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DARWIN REVIEW

Open questions in plant cell wall synthesis

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Abstract

Plant cells are surrounded by strong yet flexible polysaccharide-based cell walls that support cells while also allowing growth by cell expansion. Plant cell wall research has advanced tremendously in recent years. Sequenced genomes of model and crop plants have facilitated cataloguing and characterization of many enzymes involved in cell wall synthesis. Structural information has been generated for several important cell wall-synthesizing enzymes. Important tools have been developed including antibodies raised against a variety of cell wall polysaccharides and glycoproteins, collections of enzyme clones and synthetic glycan arrays for characterizing enzymes, herbicides that specifically affect cell wall synthesis, live-cell imaging probes to track cell wall synthesis, and an inducible secondary cell wall synthesis system. Despite these advances, and often because of the new information they provide, many open questions about plant cell wall polysaccharide synthesis persist. This article highlights some of the key questions that remain open, reviews the data supporting different hypotheses that address these questions, and discusses technological developments that may answer these questions in the future.

Keywords: Cellulose, cellulose synthase complex, cell wall biosynthesis, glycosyltransferase, hemicelluloses, mixed-linkage glucan, pectins, plant cell wall, polysaccharide, secretion.

Introduction

Plant cells are surrounded by plant cell walls, a strong yet flexible polysaccharide-based extracellular matrix that supports plant cells and defines their shapes. Plant cell walls are composed of a hydrated network of polysaccharides, including cellulose, hemicelluloses, and pectins. Cell walls also contain proteins (San Clemente *et al.*, 2022) and other molecules such as ions and phenolic compounds. Cellulose is made of β-(1,4)-linked glucose chains that coalesce to form microfibrils. Hemicelluloses and pectins are heterogeneous classes of polysaccharides, collectively called matrix polysaccharides. Hemicelluloses have a backbone of neutral sugars (e.g. glucose, xylose, and mannose), while pectin backbones include acidic sugars (particularly GalA). Both pectin and hemicellulose backbones can carry side chains of other sugars and are subject to acetylation, and pectins can be additionally modified by methylation (Fig. 1) (Anderson and Kieber, 2020). Although cell wall composition can vary between species (Popper *et al.*, 2011), cell types, developmental stages, or even subregions of the cell wall (Dauphin *et al.*, 2022), cell walls are often classified as primary or secondary. Primary cell walls are deposited before or during growth and are relatively flexible to allow turgor-driven plant cell expansion to facilitate plant growth. The primary cell wall of land plants, including the model plant *Arabidopsis thaliana*, is composed of cellulose, hemicellulose [mostly xyloglucan (XyG)], and pectins [homogalacturonan (HG), rhamnogalacturonan-I (RG-I), and RG-II] (Anderson and Kieber, 2020). Secondary cell walls are deposited

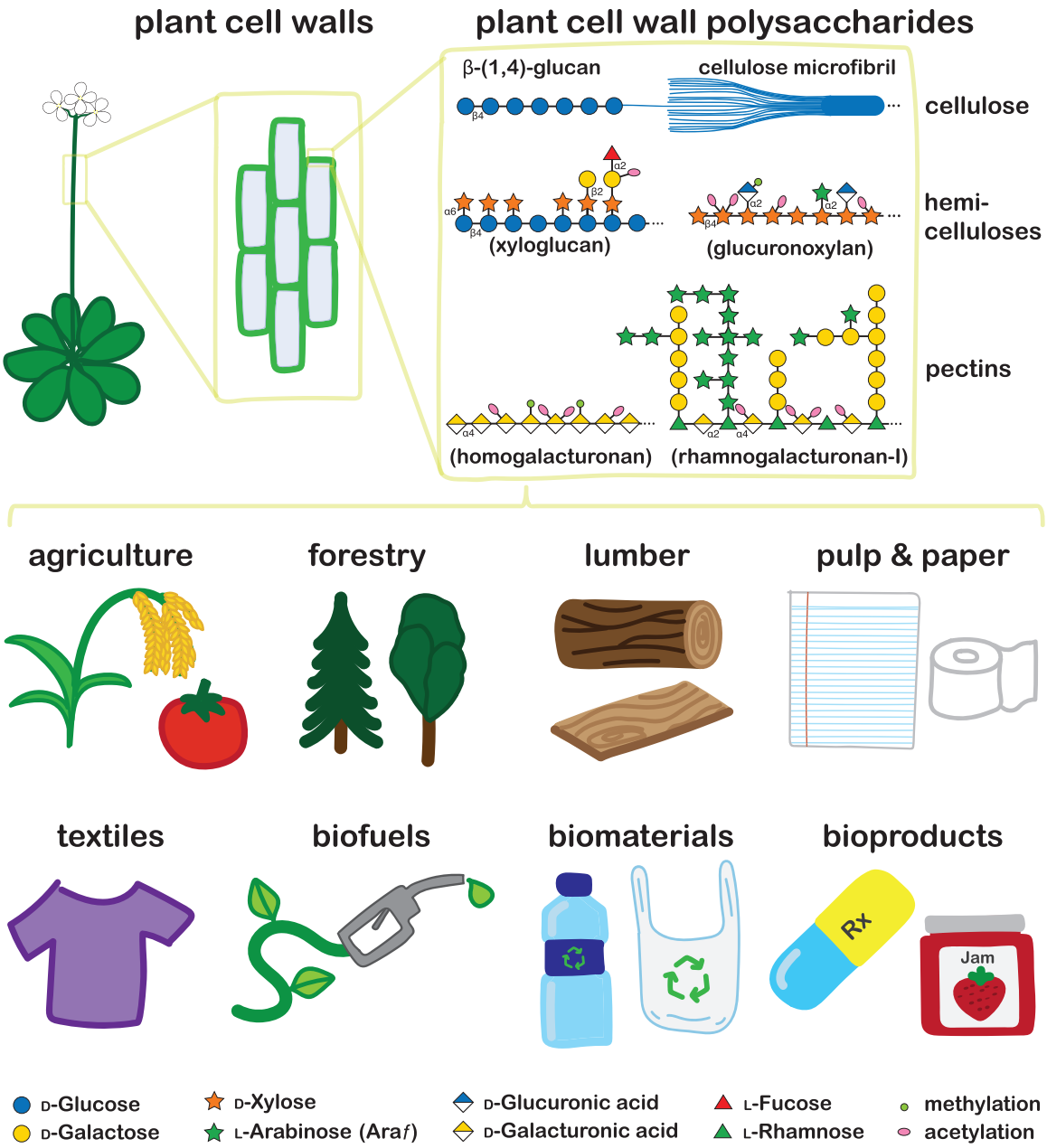


Fig. 1. Polysaccharides of the plant cell wall and uses of plant cell walls. Plant cells are surrounded by strong yet flexible polysaccharide-based plant cell walls. In the model plant, *Arabidopsis thaliana*, the primary cell wall is composed of cellulose, hemicelluloses [mostly xyloglucan (XyG) in primary cell walls and xylans in secondary cell walls], and pectins [e.g. homogalacturonan (HG) and rhamnogalacturonan-I (RG-I)]. Hemicellulose structures are after Pauly *et al.* (2013) and pectin structures are after Ropartz and Ralet (2020); the degree of acetylation and methylesterification of HG can vary and RG-I side chains are examples only since there is considerable diversity in these side chains. Plant cell walls are essential for plant growth and development, making them critical to both agriculture and forestry. Plant cell wall properties are particularly important for fruit ripening and for dietary fibre in food (Burton and Fincher, 2014). Plant cell walls make up wood; cellulose-based pulp and paper products; plant-based textiles, such as cotton, linen, rayon, and cupro; and cell wall material can provide feedstock for biofuels (Pauly and Keegstra, 2010). Hemicelluloses and celluloses can be converted into biofilms and biomaterials (Voiniciuc, 2022), and pectins are important gelling agents.

in some mature, non-growing cells, such as the tracheary elements that conduct water in vascular plants. Secondary cell walls are usually depleted in pectins, contain different hemicelluloses (e.g. xylans in Arabidopsis), and are enriched in lignin, a polyphenolic, hydrophobic compound (Meents *et al.*, 2018). Under some conditions, such as during pathogen responses, cell walls may be supplemented with callose, a β-(1,3)-linked glucan (Chen and Kim, 2009). Callose is also an important part of establishing new cell walls during cytokinesis. A transient structure that is enriched in callose, called the cell plate, develops between dividing cells and eventually matures into a cell wall that divides the two child cells (Chen *et al.*, 2018).

Plant cell walls are essential for plant growth and development, but they also provide important renewable bioproducts for human use and consumption (Fig. 1) (Pauly and Keegstra, 2010; Burton and Fincher, 2014; Voiniciuc, 2022). With increasing interest in plant-based biofuels, renewable materials, and agricultural sustainability, plant cell wall research has intensified over the last 30 years. The sequence of the *A. thaliana* genome (Box 1) (Arabidopsis Genome Initiative, 2000) revealed

Box 1. Model organisms for studying plant cell walls

Arabidopsis: *Arabidopsis thaliana* (*At*) is a model genetic plant (Krämer, 2015) and the first fully-sequenced plant genome (Arabidopsis Genome Initiative, 2000). The elongating hypocotyl and root cells are the most commonly used systems to study primary cell wall synthesis since their rapid growth requires intense cell wall synthesis (Refregier *et al.*, 2004). The lignified secondary cell walls of the stem are an excellent model system for studying secondary cell wall synthesis (Meents *et al.*, 2018), but do not constitute true wood since Arabidopsis is an annual plant.

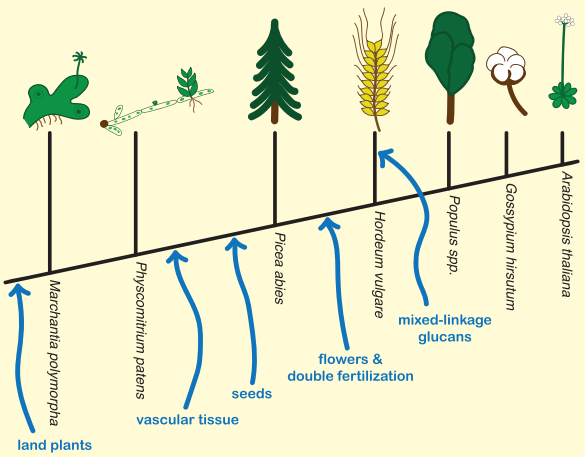
Cotton: *Gossypium hirsutum* (*Gh*) ovule epidermal cells can differentiate into long fibre cells with an extremely cellulose-rich (>90%) and lignin-poor secondary cell wall at maturity (Haigler *et al.*, 2012). They make an excellent model system for studying cellulose synthesis because they can be cultured *in vitro*, isolated in large amounts for 'omics or biochemical studies, and because both primary and secondary cell wall synthesis occur during fibre development. The paucity of molecular biology tools in cotton, relative to other model plants, makes mechanistic studies more challenging (Haigler *et al.*, 2012).

Poplar: poplar, aspen, balsam, and cottonwood trees (*Populus* spp.) are an excellent model system for wood growth because they are fast-growing trees with strong genetic resources (Tuskan *et al.*, 2006) and there is capacity to generate transgenic trees (Mellerowicz and Sundberg, 2008). Trees also form reaction wood, a specially reinforced secondary cell wall that counterbalances mechanical stress; therefore, reaction wood makes a useful model system for inducible cell wall synthesis (Tobias *et al.*, 2020). Several *Populus* species are commonly studied, including *Populus tremula*×*P. tremuloides* (*Ptt*; hybrid aspen) and *Populus trichocarpa* (*Ptr*; black cottonwood); the first structural information on a functional CESA multimer was obtained from PttCESA8 (Purushotham *et al.*, 2020), and Cas9/gRNA-targeted mutagenesis has recently been applied to study secondary cell wall biosynthesis via targeted disruption of *PtrCESA* genes (Xu *et al.*, 2021).

Barley: although the model monocot *Brachypodium distachyon* (*Bd*) (Coomey *et al.*, 2020; Hasterok *et al.*, 2022), and crops such as *Oryza sativa* (*Os*; rice) and *Zea maize* (*Zm*; maize) are frequently used in cell wall research, *Hordeum vulgare* (*Hv*; barley) is also an important model system for plant cell walls because of its high levels of mixed-linkage (β-1,3 β-1,4) glucan (MLG) (Burton and Fincher, 2014).

Spruce: *Picea abies* (Norway spruce) is a model conifer and representative gymnosperm (vascular, non-seed plant) with a high-coverage genome sequence (Nystedt *et al.*, 2013). Like angiosperm trees, gymnosperm trees also form reaction wood to counteract mechanical stress, but the composition and physiology of conifer reaction wood is distinct.

Marchantia and Physcomitrium: model non-vascular plants, including the model moss *Physcomitrium patens* (*Pp*) (Ye and Zhong, 2022) and the model liverwort *Marchantia polymorpha* (*Mp*) (Pfeifer *et al.*, 2022), offer insight into the evolution of plant cell walls since comparative studies between these non-vascular models and vascular plants can uncover features of plant cell wall synthesis that may have been shared with their last common ancestor (Donoghue *et al.*, 2021).



Model organisms for studying plant cell wall synthesis. Cartoon phylogeny of model plants used to study cell wall synthesis, with blue arrows approximately indicating key evolutionary innovations in plant cell wall synthesis. Phylogeny branch lengths and cartoon plants are not drawn to scale.

that ~10% of the genome may be involved in cell wall synthesis (McCann *et al.*, 2007) and dramatically expanded capacity to conduct functional genetic studies of these candidates. A suite of small molecule inhibitors (Larson and McFarlane, 2021) has allowed mechanistic studies of cellulose synthesis with precise control over timing, concentration, and reversibility. An elegant system to induce secondary cell wall synthesis in any cell type in Arabidopsis (Yamaguchi *et al.*, 2011) (Box 3) has allowed researchers to track this process, which is usually confined to only a small subset of cells, buried deep within the plant. A key advance was the identification of genes encoding cellulose synthase (CESA) enzymes (Pear *et al.*, 1996; Arioli *et al.*, 1998). CESAs synthesize cellulose at the plasma membrane and are organized into large, multimeric cellulose synthase complexes (CSCs) (McFarlane *et al.*, 2014). Structural information on CESAs has revealed that each CESA can synthesize a single β-(1,4)-glucan chain from UDP-glucose, and substrate addition to the growing glucan chain is coupled with its translocation through the CESA pore and into the apoplast (Purushotham *et al.*, 2020) (Fig. 1). Live-cell imaging of fluorescent protein (FP)-tagged CESAs (Box 2) has documented that CSCs are motile in the plasma membrane (Paredez *et al.*, 2006), presumably to allow elongation of the glucan chain since cellulose becomes entangled in the cell wall matrix shortly after synthesis (Diotallevi and Mulder, 2007).

Matrix polysaccharides are typically more complex than cellulose and may incorporate multiple different sugars and/or linkages into their backbone, and the backbone may be modified by additional sugar side chains, acetylation, and/or methylation (Fig. 1) (Anderson and Kieber, 2020). Most hemicellulose backbones are synthesized by multipass transmembrane domain (TM)-containing glycosyltransferases, which, like CESAs, have a cytosolic active site and are processive glycosyltransferases that couple polysaccharide synthesis with product translocation through a multipass TM pore (Scheller and Ulvskov, 2010). One exception may be xylan, a prominent hemicellulose in Arabidopsis secondary cell walls, which seems to require only single-pass membrane proteins with their active sites in the Golgi lumen (Scheller and Ulvskov, 2010), also called type-II glycosyltransferases. Pectin backbones and side chains for both pectins and hemicelluloses are also synthesized by type-II glycosyltransferases (Scheller and Ulvskov, 2010; Atmodjo *et al.*, 2013). These glycosyltransferases have been characterized using combinations of activity enrichment, homology searching, gain-of-function or loss-of-function assays, and heterologous expression (Amos and Mohnen, 2019). Fluorescent protein fusions and a suite of antibodies against cell wall polysaccharides have enabled researchers to track cell wall synthesis, secretion, and composition with subcellular accuracy (Pattathil *et al.*, 2010). Together, these tools revealed that most enzymes for pectin and hemicellulose synthesis act at the Golgi apparatus, then matrix polysaccharides are secreted to the cell wall via vesicle trafficking (Hoffmann *et al.*, 2021).

Despite these tremendous advances (and often because of the new information they provide), many open questions remain regarding plant cell wall polysaccharide synthesis. This article highlights some of these questions, reviews the data supporting different hypotheses that address these questions, and discusses new developments that may answer these questions in the future. Readers are directed to other excellent recent reviews on plant cell polysaccharide synthesis and cell wall remodelling (Anderson and Kieber, 2020; Gu and Rasmussen, 2022), polysaccharide inter-relationships in the cell wall (Cosgrove, 2022), and cell wall integrity sensing (Wolf, 2022).

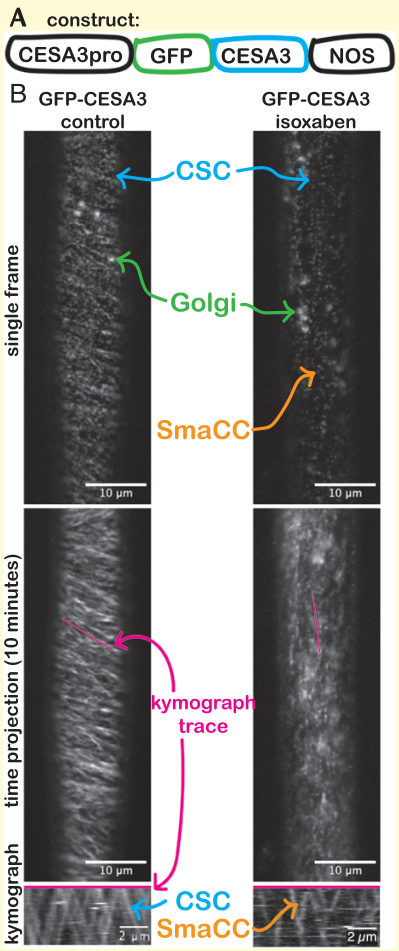
1) What other proteins are required for cell wall polysaccharide synthesis?

The Arabidopsis genome encodes at least 566 glycosyltransferases, corresponding to >2% of all genes, and this proportion is similar in other plant genomes (Drula *et al.*, 2022). While some of these are involved in protein or metabolite glycosylation, many are involved in cell wall synthesis; however, only a small proportion of plant glycosyltransferases have been characterized in detail. Other proteins are also required for cell wall synthesis, including nucleotide sugar synthesis and interconversion enzymes that make the activated 'building blocks' for polysaccharide synthesis (Figueroa *et al.*, 2021) and transporters to move nucleotide sugars into the Golgi lumen when necessary (Rautengarten *et al.*, 2014), Golgi transporters for glycosyltransferase cofactor ions (He *et al.*, 2022), acetyltransferases and methyltransferases to modify polysaccharides, plus transporters for their substrates (Temple *et al.*, 2022), and components for vesicle trafficking of polysaccharides and enzymes to the apoplast (Hoffmann *et al.*, 2021). Indeed, estimates indicate that ~10% of the Arabidopsis genome may encode proteins involved in cell wall synthesis (McCann *et al.*, 2007). Previously successful strategies for characterizing these components include activity enrichment, homology searching, gain-of-function or loss-of-function assays, and heterologous expression (Amos and Mohnen, 2019). Several recent advances in plant cell wall synthesis underscore the strengths and weaknesses of these approaches and highlight key methods that may be applied to characterize other cell wall synthesis proteins.

Some glycosyltransferases have been identified from cell types that are enriched in one cell wall polysaccharide. For example, nasturtium (*Tropaeolum majus*) seeds deposit massive amounts of the hemicellulose xyloglucan, which consists of a β-(1,4)-linked glucose backbone, substituted by xylose and other sugars (Fig. 1). A multipass TM-containing glycosyltransferase CELLULOSE SYNTHASE LIKE C (CSLC) was highly expressed in nasturtium seeds, and expression of its homologue AtCSLC4 in the yeast *Pichea pastoris* resulted in synthesis of β-(1,4)-glucan product (Cocuron *et al.*, 2007), which could correspond to either cellulose or the xyloglucan backbone

Box 2. Live-cell imaging of cellulose synthesis via fluorescently tagged cellulose synthases

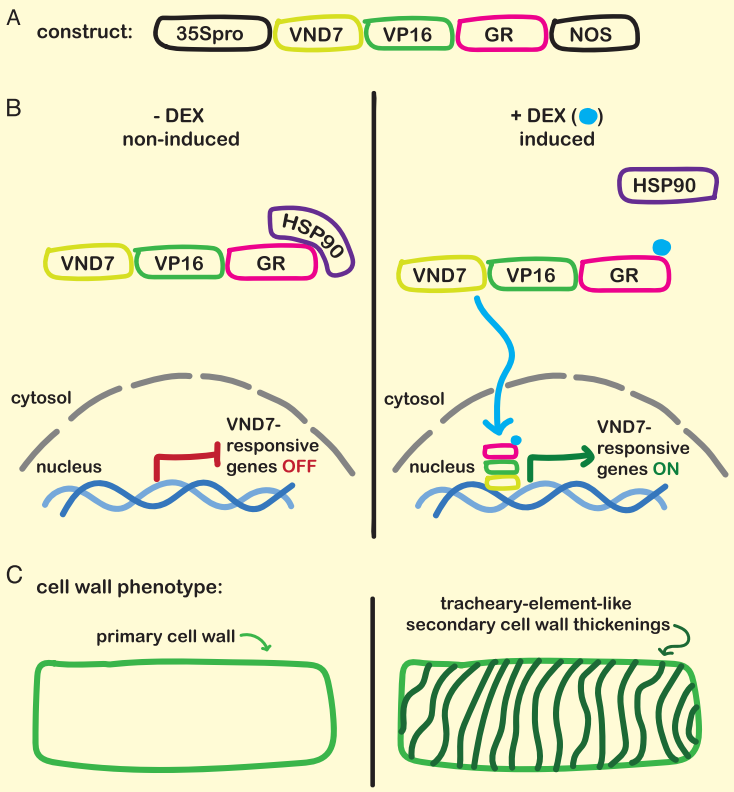
Live-cell imaging of fluorescent protein (FP)-tagged cell wall synthesis enzymes has confirmed that cellulose is made at the plasma membrane while matrix polysaccharides are synthesized at the Golgi apparatus (Hoffmann *et al.*, 2021). Native promoter-driven N-terminal FP fusions have localized CESAs to three different subcellular locations: (i) cellulose synthase complexes (CSCs) (Paredez *et al.*, 2006); (ii) Golgi bodies; and (iii) Small CESA compartments (SmaCCs) (Gutierrez *et al.*, 2009), which are sometimes associated with microtubules and called microtubule-associated cellulose synthase compartments (MASCs) (Crowell *et al.*, 2009). CSCs are small, FP–CESA-labelled puncta in the plasma membrane, which represent multiple FP-tagged CESAs incorporated into single complexes (Chen *et al.*, 2014) and move with a characteristic, consistent speed along linear trajectories, which are particularly evident in sum projections of time series data. Individual CSCs can be identified in kymographs as lines with consistent slopes, and the slope of the CSC trajectory in the kymograph can be measured to determine the speed of CSC movement in the plasma membrane (Verbančič *et al.*, 2021), which directly reflects the rate of CESA enzyme activity (Purushotham *et al.*, 2020). Golgi bodies are large, bright, usually fast-moving particles that are more frequently observed in the subcortical regions of the cell. SmaCCs are slightly larger and brighter than CSCs and most easily defined by their irregular movement in time-lapse imaging, represented as wavy lines with variable slopes in kymographs (Gutierrez *et al.*, 2009). While it is likely that SmaCCs represent a heterogeneous population of both secretory and endocytic compartments (Hoffmann *et al.*, 2021), they can be induced by various treatments, such as the cellulose synthesis inhibitor isoxaben, which causes FP–CESA depletion from the plasma membrane and an increase in SmaCCs (Gutierrez *et al.*, 2009). FP–CESA imaging is very sensitive to imaging conditions including temperature (Fujita *et al.*, 2011), osmotic conditions (Crowell *et al.*, 2009; Gutierrez *et al.*, 2009), and even mounting and imaging conditions (Verbančič *et al.*, 2021), and these and many other conditions can deplete CSCs from the plasma membrane and increase FP–CESA signal in SmaCCs. Studies of FP–CESAs have provided important insights into the mechanisms of cellulose synthesis (Paredez *et al.*, 2006), CSC guidance (Gu *et al.*, 2010; Chan and Coen, 2020), CSC assembly (Zhang *et al.*, 2016) and trafficking (Crowell *et al.*, 2009; Gutierrez *et al.*, 2009), and secondary cell wall synthesis (Gardiner *et al.*, 2003; Watanabe *et al.*, 2015).



Live cell imaging of cellulose synthesis via fluorescently tagged cellulose synthases. (A) Native promoter-driven N-terminal fluorescent protein fusions to several CESAs have been generated for AtCESA7 (Gardiner *et al.*, 2003), AtCESA6 (Paredez *et al.*, 2006), AtCESA3 (shown here) (Desprez *et al.*, 2007), AtCESA1 (Miart *et al.*, 2014), BdCESA3 (Liu *et al.*, 2017), and PpCESA5 (Tran *et al.*, 2018). (B) In all cases, including rapidly expanding Arabidopsis hypocotyl epidermal cells shown here, the construct is localized to three distinct subcellular compartments, CSCs in the plasma membrane, plus SmaCCs and Golgi bodies in the cytoplasm. Time-average images (10 s intervals for 10 min shown here) highlight the linear trajectories in which CSCs move. Kymograph analyses display every time frame for a two-dimensional region (along the magenta line) and can be used to analyse CSC speeds in the plasma membrane, which are indicative of CESA enzyme activity (Verbančič *et al.*, 2021). The cellulose synthesis inhibitor isoxaben (200 nm for 2 h shown here) rapidly depletes CSCs from the plasma membrane and induces CESA internalization into SmaCCs (Paredez *et al.*, 2006); however, stress conditions can also induce this response (Verbančič *et al.*, 2021).

Box 3. A system for induced secondary cell wall formation in Arabidopsis

There are several challenges to studying secondary cell wall synthesis: most secondary cell wall development occurs deep within the plant body, obscuring these cells from high-resolution live-cell imaging techniques that have been applied to study primary cell wall synthesis; plus, in the model genetic plant Arabidopsis, secondary cell wall-synthesizing cells make up only a small proportion of tissues and are difficult to isolate for analyses. Insights into the regulation of secondary cell wall synthesis led to identification of several master transcriptional regulators that were sufficient to induce this process; however, expression of these transcription factors also induces cell death since this is the last step in development of xylem cells (water-conducting cells with secondary cell walls) (Kubo *et al.*, 2005). Therefore, subsequent studies generated constructs for constitutive expression of these transcription factors fused to the herpes virus VP16 activation domain and the steroid-binding domain of the rat glucocorticoid receptor (Yamaguchi *et al.*, 2010). In this elegant system, the transcription factor is ubiquitously expressed, but retained in the cytosol until plants are exposed to exogenously applied glucocorticoid hormone (e.g. dexamethasone; dex), which releases the transcription factor, allowing it to move to the nucleus and trigger differential gene expression (Sablowski and Meyerowitz, 1998). Expression of one of these transcription factors, VND7, under the control of the dex-inducible system, allows secondary cell wall synthesis to be triggered in almost any cell type in Arabidopsis in large-scale and synchronized fashion. This system has allowed researchers to study transdifferentiation of epidermal cells into xylem tracheary element-like cells with helical secondary cell wall thickenings. This and another similar system (Oda *et al.*, 2010) have provided important insights into gene regulation (Yamaguchi *et al.*, 2011), secondary cell wall synthesis (Watanabe et al., 2015, 2018), and the mechanisms that pattern secondary cell wall thickenings (Oda and Fukuda, 2012; Schneider *et al.*, 2017; H. Wang *et al.*, 2022).



A system for induced secondary cell wall formation in Arabidopsis. (A) The VND7 transcription factor, fused to a VP16 activation domain and the steroid-binding domain of the rat glucocorticoid receptor (GR), is ubiquitously expressed under the control of the 35S promoter. (B) Without exogenously applied glucocorticoid hormone (e.g. dexamethasone; dex) (left) the VND7 transcription factor is retained in the cytoplasm via HSP90 interaction with GR, so VND7-responsive genes are not transcribed. Upon dex addition (right), dex binds the GR domain, releasing it from HSP90 and allowing the VND7 fusion protein to move into the nucleus to activate transcription of VND-responsive genes. (C) Expressing this construct in plant cells that normally form a primary cell wall only (left) will induce treachery element-like secondary cell wall thickenings (right) upon dex exposure.

(Fig. 1). Loss-of-function mutant analyses in Arabidopsis displayed no polysaccharide changes for *csl*c single mutants, but higher order mutants, including a quintuple mutant (affecting all five CSLCs in Arabidopsis), displayed complete loss of xyloglucan and mild growth phenotypes (Kim *et al.*, 2020), consistent with other mutants completely lacking xyloglucan (Cavalier and Keegstra, 2006; Yu *et al.*, 2022). Together, these studies highlight the power of using diverse systems to identify polysaccharide synthesis genes and combining this with mechanistic studies in model plants.

In a similar activity enrichment approach, two type-II glycosyltransferases, GAUT1 and GAUT7, were implicated in synthesis of HG, a pectin made of α-(1,4)-linked GalA, which may be partially methylesterified and acetylated (Fig. 1) (Sterling *et al.*, 2006). Although these two glycosyltransferases can physically interact (Atmodjo *et al.*, 2011), and loss of another GAUT family member resulted in HG defects (Bouton *et al.*, 2002), the nature of their combined biosynthetic activity remained elusive until detailed biochemical assays were conducted on both GAUT1 and GAUT7 isolated from human HEK293F cells. When GAUT1 and GAUT7 were incubated with different lengths of GalA polymers as acceptors for different times, longer acceptors were rapidly elongated, while shorter acceptors and *de novo* HG synthesis proceeded relatively slowly (Amos *et al.*, 2018). Based on these data, the authors propose a two-phase model in which GAUT1 alone could initiate HG synthesis and make short products, but non-enzymatic activity of GAUT7 stabilized longer acceptors, allowing for increased reaction rates of larger polysaccharides (Amos *et al.*, 2018). These results emphasize that assessing glycosyltransferase function using a wide range of acceptors can reveal additional insights into the mechanisms of polysaccharide synthesis.

In the case where Arabidopsis does not usually synthesize a polysaccharide of interest, gain-of-function studies can be particularly helpful. For example, mixed-linkage glucan is a matrix polysaccharide that is found in *Poales* (grasses including important staple crops rice, maize, wheat, and barley), but not detected in most other plants (Fry *et al.*, 2008). Mixed-linkage glucan synthesis is intriguing because the glucose polymer is made of both β-1,3 and β-1,4 linkages that are distributed in a non-random, non-repeating fashion. Overexpressing mixed-linkage glucan synthesis candidates, OsCSLF2 or OsCSLF4, in Arabidopsis resulted in mixed-linkage glucan synthesis (Burton *et al.*, 2006). While other CSL family members (CSLJ and CSLH) may be involved (Little *et al.*, 2018), CSLF6 is the major isoform based on strong mixed-linkage glucan-defective phenotypes when CSLF6 function was disrupted in wheat (Nemeth *et al.*, 2010), rice (Vega-Sánchez *et al.*, 2012), barley (Taketa *et al.*, 2012; Garcia-Gimenez *et al.*, 2020), or brachypodium (Bain *et al.*, 2021). Recently, a medium-resolution cryoEM structure of HvCSLF6 isolated from insect (Sf9) culture cells (Purushotham *et al.*, 2022), demonstrated that monomeric HvCSLF6 alone synthesized both β-1,3 and β-1,4 linkages of mixed-linkage glucan without the addition of other plant-derived factors (Purushotham *et al.*, 2022). While gain-of-function assays were critical in identifying mixed-linkage glucan synthases, the structure of HvCSLF6 provided essential information that answered many long-standing questions.

In some cases, conflicting data arise depending on the methods used to study glycosyltransferase function. For example, loss-of-function *csld* mutants revealed changes to root hair growth, cell plate maturation, and pollen tube growth, but no significant changes in monosaccharide composition of matrix polysaccharides (Yin *et al.*, 2011), while immunolabelling detected changes to xyloglucan distribution in the cell wall (Galway *et al.*, 2011). Heterologous expression of CSLD family members increased GDP-mannose utilization and incorporation into β-mannan, but no change in utilization of other substrates (Yin *et al.*, 2011). Together, these data implicated CSLD family members in hemicellulose synthesis, but data conflicted about which polysaccharides CSLDs might synthesize. In contrast, isolation of CSLD expressed in yeast (*Saccharomyces cerevisiae*) provided compelling evidence that CSLDs synthesized a β-(1,4)-linked glucose, cellulose-like product (Yang *et al.*, 2020), consistent with catalytic domain-swap complementation experiments between CSLD3 and CESA6 (Park *et al.*, 2011; Yang *et al.*, 2020). The authors propose a model in which CSLDs might synthesize a cellulose-like product, possibly in shorter and/or less crystalline fragments (Yang *et al.*, 2020), particularly in cell types that require significant flexibility and/or in cellular regions where there are fewer microtubules to guide organized cellulose synthesis (Question 7), such as cell plates (Gu *et al.*, 2016) or root hair tips (Park *et al.*, 2011). The conflicting results from loss-of-function, gain-of-function, and characterization of CSLD3 activity *in vitro* underscore the importance of using multiple methods to study glycosyltransferase function.

Beyond glycosyltransferases, substantial progress has been made characterizing the nucleotide sugar transporters, which move activated nucleotide sugars into the Golgi apparatus for use by type-II glycosyltransferases. An unbiased approach of simultaneously supplying nucleotide sugar transporters reconstituted in proteoliposomes with a wide range of possible nucleotide sugar substrates (Rautengarten *et al.*, 2014) captured unique transporter dynamics (Rautengarten *et al.*, 2017) and identified bifunctional transporters (Rautengarten *et al.*, 2014). Although potentially time-consuming and complex to interpret, these and similar unbiased experiments (Ruprecht *et al.*, 2020; Ehrlich *et al.*, 2021) avoid confirmation bias by supplying a range of substrates in competitive assays. Such experiments can uncover previously unknown functions of cell wall synthesis genes. Although similar sequence and structure suggest a conserved catalytic mechanism within each family of glycosyltransferases, proteins within one family can display differences in substrate specificity (Amos and Mohnen, 2019; Ruprecht *et al.*, 2020; Ehrlich *et al.*, 2021; Drula *et al.*, 2022) that may go undiscovered without unbiased biochemical assays.

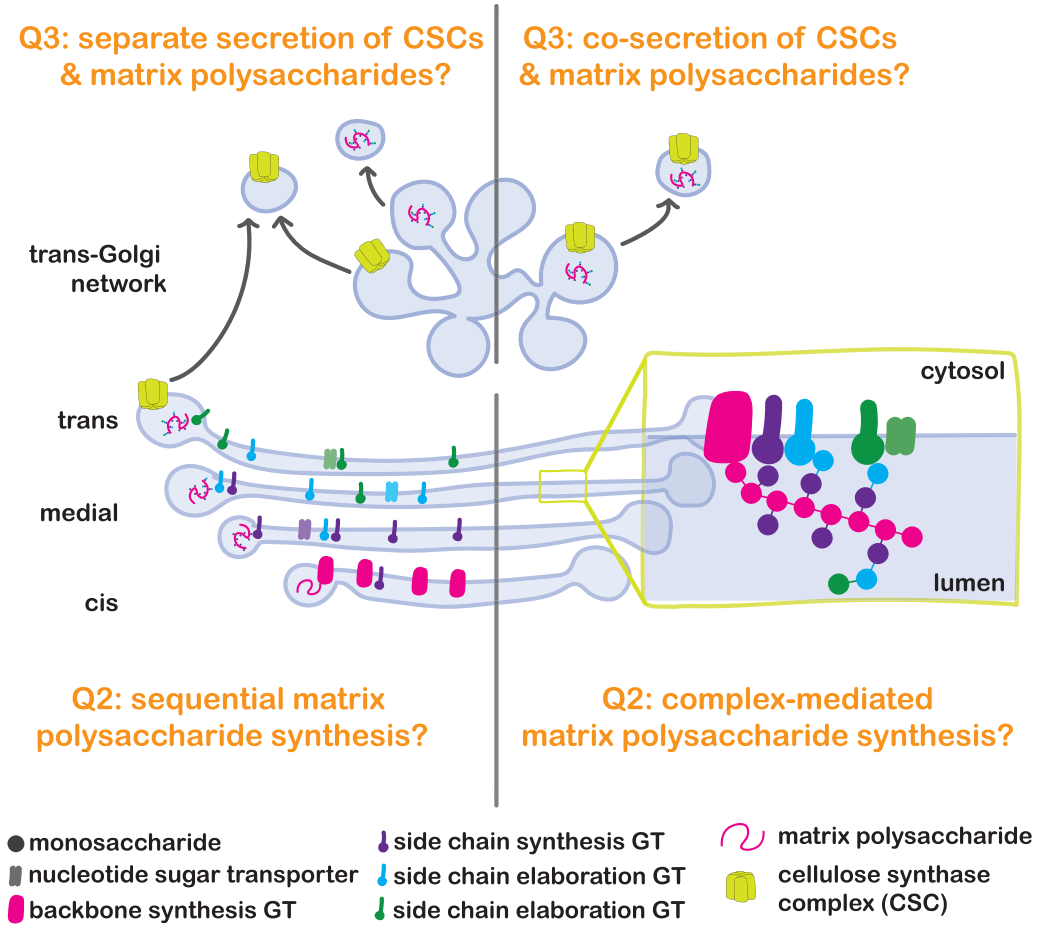


Fig. 2. Polysaccharide synthesis and regulation at the Golgi apparatus. Two non-mutually-exclusive models of matrix polysaccharide synthesis at the Golgi apparatus have been proposed (Question 2): sequential matrix polysaccharide synthesis and complex-mediated matrix polysaccharide synthesis. In the sequential model (left), backbone synthesis via backbone synthesis glycosyltransferases (GTs; magenta) occurs in earlier (more cis-) cisternae, while side chain synthesis (purple) and elaboration (blue & green) via side chain GTs occur in later (more trans-) cisternae. In the complex-mediated model (right), multiple components associate into a multiprotein complex to facilitate matrix polysaccharide synthesis. The most likely scenario is that different complexes are differentially distributed across cisternae, with backbone synthesis GTs (magenta) and side chain synthesis GTs and transporters (purple) associating in earlier cisternae, and side chain synthesis (purple) and elaboration components (blue & green) associating in later cisternae. Although cellulose synthase complexes (CSCs; yellow) are assembled in the Golgi apparatus, it also remains unclear (Question 3) whether distinct secretory vesicles are responsible for CSC and matrix polysaccharide secretion (left), or whether CSCs are packaged into the same secretory vesicles as matrix polysaccharides (right).

Together, these advances highlight the value of conducting detailed characterization of proteins involved in cell wall synthesis. Unfortunately, little structural information is available for plant glycosyltransferases (Rocha *et al.*, 2016; Urbanowicz *et al.*, 2017; Culbertson *et al.*, 2018; Purushotham et al., 2020, 2022; Prabhakar *et al.*, 2023). Additional structural information and structural modelling via AlphaFold (Jumper *et al.*, 2021) will help to fuel further predictions about glycosyltransferase mechanisms, interactions, and regulation (Moremen and Haltiwanger, 2019). Another limitation has been the incomplete availability of appropriate substrates to assess glycosyltransferase activity. Two relatively new approaches may address this limitation. First, analysis of isolates from the human gut microbiome identified bacteria capable of cleaving 20 out of 21 glycosidic linkages present in RG-II (Ndeh *et al.*, 2017); further characterization of these and similar enzymes may allow controlled hydrolysis of plant cell wall material to generate custom substrates for glycosyltransferase assays. Secondly, synthetic glycan arrays (Ruprecht *et al.*, 2017) coupled with functionalized nucleotide sugars for simple detection (Ruprecht *et al.*, 2020) can offer a variety of substrates to purified enzymes, allowing simultaneous and competitive screening of multiple substrates in a single reaction. Together with efforts to create an Arabidopsis glycosyltransferase clone library (Xuan *et al.*, 2021), plus expanding resources for comparative genomics and CRISPR technology to assess gene function in other plant species (Reynolds *et al.*, 2022), these new technologies represent promising resources for identifying and characterizing additional plant cell wall synthesis enzymes.

2) Are matrix polysaccharides synthesized by complex-mediated or sequential synthesis?

Unlike many other biological polymers, polysaccharides are synthesized without a template; so how are proteins for matrix polysaccharide synthesis organized within the Golgi apparatus to ensure reproducibility? Furthermore, the plant Golgi apparatus is split into hundreds of highly motile mini-stacks per cell (Boevink *et al.*, 1998). Each stack consists of multiple membrane-bound compartments (cisternae), organized from *cis* to *trans*, with the *trans*-most cisterna closest to the *trans*-Golgi network (Fig. 2). Each Golgi stack probably has the full capacity to synthesize all cell wall polysaccharides, since multiple enzymes (Parsons *et al.*, 2019) and products (Moore *et al.*, 1991; Zhang and Staehelin, 1992; Young *et al.*, 2008; Wang *et al.*, 2017) for synthesis of multiple polysaccharides localized to the same Golgi stacks. This raises questions as to how diverse polysaccharides are synthesized in the complicated milieu of enzymes and substrates in each Golgi stack. Two non-mutually-exclusive hypotheses for matrix polysaccharide synthesis in the Golgi apparatus are (i) sequential polysaccharide synthesis and (ii) complex-mediated synthesis (Fig. 2) (Atmodjo *et al.*, 2013; Hoffmann *et al.*, 2021; Zabotina *et al.*, 2021). In the sequential model, the enzymes for polysaccharide synthesis are distributed throughout different Golgi cisternae, allowing backbone synthesis in the *cis*- and medial-Golgi, then side chain synthesis and elaboration in the medial- and/or *trans*-Golgi cisternae. In the complex-mediated model, multiple proteins required for synthesis of a single polysaccharide associate together for optimal functionality.

The sequential model of matrix polysaccharide synthesis predicts heterogeneous distribution of enzymes and polysaccharides across different cisternae, with proteins required for backbone synthesis and products representing relatively undecorated polysaccharide backbones enriched in cisternae towards the *cis*-Golgi, and polysaccharide products with more complex side chains and proteins for their synthesis enriched in the *trans*-Golgi (Fig. 2). Consistent with this model, immunoTEM of sycamore maple (*Acer pseudoplatanus*) suspension cultured cells documented anti-xyloglucan labelling across medial- and *trans*-cisternae, plus the *trans*-Golgi network, but anti-fucose (the terminal side chain elaboration on xyloglucan) only in the *trans*-cisternae and *trans*-Golgi network (Zhang and Staehelin, 1992). Similar patterns were also detected for pectins in maple (Zhang and Staehelin, 1992), for pectins and hemicelluloses in Arabidopsis (McFarlane *et al.*, 2008; Young *et al.*, 2008), and for hemicelluloses in secondary cell wall synthesis (Meents *et al.*, 2019). Indeed, xyloglucan xylosyltransferase XXT1 preferentially localized to the *cis*-Golgi, putative galactosyltransferase MUR3 to the medial-Golgi, and fucosyltransferase FUT1 to the *trans*-Golgi (Chevalier *et al.*, 2010), which is consistent with their sequential roles in synthesizing a xylose–galactose–fucose side chain on xyloglucan. Recently, these results were corroborated by a combination of proteomics of free-flow electrophoresis separation of Golgi compartments, supported by super-resolution imaging of FP-tagged enzymes and immunoTEM of polysaccharide epitopes (Parsons *et al.*, 2019). By analogy with *N*-glycoprotein processing enzymes, which are also spread across Golgi cisternae (Saint-Jore-Dupas *et al.*, 2006; Schoberer *et al.*, 2009), these enzyme distributions across Golgi cisternae may serve to limit competition for substrates or to ensure the correct sequence of side chain elaboration.

The complex-mediated model of matrix polysaccharide synthesis predicts that polysaccharide synthesis proteins interact within the same Golgi cisterna (Fig. 2). Interactions have been documented between proteins involved in pectin (HG) and hemicellulose (xyloglucan and xylan) synthesis. In HG synthesis, GAUT1 interacted with GAUT7 to localize to the Golgi apparatus (Atmodjo *et al.*, 2011) and to efficiently synthesize long HG backbones (Atmodjo *et al.*, 2011; Amos *et al.*, 2018). Pulldown assays documented interactions between GAUT1 and GAUT7 and with QUA3 (Atmodjo *et al.*, 2011), which is similar to QUA2, a HG methyltransferase (Du *et al.*, 2020). Interestingly, *qua2* mutants displayed reduced HG levels, but wild-type proportions of HG methylesterification (Mouille *et al.*, 2007; Du *et al.*, 2020), suggesting that efficient substrate channelling from glycosyltransferases to methyltransferases ensures appropriate levels of HG methylesterification before secretion. This may be facilitated by a domain of unknown function 1068 protein that interacts with both pectin glycosyltransferases and methyltransferases, and is required for pectin synthesis, but seems to have no hallmarks of enzyme activity itself (Lathe *et al.*, 2021, Preprint). Interestingly, there is no reported evidence of interactions between pectin synthesis enzymes RRT and RGGAT, which act together to alternatively add rhamnose and GalA to the RG-I backbone, respectively (Amos *et al.*, 2022). Interactions have been documented amongst xyloglucan glycosyltransferases, including backbone synthesis CSLC4, side chain synthesis xylosyltransferases XXT1, XXT2, and XXT5, and side chain elaboration glycosyltransferases MUR3 and FUT1 via multiple methods (Chou et al., 2012, 2015; Lund *et al.*, 2015). Furthermore, the strong phenotypes of *mur3* mutants (Kong *et al.*, 2015) suggest that accumulation of xyloglucan with unusual side chains is more detrimental than complete loss of xyloglucan (Cavalier *et al.*, 2008; Kim *et al.*, 2020), suggesting that xyloglucan must be carefully regulated and efficiently modified.

There are several similarities between HG synthesis and synthesis of xylan hemicelluloses, despite the very different enzymatic reactions required, that imply that xylan may also be synthesized by enzyme complexes. Mutant phenotypes indicated that IRX9/IRX9L (Lee *et al.*, 2007), IRX10/IRX10L (Brown *et al.*, 2009; Wu *et al.*, 2009), and IRX14/IRX14L (Brown *et al.*, 2007) were all required for xylan synthesis in Arabidopsis. IRX10L catalysed β-(1,4)-xylan backbone synthesis (Urbanowicz *et al.*, 2014), but glycosyltransferase activity of IRX9 and IRX14 was not required (Ren *et al.*, 2014).

Heterologous expression detected interactions between homologues of these proteins from wheat (Zeng *et al.*, 2010) and asparagus (Zeng *et al.*, 2016), but not Arabidopsis (Lund *et al.*, 2015). Since expression of all three proteins from asparagus was required for their effective targeting to the Golgi apparatus (Zeng *et al.*, 2016), IRX9 and IRX14 might play analogous roles to GAUT7, though this remains to be determined.

The sequential and complex-mediated models of matrix polysaccharide synthesis are not mutually exclusive and may be integrated into a combined model. While protein complexes may exist to channel substrates into specific polysaccharides or to rapidly modify products before aggregation, different complexes may be distributed amongst different Golgi cisternae (Zabotina *et al.*, 2021). This could allow bifunctional or multifunctional enzymes to synthesize multiple products if they are localized to different Golgi cisternae where they encounter different substrate availabilities, pH, or ion concentrations as established by different transporters. For example, XXT1, which can add xylose to the glucan backbone during xyloglucan synthesis (Cavalier and Keegstra, 2006), can also use other substrates (Ehrlich *et al.*, 2021) or add xylose to other acceptors (Ruprecht *et al.*, 2020) *in vitro*, so localization of XXT1-containing complexes to specific Golgi cisternae may help direct enzyme activity *in vivo*. This combined model is consistent both with interactions between xyloglucan synthesis enzymes (Chou *et al.*, 2012, 2015; Lund *et al.*, 2015), and with their differential steady-state localization within Golgi stacks (Chevalier *et al.*, 2010). Testing this combined model will require localization of multiple functional, tagged enzymes expressed at native expression levels using quantitative, high-resolution methods, such as immunoTEM and super-resolution microscopy.

3) How is the balanced synthesis of different polysaccharides achieved?

Within a single plant, cell wall composition may vary across tissue types, developmental timelines (Yang *et al.*, 2016), and even regions of the cell wall (Dauphin *et al.*, 2022), yet cell wall composition is relatively reproducible for individual plants and tissue types. So, how is cell wall composition controlled and how is matrix polysaccharide synthesis at the Golgi apparatus balanced with cellulose synthesis at the plasma membrane? One possibility is that matrix polysaccharides and CESAs might be packaged into the same secretory vesicles at the Golgi apparatus (Question 4). Three other possible mechanisms to balance cell wall synthesis are transcriptional regulation, control of carbon flux into cell wall synthesis, and feedback from cell wall integrity signalling mechanisms.

Transcriptional regulation of cell wall synthesis genes plays a role in regulating cell wall composition. For example, secondary cell wall synthesis is under complex transcriptional control (Taylor-Teeples *et al.*, 2015), and ectopic expression of VND transcription factors is sufficient to induce secondary cell wall formation (Box 3) (Kubo *et al.*, 2005; Yamaguchi *et al.*, 2010). Although transcriptional control of primary cell wall synthesis is less completely understood (Pedersen *et al.*, 2023), genes involved in primary cell wall synthesis have been identified based on co-expression relationships (Persson *et al.*, 2005). However, transcriptional control of cell wall synthesis enzymes is insufficient to explain all variation in cell wall composition. For example, some glycosyltransferases showed expression variation across the Arabidopsis shoot apical meristem, but these were not strictly correlated with differences in cell wall composition (Yang *et al.*, 2016), and similar inconsistencies were documented in maize coleoptiles (Okekeogbu *et al.*, 2019).

Cell wall polysaccharide synthesis may be regulated by controlling carbon flux into cell wall precursors and/or via energy signalling mechanisms (Verbančič *et al.*, 2018). UDP-glucose may be supplied to CESAs via sucrose synthase (SUS) or the combined activity of cytosolic invertase (CIV) and UDP-glucose pyrophosphorylase (McFarlane *et al.*, 2014). SUS can co-fractionate with CESA activity in cotton (Amor *et al.*, 1995) and Azuki bean (*Vigna angularis*) (Fujii *et al.*, 2010), and SUS overexpression in hybrid poplar (*Populus alba*×*grandidentata*) xylem cells resulted in slightly increased cellulose synthesis (Coleman *et al.*, 2009). However, RNAi targeting the main SUS isoforms in developing wood of hybrid aspen did not dramatically change growth or cell wall synthesis (Gerber *et al.*, 2014). Importantly, Arabidopsis sextuple *sus* mutants with no detectable SUS activity (Fünfgeld *et al.*, 2022) displayed no significant changes in plant growth, primary or secondary cell wall morphology, cellulose content, or UDP-glucose content (W. Wang *et al.*, 2022), indicating that SUS is not essential for cell wall synthesis but may affect carbon partitioning into cell wall components (Gerber *et al.*, 2014). On the other hand, double *civ civ2* mutant seedlings displayed broad metabolic and transcriptional changes (Pignocchi *et al.*, 2021) and had reduced crystalline cellulose and slower CESA speeds, but no changes to CSC density in the plasma membrane (Barnes and Anderson, 2018). These results suggest that CIV activity may be important for substrate supply during cellulose synthesis. However, substrate availability itself is not directly regulating CSC activity at the plasma membrane since UDP-glucose levels remained constant across carbon-limiting and carbon-replete conditions, even as CSC activity at the plasma membrane fluctuated (Ivakov *et al.*, 2017), suggesting that energy signalling, rather than substrate availability, may be regulating cellulose synthesis. It remains unclear whether matrix polysaccharide synthesis may be fine-tuned via carbon availability in a similar fashion.

Cell wall integrity signalling has been described in plants (Wolf, 2022) and may offer additional mechanisms by which plants can balance and/or modify cellulose and matrix polysaccharide content. Most work on cell wall integrity signalling has been focused on the initial perception of either chemical signals, such as damage-associated molecular patterns (Bacete

et al., 2018), or mechanical signals, such as changes in cell wall resistance to turgor (Bacete and Hamann, 2020). Research on plant responses to these signals has been focused on dramatic cell wall changes, such as total loss of xyloglucan (Kim *et al.*, 2020) or major defects in cellulose synthesis (Hématy *et al.*, 2007). Once the molecular mechanisms of cell wall signalling are better characterized, it may become possible to test whether subtle cell wall changes might provide feedback to balance cell wall polysaccharide synthesis.

4) Are matrix polysaccharides and CSCs secreted via the same vesicles?

Matrix polysaccharide and cellulose synthesis could be coordinated if Golgi-synthesized matrix polysaccharides are packaged into the same post-Golgi vesicles as CSCs, since fusion of these vesicles with the plasma membrane would deliver matrix polysaccharides to the apoplast and CSCs to the plasma membrane for cellulose synthesis (Fig. 2). Since CESAs seem to cycle between the Golgi and the plasma membrane (Question 9; Fig. 3), this cycling could help balance matrix polysaccharide delivery with CESA regulation.

A series of meticulous double-labelling immunoTEM experiments detected both anti-xyloglucan and RabA4b in the same post-Golgi structures and co-localized RabA4b with SYP61 (Kang *et al.*, 2011). Since CESA6 co-localized with SYP61 in immunoTEM (Drakakaki *et al.*, 2012), these results provide indirect evidence that CESAs co-localize with matrix polysaccharides in the same *trans*-Golgi network, but it is unclear whether individual vesicles that bud from the *trans*-Golgi network carry both CSCs and matrix polysaccharide cargo. More recently, several approaches have isolated subpopulations of membrane-bound compartments from plant cells to analyse their proteome and glycome, including flotation centrifugation (Okekeogbu *et al.*, 2019), free-flow electrophoresis (Parsons *et al.*, 2019), and affinity purification of SYP61 (Drakakaki *et al.*, 2012; Wilkop *et al.*, 2019). Proteomic analysis of SYP61 affinity-purified membranes also detected CESA2, CESA3, and CESA6 (Drakakaki *et al.*, 2012), and glycomic analysis of this fraction detected xyloglucan, pectins, and cell wall glycoproteins (Wilkop *et al.*, 2019). Since similar proteins and polysaccharides were detected by the other studies (Okekeogbu *et al.*, 2019; Parsons *et al.*, 2019), these results also imply that CESAs and matrix polysaccharides may be packaged in the same post-Golgi compartments (Fig. 2). However, these analyses represent a homogenized population of subcellular compartments, which could mask subpopulations with distinct cargo.

These systems-level studies also identified proteins that may be involved in CESA and matrix polysaccharide trafficking. For example, both the ECHIDNA complex (Gendre *et al.*, 2011, 2013) and the TRAPPII complex (Qi *et al.*, 2011; Rybak *et al.*, 2014) have been implicated in secretory trafficking from the Golgi, and components of both complexes were identified in SYP61 affinity-purified membranes (Drakakaki *et al.*, 2012). While it seems that these components play distinct roles in post-Golgi trafficking (Boutté *et al.*, 2013; Ravikumar *et al.*, 2018), both complexes may be involved in cell wall secretion. Indeed, *ech* complex mutants were defective in matrix polysaccharide secretion (Gendre *et al.*, 2013) and mistargeted polysaccharides to the vacuole (McFarlane *et al.*, 2013), but CESA trafficking has not yet been analysed in these mutants. Studying CESA and polysaccharide localization in mutants affecting TRAPPII complex components is difficult because knockout mutants are seedling lethal (Rybak *et al.*, 2014), but small molecules affecting subcellular trafficking can overcome the issues of genetic redundancy, pleiotropy, or lethality (Ma *et al.*, 2022). CESTRIN is a small molecule that disrupted CESA trafficking to the plasma membrane and affected both cellulose and matrix polysaccharide synthesis (Worden *et al.*, 2014). Although CESTRIN treatment retained CESAs inside intracellular compartments, matrix polysaccharide localization was not assessed (Worden *et al.*, 2014). Together, these results provide circumstantial, but inconclusive evidence that CESAs and matrix polysaccharides may be transported by the same post-Golgi compartments.

While high-resolution immunoTEM approaches can localize CESAs or polysaccharides to individual vesicles, labelling is sparse (Moore *et al.*, 1991), so the probability of finding a statistically significant population of double-labelled vesicles is prohibitively small. On the other hand, biochemical techniques have documented the proteomic and glycomic makeup of vesicle populations enriched from different tissues and subcellular fractions, but the modest sensitivity of analyses means that samples must be pooled; therefore, these studies represent a homogenized sample population, which could mask subpopulations with distinct cargo and/or functions. Increased sensitivity of these analytical methods and application of single-cell proteomics/metabolomics to study subcellular compartments may be required to decisively address this question.

5) How are CESAs assembled into a CSC?

Cellulose is made at the plasma membrane by CSCs with 6-fold symmetry (Wilson *et al.*, 2021). Land plants encode multiple CESAs, and multiple CESAs assemble to form the CSC. The cryo-EM structure of PttCESA8 (Purushotham *et al.*, 2020), freeze-fracture of CSCs (Nixon *et al.*, 2016), and cellulose fibre measurements (Newman *et al.*, 2013) best fit a hexamer-of-trimers structure for an overall 18mer CSC (Fig. 3). However, the ancestral CSC was probably a homooligomer, and an elegant model has been proposed to explain how heterooligomers could have arisen (Haigler and Roberts, 2019). Indeed, heterologously expressed PpCESA5 synthesized

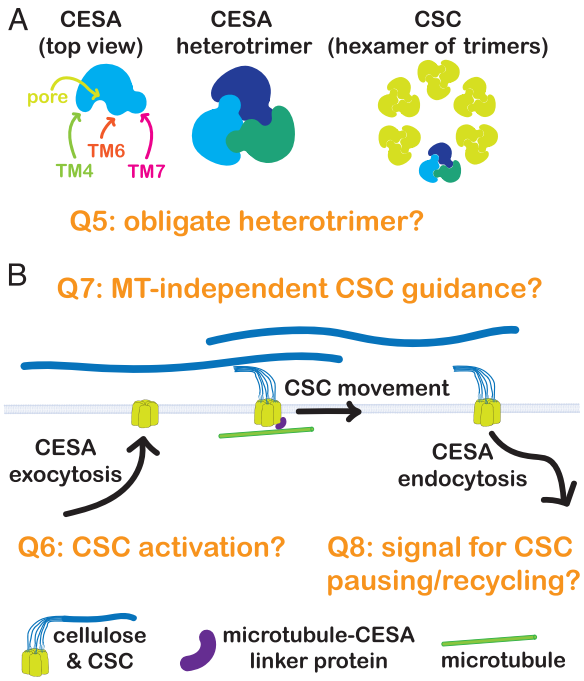


Fig. 3. The cellulose synthase complex (CSC) and its regulation. (A) Plant cellulose synthase (CESA) enzymes assemble into large, multimeric structures called the cellulose synthase complexes (CSCs). Most evidence is consistent with a hexamer-of-trimers model, in which the hexamer is made of six heterotrimers consisting of CESA1, CESA3, and CESA6-likes (CESA2/CESA6/CESA5/CESA9) in Arabidopsis primary cell walls and CESA4, CESA7, and CESA8 in Arabidopsis secondary cell walls. This heterotrimeric interaction is formed in part by interactions between transmembrane domains (TMs) that form the transmembrane pore for β-glucan chain translocation across the membrane. Here, TM7 from one CESA interacts with TM4 and TM6 from another CESA to complete translocation pore formation. However, it is unclear whether CESAs form obligate heterotrimers or whether there is some flexibility in the hexamer-of-trimers model (Question 5). (B) Although CESAs are localized to the Golgi apparatus and Small CESA compartments (SmaCCs) in the cytoplasm, plus CSCs in the plasma membrane (Box 2), the biological function of CESA cycling between the Golgi apparatus and the plasma membrane remains unclear (Question 9). The steps of CSC exocytosis to the plasma membrane are described in detail in Zhu and McFarlane (2022), but the signal that activates CESA activity and triggers CSC motility in the plasma membrane remains unknown (Question 6). Once active in the plasma membrane, CSCs will synthesize β-glucan chains and these will coalesce into cellulose microfibrils. CSCs are guided in part by microtubules via interactions with CESA–microtubule linker proteins, such as CSI1. However, it remains unclear what proportion of CSCs are actively guided by microtubules (Question 7), since microtubule-independent mechanisms of CSC guidance have also been described. While cellulose microfibrils already in the cell wall may play a role in microtubule-independent CSC guidance, other mechanisms remain possible (Question 7). The signals that trigger CESAs for endocytosis also remain uncharacterized (Question 8).

cellulose microfibril-like structures (Cho *et al.*, 2017) and ectopic PpCESA5 complemented an otherwise null CESA mutant (Li *et al.*, 2022), implying that PpCESA5 can act as a homooligomer *in vivo*. Homotrimers of vascular plant PttCESA8 (Purushotham *et al.*, 2016) and GhCESA7 (X. Zhang *et al.*, 2021) have also been isolated via heterologous expression and can make β-(1,4)-glucan *in vitro*. These data raise questions such as: are all CSCs obligate heterooligomers and how much flexibility is there in CSC assembly?

Different CESA are unique, based on sequence, interactions, and functions, implying that they may form obligate heterooligomers. Functionally, CESAs act non-redundantly; in Arabidopsis, CESA1, CESA3, and a CESA6-like (CESA2/CESA5/CESA6/CESA9) were essential for primary cell wall synthesis (Desprez *et al.*, 2007; Persson *et al.*, 2007), and CESA4, CESSA7, and CESA8 were required for secondary cell wall synthesis (Taylor *et al.*, 2003), and similar requirements exist in other plants (Pancaldi *et al.*, 2022). Furthermore, fluorescently tagged primary CESA6 and secondary CESA7 displayed differences in motility and subcellular trafficking within the same cells (Watanabe *et al.*, 2018). Different phosphorylation sites (Cruz *et al.*, 2019) and acylation sites (Kumar *et al.*, 2022) may also confer distinct functions to different CESAs. For example, phosphorylation differences may distinguish between primary CESA5 and CESA6 (Bischoff *et al.*, 2011), while acylation differences may distinguish secondary CESA7 and CESA8 (Kumar *et al.*, 2022).

Vascular plant CESAs cluster distinctly from other cellulose synthases in phylogenetic analysis, and primary versus secondary CESAs cluster separately (Pancaldi *et al.*, 2022). Plant CESAs have additional domains compared with bacterial cellulose synthases and there is more sequence divergence in these regions, suggesting that they confer CESA functional specificity. Domain-swap experiments have revealed complex relationships between these domains of CESA4, CESA7, and CESA8 (Kumar *et al.*, 2016a; Hill *et al.*, 2018a). Recombinantly expressed domains of CESAs from several species have the capacity to dimerize (Kurek *et al.*, 2002; Olek *et al.*, 2014) or trimerize (Vandavasi *et al.*, 2015; Du *et al.*, 2022) *in vitro*. It is unclear what contributions these interactions make *in vivo* with the added context of TMs and the complete three-dimensional structure of the full-length CESA. However, these interactions may contribute to CESA assembly into higher order structures (Wilson *et al.*, 2021; Pedersen *et al.*, 2023), particularly since these are disordered regions (Purushotham *et al.*, 2020), so sequence divergence in these regions may govern how CESAs assemble into heterotrimers and/or 18mers.

Physical interactions have been detected among both primary (Desprez *et al.*, 2007; Wang *et al.*, 2008) and secondary CESAs (Taylor *et al.*, 2003), and primary CESA1/3/6 and secondary CESA4/7/8 have been isolated in 1:1:1 ratios (Gonneau *et al.*, 2014; Hill *et al.*, 2014). However, these data do not rule out a hexamer-of-homotrimers model. The best evidence supporting CESA heterotrimers comes from analysis of herbicide resistance-conferring mutations within the context of CESA structure (Shim *et al.*, 2018). Several isoxaben resistance-conferring mutations map to residues in the TMs of both CESA3 and CESA6 (Larson and McFarlane, 2021). Since the structure of PttCESA8 documented that TM7 from one CESA monomer interacts with TM4 and TM6 of another

monomer to form a complete pore for glucan chain translocation into the apoplast (Purushotham *et al.*, 2020), the locations of these herbicide-resistant mutants suggest that CESAs assemble into heterotrimers, in which CESA3 and CESA6 TMs interact to form one pore and that isoxaben may interfere with this region of the CSC (Fig. 3).

On the other hand, there seems to be plasticity in CSC assembly, including diversity in CSC structures and flexibility in CESA interactions, which suggests there may be some pliancy in CSC oligomerization. Although most CSCs observed by freeze-fracture displayed regular hexameric organization, there was significant variation amongst individual CSCs (Reiss *et al.*, 1984; Nixon *et al.*, 2016). These diverse CSC structures could reflect different CESA makeups, but could also be the result of CESA association with different accessory proteins (such as those required for microtubule-mediated CSC guidance; Fig. 3), differences in CESA activation status, or CSC disassembly before endocytosis (Questions 6–8). Pairwise interaction studies also suggested flexibility in CESA interactions; although CESAs displayed differences in their capacity to homomerize, all CESAs heterodimerized, even in combinations that mixed primary and secondary CESAs (Carroll *et al.*, 2012). However, these experiments are difficult to interpret since CESA trafficking and motility were not assessed, so it is unclear whether these different CESAs were incorporated into functional CSCs. The most compelling evidence for divergence from the hexamer-of-heterotrimers model in vascular plants comes from proteomic analysis of aspen (*Populus tremula*) during secondary cell wall synthesis. Developing xylem cells displayed a ratio of 3:2:1 for PtCESA8a/b:PtCESA4:PtCESA7a/b, which was exacerbated to 8:3:1 in developing tension wood, which synthesize extremely thick cell walls to counterbalance mechanical stress, suggesting that PtCESA8a/b might form homomers *in vivo* (Zhang *et al.*, 2018). Taken together, these data imply that while the 'typical' CSC may be an 18mer of heterotrimers, there may be some flexibility in CSC assembly, particularly during unusual demands on cell wall synthesis.

Structural information on PttCESA8 (Purushotham *et al.*, 2016) and GhCESA7 (X. Zhang *et al.*, 2021) homotrimers has generated testable hypotheses about CESA assembly into trimers and higher order assemblies (Wilson *et al.*, 2021; Pedersen *et al.*, 2023). Some studies have already leveraged these data to generate domain-swap and point mutations of CESAs that may affect multimerization (Kumar *et al.*, 2022; Olek *et al.*, 2023). Analysing these mutations with assays that directly assess CSC assembly, trafficking, and activity at the plasma membrane (Huang *et al.*, 2022, Preprint) may clarify the degree of flexibility in CSC assembly.

6) What activates CESAs in the plasma membrane?

The delivery of CSCs to the plasma membrane is well characterized. Freeze-fracture experiments have demonstrated that CESAs assemble into CSCs in the Golgi apparatus, but do not seem to make cellulose until they reach the plasma membrane (Fig. 3) (Haigler and Brown, 1986). Live-cell imaging and mutant analyses in Arabidopsis have implicated myosin XIK (W. Zhang *et al.*, 2019, 2021), exocyst complex components (Zhu *et al.*, 2018; W. Zhang *et al.*, 2021), PATROL1 (a Munc13-like protein), and CSI1 (Zhu *et al.*, 2018) in CESA exocytosis. Together, these studies have established an intricate timeline of CSC delivery to the plasma membrane (Zhu and McFarlane, 2022). However, after CSCs were delivered to the plasma membrane, they did not immediately begin to synthesize cellulose and remained immotile for an additional ~80 s (~10% of their total lifetime in the membrane; see Question 9). This pause continued even after components associated with vesicle trafficking dissociated (Huang *et al.*, 2022, Preprint), suggesting that this pause is related to activation of CSCs after they are delivered. Together, these data imply that CSCs must be somehow 'activated' once they reach the plasma membrane, but the question remains: what triggers CESA activity in the plasma membrane?

One hypothesis is that biochemical conditions must be suitable for CESA activation, such as substrate concentrations or a primer for cellulose synthesis. Substrate availability is not likely to be controlling CESA activity, since UDP-glucose levels were stable within cells, even as cellulose synthesis fluctuates with carbon availability (Ivakov *et al.*, 2017) (Question 2). Biochemical evidence implicated glucose-linked sterols in initiating cellulose synthesis in membrane fractions from cotton fibres (Peng *et al.*, 2002). However, PttCESA8 isolated from either *P. pastoris* (Purushotham *et al.*, 2016) or insect-derived Sf9 culture cells (Purushotham *et al.*, 2020) can synthesize β-glucan *in vitro* when supplied with only UPD-glucose and Mn^{2+}, indicating that no additional plant-derived factors are required for PttCESA8 activity. Furthermore, mutants affecting both UDP-Glc:sterol glycosyltransferases encoded by Arabidopsis displayed wild-type levels of cellulose (DeBolt *et al.*, 2009), suggesting that a primer is not strictly necessary for cellulose synthesis. A third possibility is that the initial stages of cellulose synthesis might be slow until the product reaches a length that can be stabilized in the translocation pore, after which the rate of synthesis could reach a steady state.

Alternatively, a signalling molecule might be required to initiate cellulose synthesis. In bacteria, cellulose synthesis required cyclic-di-GMP (Ross *et al.*, 1987), and the crystal structure of a bacterial (*Rhodobacter sphaeroides*) cellulose synthase documented that cyclic-di-GMP binds to the PilZ domain to release a 'gating loop' (Morgan *et al.*, 2014). Although the PilZ domain is absent from plant CESAs, an 'FTVTxK region' that seems to be analogous to the 'gating loop' of bacterial cellulose synthase was poorly resolved in the PttCESA8 structure, implying that it may be a flexible region (Purushotham *et al.*, 2020). However, mutations affecting this 'FTVTxK region' in AtCESA7 and PpCESA5 still provided partial functionality

(Slabaugh *et al.*, 2016; Burris *et al.*, 2021), so it remains unclear whether the 'gating loop' is strictly necessary for plant CESA regulation.

Phosphorylation is another signal that could activate CESAs, since it is rapid and reversible, and phosphorylation sites have been detected on several Arabidopsis CESAs (Cruz *et al.*, 2019). Importantly, mutations abolishing putative phosphorylation sites (S/T→A) or generating phosphomimic sites (S/T→D/E) altered CESA motility in the plasma membrane (Chen *et al.*, 2010, 2016; Bischoff *et al.*, 2011; Sánchez-Rodríguez *et al.*, 2017), implying that phosphorylation can affect CESA activity. None of these phosphosite mutations completely abolished CSC activity, suggesting that either other phosphorylation sites (or combinations of sites) remain to be tested, or else phosphorylation does not strictly activate CESAs, but rather it can fine-tune CESA activity in the plasma membrane.

In the future, systematically dissecting the combined function of previously identified CESA phosphosites may decisively address whether phosphorylation can activate CESAs, but an alternative approach is to identify kinases that may act upon CESAs. Currently, BIN2 is the only candidate kinase for CESA phosphorylation; however, results implied that BIN2-mediated phosphorylation decreased CESA1 activity (Sánchez-Rodríguez *et al.*, 2017), making it an unlikely signal for CSC activation in the plasma membrane. Several other plasma membrane-spanning kinases have been implicated in cell wall synthesis and cell wall signalling, but the mechanisms by which they affect cellulose synthesis remain unknown (Wolf, 2022). Identifying and functionally assessing other reversible post-translational modifications of CESAs (such as ubiquitination; Question 8) may provide further insight into CESA activation.

7) How are CSCs guided independently of microtubules, and why are multiple mechanisms governing CSC movement in the plasma membrane?

Co-alignment between cellulose fibres and cortical microtubules was described almost 60 years ago (Ledbetter and Porter, 1963), leading to several hypotheses about how microtubules in the cell cortex could guide CSCs in the plasma membrane (Emons *et al.*, 2007). CSCs move in ordered arrays (Box 2) that co-align with microtubules (Paredez *et al.*, 2006), and strong evidence for direct, microtubule-mediated guidance was provided by characterization of several proteins that interacted with CESAs and microtubules, including CSI1, CC1, and TTLs (Gu *et al.*, 2010; Li *et al.*, 2011; Bringmann *et al.*, 2012, Endler *et al.*, 2015; Kesten *et al.*, 2019a, 2022). Mutations affecting these components displayed reduced cellulose synthesis and disorganized CSC trajectories in the plasma membrane (Gu *et al.*, 2010; Li *et al.*, 2011; Bringmann *et al.*, 2012; Endler *et al.*, 2015; Kesten *et al.*, 2019a, 2022). Additionally, changes to cellulose synthesis organization via inhibitors or disrupting CESA-interacting proteins affected cortical microtubule array organization (Himmelspach *et al.*, 2003; Chu *et al.*, 2006; Paredez *et al.*, 2008; Liu *et al.*, 2016; Schneider *et al.*, 2022), providing further support for a physical interaction between CSCs and microtubules (Fig. 3). However, even in the absence of individual CESA–microtubule linker proteins, or under other conditions disrupting cortical microtubule organization, cellulose synthesis recovered into an ordered array in models of cellulose deposition (Emons and Mulder, 2000) and during both primary (Himmelspach *et al.*, 2003; Sugimoto *et al.*, 2003; Baskin *et al.*, 2004; Paredez *et al.*, 2006; Bringmann *et al.*, 2012) and secondary cell wall deposition (Schneider *et al.*, 2017). Recent live-cell imaging in Arabidopsis documented changes in CSC trajectories independent of co-localization with cortical microtubules (Chan and Coen, 2020; Duncombe *et al.*, 2022), implying that a microtubule-independent mechanism of CSC guidance may also exist. These data prompt two questions: how are CSCs guided independently of microtubules, and why are multiple mechanisms governing CSC movement in the plasma membrane?

One hypothesis for microtubule-independent CSC guidance is that components of the cell wall might influence CSC trajectories. For example, previously deposited cellulose microfibrils might interact with nascent glucan chains emerging from the CSC. Indeed, mild cellulase treatment partially disrupted microtubule-independent CSC guidance (Chan and Coen, 2020). Other plasma membrane-localized proteins with an extracellular domain displayed significantly reduced lateral mobility in the plasma membrane, and this effect was alleviated when the plasma membrane–cell wall interface was disrupted (Feraru *et al.*, 2011; Martinière *et al.*, 2012). These results imply that many proteins, not just CESAs, may interact with the cell wall as they move in the plasma membrane. In contrast to this hypothesis, ordered cellulose synthesis recovered after disruption of both microtubule organization and organized cellulose synthesis (Himmelspach *et al.*, 2003).

It is also possible that other cell wall components, such as pectins and/or hemicelluloses, could influence CSC movement. For example, loss of a rhamnose biosynthesis enzyme, RHM1, which is required for synthesis of RG-I pectins, results in cell twisting (Saffer *et al.*, 2017). Cell twisting is also a common phenotype of microtubule disorganization, presumably due to cellulose disorganization (Furutani *et al.*, 2000); however, microtubule organization was unaffected in *rhm1* mutants (Saffer *et al.*, 2017), suggesting that changes to cellulose organization, independent of microtubule organization, may be causing the twisting phenotype in RG-I-defective plants. Similarly, changes to HG synthesis caused changes to cellulose synthesis, cellulose organization, and CSC trajectories (Du *et al.*, 2020), implying a relationship between pectins and organized cellulose synthesis. However, HG defects also resulted in changes to microtubule organization (Du *et al.*, 2020), so it is unclear

whether the cellulose defects in these mutants are a secondary consequence of changes to microtubule organization, or a primary consequence of changes in the organization of cellulose synthesis. Considering hemicellulose-mediated CSC guidance, xylan was enriched at the borders of secondary cell wall thickenings and was required for pit formation by excluding cellulose (and presumably CESAs) from these developing regions (H. Wang *et al.*, 2022). Similarly, primary cell wall xyloglucan-defective *xxt1 xxt2* mutants displayed reduced and disorganized cellulose synthesis (Xiao *et al.*, 2016). Interestingly, *xxt1 xxt2 ktn1* triple mutants, defective in both xyloglucan synthesis and microtubule organization, were seedling lethal (Zhao *et al.*, 2019). These data suggest a synergistic interaction between microtubule organization and xyloglucan synthesis, which would be consistent with a hemicellulose-mediated mechanism of microtubule-independent CSC guidance. However, interpreting these results is complicated by feedback between cortical microtubule organization and cellulose synthesis (Himmelspach *et al.*, 2003; Chu *et al.*, 2006; Paredez *et al.*, 2008; Liu *et al.*, 2016; Schneider *et al.*, 2022).

An alternative hypothesis is that the plasma membrane might influence microtubule-independent CSC guidance. There is evidence that both primary and secondary CESAs can be acylated in Arabidopsis (Hemsley *et al.*, 2013; Kumar *et al.*, 2016b, 2022), which should affect their association with membranes; therefore, a heterogeneous lipid environment could guide CSC trajectories. Indeed, sterol synthesis mutants displayed cellulose-deficient phenotypes (Schrick *et al.*, 2004). However, it is unclear whether these phenotypes are due to changes in CSC movement in the plasma membrane, or whether CESA endocytosis and recycling are affected (Question 9), since genetic or pharmacological disruption of sterols affected endocytosis (Men *et al.*, 2008).

Whatever the mechanism of microtubule-independent CSC guidance, it seems that microtubule-mediated guidance can 'over-rule' microtubule-independent guidance (Chan and Coen, 2020). Therefore, microtubule-mediated CSC guidance may be particularly important when the orientation of cellulose deposition must be reorganized. For example, microtubule trajectories underwent cell-wide reorientation over time (Chan *et al.*, 2010), presumably to produce multilamellar cell walls in which newly synthesized cellulose is deposited at an angle relative to previously synthesized microfibrils. Similarly, microtubule-mediated CSC realignment played an important role during the early phases of secondary cell wall patterning, but became dispensable in the later stages (Schneider *et al.*, 2017), implying that the microtubule-mediated CSC guidance may be particularly important during developmental transitions. Microtubule reorientation is also important during stress responses, including mechanical stress (Hamant *et al.*, 2008; Schneider *et al.*, 2022) and salt stress (Endler *et al.*, 2015; Kesten *et al.*, 2019a, 2022). Therefore, microtubule reorientation coupled to microtubule-mediated CSC guidance could allow plant cells to reorganize cellulose synthesis to reinforce the cell wall as a mechanism to counteract stress.

Future experiments will be required to directly test which components contribute to microtubule-independent CSC guidance. For example, live-cell imaging of fluorescently tagged CSC trajectories in the plasma membrane in lipid-disrupted conditions and/or imaging of point mutations affecting CESA acylation (Hemsley *et al.*, 2013; Kumar *et al.*, 2016b, 2022) is required to test whether the lipid environment can contribute to CSC guidance. Similar experiments will be required to test whether different cell wall components are directly required for microtubule-independent CSC guidance. Combining mild microtubule depletion from the cell cortex (Chan and Coen, 2020) with super-resolution imaging of fluorescently tagged CSCs (Duncombe *et al.*, 2022) may provide additional insights into microtubule-independent CSC behaviour. Once the molecular mechanisms of microtubule-independent CSC guidance have been established, it will be possible to evaluate the biological importance of this guidance mechanism. In the meantime, the importance of microtubule-mediated CSC guidance is becoming clearer, particularly in studies of complex cell shapes, such as leaf epidermal pavement cells (Bidhendi *et al.*, 2019; Schneider *et al.*, 2022).

8) What signals trigger CESA endocytosis?

When their lifetime in the plasma membrane is complete, CESAs are endocytosed (Fig. 3). Live-cell imaging, mutant characterization, and proteomics have implicated clathrin-mediated mechanisms in CESA endocytosis (Bashline et al., 2013, 2015; Sánchez-Rodríguez *et al.*, 2018; Dahhan *et al.*, 2022). Although the timing of clathrin-mediated endocytosis has been analysed in detail (Wang *et al.*, 2020), these components have not been co-localized with CESAs in time-course experiments and it remains unclear whether a complete CSC may be endocytosed or whether the CSC disassembles into trimers or monomers before endocytosis (Zhu and McFarlane, 2022). Because clathrin-mediated endocytosis components assemble as non-motile puncta in the plasma membrane (Wang *et al.*, 2020), CSCs must pause in the plasma membrane before they can undergo endocytosis. However, the molecular mechanisms mediating this pause are unclear, and the question remains: what signals trigger CESAs for endocytosis?

Signals that might initiate CESA enzyme activity in the plasma membrane (Question 6) could presumably be reversed to inactivate CESAs before endocytosis. For example, if phosphorylation increases CESA activity, dephosphorylation could decrease activity; however, CESA phosphosite mutations modulated CSC activity without a dramatic effect on CESA localization (Chen *et al.*, 2010, 2016; Bischoff *et al.*, 2011), implying that other signals might target CESAs for endocytosis. The search for these signals is complicated by the variety of stresses that depleted CESAs from the plasma membrane and induced CESA accumulation in SmaCCs, including treatment with cellulose biosynthesis inhibitors (Box 2) (Paredez *et al.*, 2006;

Crowell *et al.*, 2009; Gutierrez *et al.*, 2009; McFarlane *et al.*, 2021), salt stress (Endler *et al.*, 2015; Kesten *et al.*, 2019a, 2022), osmotic pressure (Crowell *et al.*, 2009; Gutierrez *et al.*, 2009; Fujimoto *et al.*, 2014), protein synthesis inhibitors (Crowell *et al.*, 2009; Hill *et al.*, 2018b), fungal elicitors (Kesten *et al.*, 2019b), mechanical stress (Schneider *et al.*, 2022), and even stress induced during CESA imaging protocols (Verbančič *et al.*, 2021). Since most of these treatments were effective within minutes, it is assumed that the increased CESA signal in internal compartments, such as SmaCCs, is the result of endocytosis (Box 2), rather than a block in protein secretion (Hoffmann *et al.*, 2021). It is difficult to imagine signalling pathways that could integrate all of these conditions to effectively regulate CESA endocytosis on such a short time scale.

Interestingly, two independent proteomics studies detected CESA ubiquitination (Johnson and Vert, 2016; Grubb *et al.*, 2021). Plasma membrane proteins undergo ubiquitin-mediated changes in subcellular localization (Arora and van Damme, 2021), suggesting that CESA localization could also be regulated via ubiquitination. Intriguingly, the TASH3 subunit of the TPLATE adaptor-like complex for clathrin-mediated endocytosis interacted with ubiquitin (Grones *et al.*, 2022) and OsTASH3 interacted with OsCESA4 (Sánchez-Rodríguez *et al.*, 2018); however, it is unclear whether this interaction is dependent upon OsCESA4 ubiquitination.

Whatever the signal for CSC pausing and subsequent CESA endocytosis, it is likely that glucan chains emerging from the CSC must be cleaved before endocytosis (Delmer, 1999). A prime candidate for this activity is KORRIGAN (KOR), a transmembrane protein required for cellulose synthesis in both primary (Nicol *et al.*, 1998) and secondary cell walls (Szyjanowicz *et al.*, 2004). Purified *Brassica napus* KOR hydrolysed non-crystalline cellulose, but not crystalline cellulose or hemicelluloses (Mølhøj *et al.*, 2001), implying that it could act on nascent glucan chains as they emerge from active CESAs without hydrolysing crystalline cellulose in the cell wall. Furthermore, KOR has a TM and interacted with CESAs (Vain *et al.*, 2014), indicating that it probably acts at the plasma membrane, rather than in the cell wall. *kor1* mutants displayed similar phenotypes to mutants affecting CESA endocytosis (Bashline *et al.*, 2015; Sánchez-Rodríguez *et al.*, 2018), including increased CSC density in the plasma membrane and decreased CSC speed (Paredez *et al.*, 2008; Vain *et al.*, 2014). Upon treatment with cellulose biosynthesis inhibitors that normally induce CESA internalization into SmaCCs, less intracellular CESA signal was detected in *kor1* mutants (Vain *et al.*, 2014), specifically implicating KOR in CESA endocytosis. Alternatively, KOR could be involved in removing tensional stress during microfibril formation (Delmer, 1999).

Future experiments identifying the signals that can trigger CESA endocytosis will probably overlap with experiments designed to elucidate the mechanisms of CESA activation (Question 7). Proteomics approaches may address whether CESAs are ubiquitinated, and analysis of fluorescently tagged CESAs carrying mutations affecting potential ubiquitination sites may be able to test whether CESA ubiquitination can affect CSC assembly, trafficking, or activity. These mutants could also be used to directly test whether CCESA ubiquitination affects its interaction with components of clathrin-mediated endocytosis (Grones *et al.*, 2022). Further studies of the mechanisms that regulate CESA endocytosis may also shed light on the function of KOR in cellulose synthesis.

9) Why is a large population of inactive CESAs retained in the Golgi and why do CESAs cycle between the plasma membrane and the Golgi apparatus?

A significant proportion of CESAs in a cell are retained in the Golgi apparatus, and only a fraction of CESAs are actively synthesizing cellulose at the plasma membrane (Box 2) (Paredez *et al.*, 2006; Crowell *et al.*, 2009; Gutierrez *et al.*, 2009; Watanabe *et al.*, 2015). This localization pattern is distinct from most plasma membrane-localized proteins and prompts the question: why is such a significant proportion of CESAs retained in the Golgi apparatus, even when cellulose synthesis rates at the plasma membrane are high?

There are two possible trivial explanations for this phenomenon. First, the fluorescent protein tag could interfere with CESA trafficking. However, these constructs complemented their corresponding mutants (Gardiner *et al.*, 2003; Paredez *et al.*, 2006; Desprez *et al.*, 2007; Miart *et al.*, 2014), and both freeze-fracture (Haigler and Brown, 1986) and proteomics experiments from Arabidopsis, maize, and pine (Parsons et al., 2012, 2013; Nikolovski *et al.*, 2014; Heard *et al.*, 2015; Okekeogbu *et al.*, 2019) have detected untagged CESAs in the Golgi apparatus. Secondly, CESAs could take an unusually long time to mature before secretion to the plasma membrane. During time-course studies of VND induction (Box 3), secondary cell wall *CESA* transcripts increased within 4 h (Yamaguchi *et al.*, 2011), CESA7 protein was detectable within 6 h, and new CESA7 proteins reached the plasma membrane within 8 h (Watanabe *et al.*, 2018). These data are similar to reported maturation kinetics for other plasma membrane-localized proteins (Boutté *et al.*, 2013), which are not dual localized to the Golgi and plasma membrane. Therefore, it seems likely that there is a biological explanation for CESA localization in the Golgi apparatus.

CESAs would also be detected in both the Golgi and the plasma membrane if they are cycling between these two locations; this hypothesis predicts that CESA lifetime in the plasma membrane should be much shorter than the total lifetime of CESA proteins in the cell. Indeed, CESA1, CESA3, and CESA6 are sustained for several days in cycloheximide-treated Arabidopsis seedlings (Hill *et al.*, 2018b), although shorter lifetimes have been reported for GhCESA1 in cotton fibres

(Jacob-Wilk *et al.*, 2006). Tracking CSCs in the plasma membrane documented average lifetimes of 7–15 min (Paredez *et al.*, 2006; Sampathkumar *et al.*, 2013). The degree of polymerization of cellulose has been reported from 250 up to 10 000 glucosyl units (Klemm *et al.*, 2005), but many techniques and samples converge on values of ~3000–5000 (Hallac and Ragauskas, 2011). Since one glucose molecule is ~8.6 Å (BNID 110368; Milo *et al.*, 2009), a degree of polymerization of 3000–5000 would be roughly equivalent to a cellulose polymer of 2500–4500 nm. Mean CSC speeds in the plasma membrane ranged from 150 nm min^{-1} to 500 nm min^{-1} for AtCESA6 (Paredez *et al.*, 2006), AtCESA7 (Watanabe *et al.*, 2015), BdCESA3 (Liu *et al.*, 2017), and PpCESA5 (Tran *et al.*, 2018). Assuming 250 nm min^{-1}, a CSC would synthesize a chain of 2500–4500 glucose molecules within 10–18 min, and even the largest reported values (~14 000) would result in a CSC lifetime under 1 h, which is still orders of magnitude shorter than CESA protein lifetime (Hill *et al.*, 2018b). Therefore, these data imply that CESAs may be reused as they cycle between being active CSCs in the plasma membrane and inactive CESAs in the Golgi apparatus, and this cycling may regulate the length of cellulose fibres.

Interestingly, in freeze-fracture studies of the moss *Funaria hygrometrica*, only 75% of CSC-like particles had a clear 'rosette-like' structure with 6-fold symmetry (Fig. 3) and the remaining 25% of CSC-like particles seemed to be in various states of disassembly (e.g. six subunits in an 'open circle' conformation or less than six subunits), which may represent CSCs in the process of being endocytosed or recycled (Reiss *et al.*, 1984). Several endocytosis-defective mutants showed increased CESA density, but decreased CESA motility in the plasma membrane, and decreased cellulose content (Bashline *et al.*, 2015; Sánchez-Rodríguez *et al.*, 2018). Furthermore, mutants that are defective in CSC trafficking from the Golgi to the plasma membrane are hypersensitive to treatments that disrupt cell wall synthesis (Zhang *et al.*, 2016; He *et al.*, 2018; McFarlane *et al.*, 2021; Vellosillo *et al.*, 2021), suggesting that in wild-type cells, CESAs in the Golgi apparatus can be mobilized to help plants recover from cell wall stress. Together, these data suggest that CESA cycling between the Golgi and the plasma membrane does more than just regulate the degree of β-glucan chain polymerization and that this CESA cycling is required to maintain CESA activity.

Further analysis of mutants that are defective in CSC trafficking from the Golgi to the plasma membrane (Zhang *et al.*, 2016; He *et al.*, 2018; McFarlane *et al.*, 2021; Vellosillo *et al.*, 2021) or CESA endocytosis (Bashline *et al.*, 2015; Sánchez-Rodríguez *et al.*, 2018) may clarify the biological functions of CESA cycling. For example, a thorough analysis of the degree of cellulose polymerization in these mutants could address whether CESA cycling, and therefore dual CESA localization to both the Golgi apparatus and the plasma membrane, is necessary for regulating the degree of β-glucan chain polymerization.

Conclusion

Increasing interest in plant cell wall-derived bioproducts has spurred tremendous progress in plant cell wall synthesis research. However, as new information is uncovered, new questions have arisen, and several long-standing questions remain unanswered. Key technical advances have pushed cell wall research forward, including structural information on several cell wall synthesis enzymes, a set of antibodies raised against cell wall polysaccharides and glycoproteins, herbicides that specifically affect cell wall synthesis, live-cell imaging protocols to track cell wall synthesis, and an inducible secondary cell wall synthesis system. Combining these advances with emerging technologies, such as synthetic glycan arrays to assess enzyme activity, the increasing diversity of sequenced plant genomes to identify cell wall synthesis enzymes not present in Arabidopsis, and CRISPR/Cas9 mutational approaches to assess *in planta* enzyme function, new advances and more questions are sure to follow.

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Conflict of interest

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