Cellulose Synthesis in Higher Plants

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cell wall, cellulose synthase, microtubules, transcription, regulation, mutant

Abstract

Cellulose microfibrils play essential roles in the organization of plant cell walls, thereby allowing a growth habit based on turgor. The fibrils are made by 30 nm diameter plasma membrane complexes composed of approximately 36 subunits representing at least three types of related CESA proteins. The complexes assemble in the Golgi, where they are inactive, and move to the plasma membrane, where they become activated. The complexes move through the plasma membrane during cellulose synthesis in directions that coincide with the orientation of microtubules. Recent, simultaneous, live-cell imaging of cellulose synthase and microtubules indicates that the microtubules exert a direct influence on the orientation of cellulose deposition. Genetic studies in *Arabidopsis* have identified a number of genes that contribute to the overall process of cellulose synthesis, but the role of these proteins is not yet known.

Contents

INTRODUCTION

Cellulose microfibrils are insoluble cable-like structures that are typically composed of approximately 36 hydrogen-bonded chains containing 500 to 14,000 β-1,4-linked glucose molecules. Cellulose microfibrils comprise the core component of the cell walls that surround each cell. Roughly one-third of the total mass of many plants is cellulose. The long, inelastic, microfibrils wrap around cells in spatially oriented overlapping layers that provide resistance to osmotic pressures that are similar in magnitude to the air pressure in a car tire. The pressure of the plasma membrane against the cell wall rigidifies the cell walls, providing the turgor that allows plants to adopt an erect growth habit. The mechanisms by which the embrace of cellulose is relaxed to allow cell division and expansion are an unsolved problem that has recently been described elsewhere (Cosgrove 2005, Marga et al. 2005). This review is focused on recent advances in understanding the mechanisms by which cellulose is synthesized and deposited. This topic has been under investigation for more than 40 years, and many previous reviews have recounted the technical challenges that have bedeviled research in this area (Brown 2004, Delmer 1999, Doblin et al. 2002, Kimura & Kondo 2002, Robert et al. 2004, Saxena & Brown 2005, Williamson et al. 2002). Recently, progress has been made on several fronts, and many promising new avenues of research have opened up, particularly for research on cellulose synthesis in *Arabidopsis*, for which the necessary genetic and genomic tools are well developed.

THE PROPERTIES OF CELLULOSE

To understand cellulose synthesis it is first necessary to understand the properties of cellulose. Because the topic has recently been reviewed by Brett (2000), only those aspects that are germane to understanding cellulose biosynthesis are described here.

Most investigations of cellulose structure have been carried out by chemists who typically exploit the insolubility and chemical resistance of cellulose fibrils to "purify" cellulose by extracting everything else from cell walls with strongly basic solutions that disrupt hydrogen bonds. Thus, it may be useful to bear in mind that the cellulose obtained in this way may have somewhat different properties than native cellulose. Early NMR and X-ray diffraction studies of extracted cellulose indicated that substantial variation in the spectra obtained from different samples may be understood as arising from two distinct types of cellulose called cellulose Iα and Iβ (Brown 1996). Cellulose Iα exists as a single-chain triclinic unit cell, whereas cellulose Iβ has a two-chain monoclinic unit cell. The proportion of Iα varies from approximately 64% in Valonia to 20% in cotton (Brett 2000). In both forms, the cellulose chains are parallel, and successive glucose residues are rotated 180°, forming a flat ribbon in which cellobiose is the repeating unit. The parallel chains are compatible with the idea that the chains in a microfibril are made simultaneously. The cellulose chains are held in a crystalline structure by hydrogen bonds and Van der Waals forces to form microfibrils (Nishiyama et al. 2002, 2003). It is not yet known to what extent the "crystallization" of the nascent glucan chains

to form cellulose may be facilitated by proteins other than the catalytic enzyme. Jarvis (2000) has shown that the two forms can be interconverted by bending. He suggested that the sharp bend thought to take place when cellulose emerges from the rosette and becomes appressed to the overlying cell wall may be sufficient to induce the interconversion (**Figure 1**). Nishiyama et al. (2003) also concluded that slippage of the glucan chains is the most likely mechanism for conversion of Iα to Iβ. Additional forms, which are primarily of interest in the context of industrial uses of cellulose, can be produced from natural cellulose by extractive treatments. For instance, in cellulose II, the chains are antiparallel—something that is unlikely to occur in native cellulose. Cellulose I is converted to cellulose II by extraction under strongly alkaline conditions.

The molecular weight of the individual glucan chains that compose cellulose microfibrils has been difficult to determine because the extraction of these chains may lead to degradation. Analyses of secondary wall cellulose in cotton suggest a degree of polymerization (DP) of 14,000 to 15,000 (Brett 2000). Primary wall cellulose appears to have a lower molecular weight; Brown (2004) reported a DP of 8000 for primary wall cellulose. However, Brett (2000) reported a low-molecular-weight fraction of ~500 DP and a fraction with a DP of 2000–4000 and suggested that the low-molecular-weight fraction may be chains at the surface of microfibrils, whereas the high DP fraction may be chains in the microfibril interior. Because a DP of 2000 corresponds to approximately 1 μm of length, the implication is that the primary wall cellulose fibrils, which are frequently observed to be much longer than 1 μm, must be composed of chains with breaks at various locations along the fibrils. As noted below, this is compatible with genetic evidence that a cellulase is required for cellulose synthesis in both plants and bacteria (Lane et al. 2001, Römling 2002). Whatever the exact length, in some cells the fibrils can be extremely long relative to other types of biological macromolecules.

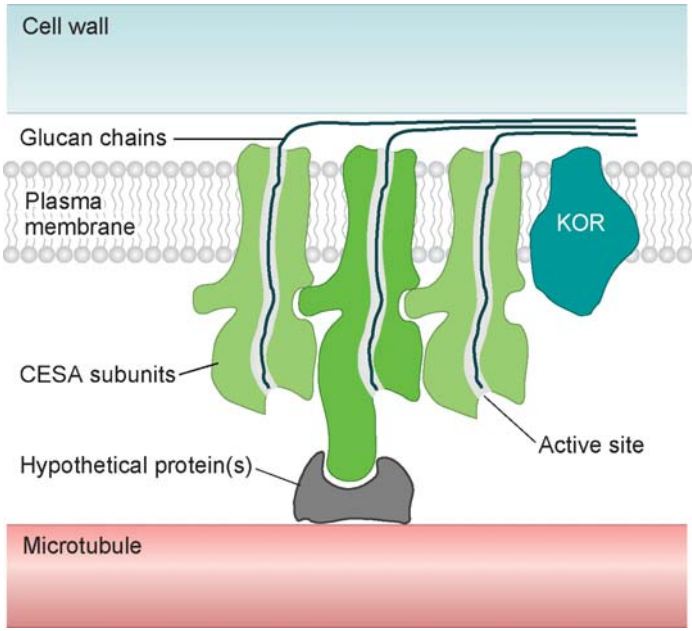

Figure 1

Schematic model of cellulose synthesis. Cellulose synthesis takes place in the plasma membrane. The plasma membrane is tightly appressed to the cell wall so that most of the cellulose synthase is in or below the plane of the membrane, which minimizes friction as the enzyme moves through the plasma membrane in response to elongation of the growing glucan chains by addition of glucan moieties from cytoplasmic UDP-glucose. The cellulose synthase complex is thought to contain as many as 36 CESA proteins, only a subset of which are illustrated. That three types of CESA proteins are required to form a functional complex suggested that different types of CESA proteins perform specific functions, such as interacting with the cortical microtubules.

Based on electron micrographs, researchers have found that the width of cellulose fibrils varies from approximately 25–30 nm in Valonia and other green algae to approximately 5–10 nm in most plants (Ha et al. 1998, Herth 1983). The variation in size may indicate that cellulose microfibrils from different sources contain different numbers of chains, and it may reflect variation in the kind or amount of hemicellulose coating on the fibrils. In a study of onion primary wall by solid-state NMR (Ha et al. 1998), the spectral interpretation was consistent with the idea that the 8-nm-wide microfibrils were composed of six 2 nm fibrils, each containing approximately

Secondary wall: a nonexpendable wall that is deposited between the primary wall and the plasma membrane; usually found in cells that are subject to mechanical stress

Degree of polymerization (DP): the number of sugar residues in a polysaccharide chain

Primary wall: an expandable polysaccharide-rich matrix surrounding all plant cells

Xylogalacturonan: a polysaccharide with a backbone of galacturonic acid residues and xylose side chains

CESA: a member of a family of related proteins that compose cellulose synthase

ten chains. Herth (1983) estimated by electron microscopy that the microfibrils of Spirogyra contained 36 glucan chains. Thus, the measurements are generally consistent with the idea that each of the six globules in a rosette is composed of a number of subunits that synthesize six to ten chains that hydrogen bond to form the 2 nm fibrils. Six of these 2 nm fibrils then bond to form the microfibrils. In certain special cases, such as for quince seed mucilage, in which 2 nm cellulose fibrils coated with xylogalacturonan are dispersed throughout the mucilage, there has evolved a variation of the basic synthetic process in which the 2 nm fibrils may become coated with xylogalacturonan as they are synthesized, thereby preventing their coalescence into large microfibrils (Ha et al. 1998).

Considered as a whole, the analyses of cellulose structure indicate that cellulose synthase is a highly processive enzyme, that it has many active sites that coordinately catalyze glucan polymerization, that alternating glucan units are inverted, and that interspecies variation exists in the number of glucan chains per fibril or possibly in the kind or amount of hemicellulose. What is not clear is whether the enzyme participates in facilitating the hydrogen bonding of the glucan chains or whether proximity of the glucan chains as they emerge from the enzyme is sufficient to cause formation of the highly ordered microfibrils. It is also unclear how cellulose microfibrils develop a regular periodic right-handed twist along the microfibril axis (Hanley et al. 1997). This suggests that the cellulose synthase complexes are under tortional stress and may rotate in the membrane to relieve the stress.

STRUCTURAL PROPERTIES OF CELLULOSE SYNTHASE

Cellulose synthase can be visualized by freeze fracture of plasma membranes in vascular plants as symmetrical rosettes of six globular complexes approximately 25–30 nm in diameter. The rosettes have been shown to be cellulose synthase by immunological methods (Kimura et al. 1999). Based on careful measurements of the dimensions of microfibrils compared with calculated dimensions, Herth (1983) proposed that each of the six subunits of a rosette may synthesize six β-1,4-glucan chains, which cocrystallize into a 36-glucan chain microfibril. In many algae, cellulose synthase appears to be in even larger terminal complexes (TCs), rectangular arrays of globules that produce ribbons of cellulose. The name refers to the fact that they were originally observed at the ends of microfibrils (Montezinos & Brown 1976). Tsekos (1999) has reviewed the different types of TCs that have been observed. Among the most extreme are those of the alga *Oocystis apiculata*, in which the TCs have a width of 30–35 nm and a length of 500 nm and are composed of three rows of approximately 30–40 particles, each 7 nm in diameter. Linear TCs have also been found in a tunicate (Kimura & Itoh 1996), the bacterium *Acetobacter xylinum* (reclassified as *Gluconacetobacter xylinus*) (Ross et al. 1991), and the slime mold *Dictyostelium discoideum* (Grimson et al. 1996). Thus, the mechanisms involved in forming cellulose appear to be readily altered during evolution to produce polymers with different properties.

The only known components of cellulose synthase in higher plants are the CESA proteins, originally identified by sequence similarity of cotton cDNA sequences to bacterial cellulose synthase (Pear et al. 1996). The completion of the *Arabidopsis* genome sequence revealed that *Arabidopsis* has 10 *CESA* genes that encode proteins with 64% average sequence identity (Holland et al. 2000, Richmond 2000). Maize has at least 12 *CESA* genes (Appenzeller et al. 2004), barley has at least 8 (Burton et al. 2004), and poplar has at least 7 (Joshi et al. 2004). The *CESA* genes in green algae show strong sequence similarity to higher plant *CESA* genes and have conserved intron structures (Roberts & Roberts 2004). Thus, it appears that all higher plants have a similar set of genes. The proteins range from 985–1088 amino acids in length and have eight putative transmembrane

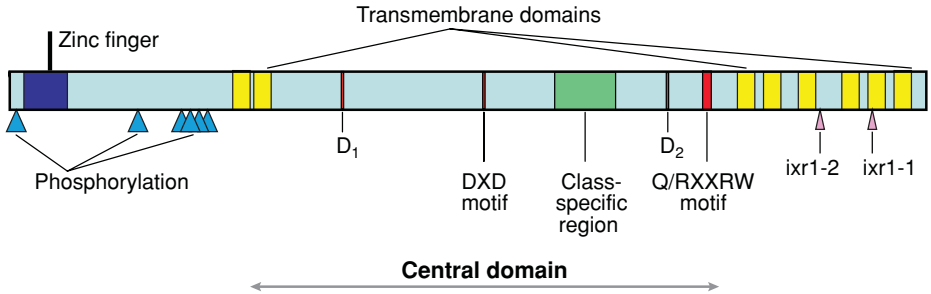

Figure 2

Illustration of the structure of CESA3, a typical CESA protein. Two motifs that have been implicated in activity of related glycosyltransferases are shown as well as the locations of several aspartate residues (D_1 and D_2) that are thought to participate in the enzyme's catalytic activity. The locations of two point mutations that confer resistance to the herbicide isoxaben are shown, as are the sites where the protein has been found to be phosphorylated.

domains. Two of the transmembrane domains are near the amino terminus, and the other six are clustered near the carboxy terminus (**Figure 2**). The N-terminal region of each protein has a cysteine-rich domain with a motif $CX_2CX_{12}FXACX_2CX_2PXCX_2CX-EX_5GX_3CX_2C$ that is a good fit to the consensus for a RING-type zinc finger. RING fingers have been implicated in mediating a wide variety of protein-protein interactions in complexes (Saurin et al. 1996). Otherwise, the N-terminal domain is structurally heterogeneous among the ten CESAs in *Arabidopsis*. The average overall sequence identity of the N-terminal domains is 40%, compared with an averge overall identity of 64%. Expression of the N-terminal domain as a glutathione-*S*-transferase fusion in *Escherichia coli* resulted in a recombinant protein that bound ^{65}Zn (Kurek et al. 2002). Because of the observation that dimerization of other types of proteins occurs via zinc fingers, Kurek et al. (2002) speculated that the CESA proteins also may dimerize by this mechanism. Indeed, the N-terminal domain of the cotton CESA1 protein interacted with itself and with CESA2 in a two-hybrid system and also in pull-down experiments (Kurek et al. 2002). Although this seems entirely believable, there may be additional points of interaction between the subunits to assemble the large complexes that compose rosettes.

A large central domain of approximately 530 amino acids lies between the two regions of transmembrane domains and is thought to be cytoplasmic (Delmer 1999). Use of this feature to anchor the topology of the protein indicates that the N-terminal domain is also cytoplasmic. The central domain is highly conserved among all the CESA proteins except for an approximately 64–91-residue region of unknown significance where there is weak sequence identity. This was originally referred to as a hypervariable region, but as CESA sequences from various species accumulated, Vergara & Carpita (2001) recognized that there is sequence conservation across species and renamed it the class-specific region. The domain contains a motif (Q/RXXRW) that is associated with bacterial cellulose synthases and other processive glycosyltransferases (Saxena & Brown 1997), such as chitin and hyaluronan synthases, and with glucosylceramide synthase (Marks et al. 2001). Additionally, a DXXD motif and two other aspartate residues have been associated with this class of enzymes and is referred to collectively as the D,D,D,Q/RXXRW motif. Site-directed mutagenesis experiments of the chitin synthase 2 of yeast showed that the conserved aspartic acid residues and the conserved residues in the QXXRW motif are required for chitin synthase activity (Nagahashi et al. 1995). Similarly, Saxena et al. (2001)

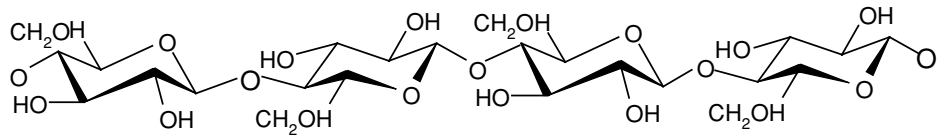

Figure 3

A fragment of β-1,4-glucan showing how alternating sugar residues are inverted.

6xHIS-tagged: a string of six histidine residues that collectively bind nickel

replaced the aspartate residues in the *A. xylinum* cellulose synthase and found that they were required for catalytic activity. Although this does not prove that the identified residues are involved directly in catalysis, it is consistent with the proposal. In the *irx1-1* mutant of *Arabidopsis*, a D683N change inactivated the enzyme (Taylor et al. 2000). Saxena et al. (2001) have presented a very thoughtful analysis of the probable function of the motif elements and also used a computer model to develop a theoretical structure for the domain. It appears that at this point it is not possible to draw any conclusions about the specific roles of the conserved residues in substrate binding or catalysis. In particular, it is not clear from the sequence whether or not the enzyme has two binding sites for UDP-glucose that might explain how alternate glucan moieties are inverted during synthesis (Albersheim et al. 1997) (**Figure 3**).

As noted below (see section on Mutations That Affect Cellulose Synthesis), analysis of mutants with defects in secondary wall cellulose has revealed that three separate CESA proteins are required in the same cell at the same time (Taylor et al. 2003). Evidence that the various CESA subunits interact was obtained by immunoprecipitation experiments in which solubilized cellulose synthase complexes from a plant containing 6xHIS-tagged CESA7 were purified on a nickel column. CESA8 protein was also found in the eluate (Taylor et al. 2000). Thus, within a cell type there may be a single type of complex containing three types of CESA subunits. The three genes required for secondary wall synthesis in *Arabidopsis* are *CESA4*, -7, and -*8*. A requirement for at least three CESA proteins in primary wall synthesis may be inferred from analysis of mutations and antisense constructs for *CESA1*, *CESA2*, *CESA3*, and *CESA6* (Arioli et al. 1998, Beeckman et al. 2002, Burn et al. 2002a, Desprez et al. 2002, Scheible et al. 2001). CESA1 and CESA3 appear to be absolutely required, whereas CESA2 and CESA6 may be at least partially redundant. The phenotype of *cesA5* mutants has not yet been reported. There is, at present, no substantiated explanation for the apparently larger number of primary wall *CESA* genes. Perhaps a different type of complex is required for formation of the new cell walls during cytokinesis than for production of cellulose during cell expansion.

The requirement for multiple types of subunits would be expected based only on geometric considerations (Perrin 2001, Scheible et al. 2001). In brief, it is not possible to make a planar rosette structure containing 30–36 subunits from a single type of subunit because there are a number of distinct protein-protein interactions required. In principle, the three types of CESA proteins in a complex allow at least three types of protein-protein interactions. Of course, it remains possible or even likely that additional protein-protein interactions are also required for the overall process.

Cellulose synthase has been suggested to be a member of a structural class, called the SGC domain proteins, that includes *Bacillus subtilis* glycosyltransferase SpsA, bovine β-1,4-galactosyl transferase 1, and *E. coli N*-acetylglucosamine-1-phosphate uridyltransferase (Unligil et al. 2000). These proteins exhibit no readily detectable sequence identity, but all reportedly show common tertiary structure, defined as the SGC domain.

Although Unligil & Rini (2000) remark that cellulose synthase is a probable member of this group, it appears to be highly speculative because there is no tertiary structural information for CESA proteins and no related proteins that would permit computational threading.

The CESA1 protein from *Arabidopsis* has five putative N-linked glycosylation sites, and mutants of *Arabidopsis* with defects in processing of N-linked glycans are deficient in cellulose synthesis (Gillmor et al. 2002). Robert et al. (2004) used MALDI TOF to measure the mass of peptides containing two of the sites from the cotton CESA1 ortholog and found that they were not modified. Gillmor et al. (2002) observed that treatment of membrane preparations with deglycosylating enzymes did not alter the mobility of CESA proteins on western blots and concluded that CESA proteins involved in primary wall synthesis do not appear to be glycosylated. However, the resolution of SDS PAGE is not adequate to exclude the possibility of glycosylation completely.

MUTATIONS THAT AFFECT CELLULOSE SYNTHESIS

During the past decade, a relatively large number of mutations that affect cellulose synthesis, directly or indirectly, have been identified in mutant screens of *Arabidopsis* for tissue swelling, drug tolerance, embryo lethality, or altered vascular morphology (Arioli et al. 1998, Robert et al. 2004, Somerville et al. 2004, Turner & Somerville 1997, Williamson et al. 2001a). The most extreme mutations, such as nulls of *CESA1*, cause embryo lethality (Beeckman et al. 2002, Gillmor et al. 2002). Homozygous mutant embryos are severely cellulose deficient, and as a result, the cells are swollen, and in some cases the primary cell walls exhibit gaps. A temperature-conditional allele, *rsw1-1*, facilitated analysis of the phenotype of the defect in more mature plants (Arioli et al. 1998, Williamson et al. 2001b). At the nonpermissive condition the cells in expansion zones swell, presumably reflecting loss of ability to restrain turgor. Importantly, rosettes disappear from the plasma membrane, suggesting that a correctly folded CESA1 is essential for assembly. Arioli et al. (1998) reported that although the mutant does not make cellulose, it makes an amorphous glucan. This implies that either the mutant CESA1 protein or other components of the complex continue to function even though they cannot assemble. This seems a bit unusual and bears additional analysis. Perhaps the amorphous glucan is produced by a wound-activated pathway as a response to a complete loss of cellulose synthesis. Indeed, a leaky mutation in the *CESA3* gene (*cev1*) was identified on the basis of enhanced disease resistance due to wound-induced jasmonate production (Ellis et al. 2002). Apparently, the defect in cellulose synthesis is perceived by a cell wall integrity signaling pathway in the plant that induces the defense responses (Pilling & Hofte 2003). In the same vein, virus-induced silencing of *CESA* genes in tobacco (Burton et al. 2000) resulted in plants with a syndrome of effects similar to leaky mutations of *CESA* genes in *Arabidopsis* (Gillmor et al. 2002), including enhanced pectin accumulation.

Null mutations in *CESA6* cause a less severe phenotype than do the *rsw1* mutations (Fagard et al. 2000). In the *procuste 1* (*prc1*) mutants, dark-grown hypocotyls are reduced in elongation and swollen, and the roots have a similar phenotype (Desnos et al. 1996). Normal hypocotyl elongation is restored in plants grown in white, blue, or red light, presumably because expression of functionally redundant CESA genes are induced. Indeed, the *cop1-6* mutation, which alters photomorphogenesis, is epistatic to the *prc* mutants (Desnos et al. 1996). Cloning of a mutant gene conferring resistance to the herbicide isoxaben, *isoxaben resistance 2* (*ixr2-1*), revealed that it carried a mutation in the *CESA6* gene at a site distal to the large cytoplasmic loop containing the proposed active site residues (Desprez et al. 2002) (**Figure 2**). Several mutations in the *CESA3*

Pectin: a polysaccharide containing uronic acids

Photomorphogenesis: light-regulated development

gene (*ixr1-1*, *ixr1-2*) also confer a high degree of recessive isoxaben resistance (Scheible et al. 2001). The recessive nature of these mutations is consistent with the idea that the presence of a sensitive CESA protein in a cellulose synthase complex may render the whole complex sensitive to the compound. However, in this respect, it is not clear why mutations in either *CESA3* or *CESA6* can confer resistance. The implication seems to be that whatever aspect of CESA function is altered by isoxaben is redundantly provided by CESA3 and CESA6. Perhaps an isoxaben-binding site is formed at the junction between the two subunits. Whatever the case, knowledge that mutations in the CESA proteins confer strong resistance to isoxaben obviates some concerns about secondary effects of the compound, something that is not true of any other inhibitor of cellulose synthesis.

Antisense constructs for *CESA1*, *-2*, and *-3* were used to reduce expression of the corresponding genes in *Arabidopsis* (Burn et al. 2002a). These studies showed that reduced expression of *CESA3* produced a severe phenotype comparable with that for *CESA1*. Reduced expression of *CESA2* produced a mild phenotype relative to those observed for *CESA1* and *CESA3*. We have also observed that insertion mutations in *CESA2* and *-5* result in relatively mild phenotypes, similar to those observed for *CESA6* (A. Paredez, S. Persson, and C. Somerville, unpublished data). Thus, although *CESA1*, *-2*, *-3*, *-5*, and *-6* are involved in primary wall synthesis, some of the enzymes are indispensable (e.g., *CESA1*, *-3*), whereas the others appear to have nonessential roles. Unfortunately, it is not yet clear whether the dispensability of *CESA2*, *-5*, and *-6* is due to functional redundancy or some other reason. Double- or triple-mutant analysis should clarify this point. Preliminary results indicated that the double *cesA2 cesA6* mutant has a more severe phenotype than does either parent, suggesting redundancy (S. Persson, A. Paredez, and C. Somerville, unpublished data). Expression of the *CESA3* gene under control of the 35S promoter did not rescue the *cesA1* mutant, whereas expression of the *CESA1* gene under the same promoter did (Burn et al. 2002a). Thus, *CESA1* and *CESA3* have distinct functions.

Mutations that alter secondary cell wall synthesis in *Arabidopsis* typically exhibit collapsed xylem cells and have been designated *irregular xylem* (*irx*) (Turner & Somerville 1997). *The irx5*, *-3*, and *-1* mutations correspond to defects in the *CESA4*, *-7*, and *-8* genes, respectively (**Table 1**) (Taylor et al. 1999, 2000, 2003). As noted above, a key observation that emerged from analysis of the *irx* mutants was that the *CESA4*, *-7*, and *-8* genes were simultaneously required for secondary cell wall synthesis (Taylor et al. 2000, 2003). The observation that the three genes are expressed in the same cells at the same time clearly indicates that the corresponding proteins are not functionally redundant.

Screens for *fragile fiber* (*fra*) mutants of *Arabidopsis*, in which interfascicular fibers exhibit reduced mechanical strength, also resulted in the identification of mutations in the *CESA7* gene (*fra5*) and *CESA8* gene (*fra6*) (Zhong et al. 2003). Interestingly, expression of the *fra5-1* allele of *CESA7* in wild-type plants caused a dominant-negative phenotype, whereas expression of the *fra6* allele of *CESA8* did not. Further analysis of this effect may be very informative about the mechanisms involved in CESA function. One possibility is that the *P557T* mutation caused a conformational change in the CESA7 protein that prevented correct assembly of the complexes. Alternatively, perhaps incorporation of a defective CESA protein into an otherwise wild-type complex caused stalling of the entire complex. The *fra1* mutation, which has reduced fiber strength but apparently normal cell wall composition, was found to encode a kinesin and to have somewhat disoriented cellulose deposition, leading Zhong et al. (2002) to propose a possible role of *fra1* in the orientation of deposition. In reviewing this intriguing mutant, Smith & Oppenheimer (2005) speculate that the protein may have a role in linking cortical

Table 1 Correspondence of mutations and genes implicated in cellulose synthesis[a]

Gene	Mutation	Phenotype	Gene ID
CESA1	*rsw1*	Root swelling (conditional), embryo lethal	At4g32410
CESA2			At4g39350
CESA3	*ixr1*, *cev1*, *eli*	Isoxaben resistance, disease resistance, enhanced lignin	At5g05170
CESA4	*irx5*	Irregular xylem	At5g44030
CESA5			At5g09870
CESA6	*prc*, *ixr2*	Procuste, isoxaben resistant 2	At5g64740
CESA7	*irx3*, *fra5*	Irregular xylem	At5g17420
CESA8	*irx1*	Irregular xylem	At4g18780
CESA9			At2g21770
CESA10			At2g25540
KOBITO	*kob*, *eld1*, *abi8*		At3g08550
KOR	*irx2*, *rsw2*, *lit*, *acw1*	Irregular xylem, root swelling, hypocotyl swelling	At5g49720
FRA1	*fra1*	Fragile fiber	At5g47820
FRA2	*fra2*, *bot*, *frc2*, *ktn1*, *ftr*, *erh3*	Fragile fiber	At1g80350

[a]Because the parallel processes of mutant analysis and gene discovery have led to duplicate names with sometimes confusing symbols, I have referred to cellulose synthase genes thoughout as *CESA*. This Table lists some of the other designations in use for *Arabidopsis*. Unfortunately, the numbering of the *CESA* genes varies from one species to another so that *CESA1* in *Arabidopsis* does not necessarily correspond to *CESA1* in another species.

microtubules to a membrane-associated scaffold, postulated by Baskin (2001) to play a role in cellulose deposition. Live-cell imaging of the behavior of cellulose synthase in this mutant may shed light on the role of the protein.

A relatively large number of *brittle culm* mutants of rice are thought to have defects in cell wall synthesis. Tanaka et al. (2003) isolated and characterized three novel cellulose-deficient mutants of this class and showed that they were due to mutations in three *CESA* genes. As in *Arabidopsis*, the three genes were found to be simultaneously required for cellulose synthesis. The phenotypes of the mutants, which included dwarfing and reduced thickness of cell walls, were suggested to be due to a reduction in primary wall cellulose.

Mutations in a number of other proteins reduce but do not completely eliminate cellulose synthesis. Mutations in the *korrigan* (*kor*) gene exhibit reduced cellulose accumulation and changes in pectin composition that presumably reflect responses to the cellulose defect (His et al. 2001, Nicol et al. 1998, Sato et al. 2001). The KOR protein, which appears to be expressed in all cells, encodes a membrane-localized β-1,4-glucanase (Nicol et al. 1998). The soluble domain of a KOR-like protein from *Brassica napus* expressed in *Pichia pastoris* showed cellulase activity but was not active on related polysaccharides such as xyloglucan (Molhoj et al. 2001). An ortholog purified from poplar had similar properties (Master et al. 2004). A number of other mutations have been found to be mutant alleles of the *KOR* gene. These include *rsw2*, which exhibits a temperature-sensitive defect in cellulose accumulation (Lane et al. 2001), as does another allele identified by Sato et al. (2001); the *irx2* mutation (Szyjanowicz et al. 2004); *lions tail* (*lit*); and *altered cell wall 1* (*acw1*) (Molhoj et al. 2002).

The role of KOR is unknown. A C-terminal green fluorescent protein (GFP) fusion to *KOR* expressed in tobacco BY-2 cells accumulated in intracellular organelles in interphase cells but was localized to the phragmoplast in dividing cells (Zuo et al. 2000).

Xyloglucan: a polysaccharide with a glucan backbone and xylose-containing side branches

GFP: green fluorescent protein

BY-2 cells: an established cell culture of tobacco

Phragmoplast: a polysaccharide-rich matrix that is deposited between daughter cells during cell division

YFP: yellow fluorescent protein

Dichlobenil (DCB): an herbicide that inhibits cellulose synthesis

Abscissic acid: a plant hormone derived from carotenoids

GPI: glycophosphatidyl inositol

Unfortunately, evidence was not presented as to whether the GFP fusion complemented a *kor* mutation, and subsequent studies failed to confirm this localization in *Arabidopsis* (Robert et al. 2005). A putative tomato ortholog was previously found to be located in both the endomembrane and plasma membrane fractions by subcellular fractionation methods (Brummell et al. 1997). Most recently, a functional GFP fusion was observed in endosomes and Golgi membranes but could not be seen in the plasma membrane (Robert et al. 2005). Thus, the localization results do not provide clear evidence for an association with cellulose synthesis. However, recent live-cell imaging of individual cellulose synthase complexes tagged with yellow fluorescent protein (YFP) has revealed that it is possible to visualize discrete complexes in the plasma membrane (Paredez et al. 2006). If fewer than approximately ten molecules of KOR were attached to each CESA complex, it would be technically very challenging to observe plasma membrane localization. Thus, based on the functional evidence, I think it likely that KOR is associated with the CESA complexes. The simplest notion at present seems to be that KOR may remove noncrystalline glucan chains and/or relieve tensional stress, which presumably is generated during the assembly of the large number of glucan chains into a microfibril (Molhoj et al. 2002). This view is generally consistent with the observation that bacterial cellulose synthesis requires a related glucanase for in vivo activity but not for in vitro activity (Römling 2002).

The cellulose-deficient *elongation defective 1* mutations (*eld1*) (Lertpiriyapong & Sung 2003), which are allelic to *kobito* (*kob*) (Pagant et al. 2002), affect a protein of unknown function. In cells within the elongation zone of *kob1* roots, microfibrils were oriented randomly and occluded completely by pectic material at the cytoplasmic side of the wall. Randomly oriented microfibrils also have been observed in elongating root cells of the cellulose-deficient mutant *rsw1* at restrictive temperatures and in the wild type treated with 1 uM dichlobenil (DCB), indicating that a severe reduction in the synthesis of cellulose alters orientation of the remaining microfibrils (Sugimoto et al. 2001).

Surprisingly, an *abscissic acid insensitive* mutation (*abi8*) was also found to be allelic to *eld1/kob* (Brocard-Gifford et al. 2004). A detailed analysis of the abscissic acid–related phenotypes did not lead to a clear explanation for the *abi* or the cellulose-deficient phenotypes. An overexpressed GFP-KOB1 fusion localized to the plasma membrane of cells in the root elongation zone but showed a punctate intracellular distribution in the cell division zone at the root tip (Pagant et al. 2002). However, a complementing ABI8-GUS fusion expressed under control of the *ABI8* promoter was limited to the root elongation zone and the more terminal portions of the zone of differentiation, where it was concentrated in punctate patches. Brocard-Gifford et al. (2004) suggested that the intracellular localization of the GFP fusion may have been an artifact caused by hyperexpression or by occlusion of a putative N-terminal signal sequence. Indeed, a C-terminal GFP fusion that complemented the mutation was localized to the cell wall (Lertpiriyapong & Sung 2003).

The *COBRA* (*COB*) gene encodes a glycophosphatidyl inositol (GPI)–anchored plant-specific protein of unknown function (Schindelman et al. 2001). The first mutant alleles described had relatively weak phenotypes and were fertile when grown on soil. However, null *cob* mutants are extremely deficient in cellulose and are strongly dwarfed (Roudier et al. 2005). Most of the protein is located in the cell wall rather than the plasma membrane (Roudier et al. 2005), suggesting that the function of the GPI anchor may be to deliver the protein to the wall by a pathway that circumvents the accumulation of the protein in the Golgi lumen. Alternatively, the protein appears to be enriched in longitudinal cell walls, and the GPI anchor may play a role in polarizing secretion. Indeed, in elongating epidermal cells, the COB protein is distributed in the cell wall in transverse bands

that parallel cortical microtubules. When microtubule organization is altered with oryzalin, the COB banding pattern becomes disrupted, implying that microtubules play a role in COB localization. A detailed analysis of the cell wall phenotype of a null allele in *Arabidopsis* concluded that the protein appears to have a role in orienting cellulose microfibril deposition (Roudier et al. 2005). However, no mechanism could be proposed. *Arabidopsis* has 11 related genes, and similar families exist in other plants (Roudier et al. 2002). A mutation in a cobra-like gene (*cobl4*) exhibits defects in secondary cell wall synthesis (Brown et al. 2005). The *brittle culm 1* (*bc1*) mutant of rice is deficient in a COB ortholog and exhibits a syndrome of phenotypes similar to those of the *Arabidopsis* mutant (Li et al. 2003).

Mutants deficient in glycosidase I (*knopf*) (Boisson et al. 2001, Gillmor et al. 2002) and glycosidase II (*rsw3*) (Burn et al. 2002b), enzymes that catalyze the early steps of N-linked glycan maturation, are severely deficient in cellulose. Putative null mutant alleles of both genes cause embryo lethality, presumably because of many pleiotropic effects on processes not directly related to cellulose synthesis. The effect on cellulose synthesis appears to be indirect because cellulose synthase does not appear to be glycosylated (Gillmor et al. 2002). Because glycosylation of KOR is required for its in vitro activity (Molhoj et al. 2001), an effect on KOR may be sufficient to explain the phenotype of the mutants (Gillmor et al. 2002).

A key issue concerning the results of mutant analysis to date is the degree to which all the genes for proteins directly involved in cellulose synthesis have been identified by mutations. Given that null alleles of *CESA1* and *CESA3* are embryo lethals, null mutations in other proteins required for primary cell wall cellulose synthesis likely are also lethal. However, screens for cellulose-deficient embryo lethals have produced relatively uninformative mutants, such as five *peanut* (*pnt*) loci, that encode genes required for GPI anchoring (Gillmor et al. 2005). Although such mutants have value in the context of understanding certain aspects of GPI anchoring, they are so pleiotropic that they do not provide significant insights into the mechanisms involved in cellulose synthesis. In view of the significant effort required to characterize such mutants, a more efficient system for identifying informative mutants is required. The most promising approach appears to be the use of statistical correlation methods to analyze DNA chip data for genes exhibiting expression patterns that are highly correlated with genes, such as *CESA*s, that are known to be involved in the process (Brown et al. 2005, Persson et al. 2005). Investigation of the phenotypes of T-DNA insertions in some of the genes highly correlated with *CESA4*, *-7*, and *-8* in *Arabidopsis* resulted in the identification of eight genes (*irx6–13*) newly implicated in secondary cell wall formation. Although none of the genes for which T-DNA insertions were readily available appears to be a good candidate for a direct role in cellulose synthesis, several of the genes for which mutations have not yet been reported, such as a rho-binding protein (At1g27380), may play interesting roles.

T-DNA: the region of the Ti plasmid from *Agrobacterium tumefaciens* that is transferred into plant cells during plant transformation; usually shows little resemblance to that found on naturally occurring Ti plasmids

ENZYMOLOGY

Attempts to measure cellulose synthase activity in vitro have been problematic. When incubated with UDP-glucose, plant membrane preparations usually yield large quantities of (1,3)-β-D-glucan (callose) but little or no cellulose (Li & Brown 1993). The similarity in the structure of cellulose and callose necessitates careful and time-consuming analysis of the products of in vitro reactions. The high level of callose synthase activity and the frequent absence of cellulose synthase activity in plant extracts led to speculation that wound-induced callose is produced by cellulose synthase as a result of some change in enzyme activity associated with cellular disruption (Brett 2000). However, the identification of genes for callose synthase and the analysis of mutants deficient in wound-induced callose

accumulation (Jacobs et al. 2003, Nishimura et al. 2003) indicate that this idea was incorrect.

Steady progress has been made in defining the conditions for assay and solubilization of cellulose synthase activity, although rates are still rather low (Kudlicka & Brown 1997, Kudlicka et al. 1995, Lai-Kee-Him et al. 2002). A recent study, which was carried out with large volumes of detergent-solubilized membranes from suspension cultures of *Rubus fruticosus* that facilitated structural analysis of the products (Lai-Kee-Him et al. 2002), provided compelling evidence for synthesis of high-molecular-weight crystalline cellulose from UDP-glucose in vitro. The cellulose was visualized by electron microscopy, and the properties characterized by linkage analysis and X-ray diffraction, leaving no doubt as to the identity of the in vitro product. Interestingly, in vitro–synthesized cellulose was significantly more resistant to the Updegraff reagent (a mixture of acids) than was cellulose extracted from plants. The authors proposed that this may indicate that cellulose synthesized in vitro is not subject to the biophysical deformations, such as bending, that are thought to be associated with in vivo synthesis and deposition (Brett 2000).

Kudlicka & Brown (1997) examined cellulose synthesized in vitro by electron microscopy and observed globular particles that have the same appearance as rosettes attached to the ends of the cellulose microfibrils. Similar structures were observed by Lai-Kee-Him et al. (2002), who localized them at the nonreducing ends of the nascent cellulose fibrils. This result is in keeping with the work of Koyama et al. (1997), who observed the addition of glucose units on the cellulose microfibrils from *Acetobacter aceti* at the nonreducing ends of the growing ribbons. The question of the direction of chain growth remains controversial, however, because cellulose chains from *A. xylinum* were described by Han & Robyt (1998) to elongate from the reducing ends. In view of evidence that β-chitin, starch, and glycogen are polymerized from their nonreducing ends (Sugiyama et al. 1999), and in view of the evidence from Lai-Kee-Him et al. (2002), polymerization of cellulose likely occurs from the nonreducing ends.

The issue of a metal requirement for catalytic activity has not yet been completely resolved by the in vitro studies. The addition of Mg was necessary for maximal rates of cellulose synthesis from *R. fruticosus* extracts that were solubilized with the detergent Brij 58, but inhibited activity of extracts solubilized with taurocholate (Lai-Kee-Him et al. 2002). Activity in the absence of a divalent metal would distinguish cellulose synthase from the SGC domain proteins in which a divalent metal must bind anew at each catalytic cycle to form the nucleotide sugar–binding domain (Unligil & Rini 2000). Because the metal is transiently bound in SGC domain proteins, trace amounts in the assay would not be expected to support significant rates of activity. Thus, I infer that cellulose synthase is not a member of the SGC domain proteins.

The mechanism of cellulose synthesis is poorly understood. One of the persistent issues about the mechanism concerns the fact that adjacent sugar residues have opposed orientations (**Figure 3**). It has been proposed that cellulose synthase has two active sites, one for each orientation, to facilitate the simultaneous polymerization and extrusion of the linear polymer (Albershein et al. 1997, Koyama et al. 1997). The same situation applies to the processive glycosyltransferases that make chitin, hyaluronan, and heparin. Recently the first test of the two-site model was reported for chitin synthase (Yeager & Finney 2004). These authors reasoned that if there are two UDP-GlcNAc-binding sites in close proximity, then dimeric nucleoside inhibitors should be more potent inhibitors of catalysis than would the corresponding monomers. Potential bivalent inhibitors were synthesized by linking together 5′-deoxy-5′-aminouridine residues connected by ethylene glycol linkers of various lengths. Certain dimers were an order of magnitude more

potent than monomeric derivatives, supporting the idea of a two-site mechanism. Conversely, UDP-chitobiose was not a substrate for chin synthesis, mediating against the idea that an accessory protein may first condense two molecules of UDP-GlucNAc as a substrate for the synthase (Chang et al. 2003). Although these results suggest a two-site model, the CESA proteins contain only one QXXRW motif, suggesting that if two sites exist, they have distinct structural features (Saxena et al. 2001).

Peng et al. (2001) made a potentially important observation, following inhibition of cellulose synthesis in cotton with the inhibitor CGA 325'615. They treated the resulting cell walls with cellulase with the intention of releasing cellulose synthase from nascent cellulose microfibrils. They observed that a tryptic peptide corresponding to residues 388–413 of *Arabidopsis* CESA1 was modified by mass amounts equivalent to the addition of two to six glucose residues. This seems to imply that a covalent attachment of glucan to the protein is involved in cellulose synthesis. Retaining glycosyltransferases may contain a transient covalent linkage between an Asp of the enzyme and the reducing end of the growing glycan chain (Unligil & Rini 2000). However, CESA proteins are considered to be members of family 2 glycosyltransferases and are proposed to function as inverting enzymes (Franco & Rigden 2003), which do not have such a predicted intermediate. Peng et al. (2001) suggest that CGA 325'615 may have caused some abnormal linkage to be created.

There has been persistent interest in the concept that cellulose synthesis is initiated from a primer. Studies of the matter using bacterial synthase are controversial (Römling 2002). Delmer and colleagues have suggested that sterol glucoside is a primer for cellulose synthesis (Peng et al. 2002). One line of evidence is that expression of cotton CESA1 in yeast caused formation of sterol cellodextrin from exogenously supplied sterol glucoside. Although this is intriguing, the ability to modify sterol glucoside at extremely low rates under highly artificial conditions does not mean that a primer is involved in vitro; many enzymes are assayed with artificial substrates that bear limited structural similarity to in vivo substrates. A second line of evidence is that treatment of cotton fibers with DCB reduces incorporation of radioactive glucose into sterol glucoside. Because the mode of action of DCB is not known, DCB may act by inhibiting formation of UDP-glucose or through some other indirect effect. Indeed, there is evidence that exogenous addition of sterol glucoside may overcome the effects of DCB on in vivo cellulose synthesis in cotton fibers (Peng et al. 2001). Although the results are interesting, the demonstrations of in vitro cellulose synthesis did not require addition of any primer. The hypothesis should be tested by analysis of *Arabidopsis* mutants with defects in the synthesis of sterol glucosides, as suggested by Peng et al. (2002). Unfortunately, analysis of an *Arabidopsis* mutant with T-DNA insertions in genes for the two known sterol glycosyltransferases indicated only a 40-fold reduction in sterol glucosides (W. Scheible, J. Milne, H. Schaller, and C.R. Somerville, unpublished results), which renders ambiguous the absence of any apparent effect on cellulose synthesis.

Ihara et al. (2002) expressed the central domain of GhCESA2 in *P. pastoris* and found that it was soluble. It catalyzed incorporation of glucose into a product in the presence of an extract from cotton ovules, but the product was not β-1,4-glucan.

Inverting enzymes: change anomeric configuration during the reaction

CELLULOSE DEPOSITION

A distinguishing feature of plant cells is the presence of cortical microtubules adjacent to the plasma membrane (Shaw et al. 2003). The orientation of cortical microtubules in expanding cells is similar to that of cellulose microfibrils (Ledbetter & Porter 1963). This observation led to the hypothesis that the deposition of cellulose is oriented by an interaction between cellulose synthase and the microtubules, an idea that was reinforced by

many observations of correlations between microtubule and microfibril organization that have been comprehensively and critically reviewed by Baskin (2001). In the model of Giddings & Staehelin (1991), as recast in an influential textbook (Alberts et al. 2002), the movement of cellulose synthase is constrained by a close association between cortical microtubules and the plasma membrane, much like a bumper car bouncing along between rails of cortical tubulin. It is generally assumed that the energy of polymerization provides the motive force that moves the cellulose synthase complex through the membrane.

However, as noted in a recent critique of the model, there is no direct evidence for involvement of microtubules in microfibril orientation, and there are many inconsistencies that mediate against the idea (Wasteneys 2004). For instance, short treatment of *Arabidopsis* with the microtubule-destabilizing drug oryzalin or the microtubule-stabilizing drug taxol caused no apparent change to the orientation of cellulose microfibrils in cells that expanded during the treatment, as visualized by field emission scanning electron microscopy (Baskin et al. 2004, Sugimoto et al. 2003). Long treatments caused changes in cellulose orientation, but these may have been due to effects on the orientation of cell division. Similarly, when microtubule polymerization was impaired by shifting the temperature-sensitive *mor1-1* mutant to nonpermissive temperature, cellulose microfibrils exhibited a similar pattern of deposition as in controls (Himmelspach et al. 2003, Sugimoto et al. 2000).

Recently, Paredez et al. (2006) produced a functional N-terminal YFP fusion to CESA6 that complemented the corresponding mutant in *Arabidopsis*. When the fusion protein is expressed under the native promoter, a substantial amount of it accumulates in the Golgi apparatus, where it assembles into distinct particles that move to the plasma membrane. This is compatible with previous electron microscopy evidence indicating that cellulose synthase rosettes assemble in the Golgi (Haigler & Brown 1986). Within less than a minute of arriving in the plasma membrane, the cellulose synthase particles begin moving in linear paths at a constant rate of approximately 300 nm min^{-1}, somewhat slower than the rate observed by Hirai et al. (1998) on tobacco membrane sheets. This is reminiscent of yeast chitin synthase III, in which activity is regulated by a specialized mechanism of vesicle sorting coupled with endocytic recycling (Valdivia & Schekman 2003). In this model, chitin synthase is maintained inside specialized vesicles called chitosomes (TGN/early endosome vesicles) and is transported to the specific sites of function where it becomes activated. Inactivation occurs via endocytosis. Because plant Golgi do not synthesize cellulose, the cellulose synthase complexes observed there are not active but become activated upon arrival at the plasma membrane. Rosettes have also been estimated to have only a 20 min lifetime in moss (Rudolph & Schnepf 1988), which may suggest that they are also dissociated or endocytosed.

When viewed in cells in which the microtubules are labeled with cyan fluorescent protein, the YFP-labeled cellulose synthase particles can be seen to move along the microtubules. Importantly, inhibition of tubulin polymerization with oryzalin rapidly leads to strong disruptions of the normal patterns of movement of the cellulose synthase particles that aggregate in patterns resembling meandering streams (**Figure 4**). Thus, from live-cell imaging it is readily apparent that microtubules exert a strong effect on the orientation of cellulose synthase movement (which presumably reflects cellulose synthesis). However, after relatively long periods of oryzalin treatment, when most or all of the cortical microtubules have depolymerized, the cellulose synthase particles resume movement in relatively straight parallel paths. The rigidity of cellulose probably explains why no guidance is necessary to ensure that cellulose synthase moves in relatively straight lines. It is not clear what orients the pattern of deposition in these cells, but models for the formation of

oriented patterns of cellulose based on geometric considerations have been proposed (Emons & Mulder 1998) and may be testable in these experimental materials. These observations suggest that both sides of the microtubule-microfibril alignment debate are correct and that the discrepancies and inconsistencies between experiments reflect the limitations of using static imaging methods and different treatment times and conditions. The availability of the new imaging tools outlined here should facilitate a resolution of the matter.

Alignment of GFP-labeled cellulose synthase with microtubules was previously reported by Gardiner et al. (2003), who used an N-terminal fusion of GFP to the xylem-specific CESA7 (IRX3) protein. Because of difficulties in viewing the vascular tissues by confocal microscopy, the images of this GFP:CESA7 construct are difficult to discern. However, it appears that the distribution of fluorescence is not uniform and there are bands of fluorescence that are perpendicular to the cells' long axis. Attempts to colocalize tubulin with CESA7 using immunofluorescence methods (Gardiner et al. 2003) indicate a similar pattern. However, the resolution of the images was not high enough to provide a critical analysis. Treatment with the microtubule assembly inhibitor, oryzalin, rapidly reduced the banding pattern. Given the technical limitations of working with xylem-localized markers, the observations of Gardiner et al. (2003) appear to be entirely consistent with the more recent work of Paredez et al. (2006).

A surprising twist to the microtubule–cellulose synthase story was the observation that in tobacco protoplasts, inhibition of cellulose synthase activity prevented the development of oriented microtubule arrays (Fisher & Cyr 1998). These data are consistent with the hypothesis that cellulose microfibrils or cellulose synthase, directly or indirectly, provide spatial cues for cortical microtubule organization. Similarly, isoxaben altered microtubule organization in spruce pollen tubes (Lazzaro et al. 2003), and DCB disrupted the orientation of microtubules in *Arabidopsis* root epidermal cells (Himmelspach et al. 2003).

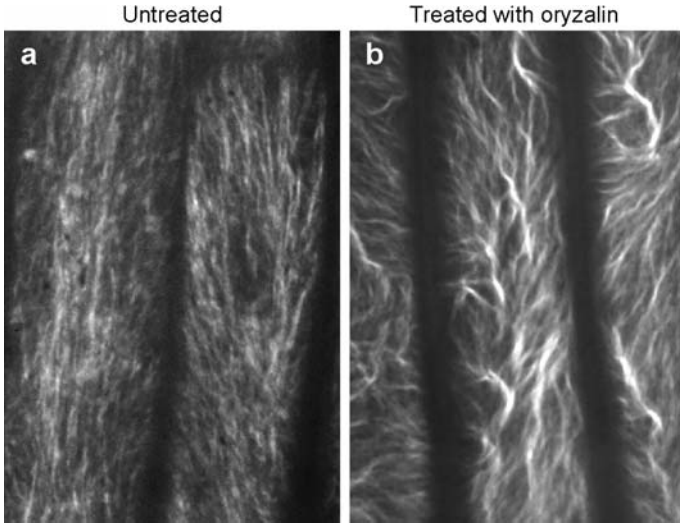

Figure 4

The effect of oryzalin on the movement of YFP:CESA6 in *Arabidopsis* hypocotyl epidermal cells. (*a*) A region of a hypocotyl cell was visualized by confocal microscopy for 10 min. A series of 60 optical sections of the plasma membrane were collected during the 10 min interval, and then the images were computationally averaged to show the pattern of movement of the YFP:CESA6 complexes, which can be seen to have moved in the vertical axis. (*b*) A hypocotyl cell was treated with 10 uM oryzalin for 45 min and then imaged in the same way as in panel *a*. The oryzalin caused strong distortions of the pattern of movement of the YFP:CESA6 complexes.

REGULATION OF CELLULOSE SYNTHESIS

In bacteria, cellulose synthase appears to be constitutively produced and is activated by the regulatory molecule Bis-(3′-5′)-cyclic dimeric guanosine monophosphate (c-diGMP) (Römling 2002, Römling et al. 2005). C-diGMP has not been found in plants, but cotton fibers were reported to have a binding protein (Amor et al. 1991). However, comparison of the sequence of the apparent binding protein with the *Arabidopsis* proteome indicates that the putative binding protein is α-tubulin or something that copurified with it.

As noted above, recent results suggest that plant cellulose synthase is activated by a process associated with secretion. In principle, a plasma membrane–localized kinase or phosphatase may alter the activation state of cellulose synthase following transfer from the Golgi, providing a mechanism for keeping it inactive in the Golgi but rapidly activating it upon arrival in the plasma membrane. A proteomics survey of plasma membrane phosphoproteins revealed that CESA1, CESA3, and CESA5 proteins were phosphorylated at a number of sites, and several of the peptides had more than one residue phosphorylated (Nuhse et al. 2004). The sites were clustered in the N-terminal domain and in the hypervariable region of the central domain (**Figure 2**). In addition, the KOR protein had at least two phosphorylated peptides. Preliminary analysis of *cesA*7 mutants with altered phosphorylation indicates that phosphorylation affects activity (Taylor 2005).

During cell expansion, cellulose synthesis is a major consumer of fixed carbon. Thus, likely whatever regulates cellulose synthesis is coordinated with other aspects of primary carbon metabolism. In plants, UDP-glucose is thought to be largely synthesized by sucrose synthase (SUSY) (Haigler et al. 2001). Amor et al. (1995) observed a form of SUSY that was associated with the plasma membranes. They also observed that sucrose supported much higher rates of cellulose synthesis by extracts from developing cotton fibers than did UDP-glucose and that sucrose synthase is very strongly upregulated in cotton fibers at the onset of fiber elongation. Haigler et al. (2001) have presented an extensive review of the hypothesis that SUSY may channel UDP-glucose to cellulose synthesis. This is an attractive idea, but direct evidence is lacking. *Arabidopsis* has six SUSY genes, so it may be challenging to analyze the effects of mutations in all these genes and to provide a direct test of the hypothesis. Transgenic suppression of several SUSY genes in developing cotton fibers prevented formation of fiber cells (Ruan et al. 2003). The effect was more profound than could be attributed solely to an inhibition of cellulose synthesis, obscuring a mechanistic interpretation of the effects. Increased expression of various forms of SUSY in transgenic tobacco plants did not result in increased cellulose per cell, suggesting that UDP-glucose is not the limiting factor in cellulose accumulation in that system (Coleman et al. 2006).

Analysis of the steady-state level of mRNA in major tissues of *Arabidopsis* with gene chips showed that the *CESA1*, *-2*, *-3*, *-5* and *-6* genes are expressed in all tissues at moderately high levels that differ by approximately fourfold at most (Hamann et al. 2004). Similar results can be compiled from the large number of public microarray datasets that are now available for *Arabidopsis* from sites such as Genevestigator (Zimmermann et al. 2004). As noted below, CESA1, -2, -3, and -6 have been implicated in primary wall synthesis by mutant analysis. Analyses of expression of CESA genes in *Arabidopsis* embryos revealed that CESA1, -2 -3, and -9 are the only CESAs expressed there (Beeckman et al. 2002). Thus, following the nomenclature of Burton et al. (2004), CESA1, -2, -3, -5, -6, and -9 are probably involved in primary wall synthesis and are referred to as Group I CESAs. By contrast, CESA4, -7, and -8 are mostly or only expressed in tissues, such as stems, in which secondary cell walls are found and are designated Group II (Hamann et al. 2004, Taylor et al. 2000). *CESA4* promoter:GUS expression studies confirmed that the *CESA4* gene was mostly or only expressed in the vascular tissues (Holland et al. 2000). Similarly, immunological staining of tissue prints with antibodies against CESA7 and CESA8 showed that the corresponding genes were expressed only in the xylem and interfascicular region (Turner et al. 2001).

Maize has at least twelve *CESA* genes (Appenzeller et al. 2004). PCR analysis of transcript levels of six of the genes in various tissues indicated that all the genes were expressed in all of the tissues examined (Holland et al. 2000). Analysis of eight of the maize genes by massively parallel signature

sequencing indicated that the levels of several of the *CESA* genes varied from one tissue type to another, but no conclusions were reached concerning functional specialization (Dhugga 2001). A subsequent analysis that included three additional genes resulted in the identification of three genes that were specifically associated with secondary cell wall formation (Appenzeller et al. 2004). Thus, maize also shows evidence for specialization of primary and secondary cell wall synthases.

Quantitative information about the relative levels of expression of the *Arabidopsis CESA* genes is lacking because the gene chips used for most studies have not been calibrated for the various *CESA* genes. By contrast, Burton et al. (2004) used quantitative PCR to measure the expression of the eight known barley *CESA* genes. They observed that the *CESA* genes could be grouped into two expression patterns (i.e., Group I and II) that were generally consistent with roles in primary and secondary wall synthesis. Additionally, they observed large differences in the relative abundance of transcripts for the various members of a CESA group. If the *CESA* genes are translated with similar efficiency, this observation would suggest that the various CESA proteins are not present in identical amounts in the CESA complexes.

Consistent with genetic evidence that at least three CESA proteins are required to produce a functional cellulose synthase complex, correlation analysis of public and private DNA chip datasets revealed that expression of the *Arabidopsis CESA4*, *-7*, and *-8* genes were indeed very highly correlated (Brown et al. 2005, Persson et al. 2005). The expression of a number of other genes was also very highly correlated with these genes, and insertion mutations in several of these genes resulted in cellulose-deficient phenotypes. Mutations in some highly correlated genes did not result in obvious effects on cellulose synthases but resulted in other defects in secondary wall synthesis. Thus, the evidence is compatible with the idea that the *CESA* genes that participate in secondary wall synthesis are under developmental control along with other genes required for secondary wall synthesis. The *CESA* genes implicated in primary wall synthesis were less highly correlated. This is consistent with the observation that there are more than three *CESA* genes associated with primary wall synthesis. This presumably indicates that some of the Group I CESAs are functionally redundant and that therefore their expression may vary from one tissue to another for unknown reasons. For instance, as noted above, CESA9 appears to be specifically expressed in embryos.

There is sparse evidence suggesting that cellulose synthesis may be regulated in response to stimuli other than developmental programs. Transgenic trees in which 4-coumarate:coenzyme A ligase expression was reduced by expression of an antisense gene exhibited up to a 45% reduction of lignin and a 15% increase in cellulose (Hu et al. 1999). Conversely, antisense-mediated reduction in expression of an α-expansin in petunia caused a significant reduction in cellulose accumulation in petals (Zenoni et al. 2004). According to current theories of expansin action (Marga et al. 2005), this presumably reflects an indirect effect from a defect in cell expansion. The properties of this mutant raise the possibility that many or all mutants with defects in cell expansion may have reduced cellulose content owing to some form of feedback regulation of cellulose synthesis.

Habituation of tobacco cells to growth on low levels of DCB led to increased accumulation of cellulose synthase, as determined by immunological methods (Nakagawa & Sakurai 1998). Nakagawa & Sakurai (1998) suggested that DCB may stabilize the CESA complexes but did not examine the effect on the rate of transcription or translation of CESA proteins. By contrast, habituation of *Arabidopsis* cell cultures to growth on isoxaben led to a decrease in the steady-state level of CESA transcripts but upregulation of a number of other genes implicated in cell wall synthesis (Manfield et al. 2004). This is obviously a topic that merits further attention.

Lignin: a polyphenolic polymer that may comprise up to approximately 30% of plant cell walls; provides strength and resistance to pathogens

Expansin: a protein that stimulates cell wall expansion

CONCLUDING PERSPECTIVES

Considering the importance of cellulose to plant growth and development, not to mention the economic value of cellulose, it is remarkable how little is known about how it is synthesized. Since the cloning of the first CESA genes approximately ten years ago, and the completion of the *Arabidopsis* genome sequence in 2000, there has been significant progress, particularly in identifying genes involved in some aspect of the process. Because it has been so challenging to understand the properties of the enzyme and the overall process by traditional enzymology, I would like to echo the sentiments expressed by Keith Roberts (2001) that cellulose synthesis should be viewed as a "cellular process" in much the same way that DNA replication and transcription are viewed as processes rather than only as "enzyme mediated reactions." In this respect, the recently developed tools for imaging individual cellulose synthase complexes in live cells may provide the next qualitative stimulus to the field by allowing sensitive investigations of the effects of mutations or other perturbations on the overall process.

SUMMARY POINTS

1. The structure of cellulose microfibrils implies that the synthesis of cellulose involved the coordinate activity of approximately 36 active sites. However, diversity of cellulose structure in various organisms implies that the enzyme complex is modular.
2. Cellulose is synthesized by a 30-nm-diameter rosette-shaped plasma membrane complex with six visible subunits.
3. The only known components of cellulose synthase are a family of CESA proteins, but mutations in genes for a number of other proteins indicate that many other proteins are involved in the overall process.
4. Recent evidence from live-cell imaging of cellulose synthase indicates that microtubules exert a direct effect on the orientation of cellulose deposition under some conditions, but the microtubules are not required for oriented deposition of cellulose under other conditions.
5. Cellulose synthase is posttranslationally regulated and is known to be phosphorylated, but the mechanisms that regulate activity are not yet known.
6. The genes for cellulose synthase are developmentally regulated, but there is relatively little evidence for environmental regulation of expression.
7. Cellulose synthase belongs to the large GT-A family 2 of glycosyltransferases, which includes chitin synthase, but the reaction mechanism is unknown.

FUTURE ISSUES

1. What is the exact composition of the cellulose synthase complex? The ability to make functional fusion proteins should enable a proteomics approach to this question.
2. How does the cellulose synthase complex interact with microtubules?
3. What regulates the activity of cellulose synthase and the lifetime of the complexes?
4. What are the roles of the various proteins implicated by mutant analysis?

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Contents