

RESEARCH PAPER

Features of the primary wall CESA complex in wild type and cellulose-deficient mutants of *Arabidopsis thaliana*

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Abstract

Evidence from genetics, co-precipitation and bimolecular fluorescence complementation suggest that three CESAs implicated in making primary wall cellulose in *Arabidopsis thaliana* form a complex. This study shows the complex has a M_r of approximately 840 kDa in detergent extracts and that it has undergone distinctive changes when extracts are prepared from some cellulose-deficient mutants. The mobility of CESAs 1, 3, and 6 in a Triton-soluble microsomal fraction subject to blue native polyacrylamide gel electrophoresis was consistent with a M_r of about 840 kDa. An antibody specific to any one CESA pulled down all three CESAs consistent with their occupying the same 840 kDa complex. In *rsw1*, a CESA1 missense mutant, extracts of seedlings grown at the permissive temperature have an apparently normal CESA complex that was missing from extracts of seedlings grown at the restrictive temperature where CESAs precipitated independently. In *prc1-19*, with no CESA6, CESAs 1 and 3 were part of a 420 kDa complex in extracts of light-grown seedlings that was absent from extracts of dark-grown seedlings where the CESAs precipitated independently. Two CESA3 missense mutants retained apparently normal CESA complexes as did four cellulose-deficient mutants defective in proteins other than CESAs. The 840 kDa complex could contain six CESA subunits and, since loss of plasma membrane rosettes accompanies its loss in *rsw1*, the complex could form one of the six particles which electron microscopy reveals in rosettes.

Key words: *Arabidopsis thaliana*, cellulose synthase, CESA complex, membrane proteins, primary wall, *rsw* mutants.

Introduction

Cellulose is a major structural polysaccharide that forms microfibrils in the plant cell wall. It is synthesized by rosettes seen by freeze etching the plasma membrane (Brown *et al.*, 1996). A microfibril has some 36 β -1,4 glucan chains and one rosette is thought to elongate all chains simultaneously. Each rosette shows six subunits in the electron microscope so that, if each glucan chain has its own glycosyltransferase, then each of those six visible subunits will themselves contain six enzymes.

The glycosyltransferase family making cellulose was identified by examining expressed sequence tags from cotton fibres that predominantly synthesize cellulose (Pear *et al.*, 1996) and by characterizing cellulose-deficient mutants (Arioli *et al.*, 1998; Taylor *et al.*, 1999, 2000, 2003; Fagard *et al.*, 2000; Burn *et al.*, 2002a; Ellis *et al.*, 2002; Caño-Delgado *et al.*, 2003; Wang *et al.*, 2006). CESA glycosyltransferases are large transmembrane proteins (*c.* 110–120 kDa) with features characteristic of processive glycosyltransferases. CESAs occur in rosettes (Kimura *et al.*, 1999) and a YFP-CESA6 fusion protein moves along linear tracks consistent with microfibril elongation (Paredez *et al.*, 2006).

Cell walls are categorized according to whether they form during cell expansion (primary walls) or after expansion finishes (secondary walls). They differ in structure and polysaccharide composition although their cellulose differs only in physicochemical properties such as chain length and crystallinity (Bertoniere and Zeronian, 1987; Brett, 2000; Lai-Kee-Him *et al.*, 2002). Reflecting those differences, known mutations in six CESA genes of *Arabidopsis* prominently affect cellulose production in primary walls in the case of CESA1, CESA3, and CESA6 (Arioli *et al.*, 1998; Fagard *et al.*, 2000; Burn *et al.*,

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Abbreviation: BN-PAGE, blue native polyacrylamide gel electrophoresis.

2002a; Ellis *et al.*, 2002; Caño-Delgado *et al.*, 2003; Wang *et al.*, 2006) or secondary walls in the case of CESA4, CESA7, and CESA8 (Turner and Somerville, 1997; Taylor *et al.*, 1999, 2000, 2003). Recent evidence (Chu *et al.*, 2006; Persson *et al.*, 2007) shows that CESA2 is more important for primary wall deposition than previously thought (Burn *et al.*, 2002a).

Mutational analysis led to the view that CESAs 4, 7, and 8 co-operate to make cellulose since mutations in any one of their genes drastically reduce cellulose deposition in the same secondary cell walls (Taylor *et al.*, 2003). The three CESAs are physically associated in a detergent-solubilized microsomal fraction and the absence of one CESA leaves the other two non-associated *in vitro* (Taylor *et al.*, 2003) and retained in the endo-membrane system *in vivo* (Gardiner *et al.*, 2003).

The situation with primary walls shows both similarities and differences. Mutations in CESAs 1, 3, and 6 all affect primary walls (Arioli *et al.*, 1998; Fagard *et al.*, 2000; Burn *et al.*, 2002a; Ellis *et al.*, 2002; Caño-Delgado *et al.*, 2003; Wang *et al.*, 2006), but it is more difficult to decide whether mutations in each gene affect precisely the same walls given the difficulties of examining very thin walls whose structure is drastically changed by cell expansion. Mutations in CESA1 (Williamson *et al.*, 2001; Gillmor *et al.*, 2002), including one expected to inactivate the enzyme by changing a critical Asp residue (Beeckman *et al.*, 2002), have very severe phenotypes but are not invariably lethal, implying that some synthesis continues. Known missense mutants of CESA3 (Ellis *et al.*, 2002; Caño-Delgado *et al.*, 2003; Wang *et al.*, 2006) have somewhat less severe phenotypes whereas even null mutants of CESA6 have a relatively mild phenotype in roots and essentially none in light-grown hypocotyls (Desnos *et al.*, 1996; Fagard *et al.*, 2000). Moreover, embryos make primary walls without expressing CESA6 (Beeckman *et al.*, 2002). Suggestions based on sequence similarities that CESAs 2, 5, and 9 might partially replace CESA6 (Robert *et al.*, 2004) are strongly supported by recent evidence (Persson *et al.*, 2007; Desprez *et al.*, 2007). Interactions were shown by bimolecular fluorescence complementation which showed *Arabidopsis* CESAs expressed in tobacco interact with one another *in vivo* and by co-immunoprecipitation of CESAs 3 and 6. Association may involve disulphide bonds between Cys residues in the zinc-binding region of the N-terminal cytoplasmic domain (Kurek *et al.*, 2002; Jacob-Wilk *et al.*, 2006) although the region of the protein from the start of the second transmembrane domain to the C-terminus is implicated in determining which site in the presumptive complex chimeric proteins access (Wang *et al.*, 2006).

This study extends other recent studies of the primary wall CESA complex by showing that CESAs 1, 3, and 6 migrate as an 840 kDa complex on blue native

polyacrylamide gel electrophoresis (BN-PAGE) and that all three CESAs (3 as well as 1 and 6) co-precipitate in pull-down experiments. Loss of the complex in a *cesal* missense mutant and the existence of a smaller, unstable complex in extracts of a *cesa6* null mutant support the complex's importance for cellulose synthesis. Given the loss of rosettes that accompanies loss of the complex in *rsw1* and arguments from stoichiometry, it is suggested that the 840 kDa complex may comprise one of the six particles that form the rosettes seen by electron microscopy.

Materials and methods

Plant materials

Mutants used were: *rsw1* (Ala549Val in CESA1/At4g32410; Arioli *et al.*, 1998); *rsw2-1* (Gly429Arg in the KOR endoglucanase/At5g49720; Lane *et al.*, 2001); *rsw3* (Ser599Phe in the α -subunit of glucosidase II/At5g63840; Burn *et al.*, 2002b); *rsw5* (Pro1056Ser in AtCESA3/At5g05170; Wang *et al.*, 2006); *rsw10* (Glu115Lys in ribose 5-phosphate isomerase/At1g71100; Howles *et al.*, 2006); *rsw13* (Gly395Glu in glucosidase I/At1g67490; PA Howles and RE Williamson, unpublished results); *prcl-19* (Tyr275stop in CESA6 of *Ws* ecotype; Fagard *et al.*, 2000); *elil-1* (Ser301Phe in AtCESA3/At5g05170; Caño-Delgado *et al.*, 2003). Columbia wild-type was used for controls except when making comparisons with *prcl-19* where *Ws* was used. *rsw1*, *rsw2-1*, and *rsw3* are available from the Arabidopsis Biological Resource Center (Ohio) as CS6554, CS6555, and CS6556, respectively.

Antibody production

The regions encoding the first hypervariable regions (HVR1) of CESAs 1, 3, and 6 were amplified from cDNAs by the PCR using the following primers: CESA1 forward 5'-CGGGATCCGAGAGT-TCAATTACGCCAGGG-3', CESA1 reverse 5'-TGAGGTACC-GACGGGTCCACGATTCTTACA-3'; CESA3 forward 5'-CGGG ATCCCAGGTACTGTTGAGTTCAACTA-3', CESA3 reverse 5'-TGAGGTACCCGACATCTGATGAATAGGGAA-3'; CESA6 forward 5'-ATTGGATCCAGTTTGAGTATGGAAATGG-3', and CESA6 reverse 5'-TAGGGTACCACCATAGGCCCTTGATGTG-3'. The products were verified by sequencing and cloned into the *Bam*HI and *Kpn*I sites of the pQE32 expression vector (Qiagen, Valencia, CA) to provide an N-terminal 6His tag.

The three peptides consisted of Glu112 to Pro196 from CESA1, Thr92 to Val173 from CESA3, and Phe113 to Met201 from CESA6, essentially comprising the HVR1 regions of the N-terminal cytoplasmic domain. Expression of these peptides in *E. coli* was induced by 1 mM isopropyl- β -D-thiogalactopyranoside and pelleted cells were freeze-thawed and sonicated in solubilization buffer (0.1 M NaH₂PO₄, 0.01 M TRIS, 6 M urea, pH 8.0) with protease inhibitors (Complete, EDTA-free protease inhibitor cocktail tablets; Roche, Basel, Switzerland). Each centrifuged extract was passed three times through a 2 ml Ni-NTA column (Qiagen) that was washed sequentially with solubilization buffer adjusted to pH 6.3 and then pH 5.9 (10 column volumes each) before the His-tagged peptide was eluted with three column volumes of pH 4.5 solubilization buffer. Each almost pure peptide was dialysed against phosphate buffered saline and injected into rabbits using standard protocols. To find strongly responding rabbits, sera were tested by immunoblotting for reaction with the His-tagged peptide and with putative CESAs (defined as an approximately 120 kDa band in

Arabidopsis extracts that had been resolved by SDS-PAGE). Crude antisera were further purified using either a protein G-Sepharose column (Amersham Biosciences, Bucks, UK) or with a column having the target peptide coupled through cyanogen bromide to Sepharose 6B (Amersham Biosciences). Purified antibodies were diluted to the same volume as the serum from which they had been purified. Affinity purified antibodies detected only 120 kDa bands when crude extracts were immunoblotted. With antibodies purified using protein G, anti-CESA3 and anti-CESA6 still stained only the 120 kDa band in crude extracts but anti-CESA1 also stained a band with a M_r significantly lower than 120 kDa. This protein was not detected in the Triton-soluble supernatants that were used for all subsequent experiments so that, given their simpler preparation, antibodies purified with protein G were routinely used.

Preparation of the Triton-soluble supernatant

Seedlings (1 g fresh weight) were routinely grown with 16 h days at 21 °C for 7–10 d in liquid culture with occasional shaking (Hoagland's liquid medium with glucose 10 g l⁻¹, inositol 96 mg l⁻¹, and thiamine hydrochloride 16.8 mg l⁻¹). Flasks growing dark-grown seedlings were wrapped in aluminium foil and placed inside a box in the same constant environment chamber. Temperature-sensitive *rsw* mutants were grown at 21 °C with the final two days (or other time as indicated) at either 21 °C or 31 °C, the permissive and restrictive temperatures, respectively. Whole seedlings were drained and ground in a mortar and pestle at 4 °C in extraction buffer (50 mM NaH₂PO₄, 300 mM NaCl, pH 8.0) with the protease inhibitor cocktail. Extracts were centrifuged at 10 000 g for 10 min and a crude microsomal pellet prepared by centrifuging the supernatant at 100 000 g for 30 min. The high speed pellet was resuspended in 1 ml of resuspension buffer (extraction buffer containing 2% v/v Triton X-100). Insoluble debris was removed by a further centrifugation (100 000 g for 30 min) and the Triton-soluble supernatant containing detergent-solubilized CESA proteins was used for pull-down experiments and BN-PAGE.

Pull-down experiments

One of the CESA antibodies (50 µl) was added to 950 µl of Triton-soluble supernatant (approximately 3 mg total protein) and incubated at 4 °C for 1 h with end-over-end rotation. After addition of 60 µl of protein G-Sepharose and a further 1 h incubation at 4 °C with end-over-end rotation, samples were centrifuged briefly to pellet the Sepharose. The pellet was washed with three changes of resuspension buffer before boiling in loading buffer (0.125 M TRIS-HCl pH 8.0, 2% SDS, 0.003% bromophenol blue, 15 mg ml⁻¹ of dithiothreitol with 5% sucrose), chilling on ice and analysing by SDS-PAGE, usually using a comb producing a single sample well covering most of the gel. Proteins transferred to Hybond-C nitrocellulose membranes (Amersham) were immunoblotted (1:500 dilution of primary antibody) by standard protocols (Harlow and Lane, 1988) with detection by horseradish peroxidase using 4-chloro 1-naphthol as substrate. In a typical experiment, adjacent strips cut from the membrane were probed with each of the three CESA antibodies.

BN-PAGE

BN-PAGE followed Swamy *et al.* (2006) using approximately 60 µg of total protein per lane and, after electrophoresis, protein complexes were denatured in the gel with heat and SDS (Kikuchi *et al.*, 2006) to facilitate electrophoretic transfer to PVDF membranes (Millipore). Coomassie blue was removed by a 1 min wash of the membrane with methanol and immunoblotting was as described for SDS-PAGE. Molecular weight standards are described in the legend to Fig. 2C.

Results

Antibody specificity

The peptides used as antigens had no significant similarity to each other in BLAST alignments and the antibodies did not recognize non-target peptides when immunoblotted (Fig. 1A). After affinity purification with the peptide antigen, they reacted only with *Arabidopsis* proteins of appropriate M_r to be CESAs (Fig. 1B) confirming that the same subset of antibodies was responsible for binding to both peptide and *Arabidopsis* protein. The approximately

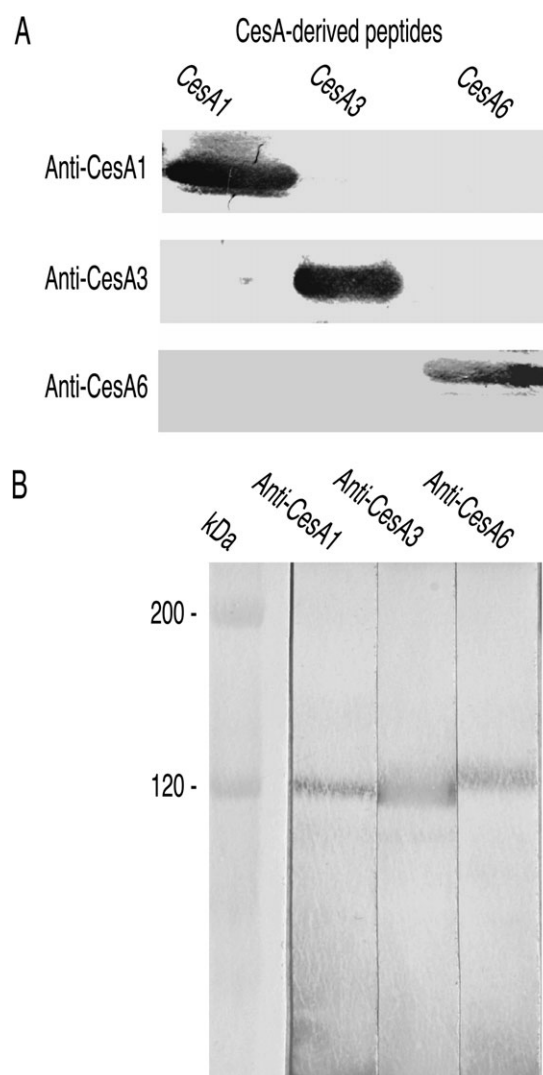


Fig. 1. Antibody specificity. (A) The three peptides used as antigens were loaded in adjacent lanes, resolved by SDS-PAGE and immunoblotted with the three antibodies. Each antibody reacts only with its own antigen. (B) Crude extracts of wild-type *Arabidopsis* seedlings grown in the light were resolved by SDS-PAGE and immunoblotted with affinity purified antibodies. Each antibody recognized only a single band of about 120 kDa (marker is β -galactosidase with predicted M_r of 116 kDa). The band stained by anti-CESA6 migrates just behind the CESA1 and CESA3 bands.

120 kDa polypeptide that anti-CESA6 recognized was immediately distinguished from those anti-CESA1 and anti-CESA3 recognized because it migrated just behind them (see, for example, Fig. 1B). Bands were not detected after omitting either primary or secondary antibody.

The CESA3 peptide showed no similarities to other *Arabidopsis* CESAs after BLAST alignments and only small regions of similarity to other *Arabidopsis* proteins. The CESA1 peptide's similarities to CESA10 are unlikely to present practical problems because CESA10 expression is very low relative to CESA1 (Burn *et al.*, 2002a; see <http://mpss.udel.edu/at/>). Anti-CESA6 detected no *Arabidopsis* proteins when CESA6 was removed by mutation in *prc1* showing that potential cross-reactions with CESAs 2, 5, and 9 were insignificant (in spite of the 44, 55, and 38 identities, respectively, with the 89 residue CESA6 peptide). It is further concluded that each antibody recognized its own CESA but not the CESA that either of the other antibodies recognized since CESAs 1, 3, and 6 precipitated independently from detergent extracts of *rsw1* (Ala549Val in CESA1) and each precipitate reacted only with the antibody that had precipitated it when subsequently analysed by immunoblotting. It is therefore considered that the antibodies were mono-specific in recognizing their target CESAs in both native and denatured states.

Preparing a Triton-soluble supernatant

Demonstrating a complex of CESA proteins required solubilizing the membrane-bound CESAs so that any association reflects protein-protein interaction rather than simply proteins remaining embedded in a common lipid bilayer. The experiments of Turner's group suggest that 2% Triton X-100 achieves this with secondary wall CESAs (Taylor *et al.*, 2000). Immunoblotting showed the fate of CESAs during the steps to produce a Triton-soluble supernatant from a crude microsomal fraction (Fig. 2A shows results using, in this case, anti-CESA1). With constant protein loadings, CESAs were only weakly detected in crude extracts (lane 1). Being membrane proteins, they were absent from the supernatant (lane 2) after centrifugation (100 000 g for 30 min) but prominent in the microsomal pellet (lane 3). After resuspending the microsomal pellet in 2% Triton X-100, most CESAs remained in the supernatant (lane 4 versus lane 5) after centrifuging again at 100 000 g for 30 min. The experiments that follow show that the targets of the three antibodies often co-precipitate from Triton extracts, but their independent precipitation from extracts of *rsw1* (Ala549Val) suggests co-precipitation is not due to the proteins still being trapped in the lipid bilayer, a conclusion further supported by the reproducible M_r that CESA complexes show on BN-PAGE. This Triton-soluble supernatant was therefore used for BN-PAGE and for antibody pull-down experiments.

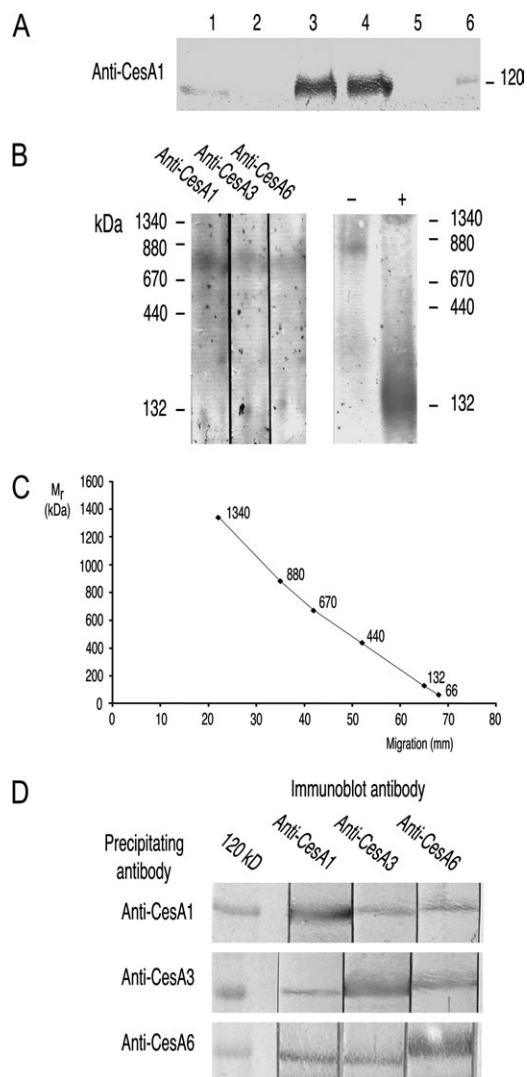


Fig. 2. Triton-soluble supernatants from light-grown wild-type; preparation and demonstration that CESA proteins occur in 840 kDa complexes. (A) Behaviour of CESAs during preparation of the Triton-soluble supernatant. Aliquots containing 50 μ g protein were resolved by SDS-PAGE and immunoblotted with anti-CESA1. The lanes are: (1) crude extract; (2) 100 000 g supernatant; (3) 100 000 g pellet; (4) Triton-soluble supernatant (100 000 g pellet resuspended in Triton and again centrifuged at 100 000 g); (5) pellet from the second 100 000 g centrifugation; (6) 116 kDa marker. (B) Proteins in the Triton-soluble supernatant resolved by BN-PAGE and immunoblotted with each of the CESA antibodies (left hand panel). Immunostaining of CESA proteins indicates a M_r of about 840 kDa. In the right hand panel, a normal sample (-) is compared with an extract which was boiled in SDS sample buffer before loading (+). A diffuse band in the vicinity of the 132 kDa marker replaces the 840 kDa complex. (C) Calibration curve showing mobility (mm) versus M_r for BN-PAGE. The markers (lowest to highest M_r) are: BSA monomer; BSA dimer; ferritin monomer; thyroglobulin monomer; ferritin dimer; thyroglobulin dimer. (D) Each CESA antibody pulls down all three CESAs from the Triton-soluble supernatant. Tightly bound proteins were resolved by SDS-PAGE and adjacent strips immunoblotted with each of the three CESA antibodies. The target of the immunoprecipitating antibody was more abundant than the other two CESA proteins.

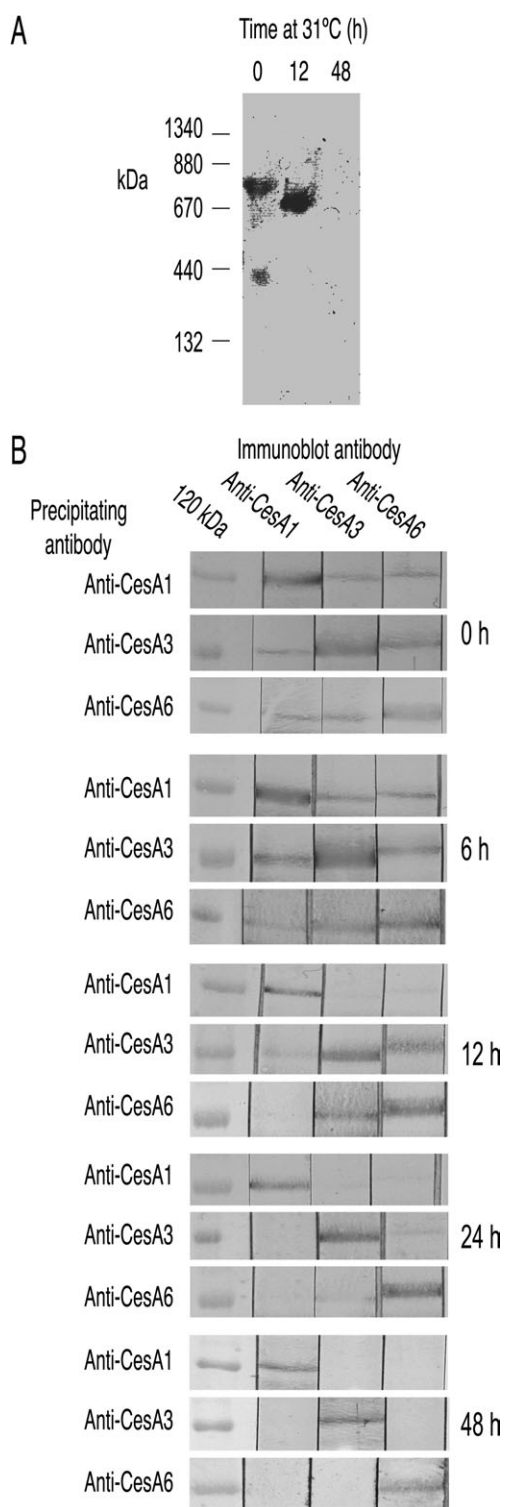


Fig. 3. The CESA complex in *rsw1* is lost at the mutant's restrictive temperature. (A) CESA proteins in extracts from seedlings grown at 20 °C migrate at 840 kDa on BN-PAGE (anti-CESA3 as primary antibody). After 12 h growth at 31 °C, the position of the band indicates a slightly lower M_r and after 48 h growth, no clear band is immunostained. (The staining at about 400 kDa in the 0 h lane was not reproducibly seen.) (B) Pull-down experiments point to progressive loss of the CESA complex at 31 °C. Triton-soluble supernatants were prepared from *rsw1* grown at the permissive temperature (0 h) and, as

CESAs 1, 3, and 6 migrate at high M_r on BN-PAGE

To see whether primary wall CESAs formed high M_r complexes in the Triton-soluble supernatant, immunoblotting was used to locate CESAs after BN-PAGE. All three CESAs migrated with an estimated M_r of 840 kDa (Fig. 2B, C). Boiling in sample buffer before electrophoresis replaced the 840 kDa bands with strong, rather diffuse staining in the vicinity of the 132 kDa standard that presumably corresponded to CESA monomers (Fig. 2B, right hand lanes).

Pull-down experiments show that complexes contain more than one CESA isoform

Migration on BN-PAGE shows all three CESAs are part of high M_r complexes, but these complexes could be homo- or hetero-oligomers. If hetero-oligomers exist (i.e. complexes containing two or more CESA isoforms), each CESA antibody should pull down all CESAs in the complex, whereas they would only pull-down their target CESA if homo-oligomers exist. All three CESAs were detected in the precipitate irrespective of which antibody performed the pull-down (Fig. 2D). The protein targeted by the pull-down antibody always stained more intensely on the subsequent immunoblot than did the other two CESA proteins. No proteins reacting with CESA antibodies on immunoblots were recovered if we omitted the CESA antibody or the protein G beads from the pull-down step.

*Extracts from *rsw1* grown at its restrictive temperature lack the 840 kDa CESA complex*

rsw1 (Ala549Val in CESA1) grown at 21 °C showed minimal phenotype (Baskin *et al.*, 1992; Arioli *et al.*, 1998; Sugimoto *et al.*, 2001) and CESAs migrated at 840 kDa on BN-PAGE (Fig. 3A). By contrast, seedlings showed a strong phenotype after 48 h at 30 °C and a high M_r complex was not detected (Fig. 3A). With seedlings grown for 12 h at the restrictive temperature, the complex migrated with a slightly lower M_r than the complex from seedlings not exposed to the restrictive temperature. Pull-down experiments were used to examine the time-course in more detail. Each antibody pulled-down all three CESAs from extracts of seedlings grown at the permissive temperature (Fig. 3B, 0 h panel), but only pulled down its target CESA from extracts of seedlings grown for 48 h at

indicated in the appropriate panels, at 6, 12, 24 and 48 h after transferring seedlings to the restrictive temperature. Proteins pulled-down with each antibody were resolved by SDS-PAGE and adjacent strips immunoblotted with each antibody. The CESA complex appears normal after 6 h (each antibody precipitates all three CESAs), but from 12 h onwards, anti-CESA1 precipitates CESA1 with minimal CESA3 and CESA6. At 12 h, there is some precipitation of both CESA3 and CESA6. At 24 h and 48 h, all three CESAs precipitate essentially independently.

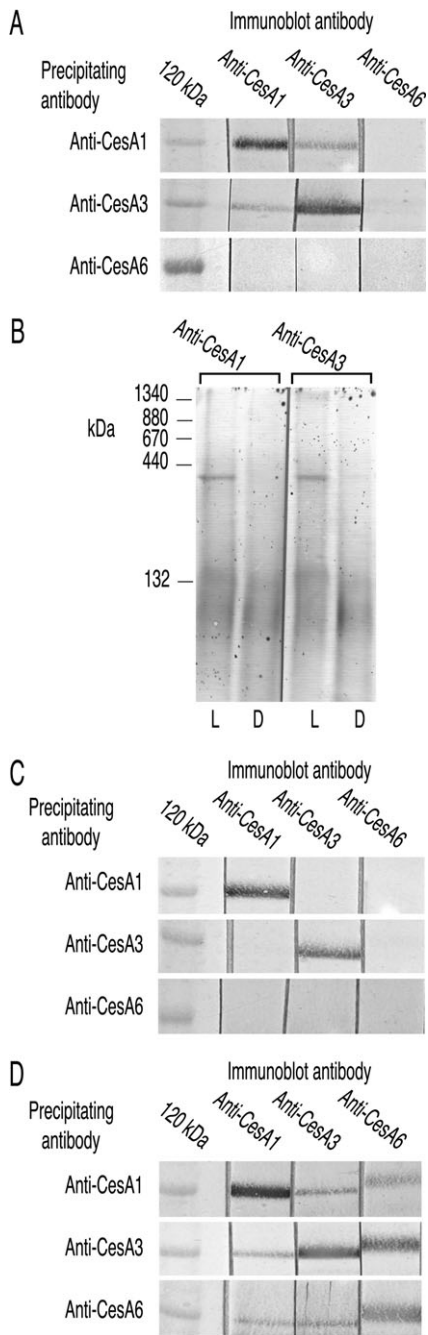


Fig. 4. CESA1 and CESA3 form a 420 kDa complex in *prc1* which is absent in dark-grown seedlings. (A) In pull-down experiments, CESA6 was not detected in the material pulled down by anti-CESA1 or anti-CESA3 and anti-CESA6 did not pull down any proteins that anti-CESA1, anti-CESA3 or anti-CESA6 recognized on immunoblots. CESAs 1 and 3 co-precipitate. Proteins pulled-down with each CESA antibody from Triton-soluble supernatants of light-grown *prc1-19* were resolved by SDS-PAGE and adjacent strips immunoblotted with all three antibodies. (B) Proteins in Triton-soluble supernatants of *prc1-19* resolved by BN-PAGE show that CESA1 and CESA3 exist in a 420 kDa rather than an 840 kDa complex using extracts from light-grown seedlings (L). This complex is not detected in extracts containing equal amounts of total protein that were prepared from dark-grown seedlings (D). Extracts of this mutant show more immunostaining in the low M_r region than seen with extracts of other mutants. The constant percentage gel used in this experiment provides better resolution in intermediate M_r ranges than the gradient gels routinely

used. (C) In pull-down experiments from dark-grown *prc1-19* seedlings, CESA1 and CESA3 precipitate only with their respective antibodies consistent with the absence of the 420 kDa complex seen in (B) with BN-PAGE. (D) In pull-down experiments from dark-grown wild-type seedlings, each antibody pulls down all three CESAs showing that, unlike the *prc1* complex, the wild-type CESA complex is stable in darkness.

Extracts from light-grown *prc1* have a 420 kDa CESA complex that extracts of dark-grown seedlings lack

The premature stop codon in *CESA6* in *prc1-19* meant that anti-CESA6 did not recognize any proteins in immunoblots of Triton-soluble supernatants, did not pull-down any CESAs (target or non-target), and did not recognize on immunoblots any proteins pulled-down by anti-CESA1 or anti-CESA3 (Fig. 4A). The more severe hypocotyl phenotype in dark-grown *prc1* seedlings than in light-grown seedlings (Desnos *et al.*, 1996; Fagard *et al.*, 2000) made it interesting to compare the CESAs in their Triton supernatants. CESA1 and CESA3 migrated at about 420 kDa after BN-PAGE of light-grown *prc1* seedling extracts rather than at 840 kDa (Fig. 4B), but only at low M_r when using extracts from dark-grown seedlings (Fig. 4B). Results of pull-down experiments were also consistent with the absence of the CESA complex in extracts of dark-grown *prc1-19*: anti-CESA1 and anti-CESA3 each pulled-down both CESA1 and CESA3 from extracts of light-grown seedlings (Fig. 4A), but pulled-down only their target CESA from extracts of dark-grown seedlings (Fig. 4C). With extracts from wild-type seedlings, pull-down experiments recovered all three CESAs in extracts of light (Fig. 2D) or dark (Fig. 4D) grown seedlings.

Extracts of two *CESA3* missense mutants retain 840 kDa CESA complexes

rsw5 (Wang *et al.*, 2006) and *elil-1* (Caño-Delgado *et al.*, 2003) are both missense mutants of *CESA3* (Pro1056Ser for *rsw5* and Ser301Phe for *elil-1*). *rsw5* was grown for the final 48 h at its restrictive temperature whereas the constitutive *elil-1* was grown throughout at 21 °C. CESAs 1, 3, and 6 all migrated with a M_r of 840 kDa on BN-PAGE (Fig. 5A) and each antibody pulled-down all

three CESAs from extracts of both mutants (Fig. 5B, C), consistent with both mutants retaining an intact complex.

Extracts of other cellulose-deficient mutants retain 840 kDa CESA complexes

Extracts prepared from other radial swelling mutants that are defective in cellulose synthesis because of mutations in genes encoding proteins other than CESAs were also examined (*rsw2-1* in the korrigan endocellulase, Lane

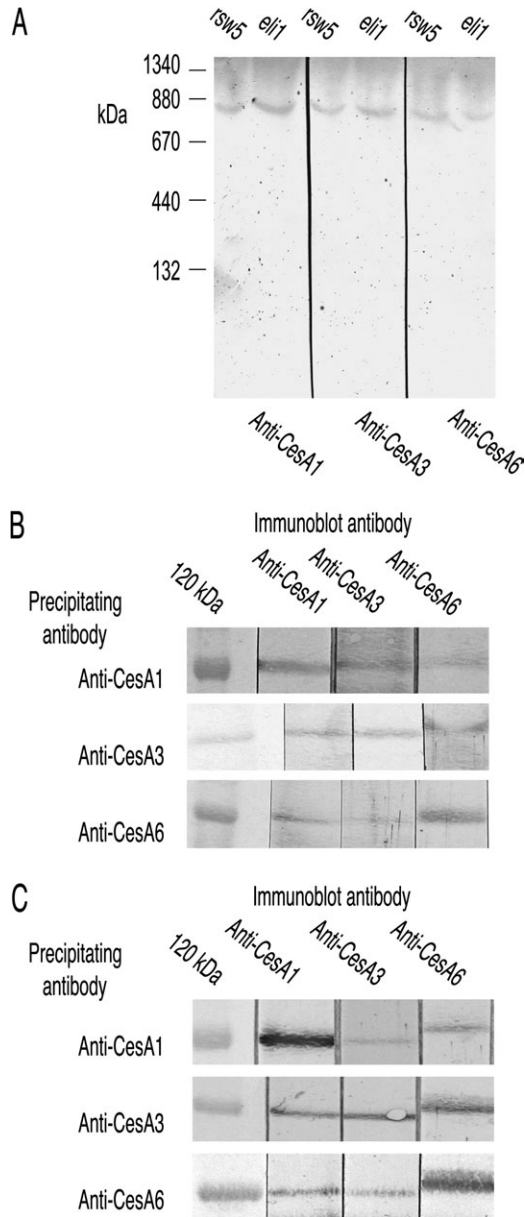


Fig. 5. The 840 kDa CESA complex remains intact in the CESA3 missense mutants *eli1-1* and *rsw5* (latter grown for 48 h at the restrictive temperature). (A) CESAs 1, 3, and 6 are detected at 840 kDa when extracts from both mutants are resolved by BN-PAGE. (B) Each CESA antibody precipitates all three CESAs from a Triton-soluble supernatant prepared from *rsw5*. (C) Each CESA antibody precipitates all three CESAs from a Triton-soluble supernatant prepared from the constitutive mutant *eli1-1* grown at 21 °C.

et al., 2001; *rsw13* and *rsw3* in the N-glycosylation/quality control enzymes, glucosidase I and glucosidase II, unpublished results of L Gebbie, P Howles and RE Williamson; Burn *et al.*, 2002b; *rsw10*, in ribose 5-phosphate isomerase required to synthesize pyrimidine nucleotides to supply UDP-glucose for cellulose synthesis, Howles *et al.*, 2006). BN-PAGE showed CESA3 migrating with a M_r of 840 kDa (Fig. 6A) and anti-CESA1 precipitated all three CESAs from extracts prepared from all mutants (Fig. 6B) showing that all mutants retained CESA complexes containing all three primary wall CESAs.

Discussion

Results from pull-down experiments and BN-PAGE point to similar conclusions regarding how CESAs 1, 3, and 6 behave in detergent extracts of wild type and several

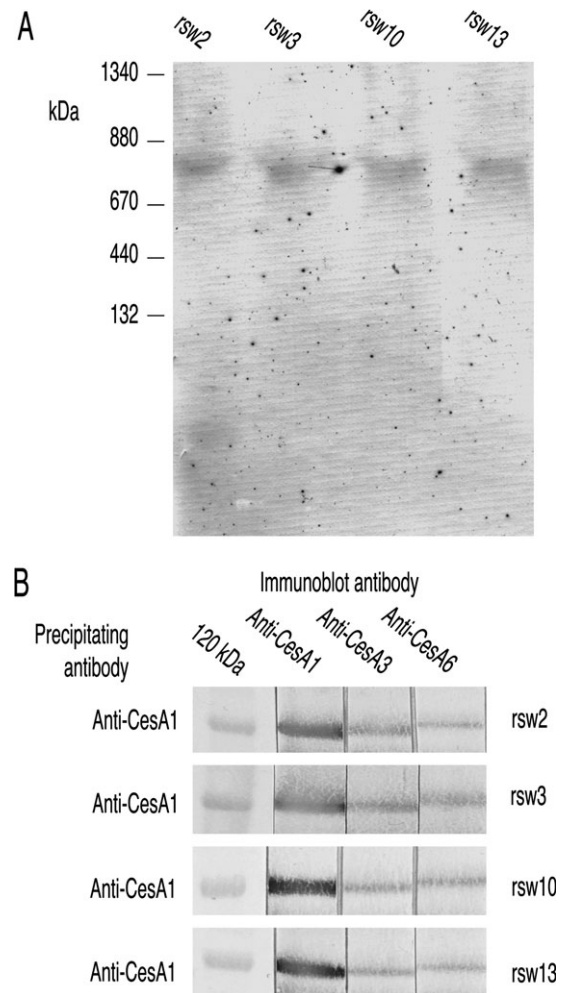


Fig. 6. The Cesa complex remains intact in *rsw2*, *rsw3*, *rsw10*, and *rsw13* grown for 48 h at the restrictive temperature before harvest. (A) BN-PAGE shows an apparently normal CESA complex persists in the Triton-soluble supernatants from these four mutants. Immunostaining with anti-CESA3. (B) Consistent with the persistence of such a CESA complex, anti-CESA1 pulls down all three CESAs from Triton-soluble supernatants.

cellulose-related mutants. The results with BN-PAGE show that wild type CESAs 1, 3, and 6 occur in complexes with a M_r of about 840 kDa and, with pull-down experiments, that those complexes contain more than one CESA isoform. Extracts of some but not all cellulose-deficient mutants show changes in the CESA complex and analysis of complex behaviour bolsters the idea that the complex contains all three CESAs rather than there being three complexes each containing only two of the three CESAs.

A primary wall CESA complex in wild type

The reliability with which pull-down experiments trap all three CESA isoforms suggests that the 840 kDa complexes seen after BN-PAGE contains more than one CESA isoform. There could be one complex with all three CESAs or three complexes each with only two of these CESA isoforms (1-3, 1-6, and 3-6), a possibility left open by bimolecular fluorescence complementation (Desprez *et al.*, 2007). Findings from *rsw1* and *prc1* show that two wild-type primary wall CESAs do not form a stable 840 kDa complex in the absence of the third: (i) *rsw1* has wild-type CESA3 and CESA6 proteins but, at its restrictive temperature, they do not form an 840 kDa CESA3-CESA6 complex; (ii) *prc1* has a complex containing wild-type CESA1 and CESA3 but it has a M_r of 420 kDa in extracts prepared from light-grown seedlings and is absent in extracts prepared from seedlings grown in the dark. This strengthens the hypothesis that the 840 kDa complex contains three CESA isoforms. Current M_r estimates lack the precision to settle the issue of the complex's protein composition. It could certainly be a hexamer as foreshadowed in various models but the presence of other protein(s) which, at the extreme, might mean it contained fewer than six CESA subunits is not excluded.

When pulled-down proteins were immunoblotted, the CESA that was the target of the pull-down antibody was reliably more prominent than the non-target CESAs. A similar effect is visible for CESAs 3 and 6 in the co-immunoprecipitation experiments of Desprez *et al.* (2007). This is consistent with the Triton-soluble supernatant containing a mixture of free and complexed CESAs; each precipitating antibody would then pull down both the free and complexed forms of its target CESA, but only the complexed form of the non-target CESAs. That hypothesis was not directly supported by BN-PAGE where there was usually little immunostaining of the regions where CESA monomers would be expected to migrate. Again, further approaches (e.g. size exclusion chromatography) will be required to settle how much of the CESAs remain outside the major complex BN-PAGE detects.

Behaviour of the CESA complex in *rsw1* extracts

The *rsw1* mutant is highly temperature conditional showing near normal growth, cellulose production, and rosettes at

the permissive temperature (Arioli *et al.*, 1998; Baskin *et al.*, 1992; Sugimoto *et al.*, 2001). Plants grown for 2 d at the restrictive temperature have less cellulose and few rosettes, changes resulting from CESA1^{rsw1} having one, slightly larger amino acid residue (Ala549Val) in its catalytic domain (Arioli *et al.*, 1998). Growing *rsw1* at either the permissive or the restrictive temperature results in the three primary wall CESAs showing dramatic differences in aggregation in identically prepared extracts. The CESAs precipitate individually in extracts from seedlings grown at the restrictive temperature with no CESA complex detected by BN-PAGE whereas the same CESAs remain part of an 840 kDa complex in extracts from seedlings grown at the permissive temperature and co-precipitate from the same extracts. It is important to stress when comparing the mutant after growth at different temperatures, that differences in the behaviour of identical CESA alleles (in this case CESA1^{rsw1}, CESA3^{WT} and CESA6^{WT}) in detergent extracts prepared identically from homogenization onwards are being observed. Differences in CESA behaviour in the extract seemingly must derive from some difference that was present *in vivo* as a result of the different growth temperatures. It would be premature to conclude that the non-aggregated state of the CESAs *in vitro* precisely indicates the *in vivo* state of the complex at the restrictive temperature although the strong *rsw1* phenotype at 30 °C makes plausible a drastic change such as extensive disassembly. Techniques such as bimolecular fluorescence complementation (Desprez *et al.*, 2007) can report on CESA aggregation *in vivo*.

rsw1 initiates radial swelling within 6–12 h of exposure to the restrictive temperature (Baskin *et al.*, 1992; Sugimoto *et al.*, 2001) and microfibrils on the wall's inner face lose their typical transverse alignment (Sugimoto *et al.*, 2001). Changes in the *in vitro* aggregation state of the CESAs are detected on a similar time scale. Pull-down experiments using extracts prepared from *rsw1* seedlings grown for 12 h at the restrictive temperature suggest that less CESA1^{rsw1} is present in any complex although CESA3^{WT} and CESA6^{WT} still remain associated with each other as judged by co-precipitation. BN-PAGE shows a CESA complex with a slightly lower M_r perhaps consistent with a complex deficient in one Cesa1^{rsw1} subunit. CESA-containing rosettes probably turn over and move between plasma and endomembrane systems on time scales shorter than those required to induce the *rsw1* phenotype (Jacob-Wilk *et al.*, 2006; Paredes *et al.*, 2006) so that changes in the behaviour of CESAs may reflect changes occurring to CESAs that are being delivered *de novo* to the plasma membrane rather than just reflecting changes occurring to pre-existing complexes already in the plasma membrane.

Behaviour of the CESA complex in prc1 extracts

A Tyr275stop mutation in *prc1-19* truncates CESA6 and the aberrant mRNA is probably processed by the

nonsense-mediated degradation pathway that removes such messages (Hori *et al.*, 2005). Immunoblots with anti-CESA6 detect no protein with a M_r of 120 kDa, good evidence that anti-CESA6 does not recognize CESAs 2, 5, and 9 which have partial sequence similarity to CESA6. Anti-CESA6 also does not pull-down enough CESA1 or CESA3 for their own antibodies to detect on subsequent immunoblots.

Two types of differences in the aggregation state of CESAs are reported in detergent extracts: those between wild type and mutant and those between mutant grown in the light and mutant grown in darkness. Again, the caveat is repeated that CESA aggregation is being observed in identically prepared detergent extracts so that differences seen here presumably reflect some difference that was present *in vivo* but does not necessarily report the exact aggregation state of CESAs *in vivo*. It is further noted that differences between *prc1* and wild-type will reflect differences caused by the absence of CESA6 in *prc1*. By contrast, differences between *prc1* grown in the light and darkness will reflect the fact that light conditions *in vivo* influence the *in vitro* aggregation behaviour of CESAs 1 and 3 when extracts come from plants lacking CESA6 (*prc1*) but have no influence when extracts come from wild-type plants containing CESA6.

In extracts of light-grown *prc1* seedlings, CESA3 and CESA1 occur in a 420 kDa complex and co-precipitate in pull-down experiments. Although *CESA6* is widely expressed, aerial parts of *prc1* show no obvious phenotype when grown in the light and only a relatively mild root phenotype (Fagard *et al.*, 2000) suggesting that they make considerable primary wall cellulose without CESA6. Genetic evidence suggests that CESAs 2, 5, and 9 partially replace CESA6 (Persson *et al.*, 2007; Desprez *et al.*, 2007). A 420 kDa complex could accommodate one of those CESAs in addition to CESAs 1 and 3, but current data do not indicate whether it does. Whatever the exact composition of the 420 kDa complex, CESAs 1 and 3 are not part of an 840 kDa complex that is stable in detergent extracts when CESA6 is absent.

Dark-grown *prc1* seedlings differ from light-grown seedlings in having a strong hypocotyl phenotype. No 840 kDa or 420 kDa complex is found in detergent extracts from dark-grown plants and, consistent with this, CESA1 and CESA3 precipitate independently. However, although drastic compared with the phenotype in light-grown seedlings, dark-grown seedlings also synthesize some cellulose (Fagard *et al.*, 2000) probably using CESAs 2, 5, and 9 instead of CESA6 (Desprez *et al.*, 2007; Persson *et al.*, 2007). If we hypothesize that cellulose synthesis *in vivo* requires an 840 kDa CESA complex containing three different CESA isoforms, then the most reasonable reconciliation between the *in vitro* behaviour seen in this study and the *in vivo* evidence from phenotype and genetics comes from proposing that the

primary wall *prc1* CESA complex or complexes are more stable *in vivo* than they are *in vitro*. This enables the complexes to support some cellulose synthesis *in vivo* although they disassemble when brought into detergent extracts, either to a 420 kDa complex when derived from light-grown seedlings or seemingly completely when derived from dark-grown seedlings. The nature of the light-related differences in the stability of complexes *in vitro* remains to be determined.

CESA complexes in other mutants

There were no dramatic changes in either CESA3 mutants examined. Both substitutions inhibited cellulose production without leading to loss of the CESA complex. Presumably both substitutions impair CESA3 function in the complex without causing its loss. Inhibition of synthesis occurs even though neither substitution is in regions of CESA3 conventionally considered important for catalysis, *rsw5* being mutated in the short sequence between the eighth transmembrane domain and the protein's C-terminus (Wang *et al.*, 2006) and *eli1* being mutated close to the cytoplasmic end of the second transmembrane domain (Caño-Delgado *et al.*, 2003).

None of the other mutants tested (*rsw2*, *rsw3*, *rsw10*, *rsw13*) showed changed CESA complexes. The KOR endocellulase mutated in *rsw2* is probably quite directly involved in glucan processing during cellulose synthesis, but is not part of the CESA complex (Desprez *et al.*, 2007). The two processing enzymes glucosidase I (*rsw13*) and glucosidase II (*rsw3*) are probably ER-localized but could affect CESAs through aberrant N-glycosylation. If they do, however, it does not detectably change the CESA complex. Ribose 5-phosphate isomerase (*rsw10*) is involved in substrate generation at some distance from the steps involving CESAs.

Possible relationship of the 840 kDa complex to rosettes and the production of readily extracted glucan

There is no direct evidence of what happens to rosette terminal complexes at tissue homogenization or when the microsomal fraction is treated with Triton but we suggest that the 840 kDa complex might derive from one of the six subunits that electron microscopy resolves in the rosette. The number of glucan chains (approximately 36) in each microfibril suggests that those six particles may each in turn contain six CESA glycosyltransferases. The 840 kDa complex that is stable through homogenization and Triton extraction plausibly contains six CESA subunits and on this hypothesis could derive from one of the six rosette particles seen by electron microscopy in the plasma membrane. Loss of the CESA complex in mutants would inevitably dismantle rosettes as is known to occur in *rsw1* (Arioli *et al.*, 1998). The converse argument, that retention of an 840 kDa complex necessarily means

retention of rosettes, does not necessarily follow. If the six particles normally clustered to form the rosette were no longer associated with each other but each remained in the plasma membrane (or even in the endomembrane system), no rosettes would be seen by electron microscopy but the 840 kDa complex might still persist *in vivo* in the separated particles and so be detectable in Triton extracts.

rsw1 at the restrictive temperature produces a readily extracted glucan which contains short chains of lipid-linked glucan that extract with ammonium oxalate and alkali (Arioli *et al.*, 1998; Peng, 1999; CH Hocart and RE Williamson, unpublished results). Glucan production does not seem related to how stable the 840 kDa complex is *in vitro* since dark-grown *prc1* does not produce glucan (Fagard *et al.*, 2000) even though it lacks a stable 840 kDa complex but *rsw2* (Lane *et al.*, 2001; Sato *et al.*, 2001) and *rsw3* (Burn *et al.*, 2002b) do accumulate the glucan even though they have stable 840 kDa CESA complexes.

In conclusion, this study demonstrates that detergent extracts contain an 840 kDa complex that is potentially big enough to accommodate six CESA monomers drawn from CESA1, CESA3, and CESA6. The complex may plausibly represent one of the six rosette particles seen by electron microscopy if the hypothesis that each rosette needs 36 glycosyltransferases is correct. Some, but not all, cellulose-deficient mutants lack 840 kDa complexes that are stable in detergent extracts. Further analysis of the complex should show the stoichiometry of its CESA components, whether it contains proteins other than CESAs and how well its *in vitro* behaviour reflects its *in vivo* behaviour.

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