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REVIEW

## Cellulose biosynthesis in plants – the concerted action of CESA and non-CESA proteins

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### Abstract

Cellulose is the most abundant polysaccharide produced by plants. In the form of rigid microfibrils surrounding the cells, cellulose constitutes the load-bearing cell wall element that controls cell growth and shape. Cellulose microfibrils are laid down outside the cell by the multimeric plasma membrane-inserted cellulose synthase complexes (CSCs), which move along underlying cortical microtubules (CMTs). In plants, CSCs are shaped as rosettes with six lobes symmetrically arranged in a hexagonal structure. In *Arabidopsis*, the CSC is composed of at least three functionally non-redundant cellulose synthase (CESA) glycosyltransferases in both primary and secondary cell walls. The number, organization, and interactions of CESA proteins within the CSC have been debated for many years on the basis of numerous lines of evidence provided by electron microscopy, biochemical and genetic approaches, spectroscopic techniques, as well as computational modeling. The *Arabidopsis thaliana* model was extremely useful in elucidating the molecular composition of CSC and enabled to elucidate the specialized functions of distinct AtCESA isoforms. Several additional, non-CESA proteins involved in cellulose synthesis and its regulation were also identified in *Arabidopsis*. This review outlines the latest findings on CSC organization, trafficking, and plant-specific proteins directly associated with the complex and interconnecting CESAs with CMTs.

*Additional key words:* *Arabidopsis thaliana*, cellulose microfibrils, cellulose synthase complex, cortical microtubules, membrane proteins, mutants.

### Introduction

Cellulose, a linear homopolymer of (1→4)- $\beta$ -linked glucose (Glc) residues, is the most abundant polysaccharide on earth because it constitutes more than half of biomass produced annually (Vanholme *et al.* 2013). Most of the cellulose is produced by vascular plants but cellulose is also synthesized by algae, bacteria, or fungi (Nobles *et al.* 2001). In nature, cellulose very rarely occurs as separate

glucan chains. From the moment of their synthesis by cellulose synthase complexes (CSCs) they associate by intermolecular hydrogen bonds into various higher-order structures in form of extracellular microfibrils of varying degrees of crystallinity and stiffness, and therefore, cellulose can play multiple functions. In *Acetobacter xylinum*, ribbons of cellulose form a raft supporting the bacteria floating at an air-water interface while in *Agrobacterium tumefaciens* cellulose is implicated in

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*Abbreviations:* AP - adaptor protein, BiFC - bimolecular fluorescence complementation, BR - brassinosteroid, CC - companion of cellulose synthase, CESA - cellulose synthase, CME - clathrin-mediated endocytosis, CMF - cellulose microfibril, CMT - cortical microtubule, CMU - cellulose synthase-microtubule uncoupling, COB - COBRA, CSC - cellulose synthase complex, CSR - class-specific region, CTL1/POM1 - CHITINASE-LIKE1/POM-POM1, CW - cell wall, FF-TEM - freeze-fracture transmission electron microscopy, GFP - green fluorescent protein, Glc - glucose, GT - glycosyltransferase, HVR - hypervariable region, KOB1 - KOBITO1, KOR1 - KORRIGAN1, MT - microtubule, P-CR - plant conserved region, PCW - primary cell wall, PM - plasma membrane, POM2/CSI1 - POM-POM2/CELLULOSE SYNTHASE-INTERACTIVE1, PTL1 - PATROL1, SAXS - small-angle x-ray scattering, SCW - secondary cell wall, SmaCC/MASC - small subcellular CESA compartment/MT-associated cellulose synthase compartment, STL - STELLO, TEM - transmission electron microscopy, TGN - trans Golgi network, TMH - transmembrane helix, UDP - uridine diphosphate, WT - wild-type, Y2H - yeast two-hybrid.

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pathogenesis by binding the bacteria to the host cell (Ross *et al.* 1991). In plant and algal cell walls (CWs), cellulose is the load-bearing component that forms a fibrillar mesh molding the shape of a cell, defining its physical and mechanical properties and controlling the direction of cell expansion (Kerstens and Verbelen 2003). Cellulose represents 10 - 30 % of dry mass of primary cell wall (PCW) and 50 - 60 % of secondary cell wall (SCW). In specialized cells as in cotton or flax fibers with thick SCWs, cellulose content reaches up to 98 %. The arrangement of glucan chains within cellulose microfibrils (CMFs) is not uniform. Current model explains that CMF is formed by a true-crystal core where chains are considered truly crystalline and by para-crystalline surface sheets with non-crystalline chains that associate with non-cellulosic polymers (Viřtor *et al.* 2002, Ding and Himmel 2006). In some cellulose-deficient mutants or in presence of cellulose biosynthesis inhibitors (*e.g.* *Isoxaben*, *CGA 325'615*), an accumulation of amorphous glucan was observed (Arioli *et al.* 1998, Peng *et al.* 2001). CMFs differ in their size depending upon the species, tissues, developmental stage as well as type of wall in which they are assembled. They are 5 - 10 nm large in PCWs and up to ~50 nm in SCWs (Herth 1983, Satiat-Jeuemaitre 1992, Donaldson 2007, Fernandes *et al.* 2011, Zhang *et al.* 2014). In cotton fibers, CMFs are ~10 nm wide and contain even ~80 glucan chains. Many authors reported, however, a diameter of ~3.5 nm or even less for a single - elementary CMF in various plant species (*e.g.*, Ha *et al.* 1998, Kennedy *et al.* 2007, Thomas *et al.* 2013).

Biosynthesis of cellulose is a complex process involving many actors and several steps: trafficking of multiple cellulose synthase (CESA) catalytic subunits to the plasma membrane (PM) and their subsequent attachment to the array of cortical microtubules (CMTs) that run along the PM cytoplasmic face, simultaneous polymerization of Glc molecules into long chains and their translocation across the PM, and parallel coalescence of multiple glucan chains into a microfibril. Apart from CESAs, other proteins necessary to cellulose synthesis and its regulation were identified counting these that directly link CSC to CMTs. The movement of the CSCs in the plane of the PM is driven by cellulose synthesis with nascent CMFs being deposited in linear trajectories along CMTs (Paredes *et al.* 2008). Uridine diphosphate-glucose (UDP-Glc) is a direct substrate for cellulose synthesis. Sucrose synthase, which produces UDP-Glc from sucrose, was shown to be associated with CSCs and involved in cellulose synthesis by channeling UDP-Glc to CESAs (Amor *et al.* 1995). Differences in CESA structure among kingdoms and taxa determine the structural variation in CSCs which adopt different shapes and sizes. Relationship exists between the geometry of the complex and the shape, size and degree of crystallinity of produced CMFs (Tsekos 1999, Roberts and Roberts 2007). Linear CSCs, arranged in single or multiple rows are found in bacteria, most algae and cellulose-synthesizing animals. For example, the alga *Valonia* has very large CSCs and synthesizes one of the largest, ~20-nm wide, highly crystalline CMFs. In contrast, charophycean algae, mosses, ferns and vascular plants have hexagonal rosette-shaped CSCs with ~25-nm

diameter in the transmembrane region and larger in the cytoplasmic region (Mueller and Brown 1980, Delmer 1999, Nixon *et al.* 2016). This review summarizes the groundbreaking achievements and the most recent findings in the field of cellulose synthesis in plants with special attention to the functions of CESA and recently identified non-CESA proteins.

## Genes *CESA* and proteins CESA

A tremendous progress has been achieved in the identification of proteins involved in cellulose synthesis, however, the biosynthetic process itself still remains tenuous. Genes responsible for cellulose synthesis were first identified in bacteria *A. xylinum* (Saxena *et al.* 1990, Wong *et al.* 1990). The product of *AxCESA* gene belongs to processive  $\beta$ -glycosyltransferases (GTs) and was shown to bind the UDP-activated Glc (Lin *et al.* 1990). Based on gene sequence similarity with *AxCESA* genes, Pear *et al.* (1996) identified two expressed sequence tags from cDNA clones obtained from RNA of cotton (*Gossypium hirsutum*) fibers at the stage of intense SCW synthesis. The two genes encoding putative CESAs were cloned and designated as *GhCESA1* and *GhCESA2*. The ability to bind UDP-Glc by CESA1 protein central fragment (containing four catalytic motifs) expressed in *E. coli*, led to the conclusion that the plant genes were true homologues of the bacterial *CESAs* (Pear *et al.* 1996). Plant *CESA* genes were larger than their bacterial counterparts and contained plant-specific insertions separating the conserved motifs from each other (Fig. 1A). Sequence alignments revealed the presence of conserved catalytic domain formed by three aspartic acid residues (D) and the QXXRW signature in bacterial and plant processive  $\beta$ -GTs (Saxena and Brown 1997). Independently, Arioli *et al.* (1998) using a map-based cloning showed that cellulose-deficient phenotype in dwarf *radial swelling1* (*rsw1*) *Arabidopsis* mutant resulted from an amino acid change (A→V) in AtCESA1. Since then, *CESA* genes have been identified in many other species. Finally, Kimura *et al.* (1999) demonstrated by immunocytochemical labeling with specific anti-CESA antibodies that rosette CSC co-localized with CESA catalytic subunit at the cytoplasmic face of freeze-fractured membranes in *Vigna angularis* proving that the PM-bound complex is the site of cellulose synthesis in vascular plants. However, first attempts to isolate active CESAs from plants (or by recombinant expression) failed. Conventional biochemical methods including detergent solubilization of intact complexes from microsomal/membrane fractions have been unsuccessful because of the high CSC instability. Only limited amounts of (1→4)- $\beta$ -glucan and little or no CESA activity was detectable above a high background of callose synthase activity *in vitro* (Kudlicka and Brown 1997, Lai-Kee-Him *et al.* 2002, Cifuentes *et al.* 2010).

Plant CESAs share a number of conserved structural features including eight transmembrane helices (TMHs), two at the N-terminus and six at the C-terminus that border a cytoplasmic domain, as well as an N-terminus with

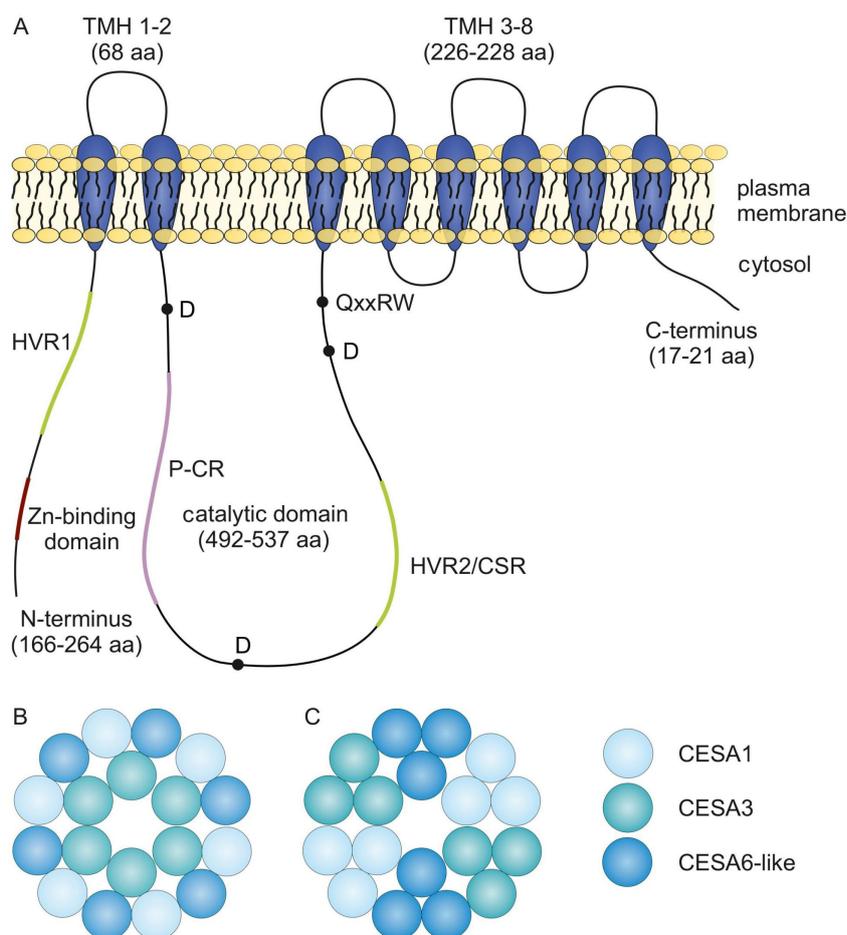


Fig. 1. A scheme of *Arabidopsis thaliana* cellulose synthase (CESA) structure (A) and the current 18-CESA model showing the putative arrangements of three distinct CESA isoforms within the complex (B and C). A - A schematic representation of the CESA polypeptide with domain length. Transmembrane helices (TMH 1-8) are marked in blue. At the N-terminal is a cysteine-rich zinc-binding domain and a hyper-variable region (HVR1) rich in acidic amino acids (aa). The central catalytic domain contains aspartic acid residues (D), which bind the UDP-glucose substrate and the conserved D, QxxRW motif characteristic for processive glycosyltransferases. The plant conserved region (P-CR) and the second hyper-variable region (HVR2), also referred to as a class specific region (CSR), located also within the cytosolic domain, are both important for plant-specific aspects of CESA function as interactions with other proteins involved in cellulose synthesis or oligomerization to form the rosette shape. B - The 'hexamer of heterotrimers' model proposed by Newman *et al.* (2013) and by Hill *et al.* (2014) in a 1:1:1 CESA ratio with consistent protein-protein interactions. The complex is composed of six lobes consisting each of three different CESAs (modified from Hill *et al.* 2014). C - The 'hexamer of homotrimers' model formulated by Vandavasi *et al.* (2016), which also fulfills the rules for the constituent number of CESA proteins within the complex described by Hill *et al.* (2014) (modified from Vandavasi *et al.* 2016).

Cys-rich Zn-binding domain which is probably involved in the dimerization of CESAs through the formation of disulfide bridges (Kurek *et al.* 2002) (Fig. 1A). Moreover, the cytosolic N-terminus contains a HVR1 region of ~150 aa that is hyper-variable between CESA paralogs but conserved between orthologs (Nakagami *et al.* 2010, Facette *et al.* 2013). A cytosolic ~540-aa domain, forming the active site for UDP-Glc substrate binding to the glucan terminus, is located between TMHs 2 and 3 (Fig. 1A). TMHs form a channel through which the newly synthesized glucan chain translocates outside the cell. The studies of the CESA crystal structure from *Rhodobacter sphaeroides* enabled to establish a model of glucan chain elongation (Morgan *et al.* 2013, Slabaugh *et al.* 2014). The authors suggested that glucan chain translocates one Glc at

a time and that each newly attached Glc molecule rotates in the opposite direction around the acetal linkage to align within the same plane of the (1→4)-β-glucan forming the 180° angle between neighbouring molecules (Morgan *et al.* 2013). GTs share a common mechanism of catalysis and, as reported by Sethaphong *et al.* (2013), a high degree of structural convergence exists between the catalytic sites of eukaryotic and prokaryotic CESAs indicating a conserved mechanism of cellulose production in plants including a single active site per CESA polypeptide chain. In AtCESAs, the cytosolic domain contains a ~126-aa plant-conserved region (P-CR) between D<sub>1</sub> and D<sub>2</sub> and another hypervariable region (HVR2) or CSR (class-specific region) of 86 - 106 aa between D<sub>2</sub> and D<sub>3</sub> (Pear *et al.* 1996, Vergara and Carpita 2001, Carroll and

Specht 2011) (Fig. 1A). Both regions are only present in rosette type of CSCs suggesting the role in CESA-CESA interactions and rosette assembly, for instance CESAs in algae with linear complexes do not have CSRs (Roberts and Roberts 2009, Sethaphong *et al.* 2016). Although a 3D atomistic model of entire plant CESA polypeptide remains unavailable, a tertiary model of cytosolic domain of GhCESA showed that these plant-specific sequences fold into distinct subdomains on the perimeter of the catalytic region indicating their role in self-oligomerization of CESAs (Sethaphong *et al.* 2013). They may also provide additional regulation sites such as phosphorylation site (Chen *et al.* 2010). Clustering of CESAs was based on sequence divergence within CSRs. Interestingly, the gene sequence similarity is greater among orthologs from different species than among paralogs of the same species (Vergara and Carpita 2001, Carroll and Specht 2011).

The availability of sequenced genomes allowed cataloguing of *CESA* genes into families (Richmond and Somerville 2000, Carroll and Specht 2011). The *Arabidopsis CESA* family members subdivide into 6 clusters with orthologous genes from other species reflecting functional specialization of distinct CESA isoforms. Their specific roles were explained on the basis of expression patterns and phenotypical analysis of corresponding *CESA* mutants. Two functional groups among AtCESAs have been distinguished: AtCESA1, AtCESA3 and AtCESA6-like necessary for PCW cellulose synthesis and AtCESA4, AtCESA7 and AtCESA8 required during SCW development (Taylor *et al.* 2003, Desprez *et al.* 2007, Persson *et al.* 2007). Four remaining isoforms are implicated in tissue-specific processes (Gardiner *et al.* 2003, Desprez *et al.* 2007, Persson *et al.* 2007). AtCESA4, -7 and -8 have no paralogs and possess at least one ortholog in other species. In rice, *OsCESA4*, *OsCESA7* and *OsCESA9* were shown to be implicated in SCW cellulose biosynthesis (Tanaka *et al.* 2003). Two functionally distinct sets of genes were also found in poplar (Djerbi *et al.* 2005), eucalyptus (Ranik and Myburg 2006), barley (Burton *et al.* 2004), or maize (Appenzeller *et al.* 2004). Cellulose synthases are evolutionary conserved and functional differentiation was anterior to the monocotyledons/dicotyledons divergence and, at least for CESA4, -7 and -8, the Angiosperms/Gymnosperms divergence (Nairn and Haselkorn 2005, Little *et al.* 2018). Moreover, the analysis of the *CESA* sequences in the moss *Physcomitrella patens* revealed that there were no orthologs for different plant *CESAs* in the moss genome indicating that the specialization of CESA isoforms evolved after the divergence of plant and moss *CESAs* (Roberts and Bushoven 2007). Since the functional specialization of *CESAs* is a more recent event than the appearance of functional hexameric rosettes, it may suggest their regulatory functions rather than geometrical and/or biochemical constraints within the rosette-CSC.

The *AtCESAs* genes are expressed in different tissues and to varying profiles throughout the life of a plant. Distinct *AtCESAs* of similar function are co-expressed in the same cell type. While *AtCESA1*, -3 and -6 transcripts are present in most tissues, *AtCESA4*, -7 and -8 are specifically

expressed during the SCW synthesis in developing xylem and interfascicular fibres. *CESA1*, -3 and -6 are necessary for the cellulose synthesis in developing seedling organs, such as root and hypocotyl. The corresponding mutants (*cesa1<sup>rsw1</sup>*, *cesa3<sup>eli1</sup>* and *cesa6<sup>prc1</sup>*) have comparable phenotypes: dwarfism with increased radial cell expansion, cellulose deficiency and/or severe inhibition of the synthesis of crystalline microfibrils (Arioli *et al.* 1998, Fagard *et al.* 2000, Caño-Delgado *et al.* 2003). Mutations in *AtCESA1* and -3 result in a much stronger phenotype than mutation in *AtCESA6*. AtCESA3 is unique but AtCESA1 has one paralog - AtCESA10 whereas AtCESA6 has three paralogs: AtCESA2, -5, -9. Transcriptome data demonstrate that *AtCESA9* is specifically expressed in stamens and embryos whereas *AtCESA10* in silicles thus both are not involved in the redundancy mechanism during seedling development. Interestingly, *AtCESA5* is specifically expressed in epidermal cells of the seed coat where it is necessary for cellulose synthesis in adherent mucilage (Sullivan *et al.* 2011). Since the *cesa1* and *cesa3* knockout alleles are lethal, the involvement of CESA1 and CESA3 in mucilage cellulose synthesis is difficult to study using a reverse-genetic approach. The strong *cesa5* mutant phenotype indicates that other isoforms do not compensate its deficiency. This stands in opposition to vegetative tissues where the isoforms redundant with CESA6 can rescue, at least partially, the growth phenotype (Fagard *et al.* 2000, Desprez *et al.* 2007). CESA2 and CESA5 partially overlap CESA6 in function but the phenotype of *cesa2cesa6* double mutant and lethality of *cesa5cesa6* indicated that the presence of CESA5 or CESA6 is mandatory for the CSC assembly (Desprez *et al.* 2007). The *irregular xylem (irx)* mutations in *AtCESA4*, -7 and -8 (*cesa4<sup>irx5</sup>*, *cesa7<sup>irx3</sup>* and *cesa8<sup>irx1</sup>*, respectively) cause a collapse of mature xylem cells due to the lack of SCW thickenings (Turner and Somerville 1997, Taylor *et al.* 2003). The *irx* inflorescence stems are characterized by a several-fold decrease in the amount of cellulose in respect to wild type (WT) and all three loci appear equally important in SCW cellulose synthesis. In these mutants, the absence of one CESA protein was neither compensated by the remaining two nor by the over-expression of other *CESAs*, which indicates that these three isoforms are not redundant. Co-purification and co-immunoprecipitation with specific antisera confirmed that CESA1, -3 and -6 as well as CESA4, -7 and -8 are parts of the same protein complex, respectively (Taylor *et al.* 2003, Desprez *et al.* 2007). The combination of three non-redundant isoforms may be necessary to form a functional CSC for several reasons: 1) they may provide distinct sites for specific intra- and intermolecular interactions for the assembly of the complex, 2) they may provide specific regulatory functions, or 3) they may catalyze initiation and polymerization reactions of distinct glucan chains. Although the experimental evidence suggests the combination of three different isoforms, a cross-functionality between some PCW and SCW isoforms occurs as *CESA1* partially complemented the defects of *cesa8* while *CESA7* partially rescued the phenotype of a weak allele of *cesa3* (Carroll *et al.* 2012). Birnbaum *et al.*

(2003) showed that PCW and SCW CESAs can be co-expressed in specific cell types which suggests that mixed PCW/SCW CSCs may exist for cellulose synthesis during the transition between the PCWs and SCWs. Moreover, yeast two-hybrid (Y2H) and bimolecular fluorescence complementation (BiFC) assays revealed a promiscuity in one-to-one CESA isoform physical interactions including formation of homodimers and heterodimers in most of possible combinations between both PCW and SCW CESAs (Desprez *et al.* 2007, Timmers *et al.* 2009, Carroll *et al.* 2012, Li *et al.* 2013). The specificity in CESA interactions within functional CSCs is determined most probably by the simultaneous interactions of three isoforms and high promiscuity observed in pair-wise assays do not reflect CESA interactions *in planta*. The short C-terminus is conserved in CESA3, -4, -6 and -7 but not in CESA1 and -8 (Carroll and Specht 2011) which suggests an additional isoform selectivity through CSC assembly (or competition between partners) and that CESAs may play different roles. Using chimeric AtCESA1 and AtCESA3 proteins (both functionally non-redundant), Wang *et al.* (2006) demonstrated in corresponding mutant backgrounds that the identity of the catalytic and/or C-terminal domains determine the conserved function and thus the position within the CSC. The experiments with chimeric CESA4, -7 and -8 with swapped protein domains showed that all three: the central domain and both N- and C-terminal regions differently contributed to class-specific functions (Hill *et al.* 2018). On the contrary, Sethaphong *et al.* (2016) showed that the variable CSRs of CESA1 and CESA3, having both the most similar aa sequence among AtCESAs, are interchangeable *in vivo* and do not determine their non-redundant functions. The AtCESA1-CESA3CSR and AtCESA3-CESA1CSR chimeric proteins restored growth in *cesa1<sup>rsw1</sup>* and *cesa3<sup>je5</sup>* backgrounds, respectively, showing that the sole CSR is not sufficient to confer the isoform specificity within native CSCs.

### Transcriptional and post-translational regulation of CESAs

Cellulose biosynthesis is highly regulated at multiple levels and synchronized with growth and differentiation. *CESA* genes vary in their transcript profiles, with isoforms specifically expressed during embryogenesis, cell elongation, or formation of the SCW. The co-expressions of PCW or SCW *CESAs* are in line with the observations that CSCs are formed by at least three different CESA isoforms (Brown *et al.* 2005, Persson *et al.* 2005). Interestingly, although *AtCESA7* transcription in stems was two to three times higher than that of *AtCESA8* (Brown *et al.* 2005), SCW CSCs showed equal CESA stoichiometry (Hill *et al.* 2014). A global *Affymetrix Arabidopsis* microarray analysis provides evidence that some genes involved in several pathways are co-regulated with two functionally distinct *AtCESA* types (Persson *et al.* 2005). For instance, genes involved in brassinosteroid (BR) synthesis showed a co-regulation with *CESA1*, -3, and -6 and not with *CESA4*, -7, and -8. The BRs control cell expansion

and morphogenesis, processes associated with PCW rearrangements. Indeed, these phytohormones control the expression of *CESA* genes and regulate cellulose biosynthesis (Xie *et al.* 2011). The authors showed that an impairment in BR biosynthesis or perception induced a cellulose synthesis deficiency and that exogenously applied BR reverted the *CESA* expression deficiency in *deetiolated2-1* mutant affected in BR synthesis. In contrast, it was not the case in BR-insensitive *br1-301*, affected in BR receptor, indicating that BR signaling is crucial for *CESA* expression regulation. Moreover, the BR-activated transcription factor BES1 binds to *CESA* promoter regions *in vivo* (Xie *et al.* 2011). On the other hand, two functionally redundant NAC domain transcription factors, SND1 and NST1, are key regulators of the transcriptional cascade that is necessary for the SCW synthesis initiation in fibers of *Arabidopsis* stems (Zhong *et al.* 2010). Over-expression of *MYB46* and *MYB83* transcription factors, which are both NAC regulators' direct targets, upregulated SCW *CESAs* expression (Ko *et al.* 2009, McCarthy *et al.* 2009). Kim *et al.* (2013) showed that MYB46 binds to SCW *CESA* promoters. Many SCW-related gene promoters in *Arabidopsis* have MYB binding motifs called SMRE (SCW MYB-responsive element) or the MYB46-responsive *cis*-regulatory elements (M46RE) which were shown to be crucial for SCW cellulose synthesis (Kim *et al.* 2012, Zhong and Ye 2012).

Other regulatory mechanisms including post-translational modifications, the control of CSC abundance and the lifetime of PM-inserted CSCs are also possible. The dimerization of CESA proteins through the redox-regulated formation of disulfide bridges of the N-terminal Zn-binding domains may be crucial for rosette assembly (Kurek *et al.* 2002). Under oxidative conditions, GhCESA1 forms a dimer which is more resistant to ubiquitin-mediated degradation than the monomeric form indicating the role of N-terminal in complex stabilization. Phosphorylation can also regulate CESA activity, interactions or signaling cascades in response to environmental and/or developmental stimuli (Speicher *et al.* 2018). Global phosphoproteomic studies of the cytoplasmic regions of integral PM proteins from *Arabidopsis* and maize identified several Ser/Thr phosphorylation sites on CESA1, CESA3, and CESA5, mostly in HVR1 (Nühse *et al.* 2003, Nakagami *et al.* 2010, Facette *et al.* 2013). They are unique among CESA paralogs but conserved among orthologs in dicots and monocots. Moreover, short aa sequences surrounding phosphorylation sites, and known as kinase recognition sites, are conserved but not between the phosphorylation sites themselves indicating that regulation of distinct CESAs by different kinases is a conserved aspect of cellulose biosynthesis (Carroll and Specht 2011). The analyses with phosphonull and phosphomimic residues in various mutants revealed functional implications *in vivo*. Studies in the *rsw1-1* background showed that phosphorylation of CESA1 is required for normal cellulose content, CSC velocity and interactions with MTs (Chen *et al.* 2010). Similarly, Bischoff *et al.* (2011) observed in their experiments with *cesa5* mutants a role for phosphorylation in the control of

CSC velocity in relation with the activation of red/far red photoreceptor (phytochrome B). The degree of CESA5 phosphorylation regulates the interactions with CMTs and thus the movement of CSC which enables the fine tuning of cellulose deposition in darkness. AtCESA4 and AtCESA7 are also phosphorylated within their HVRs and it was found that at least CESA7 is regulated *via* proteasome-dependent pathway which could play a role in the stability of SCW CSCs (Taylor 2007).

### Organization of the rosette CSC

Because the synthesis of cellulose results in highly organized crystallites, CESA catalytic subunits are believed to be also precisely organized within the complex. How many CESA proteins are necessary to form a functional CSC and how they interact has been discussed by numerous authors for decades. Given that one CESA GT polymerizes one cellulose chain, one CSC has approximately as many CESA subunits as many glucan chains are present in an elementary CMF. The long-held 36-CESA CSC model appeared in 80's mainly on the basis of the CSC size measurements and perpetuated through many years (Herth 1983, Delmer 1999, Doblin *et al.* 2002, Taylor *et al.* 2003, Ding and Himmel 2006) further supported by the pull-down and blue native (BN)/SDS-PAGE experiments on detergent-soluble extracts from *Arabidopsis* seedlings and stems (Wang *et al.* 2008, Atanassov *et al.* 2009). The authors detected different CESA oligomers including putative dimers, tetramers, and hexamers for both PCW and SCW CESAs. The largest complexes (of 740 to 840 kDa) were considered as hexamers. Nevertheless, the 36-CESA model has been often questioned on several grounds including overestimations in the CSC size using traditional freeze-fracture transmission electron microscopy (FF-TEM) as well as the calculations of a variable number of glucan chains forming elementary CMFs in different species. On the other hand, the 36-CESA CSC model was further combined with the results of genetic studies giving rise to several models explaining the putative organization and interactions between non-identical CESA proteins (Scheible *et al.* 2001, Doblin *et al.* 2002, Desprez *et al.* 2007, Persson *et al.* 2007, Timmers *et al.* 2009). Although, the genetic studies showed that functional CSCs are only formed when isoforms from each CESA class are combined (Taylor *et al.* 2003, Desprez *et al.* 2007, Carroll *et al.* 2012) they could not explain how various CESA proteins are arranged within the six lobes of the CSC, what their exact number is and how cells control their right positioning. An important step towards understanding the CESA composition within the CSC was achieved by Gonneau *et al.* (2014) and Hill *et al.* (2014) who showed the equimolar stoichiometry of CESA1, CESA3 and CESA6 in PCW CSCs and CESA4, CESA7 and CESA8 in SCW CSCs, respectively, by using co-immunoprecipitation combined with mass spectrometry and by quantitative immunoblotting with isoform-specific antibodies. However, these results did not answer the persisting questions either of the number of

CESAs building the complex ( $6 \times 3 = 18$  or  $6 \times 6 = 36$ ) or their organization.

The number of glucan chains forming an elementary CMF reflects the number of catalytically active CESAs within the complex. The hexagonal structure of rosette CSC indicates that the number of chains in an elementary CMF must be a multiple of six. It was widely assumed that typical elementary CMFs in higher plants were composed of 36 glucan chains (Herth 1983, Delmer 1999). However, several measurements in various plant species (*e.g.* early electron diffraction data from cotton and rose communicated by Chanzy *et al.* 1978, 1979) indicated that PCW microfibrils contained as few as 12 - 25 glucan chains. The number of glucan chains in an elementary CMF has not been determined in *Arabidopsis*. However, recent studies based on wide-angle X-ray scattering, small-angle neutron scattering, solid-state NMR, and computational simulations of different types of CWs including mung bean PCW, celery collenchyma and spruce wood converged on a ~3-nm diameter which would correspond to ~20 chains (Fernandes *et al.* 2011, Newman *et al.* 2013, Thomas *et al.* 2013, Oehme *et al.* 2015). CMFs are not circular and the number of constituent chains (~18 to 24) is determined by the cross-sectional shape. Given the 1:1:1 CESA ratio, the latest measurements of the size of CMFs and based on the rule that the number of glucan chains in a CMF must be divisible by six (the number of rosette lobes) and by three (non-identical CESA isoforms), Newman *et al.* (2013) and Hill *et al.* (2014) proposed the 'hexamer of heterotrimers' CSC model with 18 catalytically active CESAs. The 'hexamer of trimers' model was also formulated by Vandavasi *et al.* (2016) who observed the self-oligomerization of solubilized AtCESA1 monomers into stable trimers. The authors expressed the catalytic domain of AtCESA1 in *E. coli* and studied the structure of purified (in a folded conformation) monomers and trimers using small-angle X-ray scattering technique (SAXS). The comparison of the AtCESA1 trimer structure and size with the ~15 nm diameter of a CSC lobe at the cytosolic side from TEM images strongly supported three CESA proteins per lobe instead of six that would occupy too much area. Interestingly, the oligomers of higher order were not detected, indicating that CESA cytosolic domains are responsible for lobe formation but do not contribute to CSC assembly. Both configurations, six heterotrimers or two sets of three distinct homotrimers are possible and fulfill the rules described by Hill *et al.* (2014) (Fig. 1B,C). Further comparison of AtCESA1 trimer computational models with SAXS profiles enabled to explain the putative orientation of monomers in the trimeric lobes where CSRs are found at the periphery of the cytosolic domain, within the vertices of the triangular lobe, and do not participate in any of the interfaces within the trimer, while P-CRs form the base of a trimer and are believed to interact with other proteins involved in cellulose synthesis (Vandavasi *et al.* 2016). Such an arrangement of CESA isoforms limits the complex to six lobes. Similar conclusions were drawn by Nixon *et al.* (2016) who validated previous observations by superimposing the average size and shape of CSCs obtained from FF-TEM images from *P. patens* with

computational models for GhCESA1 TMH region as well as for the above mentioned AtCESA1 catalytic domain model based on SAXS profiling. The authors showed that trimeric lobes of the developed models correlated with individual lobe geometry and rosette morphology. In addition, they calculated that the assembly of trimers into a ring resulted in the lowest potential energy per monomer. Rosette diameters of TMH region obtained from FF-TEM pictures are variable with the mean value of 23.4 nm indicating weak interactions between lobes. This in turn suggests that forces stabilizing CSC must occur below and/or above the TMH region. Although the trimeric CESA models lacked the N-terminal region and the companion proteins, the estimated CSC diameter on the cytosolic side had in average 30 nm instead of previously proposed 45 - 50 nm (Bowling and Brown 2008). Assuming that 1 TMH region occupies an area of 1.4 nm<sup>2</sup> (Eskandari *et al.* 1998) and that one CESA protein possesses 8 TMHs, Bowling and Brown (2008) calculated that one CSC lobe would contain no more than 4 CESA proteins giving an area of 50.4 nm<sup>2</sup> perfectly matching the area of 8-nm circle (50.3 nm<sup>2</sup>) which corresponds to a single CSC lobe. Although spectroscopic and diffraction techniques support the 24-chain microfibrils, the 24-mer CSC is rather doubtful because the CESA number must be a multiple of six and divisible by three. Vandavasi *et al.* (2016) estimated a 15-nm diameter for a lobe at the cytosolic side and a similar value for the size of the AtCESA1 catalytic domain trimer model demonstrating that maximum three CESAs form individual CSC lobes. Similarly, as shown by Nixon *et al.* (2016), the space below an average FF-TEM image of the CSC could be filled by trimeric SAXS models. In contrast to large algal CMFs, production of small 18-chain CMFs by land plants may have an adaptive implication giving more possibilities for their aggregation and the size of final CMFs and CW structure.

### Regulated trafficking CESA proteins

The aptness to deliver CESAs to distinct sites at the cell surface and controlling their abundance is essential for cells to produce CMFs that control growth in a particular direction and/or under particular conditions. Indeed, the abundance and lifetime of CSCs impact the amount and properties of CMFs. CSCs were previously observed using TEM in the PM, at the periphery of the *trans* face of the Golgi apparatus and Golgi-derived vesicles (Haigler and Brown 1986). Live-cell imaging using fluorescently tagged CESAs allowed to follow the intracellular dynamics and the delivery of CESAs to the sites of cellulose production (Chan *et al.* 2007, Desprez *et al.* 2007, Crowell *et al.* 2009, Gutierrez *et al.* 2009). Green fluorescent protein (GFP)-CESA3 signals were observed in medial and *trans*-Golgi cisternae and co-localized with the *trans*-Golgi network (TGN) marker VHA-a1 (Crowell *et al.* 2009). TGN compartment plays a dual role, as a secretory sorting station it directs newly synthesized proteins to their subcellular destinations and as an early endosome it receives endocytosed recycled molecules. However, it is

not clear whether CSCs present in TGN are destined for secretion or internalized from the PM. The presence of MT-associated vesicles containing temporarily stored GFP-CESA3 was simultaneously discovered by two research groups and called respectively MT-associated cellulose synthase compartment (MASC) or small subcellular CESA compartment (SmaCC) (Crowell *et al.* 2009, Gutierrez *et al.* 2009). SmaCCs/MASCs are distinct from TGN and are not initially bound to MTs but associated with them after osmotic stress (or cellulose synthesis inhibitor treatment) and co-localized with TGN marker. SmaCCs/MASCs are thought to play a double role, as delivery vesicles, formed before the insertion of newly synthesized CSCs into the PM, and as storage/recycling vesicles of CSCs internalized upon stress (Fig. 2). However, experiments using drug treatment suggest the latter function (Crowell *et al.* 2009, Gutierrez *et al.* 2009, Wightman and Turner 2010). Vesicles containing CSC are removed from the PM *via* clathrin-mediated endocytosis (CME), another regulatory mechanism controlling the abundance and distribution of CESAs in the PM (Bashline *et al.* 2013). In accordance, the growth phenotype and cellulose content are affected in single and multiple CME-deficient *ap2m-1* and *twd40-2-3 Arabidopsis* mutants (Bashline *et al.* 2015). An AP2M, the medium subunit of the CME adaptor protein 2 (AP2) complex responsible for internalization of cargo proteins to the PM, was shown to interact with mCherry-CESA6 mediating its endocytosis (Bashline *et al.* 2013). TWD40-2, a plant-specific protein, plays a similar role and cooperates with AP2M. Interestingly, in addition to its PM localization discussed in the last section, POM-POM2/CELLULOSE SYNTHASE-INTERACTIVE1 (POM2/CSII), a CESA-interacting protein, is also associated with SmaCC/MASC compartments upon treatment with isoxaben and oryzalin (Bringmann *et al.* 2012, Lei *et al.* 2012) which suggests that POM2/CSII may play a role in CESA trafficking. Moreover, Lei *et al.* (2015) demonstrated that the protein is involved in SmaCC/MASC-mediated fast recovery of CSCs to the PM upon abiotic stress relief.

STELLO1 and 2, two other Golgi-localized proteins regulating the trafficking of CSCs were discovered in *Arabidopsis* (Zhang *et al.* 2016). In contrast to *stl1* and *stl2*, *stl1stl2* double mutant has much shorter primary root and dark-grown hypocotyl and produces less cellulose which indicates that both proteins are functionally redundant. The authors showed that the proteins interact with CESAs and that their GT activity is necessary for the proper assembly of CSCs. Changes in spatial distribution of CSCs in the Golgi and lower rate of their delivery to PM were observed in STL-deficient plants. Resulting CMFs are characterized by altered degree of crystallinity (Zhang *et al.* 2016). Apart from STLs, other actors were also shown to be cooperatively implicated in *de novo* secretion of CSCs in *Arabidopsis* including plant-specific PATROL1 (PTL1), POM2/CSII and octameric exocyst complex involved in vesicle trafficking (Lei *et al.* 2015, Zhu *et al.* 2018). While PTL1 and exocyst complex subunits are required for the delivery of CSCs to the PM, POM2/CSII functions as CMT marker defining the position of docking site.

Another example of regulated trafficking was provided

by Miart *et al.* (2014) who showed that during cytokinesis CSCs are delivered to a newly laid down crosswall following a precise spatio-temporal pattern. The authors showed that the accumulation of CESA1, CESA3, and CESA6 as well as cellulose deposition occurred already at the tubulo-vesicular membrane network formation stage. CSCs are delivered to the cell plate by phragmoplast-associated vesicles, to MT-free zones of the expanding cell plate by mobile compartments (probably Golgi bodies) and finally CSCs are redirected *via* CME from the central part of a cell plate and from the mother cell PM to the peripheral zones of a growing cell plate which is accompanied by the GFP-CESA signal attenuation at the central region of the cell plate. The *in vivo* dynamics was also studied during SCW bands formation in protoxylem cells demonstrating that CSCs are recruited at high concentration at narrow membrane domains underlying SCW thickenings and that the velocity of CESA7-containing CSCs is faster than that of PCW CSCs (Watanabe *et al.* 2015). Both Golgi-associated and independent SmaCCs/MASCs are involved in a dynamic exchange of CSCs to the SCW domains. The changes observed in SCW CSCs velocity during cell differentiation suggest a temporal, next to gene expression, regulation of cellulose biosynthesis. For more in-depth discussion of cellulose synthesis regulation mechanisms, see, Wang *et al.* (2016), Kesten *et al.* (2017), and Polko and Kieber (2019).

## Non-CESA proteins implicated in cellulose synthesis

Many genetic screens and Y2H assays enabled to identify several non-CESA proteins essential for cellulose synthesis in *Arabidopsis* including CHITINASE-LIKE1/POM-POM1 (At1g05850), COBRA (At5g60920), KOBITO1 (At3g08550), KORRIGAN1 (At5g49720), and above mentioned POM2/CSII (At2g22125) (Hauser *et al.* 1995, Nicol *et al.* 1998, Schindelman *et al.* 2001, Pagant *et al.* 2002, Gu *et al.* 2010, Sánchez-Rodríguez *et al.* 2012) (Fig. 2). Among them, two last ones have so far been found to be directly associated with CESAs (Li *et al.* 2012, Vain *et al.* 2014). The growth phenotypes observed in corresponding mutants are reminiscent of the phenotype of cellulose-deficient mutants with elongation defects or WT plants cultivated in presence of cellulose synthesis-blocking herbicides. Reduced elongation in these mutants is interpreted as a result of the loss of growth anisotropy due to the absence of the scaffold of ordered CMFs that constrain directional growth. Moreover, genes encoding non-CESA proteins are co-expressed with PCW CESAs (Persson *et al.* 2005).

KORRIGAN1 (KOR1) is the most studied member of KOR family belonging to endo-1,4- $\beta$ -D-glucanases (cellulases) (Nicol *et al.* 1998). KORRIGAN1 exhibits substrate specificity for amorphous cellulose and low-substituted carboxymethyl cellulose but not for crystalline cellulose or xyloglucans (Mølhøj *et al.* 2001). Interestingly, although its substrate is synthesized at the PM, KOR1 was

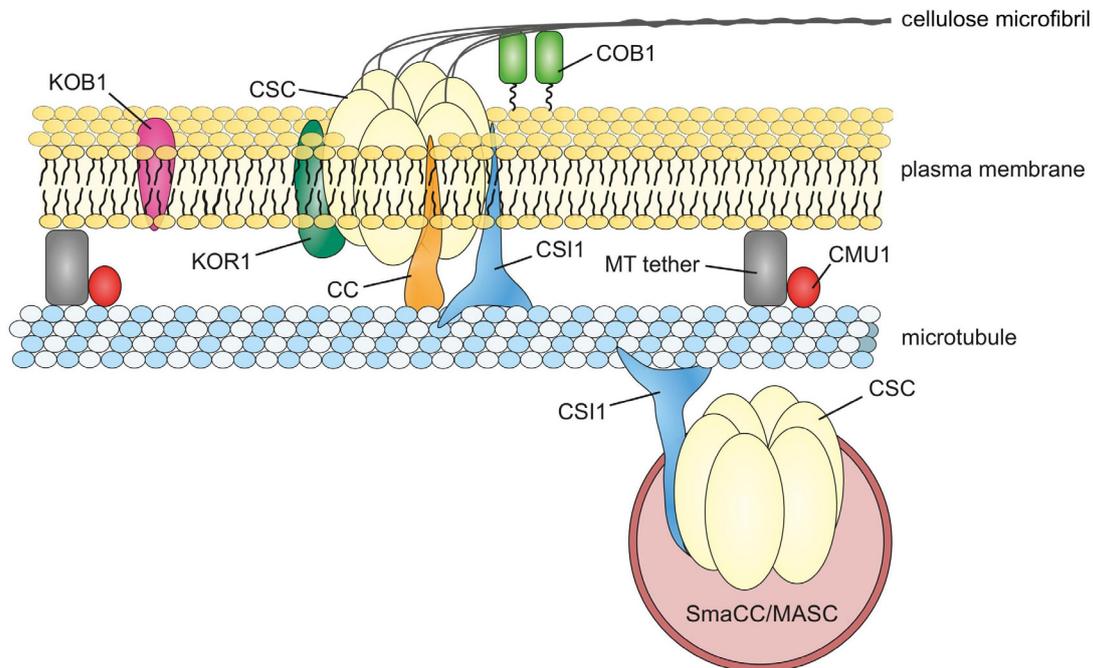


Fig. 2. A schematic representation of the plasma membrane-inserted cellulose synthase complex (CSC), its connection with a microtubule (MT), as well as some other membrane-bound proteins involved in cellulose synthesis and/or regulation. The scheme also illustrates a CSC internalized into the cell cortex in a vesicle called the small subcellular CESA-containing compartment or microtubule-associated CESA compartment (SmaCC/MASC). CC - COMPANION OF CELLULOSE SYNTHASE, CMU1 – CELLULOSE SYNTHASE-MICROTUBULE UNCOUPLING1, COB1 - COBRA1, CSII - CELLULOSE SYNTHASE-INTERACTIVE1, KOB1 - KOBITO1, KOR1 - KORRIGAN1.

shown to cycle through different intracellular compartments including Golgi stacks, post-Golgi compartments, SmaCCs/MASCs, prevacuolar compartments, vacuole, and through the PM (Robert *et al.* 2005, Vain *et al.* 2014). The *KOR1* mutants are characterized by cellulose synthesis defects including seedling lethality with cytokinesis aberrations (*kor1-2*) (Zuo *et al.* 2000), dwarfism and radial swelling in roots (*kor1-1* and *-3*, *rsw2* alleles) (Nicol *et al.* 1998, Lane *et al.* 2001), and collapsed xylem (*irx2-1* and *-2*) (Szyjanowicz *et al.* 2004). Despite numerous studies indicating its role in both PCW and SCW cellulose synthesis, precise function of *KOR1* is not understood. *KORRIGAN1* may determine the length of growing glucan chains and/or release the CMF once it was incorporated into the wall. Alternatively, *KOR1* may hydrolyze and remove disordered and aberrantly associated cellulose chains to relieve tensions arisen during CMF emergence/assembly (Newman *et al.* 2013). Y2H, life cell imaging and BiFC showed that GFP-*KOR1* co-localized with all PCW CESA proteins in the PM and constitutes an integral part of CSC, however, the interactions between CESAs and *KOR1* are more unstable than those among CESAs themselves (Vain *et al.* 2014). Indeed, the co-immunoprecipitation experiments showed a stable interaction existed between CESA3 and CESA6 in *Triton X-100*-solubilized protein extracts whereas *KOR1* did not stably interact with CESAs in these detergent-solubilized extracts (Desprez *et al.* 2007). In SCW CSCs, *KOR1* was shown to interact only with CESA4 and CESA8 (Mansoori *et al.* 2014). In addition, Vain *et al.* (2014) showed that GFP-*KOR1* moved in PM along linear trajectories defined by CMTs with a velocity similar to that of GFP-tagged CESAs and that upon treatment with oryzalin, a MT-depolymerizing drug, the trajectories of both particles GFP-*KOR1* and GFP-*CESA3* were disorganized in a comparable manner. In *kor1-1*, the velocity of GFP-*CESA3* in the PM was reduced in comparison with WT, indicating that *KOR1* is necessary for the movement of CSCs. In the presence of cellulose synthesis inhibitors, GFP-*KOR1* is, similarly to CSC, internalized into SmaCCs/MASCs (Robert *et al.* 2005, Vain *et al.* 2014). This implies that *KOR1* may indeed cleave glucan chains to detach CSC from the CW-bound CMF before internalization of the complex. Moreover, in the *kor1-1* background, the removal of GFP-*CESA3* signals from the PM to SmaCCs/MASCs upon CGA 325'615 treatment was significantly reduced in opposition to WT, showing that *KOR1* is required for the intracellular trafficking of the CSCs (Vain *et al.* 2014).

CHITINASE-LIKE1/POM-POM1 (CTL1/POM1) was identified in a 'conditional root expansion' screen for abnormal root expansion and its deficiency induces lower cellulose content, impaired CW rigidity and root swelling (Hauser *et al.* 1995). The CTL1/POM1 was detected with Golgi-localized CESAs and SmaCCs/MASCs (Sánchez-Rodríguez *et al.* 2012). Together with its close homolog CTL2, CTL1/POM1 is secreted to the apoplast and participates in cellulose assembly and interactions with hemicelluloses. The authors measured slower velocity of CSCs in the PM of elongating *ctl1-1* hypocotyls than in the WT ones resulting in reduced cellulose content.

*COBRA* (*COB*) belongs to genes that are the most highly co-expressed with PCW CESAs (Persson *et al.* 2005). It encodes a plant-specific, extracellular glycosylphosphatidylinositol-anchored membrane protein (Schindelman *et al.* 2001). *Cobra1* was identified in a screen for cell expansion regulators with a root-specific and conditional cell expansion defect (Benfey *et al.* 1993). *COB*-deficient plants are characterized by the loss of anisotropic expansion in rapidly developing organs, disordered orientation of CMFs and reduced crystalline cellulose content (Roudier *et al.* 2005). The authors detected *COB* in the Golgi, PM, and within PCW. Moreover, *COB* was specifically found in elongating cells and distributed in a banding pattern, parallel to CMTs, indicating its putative involvement in the control of the CSCs movement and oriented deposition of CMFs (Roudier *et al.* 2005). Both genetic approach and oryzalin treatment showed that *COB* distribution at the surface of elongating root cells was dependent on the CMT organization. At the onset of cell elongation, *COB* was detected in the cytosol and at the cell periphery while during the elongation phase *COB* was organized in narrow bands perpendicular to the longitudinal axis.

KOBITO1 (*KOB1*) is another plant-specific protein involved in CMF deposition in elongating cells (Pagant *et al.* 2002). The *kob1* mutant is a cellulose-deficient, severely dwarfed mutant developing short and swollen dark-grown hypocotyl, short root and reduced apical hook. CMFs in *kob1* are randomly oriented only in elongating cells where incomplete CWs are also visible indicating that the protein may coordinate cellulose synthesis and rapid cell expansion (Pagant *et al.* 2002). Similar CW gaps occur in WT seedlings treated with cellulose synthesis inhibitors. GFP-*KOB1* fusion protein was detected at the cell surface of growing epidermal and cortical cells in contrast to dividing cells where the signal was visible in the cytoplasm. Lertpiriyapong and Sung (2003) identified two mutant genes causing strong growth phenotype that are allelic to *kob1*, and named them *eld1* (*elongation defective1*). Interestingly, the ELD1-GFP signal was detected in CWs, rather than PM, indicating that ELD1/*KOB1* is a secreted protein participating in cell growth promotion.

### The connection between microtubules and cellulose synthase complex

Plant MTs are characterized by a greater intrinsic dynamicity than the animal ones (Shaw *et al.* 2003) reflecting probably the necessity to cope with changing environment in place. Highly dynamic arrays of CMTs are necessary for plant growth since they influence the CW material anisotropy that is the basis of cell elongation. In elongating cells, CMFs are transversely oriented and promote longitudinal cell expansion. CMTs serve as templates and guide the deposition of CMFs resulting in particular wall texture and effects of cell shape and size (Gardiner *et al.* 2003). CMTs change their orientation in response to a variety of signals like irradiance, gravity, or hormones (Chen *et al.* 2016)

but they also undergo cyclic rotary movements associated with cell growth that explain the changes of CMF angle in consecutive layers of the polylamellate CW (Chan *et al.* 2007). The reorientation of CMTs occurs conjointly with a reorientation of the CSCs trajectories; in consequence, the orientation of nascent CMFs changes accordingly (Paredes *et al.* 2006, Chan *et al.* 2010). Paredes *et al.* (2006) confirmed the functional association between PM-inserted CESAs and CMTs. The authors showed that CESAs move along the surface of the PM, presumably driven forward by the deposition of cellulose chains, and along linear trajectories overlaying with CMTs. In addition to orienting their trajectories, CSCs are positioned into the PM at sites determined by CMTs (Crowell *et al.* 2009, Gutierrez *et al.* 2009). Chan *et al.* (2010) showed that MT-depolymerizing or stabilizing drugs, oryzalin and taxol respectively, blocked the rotation of CESA tracks. As a result, higher variability in CMFs orientation altered the polylamellate CW texture in epidermal cells of *Arabidopsis* hypocotyls. The disruption of CMTs transiently impair the distribution of CSCs at the PM but neither the rate of CSCs insertion nor their velocity is affected proving that CMTs are required for the guidance and not the movement of CSCs *per se* (Paredes *et al.* 2006, Gutierrez *et al.* 2009, Chan *et al.* 2010). Similarly, during SCW deposition in *Arabidopsis* CSC tracks are disorganized after oryzalin treatment in protoxylem tracheary cells showing that MTs are important for SCW banding pattern (Watanabe *et al.* 2015). In contrast, they are not required either for CSC delivery to the PM or for the CSC velocity. Interestingly, as shown by the treatment with cellulose synthesis inhibitors that induce reorientation of MTs in *Arabidopsis* root cells, the organization of CMTs can be also influenced by cellulose biosynthesis (Paredes *et al.* 2008, Peng *et al.* 2013). Similarly, PCW mutants affected in cellulose biosynthesis exhibit changes in MT organization (Paredes *et al.* 2008). Finally, a very important finding on the control of CSC movement was provided by Liu *et al.* (2016) who identified a family of proteins responsible for lateral stability of CMTs in growing *Arabidopsis* seedlings. Cellulose synthase-microtubule uncoupling (CMU) proteins, distributed along the MTs as immobile puncta, are necessary for CMTs to withstand forces generated by the motile CSCs and to direct cellulose deposition during anisotropic cell growth. CMU-deficient seedlings show lateral MT displacement and affected MT-based guidance of CSC movement resulting in a cell twisting phenotype in etiolated hypocotyls.

The interactions between CSCs and CMTs are mediated by a CESA-interacting protein named POM2/CSII, identified in a Y2H screen using CESA6 catalytic domain as bait (Gu *et al.* 2010). POM2/CSII is essential for CESA movement along the PM surface and functions as a molecular bridge between CSCs and CMTs (Fig. 2). Although POM2/CSII does not share any sequence homology with structural MT-associated proteins, Li *et al.* (2012) pelleted POM2/CSII with polymerized tubulin and proved that POM2/CSII is a MT-binding protein. *POM2/CSII* encodes a large (2 151 aa) plant-specific protein containing several motifs necessary for protein-

protein interactions. POM2/CSII interacts directly with CESA1, 3, and 6 and it is the first identified non-CESA protein associated with CSC. *In vivo*, FP-POM2/CSII colocalizes with GFP-CESA3 or GFP-CESA6 in the PM but does not label any Golgi-associated CESAs indicating that POM2/CSII interacts with the CSC only upon arrival at the PM (Gu *et al.* 2010). The loss-of-function *pom2/csil* mutant is characterized by reduced cellulose content by 50 % in respect to WT and shorter and swollen roots and hypocotyls as a result of defects in control of anisotropic expansion (Gu *et al.* 2010). POM2/CSII-deficient plants not only show an abnormal distribution of CSCs but their trajectories move independently of the direction of the underlying CMTs (Bringmann *et al.* 2012, Lei *et al.* 2012). Moreover, the velocities of CESAs are reduced in POM2/CSII-deficient plants to a similar extent as in oryzalin-treated plants (Gu *et al.* 2010, Li *et al.* 2012). Two POM2/CSII-like proteins, namely CSI2 and CSI3, are encoded by *Arabidopsis* genome but in contrast to *pom2/csil* mutant, single and double knock-outs in *CSI2* and *CSI3* do not show any growth deficiencies (Bringmann *et al.* 2012).

Two plant-specific proteins, COMPANION OF CELLULOSE SYNTHASE1 and 2 (CC1 and CC2), were shown to protect CSC activity by sustaining MT dynamics during salt stress (Endler *et al.* 2015) (Fig. 2). *CC1* is co-expressed with PCW CESAs and induced by salinity. Single and double mutants do not show any changes in growth phenotype under control conditions. In contrast, under salt stress the sensitivity of the *cc1cc2* seedlings is manifested by strongly reduced hypocotyl elongation in darkness and low cellulose content indicating the functional redundancy between the two proteins. The *CC1* and *CC2* genes are members of a four-member gene family in *Arabidopsis* but only *CC1* and *CC2* are crucial for cellulose synthesis and seedling development under unfavorable conditions (Endler *et al.* 2015). Dual-labelled GFP-CC1 (or GFP-CC2) and tdTomato-CesA6 lines tracking revealed that the CSCs and CC proteins move together through the PM under control conditions hence the name of this gene family (Endler *et al.* 2015). What is more, Y2H test enabled to establish direct interactions between CESA1, 3, and 6 and CC1. Similarly to CESAs, the movement of FP-CC1 and CC2 co-aligned with the CMT array. Interestingly, GFP-CC1 signal is lost in isoxaben-treated specimens and tracks to SmaCCs/MASCs, where the CC proteins are relocated along with CESAs in response to the treatment (Fig. 2). Osmotic stress was also shown to change the distribution of CSCs by inducing their return to SmaCCs/MASCs and to diminish the rate of cellulose biosynthesis (Paredes *et al.* 2006, Gutierrez *et al.* 2009). Indeed, upon salt stress exposure, MTs depolymerize gradually but they re-polymerize under prolonged stress as a result of adaptation (Wang *et al.* 2007). In the *cc1cc2* mutants the MTs take longer to reassemble and their arrangement is unstable (Endler *et al.* 2015). Consecutively, cellulose biosynthesis is not restored in the double knockout mutant. The authors speculate that CCs play a crucial role in the re-assembly and maintenance of the MT stability and protect CESA activity during long-term ionic stress (Endler *et al.* 2015). The CCs are transmembrane proteins with the

CESA-associated C terminus located in the apoplasmic region and the MT-bound N-terminal domain. The N-terminal domain stimulates MT formation enabling the CMT array re-emergence after ionic stress (Endler *et al.* 2015). The newly established MT network allows CSCs to be re-inserted into the PM and, in effect, the resumption of cellulose synthesis.

## Concluding remarks

In recent years, a remarkable progress has been achieved in our understanding of many aspects of the cellulose-synthesizing machinery in higher plants. Latest measurements of the CSC lobes and the size of elementary CMFs support 18-CESA complex model. Smaller than algal microfibrils, 18-chain microfibrils may reflect an adaptive trait of land plants enabling more possibilities for CMF assembly, controlling architectural features of CWs. Mutant analysis demonstrated the requirement of different CESA isoforms to form a functional complex. Finally, the identification of novel non-CESA proteins involved in cellulose synthesis and regulation, especially the CSC trafficking and the MT-based guidance of CSC movements, unraveled the complexity and dynamics of cellulose production during growth and development, as well as in response to various environmental conditions. Future challenges are to discover additional regulatory mechanisms like the post-translational CESA modifications, including the functions of the kinases involved in CESA phosphorylation, or mechanisms responsible for CW integrity and remodeling control. The roles of numerous receptor-like kinases in CW sensing and feedback signaling are extensively studied and represent an exciting field in current plant cell wall research.

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