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REVIEW: PART OF A SPECIAL ISSUE ON PLANT CELL WALLS

The trafficking of the cellulose synthase complex in higher plants

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• **Background** Cellulose is an important constituent of plant cell walls in a biological context, and is also a material commonly utilized by mankind in the pulp and paper, timber, textile and biofuel industries. The biosynthesis of cellulose in higher plants is a function of the cellulose synthase complex (CSC). The CSC, a large transmembrane complex containing multiple cellulose synthase proteins, is believed to be assembled in the Golgi apparatus, but is thought only to synthesize cellulose when it is localized at the plasma membrane, where CSCs synthesize and extrude cellulose directly into the plant cell wall. Therefore, the delivery and endocytosis of CSCs to and from the plasma membrane are important aspects for the regulation of cellulose biosynthesis.
• **Scope** Recent progress in the visualization of CSC dynamics in living plant cells has begun to reveal some of the routes and factors involved in CSC trafficking. This review highlights the most recent major findings related to CSC trafficking, provides novel perspectives on how CSC trafficking can influence the cell wall, and proposes potential avenues for future exploration.

Key words: Plant cell wall, cellulose synthase, microtubule, actin, trafficking, endocytosis, exocytosis, *trans*-Golgi network.

INTRODUCTION

Polysaccharides are the major constituent of plant cell walls, with proteins comprising a much lesser portion of the wall. As one of the major cell wall polysaccharides, cellulose forms long, rigid crystalline microfibrils that provide the major load-bearing role of the cell wall. Each microfibril contains multiple β1,4-linked glucan chains (Cosgrove, 2005; Somerville, 2006; Lei *et al.*, 2012*a*). In higher plants, cellulose is synthesized by large multi-protein complexes. The hexagonal structure of the complexes and the positioning of the complexes at the ends of cellulose microfibrils that were observed in electron micrographs led researchers to refer to the cellulose-synthesizing machinery as rosettes or terminal complexes (Giddings *et al.*, 1980; Mueller and Brown, 1980; Haigler and Brown, 1986; Brown *et al.*, 1996; Kimura *et al.*, 1999). Currently, cellulose synthase (CESA) proteins are the only known permanent components of the cellulose-synthesizing rosette complexes, which has led to the coining of another name for the complexes, the cellulose synthase complex (CSC) (Kimura *et al.*, 1999; Brett, 2000; Somerville, 2006; Lei *et al.*, 2012*a*; Li *et al.*, 2014). Unlike other cell wall polysaccharides that are synthesized in the Golgi apparatus and subsequently transported to the plasma membrane for secretion through vesicular trafficking, cellulose is synthesized only when CSCs are located at the plasma membrane where the cellulose is extruded directly into the cell wall (Fig. 1) (Reiter, 2002; Scheible and Pauly, 2004; Cosgrove, 2005; Oikawa *et al.*, 2013). The prerequisite that CSCs must be localized at the plasma membrane to synthesize cellulose demonstrates the importance of understanding the pathways and mechanisms by which CSCs are trafficked to and from the plasma membrane.

This review will highlight the current observations of CSC localization and behaviour as well as the recent advancements in understanding plant trafficking pathways and mechanisms that are associated with the trafficking of CSCs. Topics of discussion will include: the current observations of CSC localization and behaviour, implications of the Golgi and *trans*-Golgi network (TGN) in CSC assembly and trafficking, and the possible mechanisms and pathways of CSC secretion, endocytosis and recycling.

THE CELLULOSE SYNTHASE COMPLEX AND ITS VISUALIZATION AT THE PLASMA MEMBRANE

CSC composition and behaviour at the plasma membrane

Prior to investigating the trafficking of CSCs, it is important to understand the composition and behaviour of CSCs in higher plants. The large size, high level of organization and multimeric nature of the CSC suggests that precise and regulated processes are involved in the assembly and subsequent handling of the complex throughout trafficking processes. CSCs were observed to be frequently associated with the ends of cellulose microfibrils in algae and higher plants (Giddings *et al.*, 1980; Mueller and Brown, 1980). In higher plants, the membrane-spanning portions of intact CSCs are visualized as six-lobed, hexagonal rosette structures that each have 6-fold rotational symmetry and a 25–30 nm diameter in electron micrographs of freeze-fracture replicas from maize and *Zinnia* (Mueller and Brown, 1980; Haigler and Brown, 1986; Brown *et al.*, 1996). Immunogold freeze-fracture electron microscopy experiments have shown the existence of CESA proteins in CSCs (Kimura *et al.*, 1999), and genetic and co-immunoprecipitation studies in arabidopsis have shown that each CSC contains multiple isoforms of

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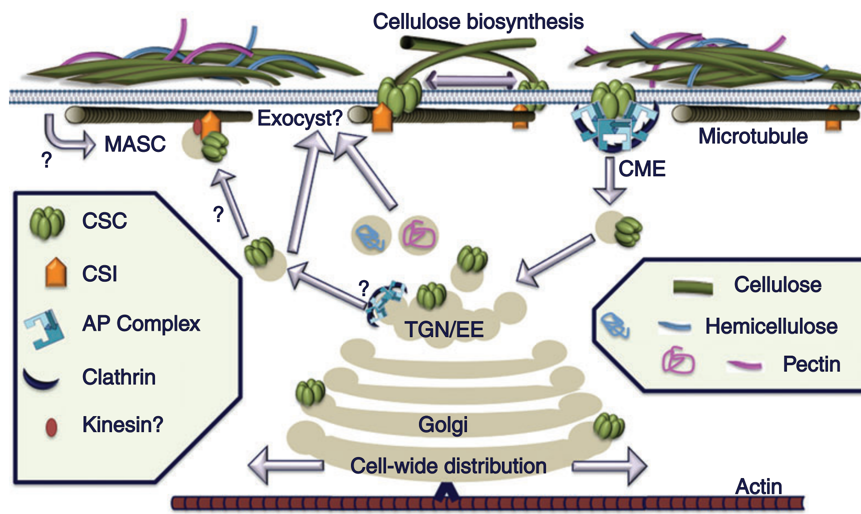

FIG. 1. An overview of the localization, trafficking pathways and proposed trafficking mechanisms of CSCs. The schematic diagram shows the many cell wall-related trafficking compartments and pathways that have been proposed. Pectin and hemicellulose are synthesized in the Golgi apparatus, and the polymers are secreted to the wall, whereas cellulose is exclusively synthesized by CSCs that are located at the plasma membrane. The CSC is thought to be assembled in the Golgi apparatus, which is responsible for the actin-dependent cell-wide distribution of CSCs. CSCs are believed to be secreted through the TGN/EE and may be partitioned into a specific domain of the TGN/EE. The mechanisms for trafficking of CSCs away from the TGN are unknown, but might involve clathrin-mediated vesicle formation. The mechanism of the fusion of CSC-containing vesicles at the plasma membrane is also unknown, but it is known that these events occur at the cortical microtubules. We speculate that the exocyst complex might be involved in the fusion event. Plasma membrane-localized CSCs move bi-directionally along cortical microtubules and are believed to be propelled by the force of cellulose polymerization. CSI proteins mediate the CSC–microtubule interaction. The endocytosis of CSCs have been shown to occur through AP2/clathrin-mediated endocytosis, and newly endocytosed material is known to be rapidly trafficked to the TGN/EE where recycling of CSCs might occur. CSC and CSI-containing cortical MASCs could be responsible for secretion, endocytosis and/or recycling, and we propose that a kinesin motor might be involved in the motility of MASCs at microtubule-depolymerizing ends.

CESA proteins (Taylor *et al*., 2000, 2003; Desprez *et al*., 2007; Persson *et al*., 2007; Wang *et al*., 2008; Atanassov *et al*., 2009; Lei *et al*., 2012*a*). Genetic studies in arabidopsis have greatly advanced our understanding of the CSC. For cellulose synthesis in both primary and secondary cell walls, three distinct CESA proteins are required to form a functional CSC (Taylor *et al*., 2000; Desprez *et al*., 2007; Persson *et al*., 2007). CESA1 and CESA3 are essential genes for cellulose biosynthesis during primary cell wall synthesis. Loss of either CESA1 or CESA3 leads to gametophytic lethality, indicating that the roles of CESA1 and CESA3 in CSC formation and function are essential and irreplaceable (Persson *et al*., 2007). CESA6 and CESA6-like proteins such as CESA2, CESA5 and CESA9 fill the role of the third CESA isoform position of primary CSCs (Desprez *et al*., 2007; Persson *et al*., 2007). Secondary cell walls are deposited in select cell types, mainly in the plant vascular tissues, after the cell expansion has ceased. For cellulose synthesis during secondary cell wall formation, CESA4, CESA7 and CESA8 are each essential to the formation of the CSC (Taylor *et al*., 2000, 2003; Atanassov *et al*., 2009). The original model of CESA protein stoichiometry within a CSC proposed that 36 CESA proteins make up each CSC, with each of the six lobes of the CSC being comprised of a CESA hexamer (Delmer, 1999; Doblin *et al*., 2002). Based on this 36-mer CSC model, each CSC would synthesize an elementary cellulose fibril containing 36 glucan chains, assuming that each CESA is simultaneously synthesizing a single glucan chain. Recently, several studies have been conducted to measure the precise dimensions of elementary cellulose fibrils and to model the number of glucan chains within each elementary fibril (Davies and Harris, 2003; Yan *et al*., 2004; Fernandes *et al*., 2011; Newman *et al*., 2013; Thomas *et al*., 2013; Zhang *et al*., 2013). These recent measurements show that elementary fibrils are approx. 3 nm in diameter, which is too narrow to accommodate 36 glucan chains, but very compatible with models in which elementary fibrils contain 18 or 24 glucan chains (Fernandes *et al*., 2011; Newman *et al*., 2013; Thomas *et al*., 2013). However, the exact number of CESA proteins in a CSC cannot be deduced simply by counting the number of glucan chains within an elementary fibre. It is possible that there is not a 1:1 ratio between the number of glucan chains in an elementary fibre and the number of CESA proteins in a CSC due to the possibilities that some of the CESA proteins within each CSC may be enzymatically inactive or that more than one CESA may be required to synthesize a single glucan chain. These scenarios have not been experimentally tested. Nevertheless, it remains clear that each CSC is comprised of many CESA proteins and that the synthesis and organization of such a large protein complex requires a vast amount of dedicated energy from the plant cell. This notion stresses the importance of proper handling, trafficking and conservation of each CSC and implies that processes for the recycling of CSC components might exist.

Many of the major advances in the understanding of cellulose biosynthesis in the past decade have been due to the development of fluorescent protein fusions with CESA proteins and the use of fluorescence microscopy to study CSC dynamics in living cells. Expression of a yellow fluorescent protein (YFP)-tagged CESA6 protein was able to complement the *cesa6*^{prc1} null mutation, which indicates that YFP–CESA6 integrates into functional CSCs (Paredez *et al*., 2006). YFP–CESA6-labelled CSCs have been visualized as diffraction-limited particles that move within the plane of the plasma membrane at a range of velocities,

with an average velocity of around 300 nm min^{-1} (Paredez *et al*., 2006; Desprez *et al*., 2007). The displacement of the CSCs is believed to correspond to the synthesis of elementary cellulose fibres by the CSCs. Therefore, several parameters of the behaviour of fluorescent CSCs including the lifetime, velocity, density and organization of plasma membrane-localized CSCs can have implications in the properties of the cellulose microfibrils being synthesized in the plant cell wall. Furthermore, many of these parameters are expected to be influenced by the trafficking of CSCs.

The influence of CSC trafficking on the length of elementary cellulose fibres

For example, the length of elementary cellulose fibres should be the product of the lifetime and the velocity of a CSC in the plasma membrane. Although it is not known exactly how plant cells control cellulose microfibril length (Somerville, 2006), it is possible that a mechanism involving the delivery and internalization of CSCs to and from the plasma membrane may control elementary fibre length. The direct measurement of the lifetime of plasma membrane-localized CSC particles is not conducive to quantification. The lifetime of plasma membrane-localized CSC particles is suspected to be $>$ 15 min (Paredez *et al*., 2006) and potentially as long as 40 min (Bashline *et al*., 2013). As of yet, it has not been possible to track confidently a single motile particle that is among a high-density population of similar particles over such a long time period. The use of fluorescence recovery after photobleaching (FRAP) techniques might be able to assist in the direct measurement of CSC lifetimes at the plasma membrane. Bleaching of plasma membrane-localized CSCs has already been used to observe the delivery of CSCs to the plasma membrane (Gutierrez *et al*., 2009; Bringmann *et al*., 2012; Bashline *et al*., 2013; Sampathkumar *et al*., 2013). After visualizing the insertion of new CSCs at the plasma membrane, a significant challenge remains in whether single CSCs can be continually tracked from the time of insertion until the time of endocytosis without confounding events taking place such as coalescence or co-localization with other CSCs that could cause misidentification of one CSC for another, migration of the CSC to the cell edge causing the CSC to leave the focal plane or photobleaching of the CSCs due to long observation times. Nevertheless, it is suspected that the rate of delivery and the rate of internalization should correlate with the lifetime of CSCs at the plasma membrane, which would also correlate with the length of cellulose elementary fibres.

The influence of CSC trafficking on the density of plasma membrane CSCs

Similarly, CSC trafficking processes probably mediate the density of plasma membrane-localized CSCs. In the epidermal cells near the apical hook of etiolated arabidopsis hypocotyls, many large intracellular compartments, which have been commonly identified as Golgi bodies, are intensely labelled by primary CESAs (Paredez *et al*., 2006; Crowell *et al*., 2009; Gutierrez *et al*., 2009). This observation suggests that a large pool of CSCs may be available for trafficking to the plasma membrane at all times in cells that are actively synthesizing cellulose and that the rates of delivery and endocytosis of CSCs, rather than the rate of *de novo* CSC synthesis, might play a large role in regulating the density of CSCs at the plasma membrane. In support of this hypothesis, an endocytosis-deficient mutant has been shown to have an increased density of CSCs at the plasma membrane in etiolated hypocotyls, suggesting that a reduced rate of endocytosis is sufficient for changing CSC density (Bashline *et al*., 2013).

The influence of CSC trafficking on the organization of plasma membrane CSCs

In addition to altering the lifetime and density of CSCs, trafficking may also affect the organization of CSCs at the plasma membrane. The orientation of cellulose microfibrils in the cell wall is determined to a large extent by the organization and direction of CSC movement in the plasma membrane. Although parallel cellulose microfibril arrays appear to be established and maintained in the absence of microtubules (Baskin, 2001; Himmelspach *et al*., 2003; Sugimoto *et al*., 2003; Wasteneys, 2004), many studies support the existence of a direct and functional link between cellulose-biosynthesizing CSCs and cortical microtubules (Paredez *et al*., 2006; Gu and Somerville, 2010; Gu *et al*., 2010; Lei *et al*., 2012*a*, *b*; S. Li *et al*., 2012, 2014). The alignment hypothesis states that cortical microtubules, which run along the inside of the plasma membrane of plant cells, determine the orientation of cellulose microfibrils (Green, 1962; Heath, 1974; Robinson, 1982; Baskin, 2001). In support of the alignment hypothesis, the simultaneous observation of plasma membrane-localized fluorescent CSCs and cortical microtubules in living plant cells showed that CSCs are co-localized with microtubules, that CSCs moved along tracks that coincided with microtubules and that the reorientation of cortical microtubules caused a co-ordinated reorientation of CSC tracks (Paredez *et al*., 2006). Furthermore, additional support for the relationship between the CSC and microtubules has arisen from the investigation of Cellulose Synthase Interactive protein 1 (CSI1), which has been characterized as a linker protein between the CSC and cortical microtubules (Fig. 1) (Gu and Somerville, 2010; Gu *et al*., 2010; Lei *et al*., 2012*b*). CSI1 was shown to interact with CESA proteins and microtubules, and the loss of CSI1 led to a disassociation between CSCs and cortical microtubules, which indicates that CSI1 probably serves as a link between CSCs and microtubules that is essential for their co-alignment (Lei *et al*., 2012*b*; S. Li *et al*., 2012). CSC delivery events at the plasma membrane have been shown to coincide with the location of cortical microtubules (Fig. 1) (Gutierrez *et al*., 2009). Therefore, the trafficking of CSCs to the plasma membrane preferentially at the sites of cortical microtubules acts as an inherent CSC organization mechanism.

CSCS IN THE GOLGI APPARATUS AND THE TGN

The assembly of CSCs within the Golgi apparatus

The Golgi apparatus plays a role in macromolecular sorting, packing and secretion, and in protein modification (Oikawa *et al*., 2013). Although CESA proteins are probably synthesized at the endoplasmic reticulum, the earliest part of the endomembrane system in which CSCs have been observed is the Golgi apparatus (Fig. 1). Rosette CSC structures have been observed in electron micrographs at the *trans* side of the Golgi and in

Golgi-derived vesicles, suggesting that the Golgi apparatus is the site of CSC assembly (Haigler and Brown, 1986). In an immunogold labelling experiment, green fluorescent protein (GFP)–CESA3 was located in the middle and the *trans* side of the Golgi (Crowell *et al*., 2009). These observations are consistent with the appearance of fluorescent protein-tagged CESAs in the Golgi in several live cell imaging analyses (Gardiner *et al*., 2003; Paredez *et al*., 2006; Desprez *et al*., 2007; Crowell *et al*., 2009; Gutierrez *et al*., 2009; Miart *et al*., 2013). While the mechanism of CSC assembly in the Golgi remains unknown, the process of assembly of CESAs into large CSCs in the Golgi raises the following questions: does the large size of CSCs require special trafficking machinery and mechanisms; does CSC assembly act as a check point in CSC secretion; and how does the structure and composition of the CSC influence its trafficking?

Although genetic analyses indicate that three distinct CESA proteins are required for adequate cellulose synthesis, it is not known whether the number and arrangement of each of the three distinct CESA proteins is the same in every CSC or whether there is some degree of heterogeneity among CSCs. A few observations appear to support the notion of a heterogeneous CSC composition. First, plasma membrane-localized CSCs exhibit a wide range of velocities (Paredez *et al*., 2006; Desprez *et al*., 2007; Gu *et al*., 2010; S. Li *et al*., 2012). Although the large variation in CSC velocities can arise from a variety of factors including differences in membrane fluidity, differences in microtubule association, differences in substrate availability, and so on, another possibility is that each CSC contains a different combination or arrangement of CESA protein isoforms that causes a variation in the enzymatic activity of the CSCs. Secondly, complementation analyses have shown that CSCs with a mixed composition of primary and secondary CESAs are functional (Carroll *et al*., 2012; Li *et al*., 2013*b*). CESA1, a primary wall CESA, can partially rescue the phenotype of the secondary wall *cesa8^{irx1}* mutant when expressed under a secondary CESA promoter (Li *et al*., 2013*b*). Similarly, CESA7, a secondary wall CESA, can partially rescue the phenotype of the primary wall *cesa3^{je5}* mutant when expressed under the control of a primary CESA promoter (Carroll *et al*., 2012). Thirdly, the partial redundancy between CESA6 and the CESA6-like proteins, CESA2, CESA5 and CESA9, suggests that these four isoforms might be interchangeable to some extent (Desprez *et al*., 2007; Persson *et al*., 2007). These studies imply that the CSC composition is dependent on the types of CESA proteins that are available during assembly. Given that some CESA proteins are essential, it may be possible that certain positions within the CSC can only be assumed by one particular isoform, while other positions are more flexible. The idea of heterogeneous CSCs would allow more flexibility in the assembly of CSCs and in the re-assembly of CSCs from a recycled population of CESA proteins, which could lead to higher efficiency in CSC assembly. If heterogeneity of CESAs within the CSC streamlines the assembly of CSCs, it could also make CSC trafficking away from the Golgi more efficient by providing CSC cargo more rapidly. However, CSC heterogeneity also comes with a cost to trafficking processes in that it requires the trafficking machinery to exhibit some promiscuity in order to recognize CSCs with variable CESA composition. Some evidence for promiscuity of CSC trafficking machinery has come from the observation that an adaptin involved in CESA endocytosis can interact with multiple CESA isoforms (Bashline *et al*., 2013).

The assembly of CSCs in the Golgi may also be influenced by other factors. Genetic screens for cellulose-deficient mutants in arabidopsis have identified genes that encode Golgi-localized proteins. Mutants within the *N*-glycosylation pathway, a mannose-1-phosphate guanylyltransferase (CYT1) mutant and an α-glucosidase I (KNOF) mutant, had deficiencies in *N*-glycosylation that led to severe phenotypes and cell wall defects (Lukowitz *et al*., 2001; Gillmor *et al*., 2002). Since CESA proteins are not thought to be *N*-glycosylated, it has been postulated that the cell wall defects of *cyt1* and *knof* may result from defects in the glycosylation of other proteins involved in cellulose biosynthesis. KORRIGAN (KOR1), a putative endo-β1,4-glucanase that is important for cellulose biosynthesis, has been shown to be glycosylated (Nicol *et al*., 1998; Lane *et al*., 2001; Szyjanowicz *et al*., 2004). In arabidopsis, KOR1 is a transmembrane protein that is localized to the Golgi, TGN and the plasma membrane (Robert *et al*., 2005), and KOR1 has been proposed to be part of the CSC (Doblin *et al*., 2002). Although orthologues of KOR1 have been shown to have cellulase activity *in vitro* (Molhoj *et al*., 2001; Master *et al*., 2004), it is possible that KOR1 is a multifunctional protein. The lethality of the KOR1 null mutant complicates the investigation into whether KOR1 plays a role in CSC assembly in the Golgi apparatus and whether glycosylation of KOR1 is important for its putative function in CSC assembly. Interestingly, mutations within polarized targeting motifs of KOR1 changed the localization of KOR1, suggesting that KOR1 trafficking is tightly regulated (Zuo *et al*., 2000). If KOR1 is associated with the CSC, KOR1 could also influence CSC trafficking; therefore, it would be interesting to investigate whether CSC localization is also affected in KOR1 polarized targeting motif mutants.

CSCs in the TGN

The TGN is a dynamic sorting station for the formation of vesicles targeted to various membranous organelles (Toyooka *et al*., 2009; Viotti *et al*., 2010; Contento and Bassham, 2012). Several observations suggest that assembled CSCs are delivered to the plasma membrane through a regulated, selective process rather than a constitutive, random exocytosis process. First, there are very few CSC-containing vesicles in a plant cell that is actively synthesizing cellulose (Crowell *et al*., 2009; Gutierrez *et al*., 2009). Secondly, a single CSC-containing vesicle can pause at different positions close to the plasma membrane to insert CSCs (Crowell *et al*., 2009; Gutierrez *et al*., 2009). Thirdly, CSC signals in the TGN are only partially co-localized with markers of the TGN, indicating that CSCs might partition into a specific, specialized domain of the TGN (Crowell *et al*., 2009; Gutierrez *et al*., 2009). Some evidence supports the existence of specialized sub-domains within the TGN based on the differential localization of TGN proteins (Bassham *et al*., 2000; Chow *et al*., 2008; Gendre *et al*., 2011, 2013; Drakakaki *et al*., 2012; Boutte *et al*., 2013). The machinery required for the formation of CSC-containing vesicles at the TGN is unknown. Clathrin-mediated vesicle formation at the TGN has recently been characterized in arabidopsis (Park *et al*., 2013; Teh *et al*., 2013; J. G. Wang *et al*., 2013), and the involvement

of clathrin-mediated endocytosis in the internalization of CESAs (Bashline *et al*., 2013) might support the idea that a clathrin-mediated mechanism could be involved in trafficking CSCs away from the TGN (Fig. 1). Other recent advances in elucidating the composition of the plant TGN may reveal important factors involved in the trafficking of specific cargos including CSCs. For example, CESA proteins were identified in a proteomic analysis of SYP61-labelled vesicles (Drakakaki *et al*., 2012; Worden *et al*., 2012). The challenge will be to identify the molecular components that are specific to CSC trafficking. If an analogous enrichment and proteomic analysis of CESA-containing vesicles could be performed, the molecular mechanisms by which CESA is trafficked might be discerned.

The early endosomes (EEs) of plants are important organelles for the regulation of plant signalling and endomembrane functions (Reyes *et al*., 2011; Contento and Bassham, 2012). Evidence has grown to support that the EE is the same compartment as the TGN in plants (TGN/EE); therefore, the TGN/EE compartment contains both secretory material and material that has been recently retrieved from the plasma membrane (Fig. 1) (Dettmer *et al*., 2006; Richter *et al*., 2009; Viotti *et al*., 2010; Reyes *et al*., 2011; Uemura and Nakano, 2013). CESA signals of the TGN/EE could be part of the *de novo* secretion pathway, the endocytic pathway or a recycling pathway. Since recently endocytosed CESAs and new CESAs both localize to the TGN/EE, the plausibly of a CESA recycling process seems rather high; however, due to the double role of the TGN/EE, the investigation of CESA recycling mechanisms has been challenging. As mentioned previously, it would be greatly advantageous for plant cells to recycle the CSC due to the energy cost of synthesizing such a large protein complex. A recent study of CESA signals during cytokinesis suggests that plant cells might relocate CSCs from the plasma membrane to the cell plate during cytokinesis and subsequently release them back to plasma membrane after the two cells have divided (Miart *et al*., 2013). Also, since CSCs are preferentially delivered to the sites of cortical microtubules, a recycling mechanism may act in organizing the plasma membrane-localized CSC population by selectively removing CSCs that are not associated with microtubules and recycling these CSCs back to the microtubules.

THE DELIVERY OF CSCS

CSC-containing vesicles near the plasma membrane

Early studies clearly demonstrated the presence of CSCs in vesicles at the cell periphery near the plasma membrane (Giddings *et al*., 1980; Haigler and Brown, 1986). Using fluorescent protein-tagged primary CESA proteins as markers, CSC-containing vesicles, referred to as small CESA compartments (SmaCCs), are detected in the cytosol at low frequency (Gutierrez *et al*., 2009). Interestingly, these vesicles are seen more frequently in cells in which cellulose biosynthesis in the primary cell wall has slowed or reached completion compared with cells that are actively synthesizing cellulose (Crowell *et al*., 2009). Since osmotic stress or treatment with isoxaben, a cellulose synthesis inhibitor that removes the plasma membrane-localized population of CSCs from the membrane, induces the formation of SmaCCs (Crowell *et al*., 2009; Gutierrez *et al*., 2009; Lei *et al*., 2012*b*), it is likely that some of these vesicles represent CSCs that have been removed from the plasma membrane. However, these vesicles are also hypothesized to represent secretory vesicles. The targeting of the CSC-containing vesicles to specific sites at the plasma membrane for CSC insertion is critical in determining the distribution pattern of plasma membrane-localized CSCs.

Cytoskeletal influence on the positioning of CSC insertion events

Transport of membranous vesicles in plant cells relies on the cytoskeleton (Reddy, 2001). The actin cytoskeleton has been shown also to affect the distribution of plasma membrane-localized CSCs (Gardiner *et al*., 2003; Wightman and Turner, 2008; Crowell *et al*., 2009; Gutierrez *et al*., 2009). The actin cytoskeleton is responsible for the cell-wide distribution of the CSC-containing Golgi organelles (Fig. 1). This is consistent with the finding that myosins are involved in the movement of the Golgi bodies in plants (Prokhnevsky *et al*., 2008; Sparkes *et al*., 2008; Avisar *et al*., 2009). A pharmacological disruption of the actin cytoskeleton causes an abnormal distribution of CSC-containing Golgi bodies in which the Golgi bodies aggregate in certain areas and are absent in other areas (Gutierrez *et al*., 2009). As a consequence, this results in an uneven distribution of CSC insertion events at the plasma membrane such that CSC insertion events occur exclusively in the areas of aggregated Golgi bodies. Thus, CSC-containing vesicles formed at the TGN are restricted to short travelling distances. Although actin is not essential for either the formation of CSC-containing vesicles or the insertion of CSCs at the plasma membrane (Gutierrez *et al*., 2009), it is not known if actin is involved in the transportation of the CSC-containing vesicles to sites at the plasma membrane.

Cortical microtubules play an important role in marking the delivery sites for the insertion of CSCs at the plasma membrane (Crowell *et al*., 2009; Gutierrez *et al*., 2009). The simultaneous visualization of CSC-containing vesicles and cortical microtubules revealed that SmaCCs are often associated with cortical microtubules. The CSC-containing vesicles that are attached to microtubules were named microtubule-associated cellulose synthase compartments (MASCs) (Fig. 1) (Crowell *et al*., 2009; Gutierrez *et al*., 2009). Interestingly MASCs exhibit sporadic motility such that MASCs either remain stationary or rapidly move by tracking the depolymerizing ends of cortical microtubules (Crowell *et al*., 2009; Gutierrez *et al*., 2009). Although it was proposed that kinesin motors are not involved in the tracking of MASCs at the depolymerizing ends of microtubules (Crowell *et al*., 2009; Gutierrez *et al*., 2009), the mechanism of MASC vesicle attachment to depolymerizing microtubule ends is unknown. Recent descriptions of kinesin motor velocities (Zhu and Dixit, 2011) suggest that kinesin motors are capable of moving at velocities that exceed the maximum velocity of MASCs. Therefore, it seems plausible to suggest that an undiscovered kinesin may indeed fulfil the role of associating MASCs with microtubules, and, upon microtubule depolymerization, the kinesin could be triggered to propel MASC motility by running along the microtubule just ahead of the depolymerizing end of the microtubule (Fig. 1).

The arabidopsis genome contains a large family of genes encoding kinesin motor proteins, and the cargos of these kinesins are largely unknown (Zhong *et al*., 2002; Lee and Liu, 2004). One candidate kinesin for MASC trafficking is FRA1 (*fragile*

fiber 1). The *fra1* mutant produces a disorganized cellulose microfibril network in secondary walls of fibre cells (Zhong *et al*., 2002). FRA1 contains a motor domain that moves towards the plus ends of microtubules at high velocity and with high processivity (Zhu and Dixit, 2011). The involvement of FRA1 or other kinesins in the transportation of MASCs on microtubules has yet to be tested. Interestingly, the rice gene *BC12* (*brittle culm 12*) encodes a kinesin, and the *bc12* mutant also has an alteration in cellulose microfibril orientation (Zhang *et al*., 2010). Although the BC12 protein has been shown to regulate gibberellic acid biosynthesis in the nucleus (Li *et al*., 2011), BC12 might also play a microtubule-related role in the cytosol that involves the trafficking of CSC-containing vesicles. A genetic approach may be helpful in identifying novel kinesins that play a role in CSC trafficking by screening kinesin mutants for cellulose deficiency phenotypes and subsequently investigating changes in CSC trafficking in the mutants.

If kinesins are involved in moving MASCs along microtubules, it will be interesting to investigate how kinesins interact with MASCs and how kinesins are recruited to MASCs. The central domains of CESA proteins are expected to face the cytosol and have been shown to interact with other proteins (Gu *et al*., 2010; Bashline *et al*., 2013). CESA central domains might also interact with kinesins. Compared with bacterial CESAs, the central domains of CESA proteins in higher plants possess additional domains called the plant conserved region (PCR) and a class-specific region (CSR) (Richmond, 2000). In a recent computationally predicted tertiary model of the central domain of a CESA from cotton, the PCR and CSR are displayed to the exterior of the catalytic core and remain exposed for interactions with other proteins (Sethaphong *et al*., 2013). To date, CSI1 and CSI3 are the only proteins, apart from CESA proteins, known to be associated with MASCs (Bringmann *et al*., 2012; Lei *et al*., 2012*b*, 2013). Since the loss of CSI1 causes CSCs to be disconnected from microtubules (Bringmann *et al*., 2012; S. Li *et al*., 2012), it remains unanswered whether CSI1 plays a role in the attachment or movement of MASCs on microtubules or whether CSI1 participates in recruiting kinesins to MASCs. The KOR1 protein is another reasonable candidate for kinesin interaction. Even though no observation has been made that KOR1 and CESA are in the same MASC, GFP–KOR1 has been visualized in small vesicles that resemble MASCs (Robert *et al*., 2005). Further investigation into the identification of the composition of CSC-containing vesicles will be important in understanding how cortical microtubules regulate MASC movement.

CSC insertion via vesicular fusion of CSC-containing vesicles with the plasma membrane

The mechanism for CSC insertion into the plasma membrane is unknown. Compositional analysis of CSC-containing vesicles might also aid in the identification of soluble *N*-ethylmaleimide-sensitive factor-activating protein receptors (SNAREs) or other tethering factors that are involved in the fusion of CSC-containing vesicles with the plasma membrane. One possible mechanism is that CSC insertion is mediated through the exocyst complex. Exocysts are octameric protein complexes for the targeting and tethering of Golgi-derived vesicles to the plasma membrane (Wang and Hsu, 2006; He and Guo, 2009). In arabidopsis, genetic analysis and sub-cellular localization studies of exocyst subunits show that the exocyst plays a critical role in exocytosis, a process that is essential for cell morphogenesis and plant development (Synek *et al*., 2006; Chong *et al*., 2010; Fendrych *et al*., 2010; Li *et al*., 2010; Wang *et al*., 2010; Fendrych *et al*., 2013). In mammals, the exocyst co-ordinates vesicular fusion at the plasma membrane with microtubules (Vega and Hsu, 2001). In plants, the relationship between exocysts and the cytoskeleton is less understood. A recent study in plants indicates that neither actin nor microtubules were required for exocyst formation. However, after long periods of pharmacological actin disruption, the distribution of exocysts on the plasma membrane was altered (Fendrych *et al*., 2013). Interestingly, mutants of EXO70A1, one of many duplicated EXO70 exocyst subunit genes in arabidopsis, exhibited strong defects in tracheary element development (Li *et al*., 2013*a*). Investigation of the exocyst in plants is still in its infancy, and plenty of opportunity exists in studying how exocyst-mediated exocytosis is involved in cell wall synthesis, including the possibility that CSC-containing vesicle tethering to the plasma membrane is a function of exocysts.

THE ENDOCYTOSIS AND RECYCLING OF CSCS

As a part of the plasma membrane, CSCs eventually need to be internalized through endocytosis. Genetic data from both arabidopsis and rice indicate that a relationship exists between endocytosis and cellulose synthesis. In arabidopsis, the *rsw9* (*radial swelling 9*) mutant exhibited a defect in endocytosis and a reduction in cellulose content (Collings *et al*., 2008). The *rsw9* mutation was mapped to the dynamin-related protein 1A (DRP1A) gene that has been shown to participate in clathrin-mediated endocytosis (CME) (Konopka and Bednarek, 2008). Similarly, in rice, a dynamin-related protein is required for secondary cell wall cellulose biosynthesis (Xiong *et al*., 2010). Together with exocytosis, endocytosis is fundamental to regulating functions of the plasma membrane. Clathrin-mediated endocytosis, the best-characterized endocytic route in eukaryotes (McMahon and Boucrot, 2011; Boettner *et al*., 2012), is also functional in plants (Holstein, 2002; Dhonukshe *et al*., 2007). Many components of the CME machinery have been identified in arabidopsis, and studies on their localizations and functions support an important role for CME in plant development (Dhonukshe *et al*., 2007; Konopka and Bednarek, 2008; Konopka *et al*., 2008; Chen *et al*., 2011; Ito *et al*., 2011; Kitakura *et al*., 2011; Van Damme *et al*., 2011; Song *et al*., 2012; Bashline *et al*., 2013; Di Rubbo *et al*., 2013; Fan *et al*., 2013; Kim *et al*., 2013; C. Wang *et al*., 2013; Yamaoka *et al*., 2013). Like in animal systems, plants also utilize clathrin-independent endocytosis routes (Onelli *et al*., 2008; R. Li *et al*., 2012). In different organisms, the size of clathrin-coated vesicles varies dependent on the properties of the cargo and membrane (McMahon and Boucrot, 2011). Endocytic clathrin-coated vesicles in plants are relatively small compared with the size of clathrin-coated vesicles in animals. One possibility for the size discrepancy might be due to the high turgor pressure in plant cells that may reduce the flexibility of the plasma membrane. Yeast cells, which also have a cell wall and turgor pressure, have endocytic clathrin-coated vesicles that are even smaller than those of plants (McMahon and Boucrot, 2011). In plants, endocytic

clathrin-coated vesicles have been commonly observed to be 60–90 nm in diameter, which is estimated to be approx. 30 nm in diameter without the clathrin coat (Dhonukshe *et al*., 2007; R. Li *et al*., 2012). Electron microscopy data indicated that the size of the cytosolic portion of a CSC could be as big as 45–50 nm in diameter (Bowling and Brown, 2008), causing much doubt that CSCs could be endocytosed through a clathrin-mediated mechanism (Crowell *et al*., 2010). However, a recent study suggests that CME may be involved in the endocytosis of CSCs. A yeast two-hybrid screen for protein interaction partners of the cytosolic portion of primary CESAs identified μ2 (AP2M) as a putative interaction partner of CESA6 (Bashline *et al*., 2013). In this study and others, μ2 (AP2M) was characterized as a component of the adaptor protein 2 (AP2) complex of arabidopsis that participates in the recognition of CME cargo and the recruitment of clathrin machinery to the sites of CME (Bashline *et al*., 2013; Di Rubbo *et al*., 2013; Fan *et al*., 2013; Kim *et al*., 2013; Yamaoka *et al*., 2013). μ2 (AP2M) was shown to interact directly with CESA proteins and play a role in the endocytosis of plasma membrane-localized CSCs. Furthermore, the loss of μ2 (AP2M) in the *μ2-1* mutant led to an endocytosis defect and an increased density of plasma membrane-localized CSCs (Bashline *et al*., 2013). It still remains to be determined how the small clathrin-coated vesicles of plants can accommodate the large CSCs. We hypothesize three possible situations. First, only a small portion of CSCs is subjected to internalization at any given moment. Likewise, μ2 (AP2M) is expected to be involved in the endocytosis of many cargos, which explains why only a small portion of μ2-labelled CME sites overlapped with CSCs at any given moment. It is possible that an intact CSC can be coated with clathrin for endocytosis in specialized, unusually large clathrin-coated vesicles that have less of a chance of being documented in electron micrographs due to the low frequency of CSC endocytosis events. Secondly, the large size of the cytosolic portion of the CSC may be contributing to the association of other proteins with the CSC that may be removed from the CSC prior to recognition for endocytosis. Thirdly, it is possible that CSCs that undergo endocytosis are deformed and disintegrate into smaller aggregations of CESAs before undergoing CME. Further investigation is required to discern how CSCs can undergo CME and whether CSCs can be endocytosed through clathrin-independent mechanisms.

CONCLUSIONS

Many achievements in the understanding of membrane trafficking in plants have been made in the last decade due to the use of fluorescent proteins. There is no doubt that newly emerging methods will add more knowledge to these important processes. The trafficking process of the CSC will be a unique anchor for dissecting trafficking between the Golgi apparatus and the plasma membrane and in other trafficking pathways. Future work is needed to identify components, general or specific, involved in each step of the CSC trafficking pathway.

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