Structure, organization, and functions of cellulose synthase complexes in higher plants

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Annually, plants produce about 180 billion tons of cellulose making it the largest reservoir of organic carbon on Earth. Cellulose is a linear homopolymer of β(1-4)-linked glucose residues. The coordinated synthesis of glucose chains is orchestrated by specific plasma membrane-bound cellulose synthase complexes (CelS). The CelS is postulated to be composed of approximately 36 cellulose synthase (CESA) subunits. The CelS synthesizes 36 glucose chains in close proximity before they are further organized into microfibrils that are further associated with other cell wall polymers. The 36 glucose chains in a microfibril are stabilized by intra- and inter-hydrogen bonding which confer great stability on microfibrils. Several elementary microfibrils come together to form macrofibrils. Many CESA isoforms appear to be involved in the cellulose biosynthetic process and at least three types of CESA isoforms appear to be necessary for the functional organization of CelS in higher plants.

Key words: cellulose biosynthesis, cellulose synthase (CESA), cellulose synthase complex (CelS), microfibril

Estrutura, organização e funções dos complexos da sintase da celulose em plantas superiores: Anualmente, as plantas produzem aproximadamente 180 bilhões de toneladas de celulose, sendo o maior reservatório de carbono orgânico no planeta. A celulose é um homopolímero linear composto por resíduos de glicose unidos por meio de ligações β(1-4). A síntese coordenada das cadeias de glicose é orquestrada por complexos específicos ligados à membrana plasmática (CelS). Postula-se que o CelS é composto por aproximadamente 36 subunidades da sintase da celulose (CESA). Cada CelS sintetiza 36 cadeias de glicose dispostas lado a lado antes de serem organizadas em microfibrilas, que são, posteriormente, associadas com outros polímeros da parede celular. As 36 cadeias de glicose presentes em uma microfibrila são estabilizadas por pontes de hidrogênio intra e inter-cadeias, conferindo grande estabilidade às microfibrilas. As microfibrilas elementares são dispostas lado a lado, permitindo a formação das macrofibrilas. Várias isoformas da CESA podem estar envolvidas no processo de biossíntese de celulose e, no mínimo, três tipos de isoformas da CESA podem ser necessárias para a organização funcional de cada CelS em plantas superiores.

Palavras-chave: biossíntese de celulose, complexo da sintase da celulose (CelS), microfibrilas, sintase da celulose (CESA)

INTRODUCTION

Cellulose is an outstanding commodity due to its abundance and distinctive structural properties. For example, its tension resistance is comparable to that of steel (Eckardt, 2003). Even though cellulose has great commercial value for the pulp, paper, and textile as well as chemical industries which use it to produce commercially important polymers, cellulose biosynthesis in trees is still not well understood. Due to the economical significance of tree cellulose for forest product industries, we have

focused our attention in this review on cellulose biosynthesis in trees. Most of the recent findings concerning the molecular mechanism of cellulose biosynthesis in higher plants resulted from research in model herbaceous plants and fiber crops and have been reviewed recently (Somerville, 2006).

Cellulose is synthesized by cellulose synthase enzymes (CESAs) and is regarded as a major sink for atmospheric carbon in plants because it is the main component of the plant cell wall (Delmer and Haigler, 2002). Many plant cell walls of commercial importance are made up of three layers: middle lamellae, primary cell wall, and secondary cell wall. The secondary cell wall is further subdivided into three sub-layers called S1, S2, and S3. All the layers present in the cell wall have two phases: microfibrilar and matrix (Brett and Waldron, 1990). The microfibrilar phase, a crystalline phase, is composed of microfibrils of cellulose and the matrix phase, a non-crystalline phase, is composed of a variety of polysaccharides (pectins and hemicelluloses), proteins, and phenolic compounds (lignin, ferulic acid, coumaric acid, and others) (Brett and Waldron, 1990).

There is a definite distinction between chemical composition of the primary and secondary cell walls. The most remarkable one is concerning the quantities of cellulose and lignin. The amount of lignin in secondary cell walls of trees like *Populus trichocarpa* (poplar) is 19-21%, whereas in the primary cell wall lignin is absent (Mellerowicz et al., 2001). The content of cellulose, expressed in dry weight, in the primary cell wall of poplars is 20-30%, whereas in the secondary cell wall it is 40-50% (Mellerowicz et al., 2001). For instance, some clones of *Eucalyptus grandis* cultivated in Brazil have a cellulose content of 43.9 – 49.7% in the secondary cell wall (Gomide et al., 2005).

In some special cases, when the developing angiosperm wood is under tension stress, a gelatinous layer (G layer) is formed instead of the S2 or S3 layer (Timell, 1969; Mellerowicz et al., 2001; Pilate et al., 2004). The G layer is composed almost exclusively of highly crystalline axially oriented microfibrils of cellulose. An increase of 10-20% in the cellulose content and a significant decrease in lignin content has been observed in tension wood in response to the deposition of the G layer. Thus tension wood may represent an excellent system to study processes involved in cellulose biosynthesis (Wu et al., 2000; Pilate et al., 2004). For example, in *Populus tremuloides* (aspen), the coexpression of three *CesAs*, namely, *PtrCesA1*, *PtrCesA2*, and *PtrCesA3*, suggests that these three *CesAs* may be important for the biosynthesis of highly crystalline cellulose present in tension wood fibers (Bhandari et al., 2006). It has been reported that KORRIGAN (KOR), a type of cellulase, is involved in cellulose biosynthesis allowing proper formation of microfibrils of cellulose I (Delmer and Haigler, 2002; Molhoj et al., 2002). The aspen *Kor* gene *(PtrKor)* has been isolated and its expression pattern has been analyzed in aspen. Similar to three aspen CESAs, a high amount of PtrKOR protein was detected in developing xylem as well as on the upper side of the bent aspen stem in response to tension stress. In contrast, a very low amount of PtrKOR protein was detected on the opposite side of the bent stem experiencing compression stress (Bhandari et al., 2006).

In this review, we will discuss the process of cellulose biosynthesis. In addition, structural characteristics of cellulose and CESAs, as well as the structural, organizational, and functional features of CESA complexes (CelS) will be discussed.

MOLECULAR AND SUPRAMOLECULAR FEATURES OF CELLULOSE

Cellulose is a linear homopolymer made up of β(1-4)-linked glucose residues and the UDP-glucose molecule acts as substrate for cellulose biosynthesis. All alternate glucose residues in the same cellulose chain are rotated 180° and are β(1-4)-linked by CESA isoforms. An anhydroglucose, one glucose residue, is a monomer of cellulose. The dimer, two glucose residues β(1-4)-linked, called cellobiose is the structural repetitive unit of the cellulose chain. The degree of polymerization is determined by the number of monomers which compose each cellulose chain (Brown et al., 1996; Delmer, 1999). Two different ending groups are found in each cellulose chain edge (Figure 1). At one end of each of the chains, a non-reducing group is present where a closed ring structure is found. A reducing group with both an aliphatic structure and a carbonyl group is found at the other end of the chains. The cellulose chain is thus a

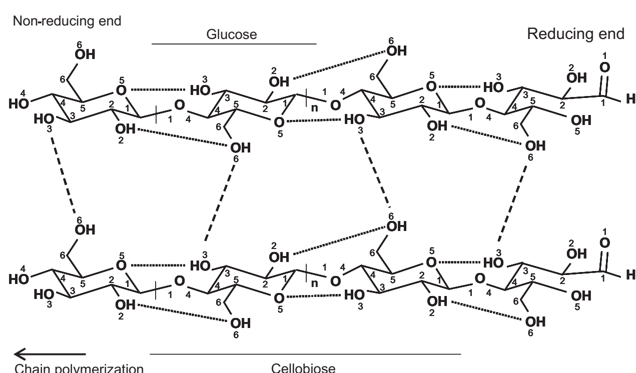

Figure 1. The structure and the inter- and intra-chain hydrogen bonding pattern in cellulose I. Dashed lines: inter-chain hydrogen bonding. Dotted lines: intra-chain hydrogen bonding.

polarized molecule. New glucose residues are added at the non-reducing end by CESAs allowing chain elongation (Koyama et al., 1997).

The β(1-4) linkage between glucose residues, in contrast to the α(1-4) linkage as occurs in starch, confers cellulose with unique structural features. Cellulose is a water-insoluble polymer with a rigid linear structure. Controlled cellulose biosynthesis allows arrangement of extensive linear chains which can be aligned side-by-side, creating fibers of great mechanical strength. As a consequence, tension resistance of cellulose is comparable to that of steel (Eckardt, 2003). In nature, cellulose is the main structural component of the cell wall and responsible for many of its distinctive traits.

Six different crystalline polymorphs of cellulose are known: cellulose I, II, III_I, III_{II}, IV_I, and IV_{II}. Cellulose I and II are found in nature. The others are obtained artificially by chemical or heat treatments. Cellulose I is the main form found in nature and it occurs as two allomorphs denominated Iα and Iβ (Figure 2). Higher plants synthesize both allomorphs, called cellulose Iβ and Iα-like chains. The cellulose Iα-like chain is conformationally similar to crystalline algal cellulose Iα, but is accommodated in a different hydrogen-bonding environment (Sturcova et al., 2004).

Cellulose II is the most crystalline thermodynamic stable form. It can also be obtained from cellulose I by two processes called regeneration and mercerization. Cellulose is found in amorphous form that is usually associated with cellulose I (O'Sullivan, 1997). Because

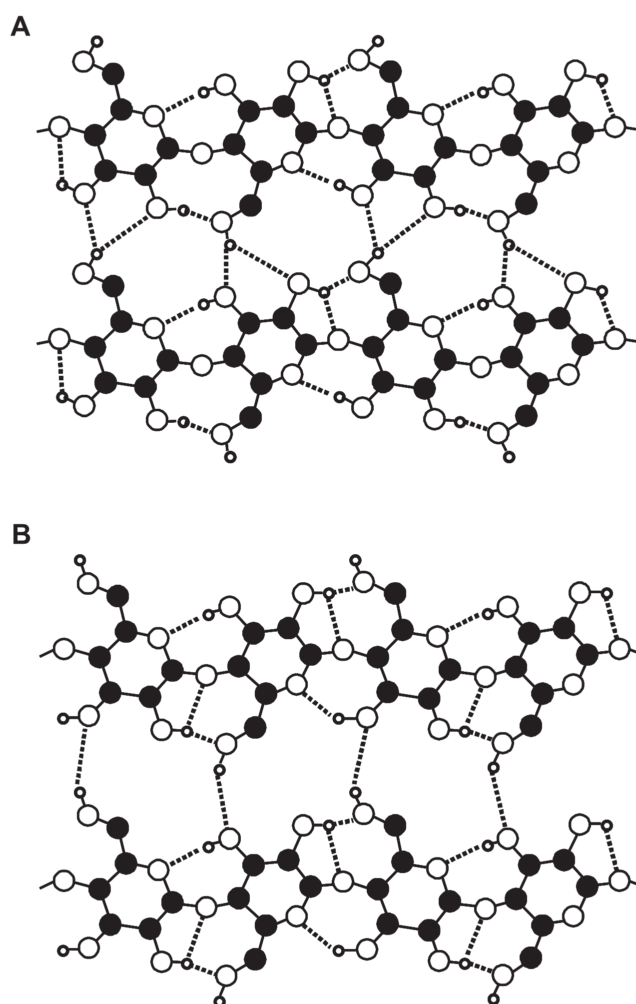

Figure 2. Hydrogen-bonding patterns in cellulose Iα (A) and Iβ (B) based on the crystal structures of Nishiyama et al. (2002, 2003). Hydrogen bonds are represented by dotted lines. Note that cellulose Iα and Iβ show a different hydrogen-bonding pattern. Carbon (●), oxygen (**O**), and deuterium atoms (**o**). Reprinted with permission from Sturcova et al. (2004). Copyright (2006) American Chemical Society.

cellulose I and II are present in nature, we shall focus our attention on these forms in this review, but more specifically on cellulose I. The inter-chain hydrogen bonding pattern differs for the two forms. The O6-H—O3 inter-chain hydrogen bonding is dominant in cellulose I, whereas O6-H—O2 is the main inter-chain hydrogen bonding in cellulose II. The O3-H—O5 intra-chain hydrogen bonding which exists in both polymorphs (I and II) is responsible for the rigid and linear shape of each cellulose chain (Figure 1) (Langan et al., 2001; Nishiyama et al., 2002, 2003).

The resolution of cellulose structure reveals that in Iβ there are two conformationally distinct chains in a monocyclic unit cell (all glucosyl residues are identical except that they face alternately in opposite directions). In contrast, in Iα there is one chain in a triclinic unit cell (alternate glucosyl residues differ slightly in conformation and hydrogen bonding) (Figure 3). The O2-H—O6 intra-chain bonding is present in both allomorphs Iα and Iβ; however, it is shorter in Iα (Nishiyama et al., 2002). The conformation of anhydroglucose residues and the β(1-4) linkages are the molecular characteristics which distinguish cellulose Iα and Iβ (Kono et al., 2002). The O2 and O6 atoms show multiple possibilities of hydrogen bonding. This may be the reason why O2 and O6 are the most reactive of the hydroxyl groups on the crystalline surface of cellulose (Figure 1). Conversely, the O3 atoms are not reactive due to the strength of the intra-chain O3-H—O5 bonding (Rowland and Howley, 1988). Cellulose Iα is less stable than Iβ and it can be converted into Iβ by heating (Hardy and Sarko, 1996; Wada, 2002).

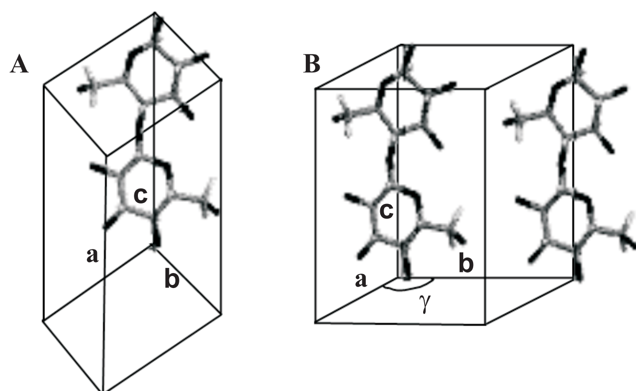

Figure 3. Schematic representation of mode of chain packing in the unit cell of cellulose. (**A**) Triclinic unit cell: cellulose Iα. (**B**) Monoclinic unit cell: cellulose Iβ. Monoclinic angle γ is obtuse. Reprinted with permission from Koyama et al. (1997). Copyright (2006) The National Academy of Sciences of the United States of America.

ELEMENTARY FIBRIL, MICROFIBRILS AND MACROFIBRILS OF CELLULOSE

The cellulose microfibrils are structural units which compose the microfibrilar phase of each layer of cell wall. Theoretically, elementary fibrils are composed of only β(1-4)-linked glucose residues synthesized by the CESA complex (Ding and Himmel, 2006). Glucose chains are set parallel and aligned side-by-side in a specific crystalline arrangement. Microfibrils are thus composed of elementary fibrils that are further associated with non-cellulosic polymers. Each cellulose microfibril has approximately 36 glucose chains. The glucose chains are stabilized by intra- and inter-hydrogen bonding which confers greater stability on microfibrils (Nishiyama et al., 2002, 2003). The degree of polymerization of cellulose chains is around 2,000-25,000 glucose residues (Brown et al., 1996). In aspen, the degree of polymerization of cellulose chains in the primary cell wall is approximately 4,200. In contrast, 9,200 glucose residues form cellulose chains in the secondary cell walls of aspen wood (Mellerowicz et al., 2001).

The coordinated synthesis of the glucose chains occurs in specific places in the plasma membrane where the CelS synthesizes glucose chains side-by-side before crystallization takes place. This process is highly organized and allows formation of cellulose I instead of cellulose II. Furthermore, it avoids the formation of non-crystalline cellulose I (Saxena and Brown, 2005). In many plants, cellulose microfibrils have 3 nm of diameter in the primary cell wall. In contrast, in the secondary cell wall, elementary microfibrils are put together side-by-side in a specific arrangement, allowing formation of macrofibrils (Delmer and Amor, 1995; Ding and Himmel, 2006). Macrofibrils are 5-10 nm in diameter (Brown et al., 1996; Jarvis, 2003). In some algae, microfibrils may have 20 nm of diameter (Jarvis, 2003); however, a much wider range in diameter of microfibrils, 10-68 nm, is found in the algae *Erythrocladia subintegra* (Tsekos, 1999; Tsekos et al., 1999).

Recently, a microfibril structural model based on direct visualization of the primary cell wall of maize parenchyma cells was proposed by using atomic force microscopy (Ding and Himmel, 2006). According to this model, 36 glucose chains are distributed into three groups based on their location (Figure 4). The first group, comprising the center true-crystal core is composed of six glucose chains (Ch1-6), forming a hexagonal cross section. This group is considered truly crystalline. The second group encompasses chains directly associated with the crystal core and is composed of 12 subcrystalline chains (Ch7-18). Finally, the third group includes 18 subcrystalline or noncrystalline chains located on the surface of the crystal (Ch19-36). The

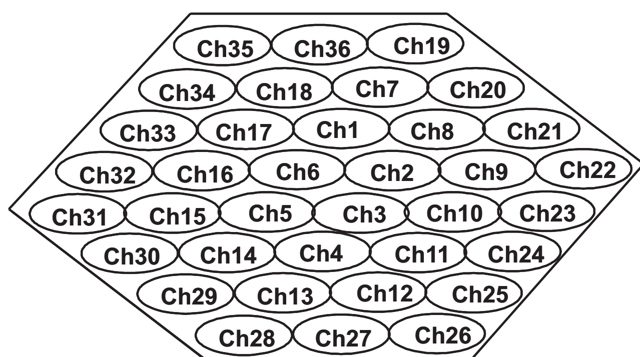

Figure 4. Cross-section of the 36-chain elementary fibril showing number of chains. Chains are numbered from Ch1 to Ch36 and categorized into three groups: group-C1 (Ch1-6) contains six true crystalline chains, group-C2 (Ch7-18) contains twelve subcrystalline chains, and group-C3 (Ch19-36) contains 18 subcrystalline or noncrystalline chains. Reprinted with permission from Ding and Himmel (2006). Copyright (2006) American Chemical Society.

groups 2 and 3 form protection and transition phases between the crystal core and later-deposited non-crystalline polymers. It is postulated that this model is structurally similar to the cellulose Iβ model (Figures 2B and 3B).

CELLULOSE SYNTHASE COMPLEX (CelS)

The CelS, usually called the terminal complex or rosette, is proposed to be composed of six subunits which are arranged in hexagonal symmetry. It is 25-30 nm in diameter (Mueller and Brown, 1980; Delmer, 1999). Each subunit of CelS has six CESA isoforms which produce six glucan chains (Brown and Saxena, 2000). The CelS is thus theoretically composed of 36 CESAs. At least three types of CESA isoforms, called α_1, α_2, and β, may be necessary for the spontaneous arrangement of CESAs in each CelS (Doblin et al., 2002; Ding and Himmel, 2006). Consistent with this hypothesis, different types of *CesA* isoforms have been found to be coexpressed in *Arabidopsis thaliana*: *AtCesA4*, *AtCesA7*, and *AtCesA8* (Taylor et al., 2003); barley: *HvCesA1*, *HvCesA2*, and *HvCesA6* (Burton et al., 2004); poplar: *PtrCesA1*, *PtrCesA2*, and *PtrCesA3* (Joshi et al., 2004; Bhandari et al., 2006); and rice: *OsCesA4*, *OsCesA7*, and *OsCesA9* (Tanaka et al., 2003). However, expression of several *CesA* isoforms in the same cell does not necessarily indicate that they interact and are part of the same CelS. According to the expression pattern and functional redundancy, three situations are possible: (i) different isoforms are expressed in different cells; (ii) different isoforms are expressed in the same cell, but they are functionally redundant; and (iii) different isoforms are expressed in the same cell, but they are essential and are not redundant (Perrin, 2001).

Several models have been proposed to explain interactions among CESA isoforms, allowing proper formation of CelS (Scheible et al., 2001; Doblin et al., 2002; Joshi, 2003a). These models are based on the hypothesis that one elementary fibril is composed of 36 glucose chains, which are synthesized by 36 CESA isoforms arranged in a planar configuration in the plasma membrane (Delmer, 1999; Kimura et al., 1999; Scheible et al., 2001). The number of CESA isoforms and the type of interactions among them are the distinctive features of each model. In the model proposed by Ding and Himmel (2006), 36 CESA isoforms are assembled into rosettes located in the plasma membrane (Figure 5). Each CelS has six identical subunits composed of six CESA isoforms: one α_1, two α_2, and three β (Figure 5A). Three types of protein-protein interactions (β-β, α_1-β, and α_2-β) may be involved in the spontaneous assembly of CelS (Figure 5B). Several CelS arranged in the plasma membrane may form a honeycomb array (Figure 5C). The honeycomb array structure could synthesize a great number of elementary fibrils to form a macrofibril.

CelS IS COMPOSED OF CESA ISOFORMS THAT ARE ENCODED BY *CesA* GENES

Several experiments were successfully conducted to suggest that CelS are made up of multiple CESA isoforms that are encoded by distinct *CesA* genes.

(i) *CelS (rosette) is associated with biogenesis of microfibrils of cellulose:* The discovery of the cotton *CesA* genes encoding potential catalytic subunits of CelS revealed the involvement of CelS in biosynthesis of microfibrils of cellulose. The identification of potential components of the plant CelS complex was achieved by a molecular approach. Two cDNA clones GhCESA1 and GhCESA2 were identified in cotton fiber enriched in cellulose (Pear et al., 1996). These cDNAs encoded

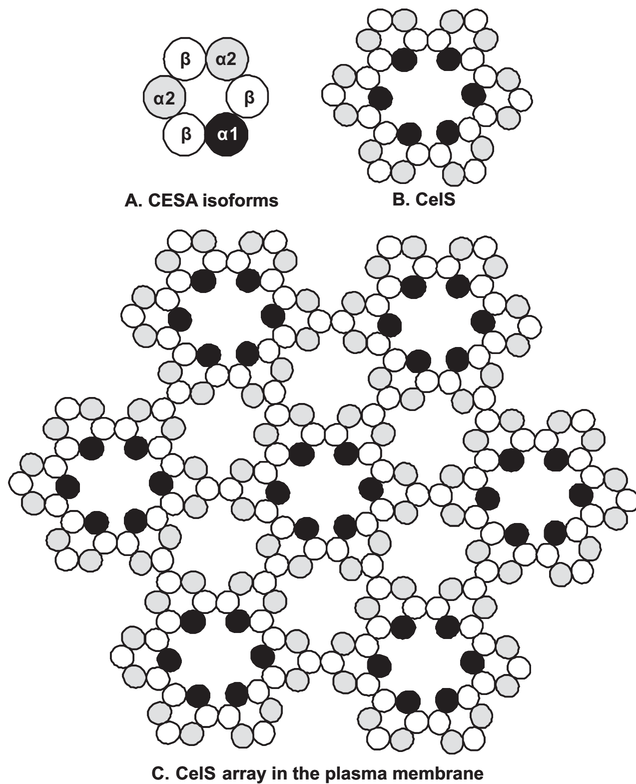

Figure 5. Schematic model structure of the cellulose synthases complex (rosettes) in higher plants. Reprinted with permission from Ding and Himmel (2006). Copyright (2006) American Chemical Society.

proteins with some similarity to the bacterial CESA proteins. Besides, sequence analysis revealed existence of the motif D, D, D, QxxRW (motifs indicative of processive glycosyltransferase) (Saxena and Brown, 1995). It was shown that GhCESA1 was able to bind to the substrate UDP-glucose and the expression patterns of *GhCesA1* and *GhCesA2* were tightly linked with the timing of cellulose biosynthesis (Pear et al., 1996). Collectively, these findings supported the hypothesis that *GhCesA1* and *GhCesA2* encoded potential catalytic subunits of CelS which may be associated with biogenesis of microfibrils of cellulose (Doblin et al., 2002).

(ii) *Proper assembly of CelS depends on protein encoded by AtCesA1:* To demonstrate that rosettes are made up of intact CESAs, the temperature-sensitive mutant *rsw1* was analyzed (Arioli et al., 1998). It displayed disassembly of CelS (or rosettes) in the plasma membrane, altered cellulose crystallinity (or production of non-crystalline cellulose), and interrupted morphogenesis. This mutant showed at ambient temperature (18°C) regular assembly of CelS. When mutant plants were exposed to high temperatures (31°C), CelS was not correctly assembled. The gene responsible for this phenotype was isolated by map-based cloning. Sequence analysis revealed that the amino acid Ala^{549} in the wild-type was substituted by Val^{549} in *rsw1* and that this gene encoded the catalytic subunit of CESA based on homology with cotton CESAs (Pear et al., 1996). Reverse genetics experiments were used to show that the cloned wild-type *AtCesA1* gene complements the *rsw1* mutant. Therefore, RSW1 locus encodes the catalytic subunit of CESA (AtCESA1) which is involved in CelS assembly. It was proposed that the *rsw1* mutant allele interrupts assembly of glucan chains into microfibrils. In the mutant, at the restrictive temperature, CelS is not properly assembled suggesting that the proper assembly of CelS depends on the presence of wild-type *AtCesA1* (Arioli et al., 1998).

(iii) *CESA is one of the components of CelS:* To show that CESA is a component of CelS, an indirect method was employed using polyclonal antisera produced against the catalytic region of cotton CESA (Kimura et al., 1999). The primary antibody was used to recognize the CESA present in the plasma membrane-localized CelS. Anti-rabbit secondary antibodies coupled to 10 nm colloidal gold were used to recognize CESAs. Once the dimension of the primary-secondary antibody complex was known (27.2 nm), it was labeled for visualization of the CESA using transmission electron microscopy. The technique of freeze-fracture of the plasma membrane was employed. After fracture, the rosettes are present in the leaflet section of the plasma membrane bilayer that is nearest the cytoplasm (the P-fracture face). The CESA antibodies specifically recognized CelS on the P-fracture face. Gold particles were observed directly over or close to CelS. It suggested that CESA is a component of CelS (Kimura et al., 1999).

(iv) *AtCESA4, AtCESA7, and AtCESA8 are co-localized in xylem cells and show interactions:* To demonstrate that three secondary wall-associated CESAs might be involved in rosette assembly, polyclonal antisera were raised against three distinct secondary wall-associated

Arabidopsis CESAs, namely, AtCESA4, AtCESA7, and AtCESA8 (Taylor et al., 2003). Tissue prints of the stem sections were hybridized with AtCESA4, AtCESA7, and AtCESA8 antibodies, indicating that the labeling in the xylem and interfascicular region in tissue prints was caused by specific recognition of AtCESA4, AtCESA7, and AtCESA8 proteins. It suggested that these genes are co-expressed in the same cells although no cellular details were visible. The next step was to verify if there is actual interaction between them. To accomplish this, a histidine tag was fused at the amino terminal of AtCESA4, AtCESA7, and AtCESA8 proteins. Such a tag allows purification of tagged protein on a Ni column and verification of interacting proteins with tagged protein can be done using Western blot provided antibodies for the interacting proteins are available. Mutant plants were transformed with the respective constructs carrying the wild-type *AtCesA* gene and the proteins were extracted from the transgenic stems. Successful complementation of the mutant with such a construct suggested that His-tag did not interfere with the biological function of CESA. The purification of AtCESA4, AtCESA7, and AtCESA8 and interacting proteins was performed by using immobilized metal affinity chromatography. These proteins were resolved by polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane and probed with AtCESA4, AtCESA7, or AtCESA8 antibodies. According to the data, there are positive interactions among them and consequently they can be part of the same complex (Taylor et al., 2003).

Taken together, these findings support that CelS is at least composed of diverse CESA isoforms encoded by distinct *CesA* genes. Read and Bacic (2002) have further suggested that there are other non-CESA components of rosettes but so far no other proteins have been proven to be part of the rosettes.

ASSEMBLY OF CelS AT THE PLASMA MEMBRANE AND BIOSYNTHESIS OF CELLULOSE

The cellulose is deposited on the external surface of each plant cell. It cannot be synthesized inside the cells because it is a water-insoluble polymer with a rigid linear structure. To accomplish this, the CelS needs to be organized throughout the plasma membrane, forming a channel through which UDP-glucose (a soluble molecule) can pass from the cytoplasm, and be further converted into cellulose (an insoluble polymer) by the action of CESA enzymes. Therefore, it is crucial that CelS is accurately assembled in the plasma membrane.

It has been postulated that two phases of assembly of the CESAs are required (Saxena and Brown, 2005). In the first phase, probably within the cytoplasmatic domain of CelS, three different homodimers could be folded, forming a linear array with six particles. A distinctive CESA may be present in each dimer. In the second phase, apparently in the endoplasmic reticulum and the Golgi apparatus, the linear arrays may be arranged in a rosette with a six-fold symmetry. The assembled CelS may then be transported to the plasma membrane for its activation and subsequent cellulose microfibril synthesis (Haigler and Brown, 1986). Two steps may be necessary for cellulose crystallization. The first one could be formation of monomolecular glucan sheets. Glucan sheets may be folded side-by-side by van der Waals forces inside of CelS rows. In the second step, six separate glucan chain sheets could pass through the CelS orifice and may then be hydrogen-bonded into the crystalline cellulose I microfibril.

In plants, besides CESAs, at least P-sucrose synthase (P-SUSY), which is associated with the plasma membrane, and membrane-bound endo-1,4-β-D-glucanase (KOR) are involved in cellulose biosynthesis (Read and Bacic, 2002). The formation of CelS takes place with the coordinate expression of *CesAs*. Even though firm evidence is not available, it is hypothesized that once the CelS is properly folded and assembled in the plasma membrane, P-SUSY might be converting sucrose into UDP-glucose and fructose. The released UDP is recycled, becoming available again to SUSY for formation of UDP-glucose (Haigler et al., 2001). The UDP-glucose molecules are then β(1-4)-linked by CESAs. The polymerization of glucose chains, assembling, processing, and formation of microfibrils of cellulose I seem to be a highly synchronized process catalyzed by CelS. During the conversion of glucose chains into the microfibril of cellulose I, it has been suggested that monitoring and editing of the microfibril of cellulose I takes place involving KOR, which is associated with the plasma membrane (Delmer and Haigler, 2002; Molhoj et al., 2002). It has been shown that the deposition of cellulose involves regulated intracellular traffic of KOR1 (Robert et al., 2005).

STRUCTURAL FEATURES OF CESA PROTEINS

The genes encoding CESAs were first discovered in *Acetobacter xylinum* (Saxena et al., 1990; Wong et al., 1990). After six years, the first plant *CesAs*, *GhCesA1* and *GhCesA2* were identified in cotton (Pear et al., 1996). Since then, *CesAs* have been identified in several species. *Arabidopsis thaliana* has at least ten *CesAs* which are denominated *AtCesA1-10* (Richmond and Somerville, 2000), following the proposed nomenclature for the genes involved in cellulose synthesis (Delmer, 1999). Nine *CesA* genes were identified in maize (Holland et al., 2000). In aspen, seven *CesA* members were extensively characterized (Joshi et al., 2004) and the presence of an additional two members was suggested (Liang and Joshi, 2004). Recently, six cDNAs encoding CESAs of *Eucalyptus grandis* were reported (Ranik and Myburg, 2006) (Figure 6). Eighteen members of the *CesA* family, the biggest known so far, were found in poplar or cottonwood genome where nine types of *CesAs* are duplicated and located on separate chromosomes (Djerbi et al., 2005; Tuskan et al., 2006). The *CesA* genes generally have 10-14 introns and encode proteins with 985-1088 amino acids (Richmond and Somerville, 2000). Several features are found in the amino acid sequence of the CESA proteins:

(i) Zinc-binding domain: the zinc-binding domain is located at the amino terminal region of protein and it is probably involved with the CESAs oligomerization (Figure 7) (Kurek et al., 2002). In oxidative conditions, it may be involved in the formation of homodimers and heterodimers among CESAs (Kurek et al., 2002). In this domain, there is a conserved motif called CxxC. It is located approximately 10-40 amino acid residues downstream from the amino terminal region: $Cx_2Cx_{12}FxACx_2Cx_2PxCx_2Cx\text{-}Ex_5Gx_3Cx_2C$, where in the x position any amino acid could be present. Only CESAs have the motif CxxC, a feature that distinguishes CESAs from cellulose synthase-like (CSL) proteins. In addition to the motif CxxC, sequence identity is used to help in the distinction of CESA and CSL (Richmond, 2000). A zinc-binding domain-like region has been observed in CSLD members (Samuga and Joshi, 2004a);

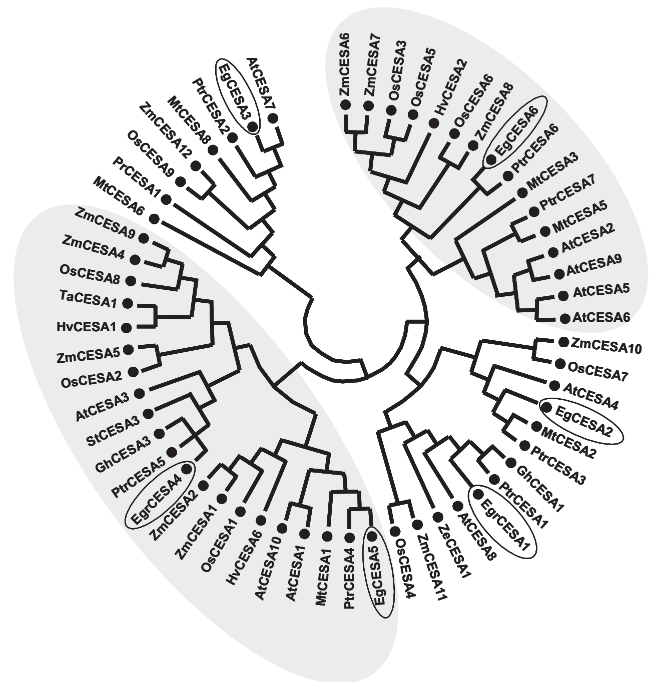

Figure 6. Tree derived from the alignment of the deduced amino acid sequences of 58 full-length CESA protein sequences. A total of 10,000 bootstrap replicates were used and only branches with at least 80% of support were considered for the development of the tree. CESAs associated with primary and secondary cell wall synthesis are respectively shown in gray and white backgrounds. The six *Eucalyptus grandis* CESAs are highlighted with black ellipses. Abbreviations: **CESA**: cellulose synthase; **At**: *Arabidopsis thaliana*; **Eg**: *Eucalyptus grandis*; **Gh**: *Gossypium hirsutum*; **Hv**: *Hordeum vulgare*; **Mt**: *Medicago truncatula*; **Os**: *Oryza sativa*; **Pr**: *Pinus radiata*; **Ptr**: *Populus tremuloides*; **St**: *Solanum tuberosum*; **Ta**: *Triticum aestivum*; **Ze**: *Zinnia elegans*; and **Zm**: *Zea mays*. Reprinted with permission from Ranik and Myburg (2006). Copyright (2006) Heron Publishing.

(ii) Hypervariable region I (HVRI) and II (HVRII): the HVRI and HVRII in the same species show low sequence conservation among *CesA* paralogs. In different species, HVRII is conserved among the *CesA* orthologs and it has been designated as a class specific region CSR (Vergara and Carpita, 2001; Liang and Joshi, 2004). CSR has been used to isolate cDNAs encoding CESAs because this region efficiently discriminates each member of a *CesA* family (Liang and Joshi, 2004; Ranik and Myburg, 2006);

(iii) Motifs indicative of processive glycosyltransferase: the CESA proteins show four motifs (D, D, D, QVLRW)

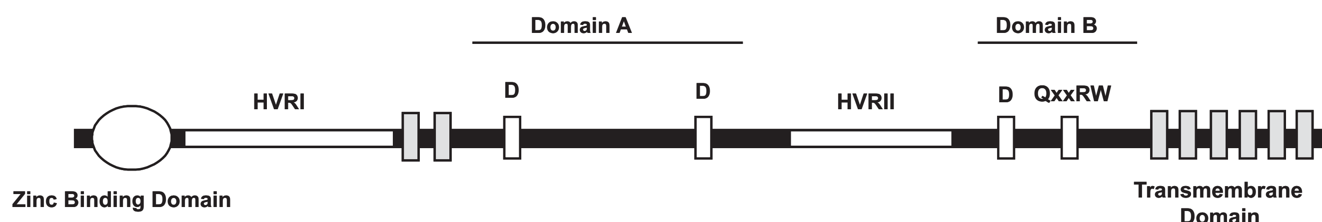

Figure 7. Schematic representation of CESA protein features. Reprinted with permission from Richmond (2000). Copyright (2006) Genome Biology.

which are essential to glycosyltransferase activity (Saxena et al., 2001). The first two D (aspartic acid) residues are located in the globular domain A. In the globular domain B is located the third D residue and the motif QVLRW;

(iv) Globular domain A and B: the globular domains A and B are highly conserved among CESAs (Pear et al., 1996; Wu et al., 2000; Joshi, 2003b; Joshi et al., 2004);

(v) Transmembrane domains: CESAs show eight transmembrane domains. Two domains are located just before the beginning of the globular domain A. The other six transmembrane domains are located just after the globular domain B, close to the carboxyl terminal region (Joshi, 2003a,b; Joshi et al., 2004).

CesA GENE FAMILY IN *Arabidopsis thaliana* AND *Populus tremuloides*

The *A. thaliana* genome has at least ten *CesAs*, denominated *AtCesA1-10*. There is no duplication of a functional *CesA* gene adjacent to any other *CesAs* on *A. thaliana* chromosomes. They are distributed all over the genome indicating that this is not a recent duplication event. The size of the *AtCesA* genes is 3.5-5.5 kb. These genes have 9-13 introns which encode proteins with 985-1088 amino acid residues and its identity is variable from 53-98 % (Richmond, 2000).

The genome of aspen has at least nine types of *CesA* genes: *PtrCesA1* (Wu et al., 2000), *PtrCesA2* (Samuga and Joshi, 2002), *PtrCesA3* (Kalluri and Joshi, 2004), *PtrCesA4* (Kalluri and Joshi, 2004), *PtrCesA5* (Kalluri and Joshi, 2003), *PtrCesA6* (Samuga and Joshi, 2004b), and *PtrCesA7* (Samuga and Joshi, 2004b) all of which encode distinct CESAs. In addition, two partial CSR regions have been isolated that are designated *PtrCesA8* and *PtrCesA9* (Liang and Joshi, 2004). These *CesA* genes and their encoded proteins are highly similar (> 95%) to nine types of *CesAs* present in the poplar genome that was completely sequenced recently (Tuskan et al., 2006). The size of the poplar *CesA* genes is 4.8-7.2 kb. These genes have 12-14 introns which encode proteins with 978-1096 amino acid residues and their identity varies from 64% to 76%. Sequence identity analysis between orthologs of Arabidopsis and aspen *CesAs* reveals that *PtrCesA1*, *PtrCesA2*, *PtrCesA3*, *PtrCesA4*, *PtrCesA5*, *PtrCesA6*, and *PtrCesA7* are respective orthologs of *AtCesA8*, *AtCesA7*, *AtCesA4*, *AtCesA1*, *AtCesA3*, *AtCesA6*, and *AtCesA6* (Figure 6) (Joshi et al., 2004). Thus Arabidopsis and poplars possess orthologous *CesAs*. All nine types of poplar *CesAs* are duplicated with the exception of the poplar *CesA* gene that is homologous to *PtrCesA3* from aspen. All duplicated poplar *CesA* genes are distributed on different chromosomes indicating that *CesA* gene duplications occurred in ancient times and overall both members of duplicated genes are expressed, but in the case of secondary wall-associated aspen *CesAs*, one member is expressed at a much higher level than the other (C.P. Joshi, unpublished observations).

Protein sequence comparisons of higher plant CESAs reveal that all CESAs are grouped into six distinct clades which contain members associated with the primary or secondary cell wall (Figure 6) (Holland et al., 2000; Vergara and Carpita, 2001; Joshi et al., 2004; Liang and Joshi, 2004; Samuga and Joshi, 2004b; Nairn and Haselkorn, 2005; Somerville, 2006). In fact, orthologs of *AtCesA1*, *AtCesA3*, and *AtCesA6* appear to be required for the synthesis of cellulose deposited in the primary cell walls (Fagard et al., 2000; Scheible et al., 2001; Burn et al., 2002; Doblin et al., 2002). In contrast, orthologs of *AtCesA4*, *AtCesA7*, and *AtCesA8* seem to be essential for cellulose synthesis in the secondary cell walls (Turner and

Somerville, 1997; Taylor et al., 1999, 2000, 2003; Zhong et al., 2003). The latter three CESA isoforms are specific to secondary cell wall formation and they actually physically interact with each other so it is highly probable that they are located in the same CelS as discussed previously (Taylor et al., 2003).

In the same way, *PtrCesA1*, *PtrCesA2*, and *PtrCesA3* are involved with cellulose deposition in the secondary cell wall and *PtrCesA4*, *PtrCesA5*, *PtrCesA6*, and *PtrCesA7* may be involved with cellulose deposition in the primary cell wall (Joshi et al., 2004; Kalluri and Joshi, 2004; Samuga and Joshi, 2004b). The gene *AtCesA8* present in the *lew2-1* mutant genome was further characterized and its disruption increases osmotic and drought stress tolerance, indicating that cellulose synthesis may be involved in stress tolerance (Chen et al., 2005), but the cause and consequence relationship needs to be delineated.

THE MOST IMPORTANT UNSOLVED TOPICS IN CELLULOSE BIOSYNTHESIS

Although some remarkable progress has been made during the past decade towards unraveling the process of cellulose biosynthesis, a number of unanswered questions still remain. In higher plants, do two distinct types of complexes of CESAs and other associated enzymes synthesize cellulose Iα and cellulose Iβ? How is cellulose synthesized and deposited during primary and secondary cell wall formation? How is each glucose residue inverted 180° with respect to its neighboring residues? Are monosaccharides or disaccharides substrates for CESAs? How are glucose chains aligned side-by-side to generate a microfibril of cellulose? How many proteins are necessary to fully assemble the CelS and synthesize cellulose? What is the exact function of each of these proteins? How are CESAs arranged in the subunits of CelS? Why are there so many *CesAs* in plants? Do the transmembrane helices of the CESA proteins form a pore in the plasma membrane through which the growing glucan chain can pass? Are *CesAs* regulated at both transcriptional and post-transcriptional phases? Would it be possible to produce wood with high contents of better cellulose and still maintain the necessary physical and mechanical properties of wood? Would it be possible to reconstitute functional CelS *in vitro* that is capable of synthesizing cellulose I? What role(s) do other proteins (such as KOR) play that have been shown to affect cellulose biosynthesis in Arabidopsis mutants? Will overexpression of SUSY or other enzymes involved in photosynthetic carbon fixation improve cellulose biosynthesis? Which proteins control economically important cellulose traits such as microfibril angle, DP, and crystallinity?

In conclusion, there are still a large number of questions that need to be answered to fully understand the process of cellulose biosynthesis in plants that has direct connection with utilization of cellulose for agricultural and forest product manufacturing. The characterization of regulatory regions of *CesAs* genes and their transcription factors might provide insights about regulatory processes involved in the specific expression patterns of *CesAs* genes and consequently cellulose production.

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