Two alleles of the AtCesA3 gene in Arabidopsis thaliana display intragenic complementation

Leonard D. Pysh

PREMISE OF THE STUDY: Cellulose is the most abundant biomolecule on the planet, yet the mechanism by which it is synthesized by higher plants remains largely unknown. In Arabidopsis thaliana (L.) Heynh, synthesis of cellulose in the primary cell wall requires three different cellulose synthase genes (AtCesA1, AtCesA3, and AtCesA6-related genes [AtCesA2, AtCesA5, and AtCesA6]). The multiple response expansion1 (mre1) mutant contains a hypomorphic AtCesA3 allele that results in significantly shorter, expanded roots. Crosses between mre1 and another allele of AtCesA3 (constitutive expression of VSP1, cev1) yielded an F1 with roots considerably longer and thinner than either parent, suggesting intragenic complementation. The F2 generation resulting from self-crossing these F1 showed three different root phenotypes: roots like mre1, roots like cev1, and roots like the F1.

METHODS: The segregation patterns of the three root phenotypes in multiple F2 and F3 generations were determined. Multiple characteristics of the roots and shoots were analyzed both qualitatively and quantitatively at different developmental stages, both on plates and on soil.

KEY RESULTS: The trans-heterozygous plants differed significantly from the parental mre1 and cev1 lines.

CONCLUSIONS: The two alleles display intragenic complementation. A classic genetic interpretation of these results would suggest that cellulose synthesis requires homo-multimerization of cellulose synthase monomers.

KEY WORDS Arabidopsis thaliana; AtCesA3; Brassicaceae; cellulose; cellulose synthase; cell wall; root

A linear polymer of glucose molecules joined through β1-4 covalent bonds, cellulose is the most abundant biomolecule on the planet, with an estimated 1.0 x 10^12 tons being synthesized annually (Duchesne and Larson, 1989; Klemm et al., 2005). Cellulose is synthesized by a variety of nonplant organisms (e.g., bacteria, fungi, and animals) (Brett, 2000), but the majority of cellulose is synthesized by higher plants and is incorporated into their cell walls (Duchesne and Larson, 1989). Because roughly one-third of a plant’s mass is cellulose (Somerville, 2006), cellulose represents a substantial commitment of photosynthetic products to nonmetabolizable carbohydrates (Lee et al., 2011). Releasing the glucosyl residues in cellulose is of particular interest in the field of renewable biofuels (Octave and Thomas, 2009). Yet, in spite of its abundance and its importance both biologically and societally, much remains unknown about the synthesis of cellulose.

Multiple cellulose chains associate through hydrogen bonding into larger cables referred to as microfibrils, which have a tensile strength comparable to that of steel (Festucci-Buselli et al., 2007). These microfibrils are embedded within a plant’s cell wall, a complex structure consisting largely of carbohydrates (cellulose, hemicellulose, and pectins) with some proteins (including the arabinogalactans and extensins) and other components such as lignin (Albersheim et al., 2011). The locations and orientations of microfibrils within the plant’s primary cell wall determine the shapes of growing plant cells and, consequently, are a major determinant of plant form. The positions of microfibrils within a plant’s primary cell walls appear to be determined by the position and orientation of the microtubules within the cells, although the mechanism by which microtubules are positioned and oriented also remains unknown (Bringmann et al., 2012).

In higher plants, cellulose is synthesized in the plasma membrane by cellulose synthase complexes referred to as rosettes due to their appearance in electron micrographs or as terminal complexes due to their locations at the ends of microfibrils. The complexes are symmetrical, between 25 and 30 nm in diameter and appear to consist of six globular subunits when visualized by electron microscopy. Each of the six globular subunits has been proposed to consist of multiple molecules of the enzyme required for the synthesis of cellulose, namely, cellulose synthase (Somerville, 2006). Although initial models based on the apparent structure and symmetry of the

---

1 Manuscript received 11 May 2015; revision accepted 13 August 2015.
Roanoke College, Department of Biology, 221 College Lane, Salem, Virginia 24153 USA
E-mail for correspondence: pysh@roanoke.edu
doi:10.3732/ajb.1500212
cellulose synthase complex proposed that a cellulose synthase complex contained 36 cellulose synthase molecules, each of which synthesized a single polymer of cellulose, all of which associated to form a microfibrill consisting of 36 linear chains (Scheible et al., 2001; Doblin et al., 2002; Timmers et al., 2009), this proposal is inconsistent with more recent analyses that indicate that microfibrils consist of only 18–24 linear chains (Fernandes et al., 2011; Newman et al., 2013; Thomas et al., 2013).

Genetic and molecular analyses of cellulose synthesis in Arabidopsis thaliana have shown that the synthesis of cellulose requires at least three isoforms of cellulose synthase. Knock-down mutations in either AtCesA1 or AtCesA3 or knock-out mutations in AtCesA6 result in a significant reduction in the size of the leaves and roots and a significant reduction in the production of cellulose in the primary cell (Arioli et al., 1998; Fagard et al., 2000; Ellis et al., 2002; Cano-Delgado et al., 2003). Knock-out and knock-down mutations in any one of the AtCesA4, AtCesA7, or AtCesA8 genes similarly affect cellulose production in the secondary cell wall, with consequent effects in the inflorescence stem (Taylor et al., 1999, 2000, 2003). Molecular analyses have shown that the products of these sets of genes can form homo- and heterodimers both in vitro and in vivo (Taylor et al., 2003; Desprez et al., 2007; Wang et al., 2008). The role these intermolecular interactions play within a cellulose synthase complex in situ is unclear, but intragenic complementation between two alleles of AtCesA1 (Fujita et al., 2013) and two alleles of AtCesA3 (present paper) may provide genetic evidence that homodimerization (or higher level homomultimerization) of CesA molecules plays an important role in the synthesis of cellulose.

MATERIALS AND METHODS

Plant material and growth conditions—The constitutive expression of vspl (cev1) mutant was kindly provided by John Turner (University of East Anglia, UK). The multiple response expansion1 (mre1) mutant was described by Pysh et al. (2012).

Arabidopsis thaliana seeds were surface sterilized by submerging seeds in undiluted bleach (5–10% sodium hypochlorite) with 0.25% Tween 20 for 3 min, then rinsing the seeds with sterile deionized water three times. Seeds were placed on 100 × 100 mm plates containing media composed of 1x Murashige and Skoog (MS) basal salt mixture (PhytoTechnology Laboratories, Overland Park, KS, USA), 0.05% 2-(N-morpholino)ethanesulfonic acid (MES), 0.7% agar, and 4.5% sucrose (hereafter referred to as MS4.5). The plates were wrapped in Parafilm, stored at 4–8 °C for 3 d, transferred to a growth chamber, grown vertically under a 16 h light/8 h dark cycle at 21°C for 1 d, then wrapped in aluminum foil and placed back in the growth chamber. Hypocotyls were measured 5 d after transfer to the growth chamber (4 d in the dark) using a DVC-1300 digital camera and Scion ImageJ software.

Leaf measurements—Seedlings grown on MS4.5 plates were scored for root phenotype and transplanted to soil 7 d after transfer to the growth chamber. The lengths of the longest leaves on each plant were determined using a ruler 24 d later. Measurements of the distance from the surface of the soil to the lowest leaf on the florescence stems were made using a ruler 12 d after the leaves were measured.

Statistical analyses—Descriptive statistics (means, standard errors of the means) were calculated using Microsoft (Redmond, WA, USA) Excel 2013. One-way analyses of variance (ANOVA) were calculated using both Microsoft Excel 2013 and Minitab 16 (Minitab, State College, PA, USA). Tukey tests to determine statistical differences between means were run using Minitab 16, with α = 0.05.

DNA and RNA analyses—DNA was isolated from 7-d-old seedlings using the DNeasy Plant Mini Kit (Qiagen, Valencia, CA, USA), as per the manufacturer’s instructions. DNA was amplified using Phusion DNA Polymerase (New England Biolabs, Ipswich, MA, USA) and primers flanking the mutations in the two parental lines. Sequences of the primers used to amplify the region including the mutation in cev1 were 5’TGATGGGACCCACACCTCGG3’ and 5’TCCAAATCGCTTCTCCAGGG3’ and including the mutation

Cellulose composition—Cellulose contents of 14-d-old roots were determined using a protocol provided by Simon Turner (University of East Anglia, UK), based on Reiter et al. (1993), Updegraff (1969), and Scott and Melvin (1953), with slight modifications. In brief, the roots were mechanically separated from the shoots with a razor blade, then treated with 70% ethanol at 70°C two times for at least 1 h each treatment, followed by rinsing with acetone and air drying at least overnight. The remaining root materials (alcohol insoluble fractions) were weighed. These materials were treated with a boiling solution of 8:4:1 acetic acid–water–nitric acid for 30 min, then rinsed three times with sterile water and allowed to air dry at least overnight. The remaining root materials were dissolved in 66% (v/v) sulfuric acid for at least an hour, then triplicate aliquots from each original sample were added to water to make a final volume of 500 µL. One milliliter of concentrated sulfuric acid containing 0.5% w/v anthrone was added to each tube. These final samples were boiled for 5 min, allowed to cool, and the absorbance determined at 620 nm. The amount of cellulose in these samples was determined using a standard curve composed using known amounts of glucose. The percentage of cellulose in the roots of each line was determined by dividing the total amount of glucose present in the final sample (that determined spectroscopically) by the dry mass of the alcohol insoluble fraction.

Hypocotyl measurements—Seedlings were surface sterilized as above and plated on MS4.5, as for the root analyses above. The plates were wrapped in Parafilm, stored at 4-8°C for 3 d, transferred to a growth chamber, grown vertically under a 16 h light/8 h dark cycle at 21°C for 1 d, then wrapped in aluminum foil and placed back in the growth chamber. Hypocotyls were measured 5 d after transfer to the growth chamber (4 d in the dark) using a DVC-1300 digital camera and Scion ImageJ software.
in mre1 were 5′CTACCCCTATCCTCCATTCC3′ and 5′GGTCC-CCATGATTTGGATCC3′. Amplified products were purified using the Gel/PCR DNA Fragments Extraction kit (IBI Scientific, Peosta, IA, USA). Amplified products were sequenced by Eurofins Genomics (Huntsville, AL, USA) using internal primers: 5′TAAG-TCACAGTTACCTATAGCGG3′ and 5′TTTGATGGTAGATGCC-TGCC3′ for cev1 and 5′TATGAGCGGCCTCATTGGGCGG3′ for mre1.

Total RNA was isolated from 7-d-old seedlings using the Total RNA Mini (Plant) kit (IBI Scientific, Peosta, IA, USA) as per the manufacturer’s instructions. Isolated RNA was treated with DNAse (DNA-Free kit, Ambion, available from ThermoScientific, Grand Island, NY, USA), then 1.5 μg was used in a reverse transcription reaction using the RetroScript Reverse Transcription kit (Ambion). The resulting cDNA was amplified using Paq5000 Polymerase (Agilent Technologies, Santa Clara, CA, USA) and the following

FIGURE 1 The root and shoot phenotypes of the trans-heterozygotes differ from the parental lines. (A, B) Twelve-day-old seedlings of wild-type Columbia 0 (Col) (left) and the segregating F2 population of a cross between cev1 and mre1 growing on plates. The F2 generations from self-pollinated F1 plants displayed three distinct root phenotypes: two short, expanded phenotypes similar to the parental lines (cross mre1 [x mre1] and cross cev1 [x cev1]) and a longer, unexpanded phenotype (cross wild-type [x wt]) similar to that observed in the F1. (B) Expanded view of panel A showing the short, expanded roots with phenotypes similar to the parental lines. (C) F2 plants with the three different root phenotypes (top) and Col (bottom) growing on soil. Image was taken 24 d after seedlings were transplanted; (D) plants shown in panel C 12 d older. (E) Wild-type, parental, and F2 segregating populations grown on plates in the dark for 5 d. cev1, uncrossed cev1 parental line; Col, wild-type Columbia 0 plants; mre1, uncrossed mre1 parental line; x cev1, F2 plants with cev1 root phenotype; x mre1, F2 plants with mre1 root phenotype; x seg, F2 segregating population; x wt, F2 plants with longer, unexpanded root phenotype. Scale bars: A, E = 10 mm; B = 5 mm; C, D = 25 mm.
Phloroglucinol staining—Roots were optically cleared with acidified methanol and basic ethanol (as above), then submerged in a solution of 1.3% phloroglucinol–63.3% ethanol–4 M HCl. Images were captured using a QColor3 digital camera and QCapture software (Olympus America, Melville, NY, USA).

RESULTS

Genetic intragenic complementation—To confirm that the phenotype observed in the multiplicative response expansion 1 (mre1) mutant was due to the mutation found in the AtCesA3 gene, mre1 was crossed to three lines containing known mutations in AtCesA3: ectopic lignification 1-1 (eli1-1), ectopic lignification 1-2 (eli1-2), and constitutive expression of VSP1 (cel1) (Ellis et al., 2002; Cano-Delgado et al., 2003). As reported by Pysh et al. (2012), the crosses between mre1 and cel1-1 and between mre1 and cel1-2 resulted in F1 plants with roots that displayed the mutant short and expanded phenotype, confirming that the mutation in the AtCesA3 gene in the mre1 line was the basis for the observed phenotype.

Unexpectedly, however, the crosses between mre1 and cev1 produced F1 plants whose roots were considerably longer than either parent and lacked the expanded tips characteristic of both of these cellulose-deficient mutants when grown under these conditions. Several trans-heterozygous F1 plants were transplanted to soil and allowed to self. The resulting F2 populations displayed three distinct root phenotypes: a phenotype similar to that observed in the cev1 parent (referred to as cross cev1 [x cev1]), a phenotype similar to that observed in the mre1 parent (referred to as cross mre1 [x mre1]), and the long, unexpanded root phenotype observed in the F1, referred to as cross wild-type (x wt) (Fig. 1A and 1B). (When included, data from the original, uncrossed parental lines are simply designated cev1 or mre1.) These three phenotypes segregated in a 1:1:2 ratio (43 x cev1: 45 x mre1: 105 x wt; $\chi^2 = 1.7$, df = 2, p > 0.05), indicating that the mre1 and cev1 alleles were demonstrating intragenic complementation.

Two additional independent crosses also displayed the same segregation (Table 1). Several F2 plants with the x wt phenotype were transplanted to soil. Upon selfing, these plants produced F3 seeds that also segregated in a 1:1:2 ratio (x cev1: x mre1: x wt) (Table 1). Several F2 plants with the x cev1 and the x mre1 phenotypes were also transplanted to soil. The F3 seeds from these x cev1 and x mre1 lines were grown on plates, and all of these F3 seeds showed the expected root phenotypes characteristic of the uncrossed parents. To further confirm their designations, the regions surrounding the two mutations were amplified from multiple F3 lines that displayed each of the three phenotypes and sequenced. The sequencing further confirmed the original assignments, as all three x cev1 lines sequenced contained the G617E mutation, all three x mre1 lines sequenced contained the G916E mutation, and all three x wt lines were heterozygous for both mutations (data not shown).

After having been transplanted to soil, the shoot phenotypes of the trans-heterozygous (x wt) plants were consistent with characters observed for the young roots grown on plates: the x wt plants had larger rosettes and longer inflorescence stems than either parental line, but smaller rosettes and shorter inflorescences than the wild-type (Fig. 1C, D). This was also true for etiolated hypocotyls grown on plates containing the same media as that used for the root analysis: the segregating population appeared to be intermediate in length between the wild-type Columbia and the two uncrossed parental lines (Fig. 1E). Due to the fact that the roots of the etiolated plants are short and those of the mre1 line do not show the expanded root phenotype under these conditions, distinguishing among the three different phenotypes and determining the lengths of the hypocotyls for each class (x wt, x cev1, and x mre1) was not possible.

Quantification of the phenotypes of the trans-heterozygotes—To confirm these qualitative indications of intragenic complementation, I quantified the differences between plants with the different phenotypes. As is shown in Fig. 2A, the lengths of the x wt roots were intermediate between those of the parental (x mre1 and x cev1) lines and those of the wild-type Columbia during early seedling development (5 to 9 d after transfer to the growth chamber): that is, the mean length of the roots of the trans-heterozygous plants was significantly greater than either the mre1 or cev1 roots, but significantly less than the wild type. The lengths of the x cev1 and x mre1 roots were statistically indistinguishable from the uncrossed parental lines (see data in Pysh et al., 2012). The lengths of the mature cortex cells in the trans-heterozygous roots were also intermediate between those of the wild type and the two mutant parents (Fig. 2B), suggesting that the root length differences observed in the x wt plants are due to alterations in cell length, not cell number (although these may also have been different). Interestingly, the amount of cellulose present in the cell walls of the trans-heterozygous roots was intermediate between that of the two mutant parents and the wild-type, indicating that the trans-heterozygotes have increased cellulose synthesis occurring in their cell walls compared with the two cellulose-deficient parental lines (Fig. 2C). The x wt roots had root hairs that were similar in length to the wild type and significantly shorter than either parental line (Fig. 2D). The x wt line also showed an absence of phloroglucinol staining in their root tips, more similar to the wild type than to the parental lines, although the x wt roots did display some ectopic lignin at more distal locations (Appendix S1, see Supplemental Data with the online version of this article).

Similar results were obtained when aspects of the shoot phenotype were quantified. Etiolated hypocotyls of the segregating
population were intermediate in length between the wild type and the two parental lines (Fig. 2E). The mean length of the longest leaves of x wt plants transplanted to soil 24 d after scoring and the mean location of the first leaf on the inflorescence stem for the same x wt plants 12 d later were also both intermediate between that of the parental mre1 and cev1 lines and of the wild-type Columbia (Fig. 2F and G). The only character analyzed in this study that does not appear to be complemented in the x wt line is the expression of the plant defensin gene PDF1.2 (At5g44420), as the expression of this gene remains high in the trans-heterozygotes, similar to that seen in the cev1 parental line (Appendix S2, see online Supplemental Data). This result might indicate that the overexpression of PDF1.2 in cev1 is due not to a decrease in cellulose production but to some special effect of the cev1 allele, particularly given the much lower expression of the gene in mre1.

**DISCUSSION**

The phenotypes observed in the trans-heterozygotes indicate that the mre1 and cev1 alleles of the AtCesA3 gene display intragenic complementation. Intragenic complementation has also been observed between two alleles of AtCesA1, radial swollen1-1 (rsw1-1, Arioli et al., 1998), and anisotropy1 (any1, Fujita et al., 2013), another of the three AtCesA genes required for the synthesis of cellulose in the primary cell wall in Arabidopsis thaliana. Several other examples of intragenic complementation have been reported in plants: in the pattern formation gene GNOM (Busch et al., 1996), the abscisic acid biosynthesis gene ABA2 (Rook et al., 2001; Merlot et al., 2002), the cytokinin receptor CRE1 (de León et al., 2004), and in the brassinosteroid receptor BRI1 (Shang et al., 2011).

Historically, intragenic complementation has been explained through one of two mechanisms. Monomeric proteins with multiple functional domains can display intragenic complementation when an allele producing a polypeptide affected in one domain is combined with a different allele producing a polypeptide affected in a different domain. The homozygous mutants display a phenotype because they lack their respective biochemical activities; combining both activities in the trans-heterozygotes can reduce or abolish the mutant phenotype. Examples of this type of intragenic complementation have been identified in the Saccharomyces cerevisiae genes his4 (Fink, 1966; Keeseey et al., 1979) and rho1 (Saka et al., 2001) and the Drosophila melanogaster Gart locus (Henikoff et al., 1986).

Proteins whose functionality depends upon the association of two or more identical subunits (i.e., proteins that consist of homodimers or higher order homomultimers) can display intragenic complementation when the product of one allele functionally compensates for the product of a second allele within the complex. In these proteins, the observed intragenic complementation has been proposed to occur through several distinct (although not necessarily exclusive) mechanisms, including interactions with a different protein or protein complex (as has been proposed for PCNA in D. melanogaster [Henderson et al., 2000]), as a result of temporal differences in the retained activities (as proposed for the EGF receptor homolog in D. melanogaster [Raz et al., 1991]), through stabilization of subunit association (as proposed for alkaline phosphatase in Escherichia coli [Hehir et al., 2000; Boulanger and Kantrowitz, 2003]), or through compensatory alterations in subunit conformation (as proposed for the suppressor of forked locus in D. melanogaster [Simonelg et al., 1996] and for human arginosuccinate lyase [Turner et al., 1997; Yu and Howell, 2000]).

Because cellulose synthases are part of large complexes and have been shown to form homodimers (Taylor et al., 2003; Desprez et al., 2007; Wang et al., 2008), the allelic complementation observed between the mre1 and cev1 alleles of AtCesA3 likely occurs due to the second mechanism, namely, through homodimeric (or higher-level) interactions. But cellulose synthesis is a complex process, requiring assembly of the cellulose synthase complex in the Golgi, transport to the plasma membrane, and regulation and maintenance of structure and activity once incorporated (Crowell et al., 2009, 2010), so the specific point at which the complementation occurs cannot be determined by these data nor can one specific mechanism be clearly identified among the multiple possibilities. Unfortunately, comparison with what is known about the two AtCesA1 alleles that display intragenic complementation (any1 and rsw1) does not provide additional insight, as rsw1 and any1 have phenotypes distinct from those of cev1 and mre1, and the mutations in rsw1 and any1 affect different amino acid residues that are predicted to be located within different regions of the AtCesA1 protein than are mre1 and cev1 within the AtCesA3 protein and therefore likely affect the conformations of the resulting polypeptides differently as well (Arioli et al., 1998; Cano-Delgado et al., 2003; Pysh et al., 2012; Fujita et al., 2013).

In light of what is understood about genetic complementation and about both mre1 and cev1, the simplest explanation for these data would be that the two gene products associate to form a dimer, and the cev1/mre1 dimer has greater activity than does either of the homoallelic dimers. This increased activity leads to synthesis of higher levels of cellulose in the cell wall and the observed increase in the sizes of the organs of the trans-heterozygous plants. In this case, the proposed dimerization might be required for proper folding of the active site(s), and the mutations in the two alleles of AtCesA3 may result in monomers whose altered conformations interact within the heteroallelic dimer to create a more functional active site(s) than does either homoallelic dimer. An alternative proposal would be that the two gene products associate...
FIGURE 2 Quantification of root and shoot phenotypes confirms complementation in the trans-heterozygotes. (A) Mean root lengths (± one standard error of the mean, SEM) of plate-grown plants having the three root phenotypes in the F2 compared with those of the wild-type Columbia 0 (Col). Horizontal labels reflect days after transfer to growth chamber. One-way ANOVA, $F_{3, 141} = 937.0$, $P < 0.001$. (B) Mean lengths (±SEM) of mature cortex cells in roots of 14-d-old Col, x wt, and the two (uncrossed) parental lines grown on plates. One-way ANOVA, $F_{3, 3673} = 1861.44$, $P < 0.001$. (C) Mean percentage cellulose (w/w, ±SEM) of the roots of 14-d-old plants grown on plates. One-way ANOVA, $F_{5, 82} = 115.77$, $P < 0.001$. (D) Mean lengths (±SEM) of
to form a dimer and the cvl/mre1 dimer is more stable than either of the homoolic dimers. Dimerization of CesA proteins might be required for cellulose synthase complex formation, and the mutations in the two alleles of AtCesA3 might result in monomers whose altered conformations interact within the heteroolic dimer to create more stable cellulose synthase complexes than does either homoolic dimer, in turn allowing for increased cellulose production.

If the AtCesA3 proteins form dimers, these data might support a proposed modification of the proposed topology of the plant CesA proteins. The commonly accepted secondary structure for plant CesA proteins includes eight transmembrane helices (TMH), with TMH1 and TMH2 separated from TMH3-8 by a cytosolic domain that includes the residues responsible for catalyzing the synthesis of cellulose (for a recent review, see Slabaugh et al. [2014a]). If this model is correct, explaining how the alteration in cvl (G617E [Ellis et al., 2002]), which affects an amino acid residue in the cytosolic domain, can be complemented by an alteration in mre1 (G916E [Pysh et al., 2012]), which affects an amino acid located in the predicted fifth transmembrane helix (TMH5) (Slabaugh et al., 2014a), is difficult. Using protein sequence alignments, Slabaugh et al. (2014a) reported, however, that TMH1-4 of plant CesA proteins align with TMH3-6 of the catalytic unit of cellulose synthase from Rhodobacter sphaeroides (BcsA), TMH5 in plants aligns with the third interfacial helix (IF3) in BcsA, and TMH6 in plants aligns with TMH7 in BcsA. They have also shown that the amino acid residues separating TMH5 and TMH6 in plants play an important role in cellulose synthesis (Slabaugh et al., 2014b), which is difficult to explain if these residues are extracellular and, therefore, spatially separated from the cytosolic catalytic region, as would be the case based on the accepted secondary structure. To explain their findings, the authors proposed that TMH3, 4, or 5 might be interfacial helices rather than transmembrane helices, because this topology would then place the residues connecting (the currently accepted) TMH5 and TMH6 in the cytosol. Our data are consistent with their proposal and more specifically indicate that TMH5 is the most likely candidate for an interfacial helix. If TMH5 were an interfacial helix, then the amino acid affected in mre1 would be placed on the cytosolic side of the plasma membrane, which then places the alterations in the mre1 and cvl mutants in the same cellular location. Our data might then also suggest an interaction between the cytosolic region containing amino acid 617 and the interfacial helix containing amino acid 916. Until the locations of the amino acid residues within a plant CesA protein and the locations and conformations of the CesA proteins within a cellulose synthase complex are known, however, these proposals remain a matter of conjecture.

Whatever the case may be, these data will require at least slight modifications of the current proposal for the organization of the cellulose synthase complex (Newman et al., 2013) because the authors proposed a model with homomeric interactions between only one of the three different isoforms of CesA found within the complex, yet we now have genetic evidence indicating that at least two of the isoforms form such interactions (AtCesA1, as reported by Fujita et al. [2013], and AtCesA3, this study). One possible way to incorporate the genetic data into the model would be to propose that the CesA proteins homodimerize and then assemble to form a complex consisting of 36 polypeptides (rather than 18). In such a case, it might be that only one of the two polypeptides within the homodimer is active so that only 18 cellulose strands are synthesized by each complex and then associate to form a microfibril. This possibility would allow for a reconciliation between the observations of Olek et al. (2014), whose data suggested that the basic building block of the complex was a dimer, and Hill et al. (2014), whose data suggested it was a trimer. Alternatively, each complex might consist of only 18 polypeptides, but at least two (and possibly all three) of the isoforms can interact with each other homomerically (instead of only one), so that plant membranes actually contain three different populations of complexes (which might have somewhat different activities).