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Identification of a cellulose synthase-associated protein required for cellulose biosynthesis

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Cellulose synthase-interactive protein 1 (CSI1) was identified in a two-hybrid screen for proteins that interact with cellulose synthase (CESA) isoforms involved in primary plant cell wall synthesis. CSI1 encodes a 2,150-amino acid protein that contains 10 predicted Armadillo repeats and a C2 domain. Mutations in CSI1 cause defective cell elongation in hypocotyls and roots and reduce cellulose content. CSI1 is associated with CESA complexes, and csi1 mutants affect the distribution and movement of CESA complexes in the plasma membrane.

Cellulose is synthesized at the plasma membrane by hexameric protein complexes with a diameter of 25–30 nm when observed by freeze-fracture electron microscopy in algae, moss, and vascular plants (1–4). The only known component of the complex is cellulose synthase (CESA), which is represented by 10 isoforms in Arabidopsis (5). Genetic studies indicate at least three Arabidopsis CESA isoforms are required for primary cell wall synthesis (6, 7). Lesions in CESA1 (rsw1), CESA3 (cov1), or CESA6 (prc1) lead to a deficiency in elongation in dark-grown seedlings (8–10). Genetic and biochemical studies demonstrating interactions between CESAs led to a heteromeric model of cellulose synthesis (11, 12) in which the complexes are composed of at least three functionally nonredundant CESA isoforms. It generally is accepted that in Arabidopsis CESA1, CESA3, and CESA6 or CESA6-like proteins are required for functional primary cell wall complexes, whereas CESA4, CESA7, and CESA8 are required for functional secondary cell wall complexes. However, the exact number of CESA proteins contained within the complex, their stoichiometry, and their specific interactions are unknown, and no other components of the complex have been reported.

Recent advances in cell biology and microscopy allow imaging of CESA complexes in live tissues. At least two of the three primary CESAs (CESA3 and CESA6) are functional when labeled with GFP and its derivatives. Both GFP-CESA3 and YFP-CESA6 were observed at the plasma membrane as discrete particles that move along linear trajectories coincident with underlying cortical microtubules (7, 13). CESA particles move bidirectionally with an average velocity of about 350 nm/min corresponding to the addition of ~700 glucose residues per glucan chain per minute (13). CESA particle dynamics are sensitive to osmotic stress and to several drugs that affect cytoskeleton and cellulose synthesis. The observation that perturbation of microtubule polymerization by oryzalin affects the overall distribution and motility of CESA particles supports models in which the microtubules guide the deposition of cellulose. However, CESA particles appear to have an intrinsic level of organization that is evident when microtubules are completely depleted (13).

Genetic screens for mutants deficient in cellulose have identified a number of proteins in the overall process of cellulose biosynthesis. Mutations in KORRIGAN (KOR), which encodes an endo-β-1,4-glucanase, exhibit deficiencies in cell elongation and reduced cellulose production (14). KOR-like proteins from Brassica napus and poplar exhibit cellulase activity in vitro. However, the exact role of cellulase in cellulose synthesis is unknown. Additional cellulose-deficient mutants include cobra, kobito, pom1, rsw3, fragile fiber1, and fragile fiber2, none of which has been assigned a clear mechanistic function in cellulose synthesis. Here we report the identification of a protein involved in cellulose synthesis that appears to be associated with primary CESA complexes. Identification of this protein opens an avenue in ongoing efforts to understand the mechanism of cellulose synthesis.

Results

Cellulose Synthase-Interactive Protein 1 Interacts with Multiple Primary CESAs. To explore whether additional proteins may be required for cellulose biosynthesis, we performed yeast two-hybrid screens to identify proteins that physically interact with CESA1, -3, and -6. Using 541 amino acids of the putative catalytic domain of CESA6 as bait, we identified a protein referred to as “cellulose synthase-interactive protein 1” (CSI1; At2g22125) (Fig. 1). To confirm the interaction of CESA6 and CSI1, we subcloned the prey into a GAL4 activation domain (GAL4-AD) and fused the putative catalytic domain of CESA6 with GAL4 binding domain (GAL4-BD). Coexpression of these constructs in yeast resulted in the appearance of β-galactosidase (GUS) activity, confirming the interaction between CESA6 and CSI1. CESA1 and CESAs also showed positive interactions with CSI1, although the CESAs interaction appeared to be weaker than the CESA1 or CESA6 interaction (Fig. 1B).

CSI1 was identified previously as one of the genes that is coregulated transcriptionally with the primary CESAs (15). In addition to CSI1, several genes that affect cellulose deposition, including COBRA and CTL1/POM1, also are coexpressed with the CESAs genes (Fig. 1C). Consistent with coexpression analysis, transgenic plants in which GUS was placed under the control of the 1.4-kb promoter region upstream of the CSI1 gene exhibited a pattern of GUS activity (Fig. 1 D, F, and H and Fig. S1) similar to that seen with promoter:GUS fusions of primary wall CESA genes (Fig. 1 E, G, and I). In addition, the CSI1 promoter drives GUS expression in floral tissues, rosette leaves, roots, and pollen (Fig. S1), indicating that the CSI1 expression pattern is similar to that of CESAs throughout development.
CSI1 Encodes an Armadillo Repeat-Containing Protein. Protein sequence homology searches identified CSI1-related sequences in a variety of dicots, monocots, and the moss Physcomitrella patens (Fig. S2). The Arabidopsis genome contains two closely related genes, which we refer to as “CSI2” and “CSI3” and which share about 55% sequence similarity with CSI1. No CSI1-like proteins were identified outside the land plants. The CSI protein contains multiple tandem copies of a degenerate protein sequence motif, the armadillo (ARM) repeat. The ARM repeat is an ≈40-aa long, tandemly repeated sequence first identified in the Drosophila segment polarity gene, armadillo (16). ARM repeats are found in more than 240 proteins which are predicted to share a conserved 3D structure and often participate in protein–protein interactions (17–19). CSI1 also contains a C2 domain at its C terminus. Some C2 domains have been shown to bind phospholipids in a calcium-dependent or -independent manner and are involved in targeting proteins to cell membranes (20, 21). Other C2 domains have been shown to mediate protein–protein interactions (22).

csi1 Mutants Have Defects in Expansion. To investigate the biological function of CSI1, we analyzed six independent homozygous transfer DNA (T-DNA) insertion lines with insertions in either exons or introns of CSI1 from the Salk Institute Genomic Analysis Laboratory (SIGnAL) collection (Fig. 2A) (23). At least five of the lines probably were null alleles for CSI1, because no CSI1 mRNA was detectable by RT-PCR (Fig. S3A). Hypocotyls in etiolated csi1 mutants were ≈30% shorter and ≈80% wider than in wild-type plants (Fig. 2B, C, and E) and elongated less rapidly than in wild-type but more quickly than in prc1-1 (a CESA6 mutant) plants (Fig. 2D). The reduced hypocotyl length and increased diameter indicate that csi1 mutants have defects in the control of anisotropic expansion (highlighted cells in Fig. 2E). The etiolated seedlings of csi1 alleles had a 50% reduction in crystalline cellulose (Fig. 2F). Several cellulose-deficient mutants, such as cob-6, csl1/pom1, and kor1, display similar cell elongation phenotypes (14, 24, 25).

csi1 mutants also exhibited short, and swollen, seedling roots. In 8-d-old light-grown seedlings, roots in csi1 mutants were ≈25% shorter than in wild-type plants (Fig. S3B and C). The roots in csi1 mutants also exhibited epidermal cell swelling and were 80% wider than wild-type roots (Fig. S3D–F). Additionally, adult csi1 mutants were dwarfed and had shorter siliques than wild-type plants (Fig. S3G). To test whether the smaller siliques were caused by partial sterility, we conducted reciprocal backcrosses to wild-type plants using heterozygous plants. The progeny of these crosses clearly showed that the transmission of the csi1 allele occurred at much lower frequency from the male gametophytes than from the female, indicating that the reduced
CSI1 is required for normal cell expansion. (A) Schematic representation of six T-DNA insertion sites in csi1. Exons are represented by black lines, and introns are shown by breaks. (B) Morphology of 4-d-old dark-grown seedlings: (Left to Right) Col-0 (wild-type) and csi1-1, csi1-2, csi1-3, csi1-4, csi1-5, and csi1-6 mutants. (Scale bar: 2 mm.) (C and D) Hypocotyl length (C) and growth rate (D) of dark-grown wild-type (Col-0) plants and csi1-3, csi1-6, and prc1-1 mutants. Data were collected from the measurement of ≈50 seedlings for each genotype. Error bars represent SE (absent error bars were obscured by symbols). (E) SEM of dark-grown hypocotyls in wild-type plants and csi1 mutants: (Left to Right) Arabidopsis thaliana Columbia (Col-0), csi1-3, and csi1-6 mutants. Colors outline one epidermal cell. (Scale bar: 100 μM.) (F) Cellulose content was reduced in csi1 mutants. n = 5. Error bars represent SE.
To mutations appeared in cell walls (27), and there was no significant difference between the genotypes in the three delineated zones of the root (Fig. 5C). Similarly, in the three root zones of both genotypes, the azimuths averaged to 90° transverse to the long axis of the root. However, for the elongation and mature zones, the variability among azimuth measurements made in individual roots was significantly greater in csi1-1 mutants than in wild-type roots, suggesting that microfibril alignment in these cell walls had become less uniform.

Discussion

The CSI1 protein is the first non-CESA protein associated with CESA complexes. Several lines of evidence led us to hypothesize that CSI1 exerts a direct effect on cellulose synthesis through its association with CESA complexes. First, CSI1 physically interacts with multiple primary CESA genes in yeast two-hybrid assays, and CSI1 is transcriptionally coregulated with several of the CESA genes involved in primary cell wall synthesis, but there was no obvious association with CESA genes involved in secondary cell wall synthesis. Additionally, CSI1 colocalizes with primary CESA complexes, and csi mutations affect the distribution and movement of CESA complexes, resulting in strongly reduced rates of CESA complex movement. If we assume that the length of cellulose microfibrils is affected by the velocity and lifetimes of CESA particles, the cellulose defect and the associated swelling phenotype can be attributed to the effect of the csi mutations on the activity of the CESA complexes. Additionally, the csi mutations appeared to decrease the degree to which cellulose microfibrils are coaligned. Polarized light is sensitive to the azimuth at which the optical axis of the crystalline sample is oriented. In both csi mutants and wild-type roots the average azimuth of cellulose microfibrils was transverse to the long axis of the root. However, the SD of the azimuth measurements was larger in csi mutants than in wild-type roots. In other words, microfibril alignment, on a scale greater than that of a wavelength, is noisier in csi mutants than in wild-type roots. A similar reduction in the uniformity of microfibril alignment across the root has been reported for treatment with low concentrations of the microtubule inhibitor oryzalin (27). The decrease in cellulose organization in csi mutants indicates that CSI1 may participate in the mechanisms responsible for organizing the deposition of cellulose microfibrils in primary walls.

CSI1 belongs to a family of highly conserved land plant-specific proteins. CSI1 contains multiple predicted ARM repeats and a single C2 domain. Three-dimensional structures of previously characterized ARM repeats comprise three α-helices. For example, yeast importin-α contains a central region of 442 aa that contains 10 ARM repeats of 42 aa, forming a right-handed superhelix of helices that creates a surface for protein–protein interactions (18). By comparison, CSI1 has 10 predicted ARM repeats distributed unevenly across the entire protein (2,151 aa). We are not able to draw direct structural comparisons between CSI1 and other proteins containing ARM repeats.

Methods

Plant Materials and Growth Conditions. Arabidopsis thaliana Columbia (Col-0) seeds were sterilized and germinated on Murashige and Skoog plates (1/2 × MS salts, 0.8% agar, 0.05% monohydrate 2-(N-Morpholino) ethanesulfonic acid, pH 5.7). Seedlings were transferred to pots in a greenhouse at 22 °C in darkness for 3 d before imaging. For soil-grown plants, seedlings were germinated on MS plates containing 1% sucrose and then transferred to pots in a greenhouse at 22 °C under 16-h light and 8-h dark.

Yeast Two-Hybrid Assay. The yeast two-hybrid screen was carried out by Hybrigenics. CSI1 was identified as a CESA6-interactive protein. To confirm the interaction between CSI1 and CESA6, we subcloned the prey fragment into pACTII (28). The catalytic domains of CESA1, CESA3, and CESA6 were cloned into pAS1-CYH2 (28) using primers indicated in Table S2. The resulting constructs were cotransformed into yeast strain Y190 as pairs (Fig. 18). Transformants were selected on SC-Tryp-Leu-His plates. Positive interactions were tested by their ability to grow on SC-Tryp-Leu-His plates containing 1% sucrose.
supplemented with 100 mM 3-aminotriazole (Sigma) and for GUS activity using a filter assay.

Coexpression Analysis. A coexpression network for CSI1 (Cluster 86) was obtained from AraGenNet at http://aranet.mpimp-golm.mpg.de/aranet/ AraGenNet (29) and trimmed to facilitate readability.

GUS Construct and Staining. A genomic DNA fragment (1.4 kb) upstream from the ATG start codon of CSI1 was cloned into pCAMBIA1305 GUS-Plus (Table S2) using BamHI and NcoI. The construct was transformed into Arabidopsis using Agrobacterium-mediated transformation. Transgenic plants were selected on hygromycin, stained for GUS activity in 100 mM sodium phosphate (pH 7.0), 10 mM EDTA, 1 mM ferricyanide, 1 mM ferrocyanide, and 1 mM 5-bromo-4-chloro-3-indolyl β-D-glucuronic acid at 37 °C, cleared in 70% ethanol, and observed under a Leica MZ12.5 stereomicroscope (Leica DFC420 digital camera).

Cellulose Measurement. Cellulose was measured from 4-d-old etiolated seedlings using the Updegraff method (31). Data were collected from five technical replicates for each tissue sample.

Microscopy. Anthers from 5-wk-old plants or hypocotyls of 4-d-old dark-grown seedlings were mounted on stubs under a pressure of 50 Pa in an Hitachi TM-1000 scanning electron microscope. Roots of 8-d-old light-grown seedlings were wet-mounted on slides and viewed and photographed with a Leica DMS5000B epifluorescence microscope (JH Technology). Images software (W. Rasband, National Institute of Health, Bethesda, MD) was used for measurement of hypocotyl and root length. Whole 5-d-old seedlings were viewed on M5 plates using a Leica MZ12.5 stereomicroscope (Leica DFC420 digital camera).

Polarized Light Analysis. Roots were prepared for polarized light analysis as described previously (27). Briefly, root tips were cryofixed in liquid propane, freeze-substituted in ethanol, embedded in butyl-methyl-methacrylate, and sectioned at 2-μm thickness. Sections were imaged on an Interphako polarized-light microscope (Zeiss) equipped with an LC Polscope quantification system (Cambridge Research Instruments) implementing the universal compensator (26). This instrument operates in circularly polarized light and generates two images. The intensity of each pixel is proportional to birefringent retardance in the first image and to the azimuth of the optical axis of the crystalline elements in the second image. For display (Fig. 5) the two images are superimposed with pixel intensity giving retardance and color giving azimuth. Measurements were taken from subcellular areas of cell wall in cortex and epidermis, as viewed in longitudinal sections. Approximately 20 sections per root and three roots per genotype were measured. For analysis, the root was divided into meristem, elongation, and mature zone based on cell length. Azimuth was defined with respect to the local midline (longitudinal axis) of the root.

To assess the statistical significance of the polarized light observations, circular statistical techniques were used (32). Specifically, microfibril orientation was expressed as a unit vector corresponding to the doubled azimuth angle; to obtain the mean orientation, this unit vector was averaged over

Fig. 5. Polarized light analysis of csi1 mutants. (A and B) Polarized-light micrographs of (A) wild-type and (B) csi1-1-mutant roots. Images are of longitudinal-tangential sections through cortex and epidermis. Intensity is proportional to retardance, and color represents optical axis (azimuth) of the crystalline elements, as shown by the color wheel. Note the similar intensity and color for the two genotypes. (C) Quantification of retardance and azimuth (90° is transverse to the root’s long axis). Top panels show means ± SEM (n = 3 roots); bottom panels show the average SD (n = 200 measurements) ± SEM for each of the roots. Note the larger SDs for csi1-1 mutants in the elongation and mature zones.
each observation zone, and the result was halved. The angular deviation was calculated as half the (nonnegative) angular distance between the unit vectors for an individual patch, a, and the mean, b, arcsin(cos(a − b)). Zone-wise mean angular deviations of microfibrils were compared between genotypes using a Mann–Whitney U test, similar to the approach used to compare sample angular deviations reported by Wallraff (32). The distribution of zone mean angular deviations was skewed right, but the distribution became approximately normal after log transformation. Therefore, logarithms of mean angular deviations also were compared using an independent sample t test. The results of the t test on log-transformed data and the u test were the same.

Isolation of T-DNA Insertion Line. The identification ofcsi1 knockout lines from the SIGnAL (Salk Institute Genomic Analysis Laboratory; http://signal.salk.edu/cgi-bin/tdnaexpress) collection was based on a combination of database searches and PCR amplification of T-DNA flanking regions. For T-DNA lines identified from the SIGnAL collection, seeds were obtained from the Arabidopsis Biological Resource Center (Ohio State University; http://www.biosci.ohio-state.edu/plantbio/facilities/abr/abchome.htm). PCR reactions were carried out to identify single plants for the T-DNA insertion. Primers used for T-DNA genotyping of csi1 alleles are listed in Table S2.

Confocal Microscopy and Image Analysis. For analyses of microtubule dynamics, seeds were germinated on MS agar plates and grown vertically in darkness for 3 d at 22 °C. Seedlings were mounted between two coverslips in water. Imaging was performed on a Yokogawa CSU1Xspinning disk system featuring the DM16000 Leica motorized microscope (13) and a Leica 100×/1.4 NA oil objective. YFP was excited at 488 nm, and a band-pass filter (520/50 nm) was used for emission filtering. Image analysis was performed using Meta Morph (Molecular Devices) and Imaris (Bitplane) software.

For Imaris analysis, the contrast was enhanced and normalized for each slice within a movie using Imaris. The enhanced movie was processed in Imaris 6.2.1 from Bitplane. Automated particle detection was performed to find particles with a diameter of ~230 nm, and tracks were generated over the lifetime of the particle. To filter noise in particle detection, only particles detected for 14 s (seven frames) were analyzed. The data for the total displacement and duration for each track in a movie were exported. Directional bias was analyzed by summing the vectors of all particles to determine the direction of greatest particle flux. The dot product of each particle’s trajectory against this direction was calculated, and the velocity of each particle was plotted against its dot product. Greater differences between particle velocities going with and against the major axis result in a larger slope in a linear regression of this plot.

Database Search and Sequence Alignment. The predicted amino acid sequence ofCSI1 (At2g22125) was retrieved from the Arabidopsis Information Resource (TAIR) database (www.arabidopsis.org). This protein sequence was used to identify full-length CSI1-like proteins in the National Center for Biotechnology Information GenBank protein database using BLASTP (www.ncbi.nlm.nih.gov/BLAST). CSI1-like proteins (Table S3) were aligned using ClustalW implemented in MegAlign (DNASTAR); protein alignments then were used to generate the phylogenetic tree of CSI1-like proteins (MegAlign; DNASTAR).

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