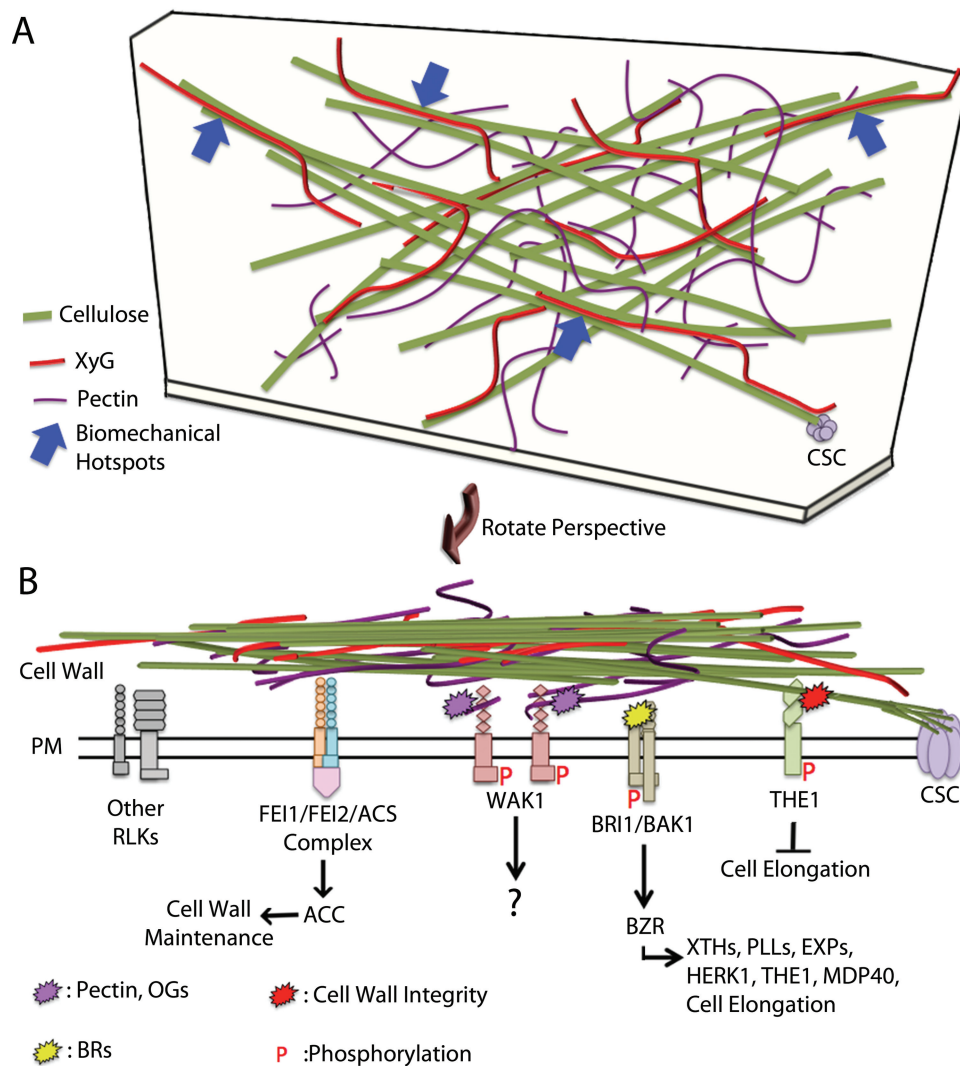


Figure 1. The Schematic Diagram Highlights Some Aspects of the Architecture, Hormonal Regulation, and Integrity Sensing of the Cell Wall.

(A) The polysaccharide composition of primary cell walls is mostly cellulose, xyloglucan, and pectin. Blue arrows highlight biomechanical hotspots in which XyG acts as an adhesive between a short stretch of two adjacent cellulose microfibrils.

(B) An actively synthesizing CSC is displayed as one physical link between the plasma membrane and the cell wall. A few cell wall integrity sensors are also shown. The THE1 RLK represses cell elongation when cellulose biosynthesis is disrupted. WAK1 can bind to pectin and OGs and initiate unknown downstream signals through its kinase domain. FEI1 and FEI2 have been speculated to bind to the ACS complex to aid in ACC synthesis, which is important for cell wall maintenance. Other cell wall integrity sensing RLKs will likely be characterized in the future. Through BRI1/BAK1, BR signaling is shown to initiate cell elongation by transcriptionally activating cell wall-related BZR target genes.

cell wall matrix model and improve our understanding of cell wall expansion mechanisms.

MICROTUBULE ARRANGEMENT AND CELL WALL ARCHITECTURE

The pioneering work of Roelofsen and Houwink (1951, 1953) showed that cellulose microfibrils were laid down in a transverse orientation that is perpendicular to the growth axis at the inner primary cell wall layer of *Tradescantia* stamen hairs, thereby establishing the basis of the multi-net growth hypothesis (MGH). The MGH was later expanded to explain the cell

wall architecture in various cell types (Green, 1960; Roelofsen, 1965). The MGH proposes that new layers of cellulose microfibrils are continuously deposited in a transverse orientation at the inner-most cell wall layer that is adjacent to the PM and that older layers of cellulose microfibrils that are farther from the PM reorient gradually during cell growth. Thereby, a gradient develops in which cellulose microfibrils are transverse in the inner layers, oblique in the intermediate layers, and longitudinal in the outermost layers of the cell wall. The arrangement of cortical microtubules, which lie just beneath the PM, are oriented in parallel to cellulose microfibrils at the inner-most layer of the cell wall in various cell types, which

is consistent with the MGH (Hepler and Newcomb, 1964; Quader et al., 1987; Seagull, 1991; Baskin, 2001). Along with the observation that disruption of the cortical microtubules disorganizes the pattern of cellulose microfibril deposition, a microtubule–microfibril alignment hypothesis was formed to explain the causative relationship between microtubules and cellulose microfibrils, which will be discussed in a later section.

The MGH does not, however, explain the existence of crossed-polylamellate wall patterns where layers of near-transverse microfibrils alternate with layers of near-longitudinal microfibrils. Crossed-polylamellate walls have been observed in filamentous green algae and in plant tissues that provide mechanical support such as phloem fibers, sclerenchyma, tracheids, and the epidermis (Chafe and Doohan, 1972; Chafe and Wardrop, 1972b; Itoh, 1975; Itoh and Shimaji, 1976). However, in contrast to discontinuously alternating transverse and longitudinal orientations that were proposed to occur in crossed-polylamellate walls, Roland et al. (1982) were able to observe multi-angle cellulose microfibrils by using a silver proteinate staining method to label cellulose microfibrils that changed orientation gradually. It was suggested that the intermediate cellulose layers existed within crossed-polylamellate walls that created helical wall patterns. Although the helical wall patterns could be observed using the silver proteinate staining method, they may have been missed in earlier thick EM sections. Arrays of microtubules have been shown to undergo similar rhythmic cycles of gradual rotary reorientation between transverse and longitudinal orientations that are consistent with the gradual changes in cellulose microfibril orientation in each layer of the cell wall (Chan et al., 2007).

In *Arabidopsis* epidermal cells of the root elongation zone, measurements of cellulose microfibril rotation rates suggest that it takes more than 9 h for cellulose microfibrils to rotate from a transverse to a longitudinal orientation (Anderson et al., 2010). In light-grown hypocotyl epidermal cells, the rate of microtubule rotation varied considerably on a cell to cell basis, taking about 200–800 min to rotate through 360°, reflecting different physical characteristics in different regions that may have been influenced by the cell elongation rate (Chan et al., 2007). The biophysical theory that supports the concept of transverse hoop reinforcement suggests that transverse microtubule arrangement would better accommodate rapid cell elongation and that microtubule reorientation from transverse to oblique or longitudinal is more likely to occur when cells slow their growth (Lloyd, 2011). However, transverse microtubules are not always correlated with a rapid growth rate. In most cases, microtubule reorientation follows a decrease of growth rate, suggesting that microtubule reorientation is not a prerequisite for a decrease of growth rate (Chan et al., 2011; Crowell et al., 2011). The independent factor(s) that regulate microtubule

orientation may be sensitive to the growth acceleration/deceleration rather than growth itself. While it remains to be determined how microtubule orientation is regulated, it is evident that the rotation of the cellulose microfibril deposition is dependent on microtubule rotation as both microtubule-stabilizing taxol and microtubule-depolymerizing oryzalin stopped the rotation of the cellulose synthase trajectories (Chan et al., 2007, 2011; Crowell et al., 2011). Importantly, the study of Chan et al. suggests that the dynamics of microtubules drives their own rotation rather than a passive mechanism of rotation that stems from feedback from cell wall self-assembly. Although these findings did not take several factors into account, including the incorporation of other wall polymers, turgor-driven expansion, and other biophysical forces, it is clear that microtubules play a primary role in determining the overall pattern of wall architecture during initial cellulose deposition by cellulose synthase complexes.

Since the growth rate of a given cell is not strictly correlated with the orientation of its microtubules or cellulose microfibrils, a broader approach that investigates the behavior of microtubules and cellulose microfibrils between neighboring cells or within inner tissue layers rather than within a single cell may be more informative. In this regard, it has been postulated that the wall of the inner face of epidermal cells plays a different role than the wall of the outer face of the same cells. Recent studies suggest that microtubules and cellulose synthase trajectories show parallel transverse orientation on the inner face of epidermal cells in hypocotyls, whereas microtubules and cellulose synthase trajectories on the outer face of the same epidermal cells have variable orientations (Chan et al., 2011; Crowell et al., 2011). A model has been established in which the microtubules of internal cells and of the inner face of epidermal cells control the anisotropic growth direction, orienting perpendicular to the growth axis. In contrast, the orientation of microtubules on the outer face of epidermal cells cycle through various orientations without inhibiting growth (Chan, 2012). Supporting this hypothesis, the addition of ethylene changed the direction of growth by affecting the orientation of microtubules and cellulose microfibrils of cortical cells of the inner tissues while the orientation of microtubules and microfibrils of the outer face of epidermal cells was less correlated with the growth effects of ethylene (Lang et al., 1982). As an extension of this model, it is believed that the orientation of microtubules on the external face of epidermal cells tends to promote an acceleration of growth rate when oriented in parallel with the microtubules of the inner face and internal tissues, which suggests growth direction can be uncoupled from growth rate (Chan, 2012). In the future, much can be learned by investigating how cells achieve polarized orientation on the inner versus outer faces of epidermal cells and the influences that hormones, biophysical forces, and cell geometry have on this process.

CELL EXPANSION AND HORMONAL REGULATION

Many hormones control cell expansion, which is accompanied by a change in microtubule organization in many cases. Early studies using pea stem and etiolated pea internodes suggest that ethylene induces radial cell expansion primarily through an effect on the reorientation of cellulose microfibrils from a transverse to a longitudinal orientation (Steen and Chadwick, 1981; Lang et al., 1982). Microtubules were also observed to reorient upon ethylene treatment and treatment with the microtubule-depolymerizing drug, colchicine, reversed the effects of ethylene treatment (Steen and Chadwick, 1981). A similar reorientation of microtubules to a predominantly longitudinal orientation was observed upon treatment with abscisic acid and cytokinins (Sakiyama and Shibaoka, 1990; Shibaoka, 1994). The effect of auxin, gibberellins (GA), and brassinosteroids (BRs) on the elongation of hypocotyls and on the orientation of microtubules appear to be opposite to that of ethylene (Shibaoka, 1993; Fujino et al., 1995; Le et al., 2005; Polko et al., 2012; Wang et al., 2012). It remains unclear whether hormone-induced microtubule reorientation is always accompanied by reorientation of cellulose microfibrils. At least in short-term hormone treatments (within 2 h), induced cell elongation might not involve concomitant reorientation of microtubules and cellulose microfibrils (Shibaoka, 1994). For example, microtubule-disrupting agents such as colchicine or ethyl N-phenylcarbamate do not inhibit IAA-induced cell elongation in azuki bean epicotyls (Shibaoka, 1972; Shibaoka and Hogetsu, 1977). Moreover, treatment with the cellulose synthesis inhibitor, 2,6-dichlorobenzonitrile, does not inhibit auxin-induced cell elongation in maize coleoptiles (Hogetsu et al., 1974b; Edelman et al., 1989). However, GA-induced microtubule reorientation is reversed by microtubule-disrupting agents and by inhibitors of cellulose synthesis, indicating that hormone-induced microtubule reorientation involves different and complex mechanisms (Shibaoka, 1972; Hogetsu et al., 1974a; Shibaoka and Hogetsu, 1977).

Progress has been made in understanding how BRs control cell expansion, particularly in hypocotyl cell elongation. BRs act through the PM receptor-like kinase (BRI1) to activate members of the BRASSINAZOLE-RESISTANT (BZR) family of transcription factors (Sun et al., 2010). BR-deficient/insensitive mutants affect hypocotyl cell elongation. For example, the BR biosynthesis-deficient mutant *det2* and BR receptor mutant *bri1* have shorter dark-grown hypocotyls whereas the dominant BZR1 mutant *bzr1-1D* has longer dark-grown hypocotyls (Chory et al., 1991; Li et al., 1996; Wang et al., 2002, 2012). The observation that *bri1* mutants had reduced expression of xyloglucan endotransglucosylase/hydrolase (XTH) 22 and 24 raised the hypothesis that BR might control cell expansion by changing the mechanical properties of cell walls (Kauschmann et al., 1996). Consistently with this hypothesis, brassinolide-treated soybean epicotyl sections showed increased cell wall extensibility (Zurek and Clouse, 1994). Recent studies

utilizing global expression profiling showed that BRs induce the expression of seven *XTHs*, two pectin lyase-like (*PLLs*), and seven expansins (*EXPs*) (Guo et al., 2009). Moreover, two BZR1 targets, HERK1 receptor kinase and THESEUS1 (THE1) receptor kinase, are required for BR-induced expression of one *XTH* and five *EXPs* (Guo et al., 2009). Some evidence also suggests that BR signaling affects microtubule organization. Dark-grown hypocotyls treated with exogenous brassinolide showed reorientation of microtubules in *det2* mutants but not in *bri1* mutants, indicating that reorientation of microtubules from longitudinal to transverse orientation is regulated by BR signaling (Wang et al., 2012). This study also identified microtubule-destabilizing protein 40 (MDP40) as a BZR1 target and as a positive regulator in BR-mediated hypocotyl cell elongation. However, overexpression of MDP40 only partially rescued the *det2* hypocotyl phenotype. Therefore, it remains to be determined whether MDP40 mediates cortical microtubule orientation by destabilizing microtubules and whether reorientation of microtubules through BR-mediated signaling has any effect on cellulose microfibril organization. Interestingly, although hypocotyl elongation is dramatically affected in *det2* and *bri1* hypocotyls, cellulose content in *det2* and *bri1* mutants was only reduced by about 3%–5% (Xie et al., 2011). Thus, it is unlikely that the defect in cell elongation is attributed entirely to deficient cellulose biosynthesis. Recently, *bri1* was identified to be a suppressor of a pectin methylesterase inhibitor overexpression (PMElox) line in a forward genetics screen. PMElox presumably compromises cell wall integrity, sensing signaling more than it affects cell wall mechanics by altering the pectin methylesterification patterns. This study suggests that a feedback mechanism exists in which BR balances cell wall homeostasis (Wolf et al., 2012b) (Figure 1B).

CELL WALL INTEGRITY SENSING

During cell expansion, a delicate balance between cell wall biosynthesis and cell wall remodeling needs to be maintained such that cell walls are loosened enough to allow turgor-driven cell wall extension to occur but not so much that the integrity of the cell is jeopardized. In principle, a sensing mechanism is needed that allows cells to monitor functional and structural alterations in the cell wall and translate them into cellular responses. In yeast, a cell wall integrity (CWI) sensing mechanism has been characterized in great detail. The CWI signaling pathway is initiated at the PM by the cell surface sensors WSC, MID, and MTL, and transduced to the downstream targets through a Rho GTPase and a MAP kinase cascade (Levin, 2005). Plants do not have obvious homologs of yeast cell surface sensors such as WSC, MID, and MTL, but emerging evidence suggests that plants do have signaling pathways that lie between CWI and growth control (Xu et al., 2008; Guo et al., 2009; Tsang et al., 2011). Potential cell surface sensors in plants include several members of the receptor-like

Ser/Thr protein-kinase (RLK) family. Wall-associated kinases (WAKs) are the only known RLKs that directly bind to polysaccharides in the wall. WAKs are composed of an extracellular epidermal growth factor-like repeat domain, a transmembrane region, and a cytosolic Ser/Thr kinase domain. The extracellular domain of WAK1 binds to calcium crosslinked pectin-derived oligogalacturonides (OGs) *in vitro* (Decreux and Messiaen, 2005; Decreux et al., 2006). Experiments using a chimera between WAK1 and EFR, a receptor for recognition of the microbe-associated molecular pattern EF-Tu, demonstrated that WAK1 is a receptor of OGs *in vivo* (Brutus et al., 2010). The perception of OGs through WAK1, together with genetic evidence that WAKs are involved in cell elongation, stress tolerance, and pathogen resistance, indicates that WAK1 is a potential sensor for cell wall signaling (Lally et al., 2001; Wagner and Kohorn, 2001; Diener and Ausubel, 2005; Kohorn et al., 2006; Li et al., 2009) (Figure 1B).

In addition to WAKs, several members of the RLKs have been implicated in CWI sensing. A mutation in a PM RLK of the CrRLK1 (*Catharanthus roseus* protein-kinase-1-like) family member, *thesus1* (*the1*), was identified in a suppressor screen of *cesa6^{prc1}* that suppressed a set of growth phenotypes, including hypocotyl growth inhibition, ectopic lignin accumulation, and altered transcript levels for a number of genes without recovering the cellulose content deficiency of *cesa6^{prc1}* (Hematy et al., 2007). Moreover, *the1* suppressed hypocotyl growth inhibition in other cellulose-deficient mutants including *cesa3^{eli1}*, *cesa1^{rsu1}*, and *pompom1*, suggesting that the perturbation of cellulose synthesis leads to activation of THE1. It has been proposed that THE1 is part of a surveillance system in growing cells and that THE1 is activated upon cell wall perturbation and in turn results in the activation of downstream target genes that control cell elongation and defense against pathogens (Hematy et al., 2007; Hematy and Hofte, 2008). The ligand of THE1 and details of the signaling cascade of THE1-mediated integrity sensing remains to be determined (Figure 1B).

FEI1 and FEI2 belong to RLK subfamily XIII, which is distinct from the WAK and THE1 subfamilies. Genetic evidence suggests that FEI1 and FEI2 are required for anisotropic cell expansion in non-permissive conditions such as high sucrose or high salt (Xu et al., 2008). Interestingly, although FEI1 and FEI2 have kinase activity, kinase inactive versions of FEI1 or FEI2 complemented the *fei1fei2* mutant phenotype, which suggests the kinase activity of FEI1 and FEI2 is dispensable. Distinct from *the1*, *fei1fei2* exhibits cellulose biosynthesis deficiencies under non-permissive conditions and *feifei2^{prc}* and *fei1fei2cob* triple mutants display enhanced root phenotypes, suggesting that FEI1 and FEI2 act in a pathway that is distinct from cell wall surveillance sensing. Although FEI1 and FEI2 interact with ACS5, an enzyme responsible for the synthesis of the ethylene precursor, ACC, ethylene production in *fei1fei2* is not drastically different from that of wild-type. Interestingly, the *fei1fei2* root swelling and cellulose deficiency phenotypes can be reverted by blocking ethylene

biosynthesis using either α -aminoisobutyric acid (AIB) or aminooxyacetic acid (AOA) but not by blocking ethylene perception. This evidence provides a model in which FEI1 and FEI2 function as a scaffold to localize and assemble the ACS complex which in turn generates a localized ACC signal to regulate cell wall biosynthesis (Xu et al., 2008) (Figure 1B). If this model can be supported, it may represent an unconventional ACC-dependent mechanism that acts independently from ethylene signaling. To test this cell wall-sensing mechanism, Tsang et al. (2011) induced cell wall perturbations by short-term treatments with the cellulose synthesis inhibitor, isoxaben, or the cellulose-binding dye, Congo red, in combination with treatments with ACC biosynthesis inhibitors or ethylene perception inhibitors and compared the effects on cell elongation. While isoxaben treatment caused a reduction in cell elongation in control seedlings, in seedlings treated with silver ions to block ethylene perception, and in the ethylene-insensitive mutant, *ein3eil1*, isoxaben treatment did not significantly change cell elongation in roots treated with the ACC biosynthesis inhibitor aminoethoxyvinylglycine (AVG) (Tsang et al., 2011). Although it could be argued that some ACC biosynthesis inhibitors are promiscuous, these data are consistent with the work of Xu et al. and suggest that isoxaben-induced inhibition is ACC-dependent but not dependent on ethylene perception and that CWI sensing involves a novel non-canonical signaling function of ACC. Interestingly, the isoxaben-induced acute growth response does not require FEI1, FEI2, and THE1.

Other RLKs involved in cell wall function include Pro-rich extension-like receptor kinase (PERK) 4, STRUBBELIG (SUB) and SUB RECEPTOR FAMILY (SRF) LRR-RLKs, ERECTA (ER) and ERECTA-LIKE (ERL) LRR-RLKs, and Leguminous L-type lectin RLKs (Haffani et al., 2006; Eyuboglu et al., 2007; Bai et al., 2009; Bouwmeester and Govers, 2009; Sanchez-Rodriguez et al., 2009) (Figure 1B). However, the current studies are in their infancy and it is too early to assign specific signaling roles to those RLKs. It is anticipated that many aspects of CWI sensing will continue to emerge including identification of elusive signals, kinase cascades, and downstream targets. It is important to note that signaling pathways are often complex and converge with many other signaling pathways and biological functions. Therefore, the effects of any particular signaling pathway on cellulose biosynthesis are not necessarily a direct effect of the stimulus of study unless the pathway is exhaustively studied.

CELLULOSE, HEMICELLULOSE, AND PECTIN SYNTHESIS AND TRAFFICKING

Intracellular trafficking of cell wall material and cell wall-modifying or -biosynthesizing machinery to and from the PM is critical to the synthesis, dynamicity, and architecture of the extracellular cell wall. Hemicellulose and pectin polysaccharides are synthesized in the lumen of the Golgi before being secreted to the apoplast (Cosgrove, 2005; Driouich et al.,

2012; Worden et al., 2012) (Figure 2). The position of non-cellulosic polymer exocytosis in relation to the sites of cellulose biosynthesizing CSCs has been proposed to influence the physical properties of the cell wall and the efficiency of cell expansion (Fujita et al., 2012). Internalization of hemicellulose and pectin back into the cell may also be an important process in cell wall remodeling (Baluska et al., 2002; Samaj et al., 2004; Baluska et al., 2005). Since cellulose biosynthesis is thought to occur exclusively at the PM, control of the abundance of CSCs at the PM through exocytosis and endocytosis likely acts as a mechanism by which cellulose incorporation in the cell wall is modulated. Recently, the development of new technologies including a system in which CESAs can be visualized in living cells, methods for labeling specific cell wall polysaccharides for fluorescence and immunogold imaging, and large-scale proteomic and oligosaccharide analyses on isolated plant fractions has great potential in detailing the trafficking routes and mechanisms of cell wall polysaccharides and cell wall-biosynthesizing and -modifying enzymes.

The importance of proper trafficking in the maintenance of CWI and proper cell expansion has been exhibited through genetic studies in which trafficking is disturbed. Disruption of the TGN/EE-localized H⁺-ATPase, VHA-a1, causes a disruption in both endocytic and secretory trafficking, which leads to a cell elongation defect and cellulose deficiency (Dettmer et al., 2006; Brux et al., 2008). A mutation in ECHIDNA, a TGN-localized protein that is critical for post-Golgi trafficking, causes intracellular accumulation of XyG and pectin and a consequential decrease of these polysaccharides in the cell wall (Gendreau et al., 2013). As a result, the *echidna* mutant displays a severe dwarfism phenotype and a cell elongation defect that stems from deficiencies in cell wall polysaccharides (Gendreau et al., 2011, 2013). A mutation in *ap1m2/HAP13*, a subunit of an adaptor protein complex (AP1) that is

involved in clathrin-mediated trafficking away from the TGN, causes a cell elongation and a dwarf phenotype that is similar to that of the *echidna* mutants (Park et al., 2013; Teh et al., 2013; Wang et al., 2013a). Although the cell wall composition of *ap1m2/HAP13* mutants has not been investigated, it is possible that these phenotypes stem from inadequate secretion of cell wall polysaccharides. It is important to note that, in mutants with widespread defects in trafficking, a combination of many factors may be at play to contribute to the cell wall defects that are observed including but not limited to direct effects on the trafficking of cell wall polymers and cell wall biosynthesizing and modifying enzymes, changes in cell wall pH, disruption of hormone or signaling pathways that affect the cell wall, and other pleiotropic effects on plant cell health.

Live cell imaging of fluorescent protein (FP)-labeled CESAs using spinning disk confocal microscopy has been influential in the analysis of CESA localization and behavior as well as the association of CESA with other proteins—capabilities that may aid in detailing CESA trafficking routes and mechanisms (Paredes et al., 2006; Crowell et al., 2009; Gutierrez et al., 2009; Bringmann et al., 2012; Li et al., 2012a; Bashline et al., 2013; Sampathkumar et al., 2013). In agreement with the localization of rosette structures in transmission electron micrographs of freeze fracture replicas (Haigler and Brown, 1986), FP-CESAs localize to intracellular Golgi bodies and to distinct foci at the PM that travel laterally in the plane of the membrane at constant velocities along linear trajectories (Paredes et al., 2006). Microtubule-associated cellulose synthase compartments (MASCs) and small CESA-containing compartments (SmaCCs) have also been described, and have been speculated to represent vesicles containing CESAs that are being delivered to and/or internalized from the PM (Crowell et al., 2009; Gutierrez et al., 2009) (Figure 2). Cortical SmaCCs/

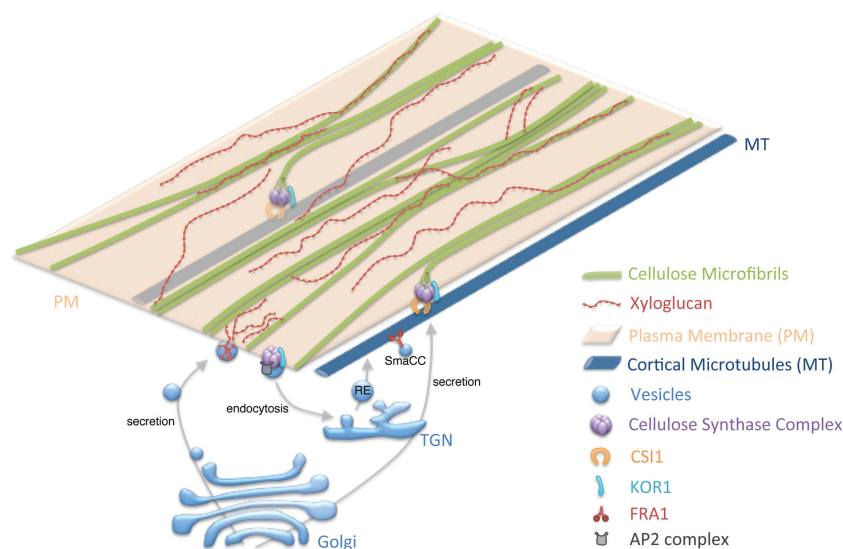


Figure 2. A Schematic Representation Shows Some of the Important Trafficking Pathways that Are Relevant to the Cell Wall and Some of the Key Components that Are Important for Cell Wall Synthesis and Organization.

MASCs display episodes of static behavior interrupted by episodes of rapid dynamics that can be attributed to the tracking of depolymerizing microtubule ends, and a sub-cortical population of SmaCCs/MASCs has also been described that moves in an actin-dependent manner (Sampathkumar et al., 2013). The distribution of FP-CESAs to SmaCCs/MASCs can be induced by various conditions including osmotic stress and treatment with cellulose biosynthesis inhibitors, but a few SmaCCs/MASCs are also detectable without treatment. The dense population of PM-localized CESAs in native conditions may hinder the detection of SmaCCs/MASCs. Care should be taken in the interpretation of the role of SmaCCs/MASCs, especially when inferring the function of all SmaCCs/MASCs based on the behavior of SmaCCs/MASCs that have been induced by artificial treatments. FP-CESAs have also been documented to co-localize with markers of the *trans*-Golgi Network/Early Endosome (TGN/EE) (Crowell et al., 2009). It is unclear whether TGN/EE-localized FP-CESAs are in the secretion or endocytic pathway because the TGN/EE has been shown to be a single compartment that is part of both the secretion and endocytic pathways in *Arabidopsis* (Dettmer et al., 2006; Viotti et al., 2010). Although CESAs have been visualized in several compartments, much ambiguity remains in the function of several compartments and in understanding the mechanisms by which CESAs are allocated to different locations. Recently, a study by our group unveiled one mechanism by which CESA proteins are endocytosed (Bashline et al., 2013) (Figure 2). In this study, the medium subunit of the clathrin-mediated endocytosis (CME) adaptor protein (AP2) complex, μ 2 (AP2M), was characterized as a significant player in CME and in the internalization of CESA proteins from the PM. Upon endocytosis, CESAs are likely trafficked to the TGN/EE, but the dual function of the TGN/EE in the secretion and endocytic pathways complicates the analysis of whether CESAs are subsequently recycled back to the PM or destined for degradation. The sheer magnitude of the amount of protein dedicated to the formation of a single CESA complex would lend support to the idea that CSCs are recycled from the TGN/EE back to the PM. The mechanism by which CESAs are trafficked from the TGN/EE remains unsolved. A μ 2 homolog, AP1M2/HAP13, has recently been implicated in trafficking away from the TGN (Park et al., 2013; Teh et al., 2013; Wang et al., 2013a). It remains to be investigated whether AP1M2 plays a role in clathrin-mediated secretion of CESAs. In addition, the dissection of the molecular components that comprise the SmaCC/MASC compartment may lead to the identification of new secretion or endocytosis mechanisms for CESAs or to the establishment of SmaCCs/MASCs as intermediate vesicles between the TGN/EE and the PM.

The importance of the cytoskeleton in CSC trafficking has been addressed by several studies (Crowell et al., 2009; Gutierrez et al., 2009; Sampathkumar et al., 2013). Actin has been shown to be important for the cell-wide distribution of CESA-containing Golgi bodies through its role in cytoplasmic streaming (Crowell et al., 2009; Gutierrez et al., 2009).

Pharmacological loss of actin leads to the aggregation of immobile CESA-containing Golgi bodies and a distribution pattern of PM-localized CSCs that is limited to an area that is in close proximity to the immobile Golgi body aggregates (Crowell et al., 2009; Gutierrez et al., 2009). Genetic disruption of actin has also been shown to reduce the delivery rate of CSCs to the PM and to increase the lifetime of CSCs at the PM, suggesting that actin might play a role in the exocytosis and endocytosis of CSCs (Sampathkumar et al., 2013). In addition to the important role of cortical microtubules in guiding PM-localized CSCs motility during cellulose biosynthesis (Paredes et al., 2006; Gu et al., 2010; Fujita et al., 2011; Li et al., 2012a), cortical microtubules have also been shown to mark the sites of CSC insertion at the PM (Crowell et al., 2009; Gutierrez et al., 2009) (Figure 2). Pharmacological disruption of cortical microtubules did not affect the delivery rate of CSCs to the PM (Gutierrez et al., 2009). Although cortical microtubules are not necessary for the delivery of CSCs, cortical microtubules play a role in organizing the insertion of CSCs. If the cortical microtubules similarly organize the sites of hemicellulose and pectin exocytosis, the cortical microtubules might act in targeting all of the nascent cell wall material to a common location to encourage the incorporation of all of the polysaccharide cell wall components into the nascent cell wall matrix at the point of cellulose crystallization. The incorporation of non-cellulosic polysaccharides into a crystallizing cellulose microfibril has been hypothesized to reduce the cellulose crystallinity, and may be dependent upon the microtubule-assisted organization of nascent cell wall polymers (Fujita et al., 2012). In support of this hypothesis, temperature-sensitive *microtubule organization 1* (*mor1*) mutants, which display cell expansion defects that stem from reduced cortical microtubule density and dynamics synthesize cellulose of higher crystallinity when at the restrictive temperature (Whittington et al., 2001; Kawamura and Wasteneys, 2008; Fujita et al., 2011, 2012). The decreased density of cortical microtubules in *mor1* leads to an increased number of non-microtubule-associated CSCs, which might consequently lead to higher cellulose crystallinity due to the inefficient targeting of non-cellulosic polysaccharides to the sites of cellulose biosynthesis and crystallization (Fujita et al., 2012). While this hypothesis represents just one putative explanation for the observations of increased cellulose crystallinity of the *mor1* mutants, it shows the many ways in which trafficking can influence characteristics of the cell wall and cell expansion.

The development of new technologies might help to better analyze the influence of trafficking on hemicellulose and pectin biosynthesis and delivery. The localization of hemicellulose and pectin in plant Golgi compartments was first discovered using biochemical staining techniques (Pickett-Heaps, 1968), and later the role of the Golgi in the synthesis of these polysaccharides was implied from the co-fractionation of polymers containing radiolabeled sugars with Golgi fractions (Harris and Northcote, 1971). Many antibodies have been

developed that recognize various polysaccharide epitopes (Pattathil et al., 2010), which can be helpful in dissecting the sequential maturation of hemicellulose and pectin polymers with sub-compartment resolution within Golgi bodies using immunogold electron microscopy (Lynch and Staehelin, 1992; Zhang and Staehelin, 1992). The progressive maturation of polymer synthesis might be interesting to observe in conjunction with immunogold localization of specific glycosyltransferases (GTs) involved in hemicellulose or pectin biosynthesis (Chevalier et al., 2010; Driouich et al., 2012). The model of progressive maturation of cell wall polysaccharide biosynthesis raises interesting questions relating to trafficking. For instance, how are the GTs that are responsible for particular stages in polysaccharide biosynthesis segregated to and subsequently maintained in different Golgi stacks? Expanding immunological imaging techniques to the analysis of post-Golgi secretory vesicles (Toyooka et al., 2009) might be useful in detailing whether hemicellulose and pectin share secretory vesicles with one another, with CESAs, and/or with cell wall modifying enzymes such as XyG endotransglucosylases (XETs), expansins, or endoglucanases. More recently, several new approaches to investigating hemicellulose and pectin have been developed that may also be suitable for using to investigate the post-Golgi trafficking of cell wall polysaccharides. An oligosaccharide mass profiling (OLIMP) technique was successful in analyzing intricate differences in the XyG structure of polymers from Golgi fractions as compared to XyG in the cell wall (Obel et al., 2009). Immuno-isolation and proteomic analysis of trafficking compartments have also been useful in identifying the protein composition of specific compartments, such as SYP61-containing Golgi compartments (Drakakaki et al., 2012). By combining these new technologies, it may be possible to perform proteomic and OLIMP analyses of immuno-isolated compartments concomitantly. If possible, this approach would be helpful not only in investigating the GTs that are involved in hemicellulose and pectin biosynthesis, but also in identifying factors that are involved in the sorting of GTs within Golgi stacks and targeting the trafficking of cell wall material and machinery to sites at the PM. A revolutionary method that uses click chemistry to attach small molecule probes to polysaccharides has provided the ability to fluorescently label and visualize pectin arabinogalactans within living cells and cell walls (Anderson and Wallace, 2012). This technique, which might be expanded to incorporate the analysis of other polysaccharides, can be used to analyze the post-Golgi trafficking of wall components in real time, to detect the specific sites of polysaccharide delivery to the cell wall, and to measure the dynamics of the polysaccharides *in muro*. Several studies have shown that CSCs are selectively delivered to sites that coincide with cortical microtubules and that cellulose biosynthesis occurs along microtubules (Paredes et al., 2006; Crowell et al., 2009; Gutierrez et al., 2009; Li et al., 2012a). It would be intriguing to analyze whether cell wall polysaccharides are selectively delivered to sites that coincide with

microtubules and/or CSCs that are actively synthesizing cellulose at the PM.

KEY PLAYERS FOR CELLULOSE SYNTHESIS AND MICROFIBRIL ORIENTATION

The composition and stoichiometry of the cellulose biosynthesizing CSCs has been of much interest. Early electron microscopy studies of freeze-fractured membranes identified that the transmembrane portion of the CSC forms a rosette ring structure that is composed of six particles with an outside diameter of about 25 nm (Mueller and Brown, 1980; Herth, 1983; Haigler and Brown, 1986). The most widely cited model of CSC composition postulates that 36 CESA enzymes comprise each six-lobed rosette with each lobe comprising a hexamer of CESAs (Brown et al., 1996; Delmer, 1999; Scheible et al., 2001; Somerville, 2006). If all 36 CESA subunits of the 36mer model are actively synthesizing cellulose, each cellulose elementary fiber would contain 36 glucan chains. Recently, several studies have measured the diameter of cellulose elementary fibers in either secondary or primary cell walls to be approximately 3 nm and have suggested that the fibers are more likely to contain 24 or 18 glucan chains (Fernandes et al., 2011; Thomas et al., 2013; Zhang et al., 2013). Genetic evidence regarding the CSC in *Arabidopsis* suggests that CESA1, CESA3, and CESA6-like (CESA6, 2, 5, and 9) isoforms are responsible for cellulose biosynthesis of the primary cell wall while CESA4, CESA7, and CESA8 are required for cellulose synthesis in the secondary cell wall (Taylor et al., 2000, 2003; Desprez et al., 2007; Persson et al., 2007). In addition to CESA, many other putative components of the CSC have been analyzed. Although genetic screens have identified many mutants that cause cell wall defects and therefore many proteins that may be involved in cell wall biosynthesis, we will limit our discussion to two proteins of recent and significant interest: cellulose synthase interactive 1 (CSI1) and KORRIGAN (KOR1) (Figure 2).

CSI1 has been revealed as a molecular linker between the CSC and cortical microtubules, thereby providing a mechanism for the co-alignment of cellulose microfibrils and cortical microtubules (Gu and Somerville, 2010; Gu et al., 2010; Bringmann et al., 2012; Li et al., 2012a). Fluorescent protein-labeled CSI1 particles co-localize with and share similar dynamics with labeled CESA3 and CESA6 particles at the PM (Bringmann et al., 2012; Li et al., 2012b). These observations, together with split-ubiquitin yeast two-hybrid data that confirmed the interaction between full-length CESAs (CESA1, CESA3, and CESA6) and full-length CSI1, suggest that CSI1 closely associates with CSCs. CSI1 was also shown to bind to microtubules *in vitro* with a disassociation constant of about 1 μ M, which is comparable to that of typical microtubule-associated proteins (MAPs) (Li et al., 2012a; Mei et al., 2012). The connection between CESAs, CSI1, and microtubules was further indicated by the disruption of the alignment between

CSCs and microtubules in *csi1*-null mutants, which suggests that CSI1 is indispensable for the co-alignment between CSCs and microtubules. Also, pharmacological removal of either CSCs by cellulose synthesis inhibitors or microtubules by microtubule-depolymerizing drugs causes a depletion of CSI1 particles (Li et al., 2012b). Importantly, CSI1 does not co-localize with Golgi-associated CSCs, which indicates that the role of CSI1 is restricted to PM-localized CSCs, where CSI1 regulates the synthesis of cellulose microfibrils under the guidance of cortical microtubules. Recently, a homolog of CSI1, CSI3, has been shown to exhibit many CSI1-like characteristics including interaction with CESAs, co-localization with active CSCs, and co-localization with cortical microtubules. Interestingly, although *csi1* mutants are short and swollen due to cellulose synthesis disruption, *csi3* mutants do not exhibit cellulose synthesis defects or a morphological phenotype. However, all cellulose biosynthesis-related phenotypes that are associated with *csi1* are exacerbated in the *csi1csi3* double mutant, suggesting that CSI3 plays a distinct role in cellulose biosynthesis that is dependent on the function of CSI1 (Lei et al., 2013).

Another protein that might fit the criteria of a CSC component is KOR1, which has been characterized as a type II integral membrane endo- β -1,4-glucanase. Transcription of KOR1 is co-regulated with primary and secondary cell wall CESAs, and mutations in KOR1 result in cellulose deficiency in both primary and secondary cell walls (Nicol et al., 1998; Zuo et al., 2000; Szyjanowicz et al., 2004). KOR1 has been speculated to co-localize with CSCs at the PM (Crowell et al., 2010); it may be tempting to speculate that KOR1 is an integral part of the CSC. However, co-immunoprecipitation experiments with either primary or secondary CESAs have failed to indicate a stable interaction between KOR1 and CESAs (CESA3, 6, 7, 8) in detergent-solubilized extracts (Szyjanowicz et al., 2004; Desprez et al., 2007). Contrary to these observations, KOR1 has been alluded to interact with CESAs in both a yeast two-hybrid assay and in a bimolecular fluorescence complementation (BiFC) experiment (Crowell et al., 2010). Nevertheless, the importance of KOR1 in the regulation of cellulose synthesis is indisputable. While further characterization of KOR1 is expected to shed light on the exact role of KOR1, three functions have been proposed: (1) a proofreading function of removing disordered glucan chains; (2) generation of short primers during the initial step of cellulose polymerization; and (3) termination of cellulose synthesis (Molhoj et al., 2001, 2002; Peng et al., 2002).

It remains to be determined how the loss of CSC and microtubule co-alignment affects the orientation of newly synthesized cellulose microfibrils. Several mutants show a disruption of cellulose microfibril orientation. A recent phenotypic analysis of the *csi1* mutant suggests that the uncoupling of cortical microtubule and cellulose microfibril orientations leads to a helical microfibril arrangement that causes torsion and abnormal phyllotaxis (Landrein et al., 2013). Field emission

scanning electron micrographs (FE-SEM) of cellulose microfibrils in the primary wall of petiole mesophyll cells in the KOR1 mutant, *acw1*, at the restrictive temperature showed mis-oriented cellulose bundles, indicating that KOR1 is important for cellulose microfibril orientation (Sato et al., 2001). FE-SEM analysis of cellulose microfibrils in secondary walls of mature fibers in *fragile fiber 1* (*fra1*) and *fragile fiber 2* (*fra2*) mutants showed aberrant deposition of cellulose microfibrils (Burk and Ye, 2002; Zhong et al., 2002). FRA2 encodes a homolog of katanin p60, which uses ATP hydrolysis to sever microtubules. As a result of the loss of microtubule severing activity, *fra2* leads to a loss of transverse microtubule arrays, which may be the cause of the aberrant deposition of cellulose microfibrils. FRA1 encodes a kinesin-like protein, which has been shown to be an authentic microtubule motor protein that moves as a dimer towards the plus-end microtubules (Zhu and Dixit, 2011). Interestingly, the *fra1* mutant appears to have normal cortical microtubules, which suggests that FRA1 plays a role in mediating cellulose microfibril orientation in a manner that is independent of the microtubule-microfibril alignment. It is important to note that a strong alkali (4N KOH) extraction was often applied before the FE-SEM imaging. Therefore, the cellulose structure observed in FE-SEM may not reflect the native cell wall architecture. Recently, atomic force microscopy (AFM) has been developed as a method to visualize the inner surface of cell walls, which allows the visualization of cellulose microfibrils with minimal disturbance and in near native conditions (Zhang et al., 2013). It is expected that AFM analysis will become a useful tool for analyzing cell wall material in the future characterization of cell wall altering mutants.

CONCLUSIONS

The cell walls of plants are unmistakably complex in many aspects (Figure 2). The structure of the cell wall matrix, with many polysaccharide and protein components linked in a convoluted meshwork, is certainly intricate. Even more confounding is the synthesis, trafficking, and assembly of all of the cell wall components into a dynamic, adjustable, yet rigid structure that dictates cell expansion. Additional levels of complexity exist in the active organization of cell wall components by intracellular components such as the microtubules as well as in the responsiveness of the cell to the integrity of the cell wall and the hormonal regulation of cell wall synthesis and modification. Encouragingly, advancement in many of these areas has been discussed here as well as several innovative tools that may lead to more progress in the understanding of plant cell walls.

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