Elucidating the Mechanisms of Assembly and Subunit Interaction of the Cellulose Synthase Complex of Arabidopsis Secondary Cell Walls

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Cellulose is the most abundant biopolymer in nature; however, questions relating to the biochemistry of its synthesis including the structure of the cellulose synthase complex (CSC) can only be answered by the purification of a fully functional complex. Despite its importance, this goal remains elusive. The work described here utilizes epitope tagging of cellulose synthase A (CESA) proteins that are known components of the CSC. To avoid problems associated with preferential purification of CESA monomers, we developed a strategy based on dual epitope tagging of the CESA7 protein to select for CESA multimers. With this approach, we used a two-step purification that preferentially selected for larger CESA oligomers. These preparations consisted solely of the three known secondary cell wall CESA proteins CESA4, CESA7, and CESA8. No additional CESA isoforms or other proteins were identified. The data are consistent with a model in which CESA protein homodimerization occurs prior to formation of larger CESA oligomers. This suggests that the three different CESA proteins undergo dimerization independently, but the presence of all three subunits is required for higher order oligomerization. Analysis of purified CESA complex and crude extracts suggests that disulfide bonds and non-covalent interactions contribute to the stability of the CESA subunit interactions. These results demonstrate that this approach will provide an excellent framework for future detailed analysis of the CSC.

Cellulose, a polymer of β(1–4)-linked glucose, is the major component of most plant cell walls. In higher plants cellulose is synthesized at the plasma membrane by a very large complex that is believed to simultaneously synthesize many individual glucose chains that hydrogen bond together to form a microfibril (1–4). The cellulose synthase complex (CSC)3 has been visualized in freeze fracture studies as a rosette structure resembling a hexamer with six distinct lobes (5–7). Most estimates suggest that each lobe of the rosette is itself a hexamer, such that the CSC is a 36-mer (4, 8, 9). Although the CSC is likely to be a major factor in determining microfibril structure, very little information is known about its organization. The only components of the CSC that have been identified to date are the cellulose synthase A (CESA) proteins that are presumed to be the catalytic subunits of the CSC (1, 4, 10–12).

The CESA family has been most intensively studied in Arabidopsis, and these studies have utilized the characterization of a wide range of mutants (4, 11). In primary cell walls a number of CESA proteins have been shown to be required for cellulose synthesis. Both CESA1 and CESA3 are essential (13–17). In contrast, CESA2, CESA5, and CESA9 appear to be partially redundant with CESA6 (18, 19). The situation is different in the secondary cell walls of developing xylem vessels where three nonredundant CESA proteins are required for cellulose synthesis: CESA4 (20), CESA7 (21), and CESA8 (22). Mutations compromising the function of any one of these three CESA proteins cause a severe deficiency in the deposition of cellulose in secondary cell walls, which exhibit a characteristic irregular xylem (irx) phenotype caused by a collapse of xylem vessels (23). The irx1, irx3, and irx5 mutants are caused by mutations in the CESA8, CESA7, and CESA4 genes, respectively. A study of Arabidopsis secondary cell wall CSC composition will therefore have the significant advantage that only three CESA subunits are likely to be present. Furthermore, the irx mutants, which are only deficient in secondary cell wall CSCs, will be a very useful tool in such a study.

The nonredundant CESA isoforms in both primary and secondary cell walls have been shown by co-immunoprecipitation to interact with one another (18–20). In a recent study using blue native polyacrylamide gel electrophoresis (BN-PAGE) of solubilized cell extracts, Wang et al. (24) demonstrated the presence of primary cell wall CESA proteins as part of a large (840 kDa) complex. This study did not, however, attempt to purify this complex or determine its composition. Although it is widely assumed that a minimum of three CESA proteins are required for and involved in CSC assembly, there is currently little information on whether proteins other than CESAs are present in the complex or how the CSC is organized and assembled.

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2 The abbreviations used are: CSC, cellulose synthase complex; BN, blue native; CESA, cellulose synthase A; DDM, dodecyl β-D-maltoside; HRP, horseradish peroxidase; irx, irregular xylem; LC-MS/MS, liquid chromatography-tandem mass spectroscopy; DTT, dithiothreitol.
The only definitive method of answering many of the outstanding questions of cellulose synthesis is to isolate an intact, pure, and fully functional complex. Although this goal is a prerequisite to proper biochemical and structural analyses by techniques such as single particle averaging, to date, it remains elusive. Various attempts to purify the complex biochemically have identified a number of potential components but have failed to identify the CESA proteins, the only known components of the complex (3). More recently cellulose synthase activity has been unambiguously identified in a solubilized membrane preparation from blackberry (25); however, because these preparations do not constitute a purified CSC, further analysis is impeded.

We have employed an epitope tagging approach to purify a secondary cell wall CESA-containing complex under non-denaturing conditions. In combination with nondenaturing gel electrophoresis, this approach has been used to elucidate the subunit composition, determine how CESA proteins assemble, and examine the nature of the interactions between subunits.

**EXPERIMENTAL PROCEDURES**

**Plant Material**—Wild type Arabidopsis (Landsberg erecta ecotype), irx1-1, irx3-1, and irx5-2 mutant plants and all transgenic plants were grown as described previously (20, 23).

**Vectors and Plant Transformation**—An 8.3-kb Xhol-MnuI genomic DNA fragment carrying the entire CESA7 coding region and the 1.7-kb promoter sequence was cloned into pCB2300 (22). To insert the RGSHEHHHHHH (His)-FLAG epitope, a double-stranded oligonucleotide (5’-CTAGCAGGGGATCCCCATACCAACCACGTACAAGGATGAGATGACAAG-3’ and 5’-CTAGCCCTCTGATCATGTCATCTGCTCTTTGATATGAGATGATGGTAGTGATGATGGAGATTCCCATCACCATCACGTACTACAAGGGATCC-3’) was ligated into a unique NheI site of pCB1300 by insertion of the 1.7-kb promoter sequence was cloned into p3HSC, producing expression vectors that contained tagged versions of CESA7, CESA4, and CESA8. The cDNAs were amplified cDNA fragments into pDONOR-Zeo using PCR primers for CESA4 (primers 5GF, 5’-AAAAAGCAGGCTTTGCGATGACACGACCAACC-3’; 5GR, 5’-AGAAGGCTGGTTATCTGTGTAATCTTTTACCTGTG-3’), CESA7 (primers 3GF, 5’-AAAGCAGGCTTTCTATGGAAGCTAGCGC-3’; 3GR, 5’-CGGATCCATCACCATCACCATCACGACTACAAGGATCC-3’), and CESA8 (primers 1GF, 5’-AAAAAGCAGGCTTTGCGATGACACGACCAACC-3’; 1GR, 5’-AGAAGGCTTTGCGATGACACGACCAACCACGTACAAGGATGAGATGACAAGGACGATGACAAG-3’; 1GR, 5’-CTAGCCCTCTGATCATGTCATCTGCTCTTTGATATGAGATGATGGTAGTGATGATGGAGATTCCCATCACCATCACGTACTACAAGGGATCC-3’). The cDNAs were subcloned into p3HSC, producing expression vectors that contained tagged versions of CESA4, CESA7, and CESA8, and then irx5-2, irx3-1, and irx1-1 plants, respectively, were transformed by Agrobacterium-mediated transformation (28).

**Phenotype and Cellulose Analysis**—Stem sections were stained with toluidine blue to determine the structure of the vascular bundle. The bottom 5 cm of the mature stem was used for cellulose measurements, as described (23).

**Microsome Extraction and Solubilization**—The stems were ground at 4 °C in two volumes of buffer A (50 mM NaH2PO4, pH 8.0, 200 mM NaCl, 1× protease inhibitor Complete (Roche Applied Science), 1 mM phenylmethylsulfonyl fluoride, 1 mM EDTA). Following centrifugation for 1 min at 500 × g and the addition of ~10% (v/v) glycerol, the extracted samples were frozen in liquid nitrogen and stored at −80 °C until use. Before purification, the samples were rapidly thawed and loaded on the top of a 30% (w/v) sucrose cushion. The microsome fraction was collected after ultracentrifugation for 45 min at 100,000 × g at 4 °C, and the pellet was resuspended in buffer A. The microsome fraction was solubilized with Triton X-100 or dodecyl β-d-maltoside (DDM) at a protein:detergent ratio of ~1:2 at 4 °C for 30 min with rotation.

**His/Strep Tag Affinity Purification**—Ten ml of the solubilized microsome preparation was diluted to 1% (w/v) detergent with buffer A and then incubated with 0.6 ml of nickel-nitrotriacetic acid Superflow (Qiagen) at 4 °C for 1 h with rotation. After three washes with 6 ml of buffer B (buffer A, 0.2% (w/v) detergent) with 30 mM imidazole, the bound proteins were eluted with 3 × 0.6 ml of buffer B containing 250 mM imidazole. Eluted proteins were diluted with buffer B to a final imidazole concentration of 100 mM (to give the His sample). Up to 10 ml of the His sample was passed slowly through a 1-ml gravity flow Strep-Tactin Sepharose column (IBA). After five washes with 1 ml of buffer B, the bound proteins were eluted with 6 × 0.5 ml of buffer B containing 2.5 mM desthiobiotin. Fractions 2–5 were pooled to obtain the purified His/Strep sample. When necessary, the purified sample was concentrated by centrifugation in a Microcon YM100 column (Millipore) or by incubation with 0.1 ml of nickel-nitrotriacetic acid Superflow and eluting the protein directly from the resin by incubation with SDS/DTT loading buffer at 65 °C.

**His/FLAG Tag Tandem Affinity Purification**—One volume of streptavidin magnetic beads M-280 (Dynabeads, Dynal Bio-tech) were mixed with 10 volumes of 10 µg/ml anti-FLAG BioM2 biotin conjugated antibody (Sigma), incubated for 30 min at room temperature, and washed five times with buffer B. The protein sample eluted from the His tag purification step was incubated with anti-FLAG BioM2/magnetic beads for 1 h at 4 °C, washed three times with buffer B05 (buffer B containing 0.5% (w/v) detergent), and subsequently eluted with different concentrations of FLAG and 3× FLAG peptides (Sigma) ranging from 200 to 2000 µg/ml in buffer B05 alone or in combination with other elution agents. For SDS/PAGE and LC-MS/MS analysis, concentrated magnetic beads with the bound proteins were incubated in SDS/PAGE sample buffer at 65 °C for 15 min.

**Pull-down Assay**—Equal volumes of the solubilized microsome fraction were treated for 30 min at 4 °C with various concentrations of DTT, NaCl, and Sarkosyl (sodium lauroyl sarcosinate) prior to incubation with Strep-Tactin resin. The final volumes of all samples were adjusted to 4 ml with buffer B. The treatments with NaCl and Sarkosyl were performed with and without 10 mM DTT. The samples were then incubated with 100 µl of Strep-Tactin Sepharose (IBA GmbH). After incubation for 1 h at 4 °C with rotation, the samples were washed three
times with buffer B, pelleted, and incubated in SDS/PAGE sample buffer at 65 °C for 15 min.

**Gel Electrophoresis and Blotting**—BN gels (3.5–15% (w/v) acrylamide, 250 mM 6-aminohexanoic acid, and 0.1% (w/v) DDM, when DDM solubilized) were prepared and run at 4 °C as described (29) using of ~80 µg of protein/lane. Vertical strips of the gel corresponding to the individual lanes were incubated with 100 mM Tris-HCl, pH 7.5, 3% (w/v) SDS, 100 mM DTT for 30 min at 65 °C and run on a second dimension of SDS-PAGE. For one-dimensional SDS-PAGE, the protein samples were incubated in sample buffer (100 mM Tris-HCl, pH 7.5, 3% (w/v) SDS, 50 mM DTT) for 20 min at 65 °C. After separation the proteins were transferred to immunoblot PVDF membrane (Bio-Rad) and subjected to immunoblot analysis with CESA4-, CESA7-, and CESA8-specific antibodies and detected using an alkaline phosphatase-conjugated secondary antibody as previously described (20). For chemiluminescent detection of the horseradish peroxidase (HRP)-conjugated secondary antibody, a SuperSignal West substrate mixture (Pierce) was used. Silver staining was performed as described (30), and colloidal Coomassie staining was by PageBlue protein staining (Fermentas). The stain intensity in the Western blots and stained gels was quantified using Imaging Densitometer GS710 and Quantity One software (Bio-Rad).

**Mass Spectrometry**—The protein bands of interest were excised from the gel, washed, reduced, alkylated with iodoacetamide, and digested with trypsin using a modified version of the procedure described (31). The extracted peptides were characterized by LC-MS/MS on a Q-TOF Micro (Waters), and the data produced were searched against the Mass Spectroscopy Database using Mascot searching software (Matrix Science).

**RESULTS**

**Analysis of CESA Proteins Using Two-dimensional BN/SDS-PAGE**—The secondary cell wall CESA proteins can be effectively solubilized from a stem microsomal fraction with 2% DDM or 2% Triton X-100 (approximate protein:detergent ratio of 1:2). BN-PAGE and immunoblotting using a CESA7-primary antibody and an alkaline phosphatase-conjugated secondary antibody detected two broad bands with approximate molecule masses of 700 and 440 kDa and diffuse bands of ~100 kDa (Fig. 1A). It was hard to reliably detect low levels of CESA protein; therefore to improve the sensitivity, a HRP-conjugated secondary antibody and chemiluminesence detection was used (Fig. 1B). The interpretation of these data was hampered by the appearance of background bands that were visible in the absence of both primary and secondary antibodies and that were generated from a small unknown protein (molecular mass, ~30 kDa) with inherent peroxidase activity that formed part of a larger complex (supplemental Fig. S1). To overcome this problem, a second dimension of conventional SDS-PAGE was utilized. The results from immunoblots after two-dimensional BN/SDS-PAGE of a DDM-solubilized stem microsomal fraction showed that the three CESA proteins (CESA4, CESA7, and CESA8) each possessed a similar pattern corresponding to the presence of a CESA monomer of ~120 kDa as well as various oligomers (Fig. 1C). The largest observed CESA oligomers had a molecular mass ~700–730 kDa. The patterns of all three CESA subunits contained oligomers with molecular masses of 440 and 220 kDa. The CESA8 protein was slightly less well resolved, possibly because of poorer detection of the protein, but the general pattern of monomeric and oligomeric forms was equivalent to CESA4 and CESA7. A truncated CESA7 form (a specific proteolytic product), observed previously in SDS-PAGE immunoblots (32), was also present in multiple oligomeric forms (Fig. 1C). Similar, but less intense truncated forms were observed for CESA8 and for the 440-kDa oligomer of CESA4.

**Analysis of CESA Proteins in irx3 and irx5 Mutants**—The irx3-1 and irx5-2 mutations are caused by premature stop codons that result in protein truncation of CESA7 and CESA4, respectively (20, 21). In the irx3-1 background, interaction
between CESA4 and CESA8 is greatly reduced, and the proteins fail to localize to the plasma membrane (20, 33). Consequently, it is assumed that these mutants do not assemble a functional CSC. To examine the organization of CESA proteins in these mutant backgrounds, two-dimensional BN/SDS-PAGE and immunoblotting was performed on stem protein extracts from irx3-1 and irx5-2. CESA4 and CESA8 migrated with predominant bands with molecular masses of 120 and 240 kDa in the irx3-1 mutant, corresponding to the size of a CESA monomer and dimer (Fig. 2). Similarly CESA7 and CESA8 migrated in a similar manner in extracts from the irx5-2 mutant (Fig. 2), suggesting that homodimerization precludes complex assembly.

**Tandem Tag Purification of CESA Proteins**—CESA7 can incorporate a His tag insertion at the N terminus and remain functional (22, 33). Although His-CESA7 can be purified using this single tag, it is not sufficient to generate a purified CESA complex (32). As an alternative approach for CESA complex purification, a functional CESA7 construct carrying N-terminal His and FLAG tags arranged in tandem was expressed in irx3-1 plants, which lack CESA7. The FLAG tag allows competitive elution with a FLAG peptide under nondenaturing conditions for various types of protein (34). It was possible to obtain a highly purified preparation of His/FLAG-CESA7; however, elution of native CESA proteins from anti-FLAG antibody beads using FLAG peptides alone or in combination with other reagents (including 1 M urea or glycine-HCl pH 2.8), was very inefficient (data not shown). In contrast, resin-bound His/FLAG-CESA7 was efficiently released using an SDS/DTT denaturing buffer (supplemental Fig. S2).

The His/FLAG-purified sample was compared with a wild type extract subjected to the same purification procedure. Following separation using SDS-PAGE, a very strong band in the His/FLAG-CESA7 fraction was clearly visible at 110 kDa, the size predicted for tagged CESA7 (supplemental Fig. S3) with several fainter bands that were not present in the wild type control. Both CESA4 and CESA7 were identified in the major 110-kDa band by LC-MS/MS analysis, indicating that they were co-purified. CESA8 was not identified in this experiment most likely because it is masked by an excess of CESA7. The presence of CESA8 was, however, demonstrated by Western analysis. A further 13 bands were analyzed that were present in the tagged extract, but not in the wild type (supplemental Fig. S3). These bands contained either CESA7 and/or CESA4 (supplemental Table S1). Other proteins were also detected such as keratin and the Ig γ chain, which are contaminating proteins with similar gel mobility to the CESA7 and/or CESA4 bands. CESA4 and CESA7 degradation products were detected in bands with molecular masses below 110 kDa, whereas CESA proteins that aggregated with one another or with other proteins were present in bands that migrated with a molecular mass greater than 110 kDa.

**CESA Purification Using a Dual Tagging System**—A major limitation of the His/FLAG tag purification system is that elution with SDS/DTT buffer precludes purification of native CESA proteins. Also, this system cannot discriminate between the monomeric forms of His/FLAG-CESA7 and those associated with larger oligomeric forms that presumably form part of the CSC. The excess of CESA7 compared with CESA4 or CESA8 is likely to result from preferential purification of the monomeric and lower oligomers of His/FLAG-CESA7 rather than the intact complex, because of their much smaller size. To overcome these problems, a dual His/STREP tag system was developed. It consists of a His and StreptII (STREP) tag inserted separately into two different CESA isoforms. A STREP-tagged protein is eluted with the low molecular weight agent (desthio)-biotin rather than by antibody binding (26, 35). Successful STREP-tagged protein purification was recently reported for membrane proteins and protein complexes (35, 36). The STREP tag was inserted at the N terminus of CESA4, CESA7, and CESA8, and the recombinant proteins were expressed in irx5-2, irx3-1, and irx1-1 mutant backgrounds, respectively. Cellulose contents of irx5-2/STREP-CESA4 and irx1-1/STREP-CESA8 plants were much lower than wild type and as such were not fully functional (supplemental Table S2). irx3-1 plants expressing STREP-CESA7 had wild type cellulose content and xylem phenotypes. Because multiple CESA7 proteins are present in the CSC, we utilized STREP-CESA7 and His/FLAG-CESA7 in a dual tag system.

irx3-1/STREP-CESA7 Plants Were Crossed with irx3-1:His/FLAG—CESA7 plants and homozygous progeny expressing both transgenes were selected and used for purification under non-denaturing conditions. Double transgenic (His/STREP-CESA7) plants had wild type cellulose content and normal xylem phenotype (supplemental Table S2). Stem microsome fractions from His/STREP-CESA7 plants solubilized with Triton X-100 or DDM were used for tandem His/STREP affinity purification under native conditions. Successive His and STREP purification yielded highly purified preparations predominately composed of proteins of ~110 kDa (Fig. 3A) that contained CESA4, CESA7, and CESA8, as determined by immunoblotting (Fig. 3B). The dual His/STREP tag purification procedure yielded 3–15 µg of protein samples from microsome fractions extracted from 50–70 g of stem material. Protein bands purified from concentrated His/STREP-CESA7 samples from Triton X-100- or DDM-solubilized microsomes were subjected to analysis using LC-MS/MS. Only CESA4, CESA7, and CESA8 proteins were identified, including the prominent degradation product of CESA7 and a high molecular weight form.
of CESA7 that ran at ~220 kDa (Fig. 4). No other CESA family members or any other proteins were identified. Some very faint bands were visible in the highly concentrated sample, which were below the MS detection limit.

Wild type and tagged CESA isoforms possess very similar molecular weights and migrate almost as a single band on a 10% acrylamide gel (Fig. 4A). Following electrophoresis on a 4–8% gradient acrylamide/SDS gel, only CESA4 and truncated CESA7 could be separated, but not the CESA4 and tagged CESA7 subunits (Fig. 4B). Although this meant it was not possible to obtain accurate stoichiometry for the different CESA subunits, measurements of protein band intensities provided a mean ratio of CESA8:CESA4 + CESA7 of 1:8.7 ± 0.53, as determined from three independent CESA complex purifications. This demonstrates that tagged CESA7 was consistently present at an equal abundance in all stem preparations. Further analysis of the His/STREP-purified samples by two-dimensional BN/SDS-PAGE and immunoblotting showed that CESA4 and CESA8 migrated predominantly at a molecular mass of 440–700 kDa, indicative of the CESA oligomers, whereas there was still a considerable proportion of CESA7 migrating at ~220 kDa (Fig. 5). These were probably CESA7 dimers composed of His-CESA7- and STREP-CESA7-tagged variants.

Disulfide Bonds Contribute to CESA Protein Association—To study the interactions between CESA subunits, measurements of protein band intensities provided a mean ratio of CESA8:CESA4 + CESA7 of 1:8.7 ± 0.53, as determined from three independent CESA complex purifications. This demonstrates that tagged CESA7 was consistently present at an equal abundance in all stem preparations. Further analysis of the His/STREP-purified samples by two-dimensional BN/SDS-PAGE and immunoblotting showed that CESA4 and CESA8 migrated predominantly at a molecular mass of 440–700 kDa, indicative of the CESA oligomers, whereas there was still a considerable proportion of CESA7 migrating at ~220 kDa (Fig. 5). These were probably CESA7 dimers composed of His-CESA7- and STREP-CESA7-tagged variants.

**FIGURE 4.** SDS-PAGE of concentrated His/STREP-purified samples and LC-MS/MS identification of CESA proteins. A, concentrated protein samples obtained after His/STREP affinity purification from microsomal fractions were solubilized with Triton X-100 (TX100) and DDM and then separated by SDS-PAGE on an 8–16% gradient and visualized by silver staining. B, a highly concentrated protein sample after a long duration run on a 4–8% gel and visualized by silver staining. In A and B the LC-MS/MS identified proteins are designated. The band identified as CESA7d is the size predicted of a CESA7 dimer. The CESA7tr band is the size of the previously identified truncated proteolytic product of CESA7.
venting disulfide bond formation, gave similar results (data not shown).

To further investigate the basis of the CESA interactions, pull-down experiments were performed on crude extracts to test the stability of the CESA oligomers following disulfide bond reduction. Even after treatment with up to 45 mM DTT, a substantial amount of CESA4 and CESA8 still co-precipitated with CESA7, suggesting that reduction of disulfide bonds alone is not sufficient for substantial dissociation of CESA subunits (supplemental Fig. S4). Therefore to test CESA interaction following disulfide bond reduction in combination with other denaturing agents, equal amounts of Triton X-100-solubilized microsomes were treated with DTT and different concentrations of NaCl (Fig. 7, A–C) or the anionic detergent Sarkosyl (Fig. 7, D–F) before incubation with Strep-Tactin resin. The relative amounts of CESA4, CESA7, and CESA8 proteins in the pull-down samples were determined by immunoblotting. This allowed the stability of CESA forms to be assessed immediately after solubilization and excluded the possibility that some associations would occur while the proteins were highly concentrated on the surface of the resin during the sample purification or concentration. There were higher rates of CESA dissociation when DTT was used in combination with NaCl at 0.5–2.0 M or with Sarkosyl at 0.1–0.9% (Fig. 7). The amount of CESA4 and CESA8 pulled down with STREP-CESA7 exhibited similar trends. Low levels of CESA protein dissociation after treatment with DTT, NaCl, and Sarkosyl alone contrasts with nearly complete dissociation after DTT reduction in combination with NaCl or Sarkosyl, suggesting that the association of CESA proteins occurs through both disulfide bonds and with noncovalent intermolecular interactions.

**Stability of the CESA Complex**—The purified or pulled down CESA complexes contain a mixture of CESA dimers and higher order oligomers that make the evaluation of the stability of individual oligomers difficult. Having established that SDS treatment alone at 25 °C without disulfide bond reduction did not liberate detectable amounts of CESA monomers from the purified sample (Fig. 6), we tested whether CESA oligomers were resistant to SDS denaturation at 65 °C using the two-dimensional BN/SDS-PAGE approach. Following BN-PAGE of identical stem samples, gel strips containing the high molecular CESA oligomers (molecular mass, >670 kDa) were treated with increasing SDS concentrations, with and without DTT reduction, at either 25 or 65 °C (Fig. 8). After treatment, the gel strips were placed on the top of a gradient SDS gel and subjected to a second dimension of SDS-PAGE. The immunoblot analysis showed liberation of significant amounts of CESA7 monomers from all samples where the SDS treatment was combined with disulfide bond reduction and incubation at 65 °C. The treatments at nonreduced conditions were less effective and resulted in the appearance of much lower amounts of CESA7 monomers. The treatment at 25 °C produced only detectable liberation of truncated CESA7 monomers at the higher (2.7%) SDS concentration (Fig. 8).

**DISCUSSION**

Here we have shown by immunoblotting with *Arabidopsis* secondary cell wall CESA antibodies that DDM-solubilized stem microsomes separated by two-dimensional BN/SDS-PAGE contain a mixture of CESA monomers and oligomers with molecular masses ranging from ~100 to 750 kDa (Fig. 1C). The observed oligomeric CESA forms probably represent CESA dimers (approximate molecular mass, 230 kDa) and tetramers (approximate molecular mass, 460 kDa), whereas the ~720-kDa forms may correspond to the CESA complex consisting of six CESA subunits. Preliminary electron microscopy level structural analysis of CESA protein complex by cryo-electron microscopy and negative staining of DDM-solubilized His/STREP-purified...
CESA7 has identified single particles of a range of sizes. These particles appear to correspond to the various oligomeric forms of CESA observed by two-dimensional BN/SDS-PAGE and would be consistent with the conclusion that the majority of the CESA proteins purified in this study do not exist as intact cellulose synthase complex. Such larger complexes appear to be absent prior to purification, and it is likely that solubilization is sufficient to cause breakdown of the CSC into units that consist of a single lobe or smaller. A recent study of primary cell wall CESA proteins using BN-PAGE to analyze Triton X-100 solubilized microsomes (24) reported the existence of CESA proteins within a similar high molecular mass protein complex with molecular mass of 840 kDa. Although no data on the composition of this complex is reported, it is likely analogous to the 720-kDa complex found in this study. Unlike in this study, no lower molecular mass primary cell wall CESA oligomers were observed by Wang et al. (24), possibly because of the insufficient sensitivity of immunoblots after BN-PAGE or lower abundance of such CESA oligomers during primary cell wall biogenesis. The presence of lower molecular mass oligomers may be a consequence of the stem material used in this study, which contains cells at different stages of differentiation and as such, is still in the process of assembling large numbers of CSC prior to cell wall biosynthesis, and consequently contains many CSC assembly intermediates. This is supported by live cell imaging of the CSC in developing xylem where the CSC is largely visualized within the cell, presumably where it is being assembled but rarely at the plasma membrane where cellulose is synthesized.

Despite the abundance of cellulose and its central role in plant cell wall architecture, the isolation of a highly purified CESA complex that retains high rates of cellulose synthase activity has remained elusive. Cellulose synthase activity has been reported in vitro from detergent (Brij 58 or taurocholate)-solubilized microsomal fractions from blackberry (25), but this did not involve purification of the CSC. One strategy is to use epitope tagging under non-denaturing conditions that favor the efficient purification. The results from the tandem His/FLAG purification procedure together with the results from the two-dimensional BN/SDS-PAGE experiments demonstrate the

3 I. I. Atanassov, R. C. Ford, and S. R. Turner, unpublished observations.
4 R. Wightman and S. R. Turner, unpublished observations.
problems and challenges related to the development of efficient epitope tagging of the native CESA complex. The poor elution of His/FLAG-CESA7 from the anti-FLAG or anti-CESA8 antibodies has shown that high affinity antibodies do not allow efficient purification of native CESA complexes. Secondly, the two-dimensional BN/SDS-PAGE demonstrates that beside the oligomeric forms of the CESA protein, the stem protein extract contains substantial amounts of CESA7 monomers that will be efficiently tagged and co-purified with any intact oligomeric forms using this method. The results from tandem His/FLAG purification confirm this, showing purification of much higher amounts of tagged His/FLAG-CESA7 than nontagged CESA4 and CESA8. A dual tag system, in which His and STREP tags were placed upon separate CESA7 molecules was therefore developed for more efficient purification of the CESA complex. Ideally, the tags would have been placed on CESA7 and CESA4 or CESA8, but the insertion of the STREP tag at the N terminus of CESA4 and CESA8 significantly impaired their activity (supplemental Table S2).

Using the dual His/STREP tag system, we were able to isolate a highly purified sample containing all three CESA subunits with virtually no contaminating proteins (Figs. 3 and 4). SDS-PAGE immunoblotting showed, however, a major loss of the tagged CESA proteins and complexes during the second STREP tag purification step, which significantly reduced the efficiency of the entire purification procedure. Further immunoblots after two-dimensional BN/SDS-PAGE demonstrated that the purified sample possesses CESA4—, CESA7—, and CESA8-containing CESA oligomers with molecular mass in the range of 400 to 700 kDa, corresponding to CESA tetramers and hexamers. The purified sample also contained substantial amounts of smaller CESA7 oligomers in the range of 240 kDa, most probably consisting of His-CESA7/STREP-CESA7 dimers. The results unambiguously demonstrate that only the CESA proteins CESA4, CESA7, and CESA8 form the core complex, and any interactions with other proteins such as microtubule-associated proteins (12) are likely to be transient and relatively unstable.

Our analysis of stem extracts from two irx mutants, irx3–1 and irx5–2, which do not form detectable amounts of CSC (20, 33), provided some insight into the early stages of CESA complex assembly (Fig. 2). These mutants are ideal for this kind of analysis because irx3–1 contains no detectable CESA7 protein, yet the plant remains viable. This is in contrast to the primary cell wall studies where analysis is complicated by partial redundancy among CESA subunits and lethality in primary wall CESA mutants (12, 18). The results from the immunoblots following two-dimensional BN/SDS-PAGE showed the formation of monomers and dimers of CESA4 and CESA8 in irx3–1 and of CESA7 and CESA8 in irx5–2, but no higher molecular weight forms (Fig. 2). Consistent with this, the dual tag purification demonstrated purification of low MW complexes that are likely CESA7 homodimers. This demonstrates that CESA proteins can undergo dimerization independently, but the presence of all three CESA subunits is required for higher order oligomerization. Because the three CESA subunits possess very similar molecular masses, we could not determine whether the CESA dimers were homo- or heterodimers; however, given that previous experiments have shown no detectable co-precipitation of CESA7 and CESA8 in an irx5–1 mutant (20), it is likely that dimers are homodimeric. Consequently, this is the first data that support a model for assembly for the CSC in which the first step is homodimerization between CESA proteins prior to assembly into larger CESA oligomers. Because larger oligomers all appear to contain all three CESA proteins, it is tempting to speculate that the largest oligomers represents a hexamer composed of CESA4, CESA7, and CESA8 homodimers.

Our dual tagging system has thus allowed a significant advance toward CESA complex purification at non-denaturing conditions. Future enhancements to the purification procedure may require the use of a more efficient tagging system as a second purification step, as well as different CESA tag configurations to further improve the selectivity and efficiency of the CESA complex purification.

The SDS-PAGE analysis of all dual His/STREP tag purified samples consistently found three prominent bands in the range of 90–120 kDa (Fig. 4). The LC-MS/MS analysis of these bands showed that they contain either CESA4, CESA7, or CESA8, which have previously been shown to interact (20). In addition, a protein band migrating at ~220 kDa was also observed with increasing intensity in the more concentrated samples. Based on previous analysis, this is likely to be a very stable CESA7 dimer. Because the purified sample contains lower CESA oligomers together with the high molecular weight CESA complex, it has not been possible to determine the stoichiometry of the three CESA protein based on the comparison of their protein band intensities. Likewise, the close molecular mass of CESA4 and CESA7 further hampered the direct analysis of CESA complex stoichiometry because they could not be separated as distinct bands even after continuous electrophoresis on a longer gel. No other proteins were identified in the His/STREP tag purified bands. Consistent with previous observations (37), the present study does not provide evidence for any association between CESA7 and KORRIGAN or any other proteins that are known to be involved in cellulose synthesis (4, 11). Clearly, it is possible that other proteins may transiently interact with the CESA proteins or that they are only very weakly bound and lost during purification; however, the data presented strongly suggest that the CESA proteins are able to assemble into large oligomers that contain no or very few other proteins.

Based on an earlier study of dimer formation from heterologously expressed cotton fiber CESA proteins, Kurek et al. (38) propose a model in which oxidative dimerization of the N-terminal zinc-binding domain contributes to CESA association. In this study we present evidence that disulfide bonds along with very stable noncovalent interactions are involved in the CESA complex assembly and stability. The treatment of purified CESA complex samples with DTT or N-ethylmaleimide found that the dissociation of CESA proteins in part required the reduction of disulfide bonds (Fig. 6); yet DTT reduction alone was not sufficient to completely dissociate CESA oligomers and had to be combined with a higher ionic strength buffer or a strong anionic detergent (Fig. 7). We cannot currently distinguish whether these disulfide bonds are intramolecular and act in parallel to strong noncovalent interactions or whether they
are intramolecular and determine a specific CESA quaternary folding structure related to the observed strong noncovalent CESA interaction.

In addition we demonstrated that treatment of CESA complex hexamers with DTT and SDS at high (65 °C) temperature is necessary for the efficient dissociation of the CESA complex and release of CESA subunits (Fig. 8). Moreover, all of the CESA oligomers possess moderate disulfide bond-mediated stability to SDS denaturation (data not shown). Although SDS is considered as a universal protein denaturating agent, several protein complexes have been reported to possess moderate to high stability in the presence of SDS, in many cases combined with a degree of thermal stability, such as major histocompatibility complex class II (39), SNARE complexes (40), and integrins (41). Although the parameters of the observed CESA complex stability in the presence of SDS require more detailed characterization, there is now an opportunity to further these studies by probing the stability of CESA associations in various cellulose mutants and transgenic CESA substitution lines.

In conclusion, the results from this study have demonstrated that 1) in detergent-soluble extract we have been able to identify and purify complexes containing only oligomers of CESA4, CESA7, and CESA8, with no other CESAs or other proteins associated; 2) the detergent-solubilized stem protein extract contains different CESA oligomers, estimated to be CESA dimers, tetramers, and hexamers; 3) the early stages of CESA complex assembly involve CESA dimerization, and the presence of all three functional CESA subunits is required to proceed with the next oligomerization step; and 4) the analysis of the CESA monomer release following various denaturizing treatments suggests that CESA association within the CESA complex involves disulfide bonds together with very stable noncovalent interaction. The approaches we have described will provide an attractive opportunity for further study of CESA complex structure, biogenesis, and function. Very few studies have been able to demonstrate in vitro cellulose synthase activity (25), and such studies have not attempted purification of the CSC. The purification of an intact, active complex therefore remains a major goal for the field. This report has described significant progress toward the native purification of a functional CSC, which is required to take us a step closer to this goal of being able to efficiently synthesize large quantities of cellulose in vitro.

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REFERENCES

1. Delmer, D. P. (1999) Annu. Rev. Plant Physiol. Plant Mol. Biol. 50, 245–276
2. Doblin, M. S., Kurek, I., Jacob-Wilk, D., and Delmer, D. P. (2002) Plant Physiol. 130, 1407–1420
3. Kudlicka, K., and Brown, R. M. (1997) Plant Physiol. 115, 643–656
4. Somervelle, C. (2006) Annu. Rev. Cell Dev. Biol. 22, 53–78
5. Mueller, S. C., and Brown, R. M. (1980) J. Cell Biol. 84, 315–326
6. Schneider, R., and Herth, W. (1986) Protoplasma 131, 142–152
7. Haigler, C. H., and Brown, R. M. (1986) Protoplasma 134, 111–120
8. Kimura, S., Laosinchai, W., Itoh, T., Cui, X. J., Linder, C. R., and Brown, R. M. (1999) Plant Cell 11, 2075–2085
9. Saxena, I. M., and Brown, R. M. (2005) Ann. Bot. 96, 9–21
10. Joshi, C. P., and Mansfield, S. D. (2007) Curr. Opin. Plant Biol. 10, 220–226
11. Taylor, N. G. (2008) New Phytol. 178, 239–252
12. Mutwil, M., Debolt, S., and Persson, S. (2008) Curr. Opin. Plant Biol. 11, 252–257
13. Arioli, T., Peng, L. C., Betzner, A. S., Burn, J., Wittke, W., Herth, W., Camilleri, C., Hofte, H., Plazinski, J., Birch, R., Cork, A., Glover, J., Redmond, J., and Williamson, R. E. (1998) Science 279, 717–720
14. Cano-Delgado, A. I., Metzlaff, K., and Bevan, M. W. (2000) Development 127, 3395–3405
15. Cano-Delgado, A., Penfield, S., Smith, C., Catley, M., and Bevan, M. (2003) Plant J. 34, 351–362
16. Ellis, C., Karafyllidis, I., Wasternack, C., and Turner, J. G. (2002) Plant Cell 14, 1557–1566
17. Scheible, W. R., Eshed, R., Richmond, T., Delmer, D., and Somerville, C. (2001) Proc. Natl. Acad. Sci. U. S. A. 98, 10079–10084
18. Desprez, T., Juraniec, M., Crowell, E. F., Jouy, H., Pochyllova, Z., Parcy, F., Hofte, H., Gonneau, M., and Venhettes, S. (2007) Proc. Natl. Acad. Sci. U. S. A. 104, 15572–15577
19. Persson, S., Paredes, A., Carroll, A., Palsdottir, H., Doblin, M., Poindexter, P., Khitrov, N., Auer, M., and Somerville, C. R. (2007) Proc. Natl. Acad. Sci. U. S. A. 104, 15566–15571
20. Taylor, N. G., Howells, R. M., Huttly, A. K., Vickers, K., and Turner, S. R. (2003) Proc. Natl. Acad. Sci. U. S. A. 100, 1450–1455
21. Taylor, N. G., Scheible, W. R., Cutler, S., Somerville, C. R., and Turner, S. R. (1999) Plant Cell 11, 769–780
22. Taylor, N. G., Laurie, S., and Turner, S. R. (2000) Plant Cell 12, 2529–2540
23. Turner, S. R., and Somerville, C. R. (1997) Plant Cell 9, 689–701
24. Wang, J., Elliott, J. E., and Williamson, R. E. (2008) J. Exp. Bot. 59, 2627–2637
25. Lai-Kee-Him, J., Chanzy, H., Muller, M., Putaux, J. L., Imai, T., and Bulone, V. (2002) J. Biol. Chem. 277, 36931–36939
26. Skerra, A., and Schmidt, T. G. (2000) Methods Enzymol. 326, 271–304
27. Becker, D., Kemper, E., Schell, J., and Masterson, R. (1992) Plant Mol. Biol. 20, 1195–1197
28. Clough, S. J., and Bent, A. F. (1998) Plant J. 16, 735–743
29. Wittig, I., Braun, H. P., and Schagger, H. (2006) Nat. Prot. 1, 418–428
30. Shevchenko, A., Wilm, M., Vorn, O., and Mann, M. (1996) Anal. Chem. 68, 850–858
31. Nomura, E., Katsuta, K., Ueda, T., Toriyama, M., Mori, T., and Inagaki, N. (2004) J. Mass Spectrom. 39, 202–207
32. Taylor, N. G., Gardiner, J. C., Wightman, R., and Turner, S. R. (2004) Cellulose 11, 329–338
33. Gardiner, J. C., Taylor, N. G., and Turner, S. R. (2003) Plant Cell 15, 1740–1748
34. Einhauer, A., and Jungbauer, A. (2001) J. Biochem. Biophys. Methods 49, 455–465
35. Junttila, M. R., Saarinen, S., Schmidt, T., Kast, J., and Westermarck, J. (2005) Proteomics 5, 1199–1203
36. Chang, I. F. (2006) Proteomics 6, 6158–6166
37. Szyjanowicz, P. M., McKinnon, I., Taylor, N. G., Gardiner, J., Jarvis, M. C., and Turner, S. R. (2004) Plant J. 37, 730–740
38. Kurek, I., Kawagoe, Y., Jacob-Wilk, D., Doblin, M., and Delmer, D. (2002) Proc. Natl. Acad. Sci. U. S. A. 99, 11109–11114
39. Schafer, P. H., Malapati, S., Hanfelt, K. K., and Pierce, S. K. (1998) J. Immunol. 161, 2307–2316
40. Kubista, H., Edlbauer, H., and Boehm, S. (2004) J. Cell Sci. 117, 955–966
41. Zolotarjova, N. I., Hollis, G. F., and Wynn, R. (2001) J. Biol. Chem. 276, 17063–17068