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Sucrose Synthase is an Integral Component of the Cellulose Synthesis Machinery

Satoshi Fujii[1,*], Takahisa Hayashi[2] and Koichi Mizuno[1]

[1]Department of Biological Sciences, Graduate School of Science, Osaka University, Osaka, 560-0043 Japan
[2]Research Institute for Sustainable Humanosphere and Institute of Sustainability Science, Kyoto University, Gokasho, Uji, Kyoto, 611-0011 Japan
*Corresponding author: E-mail, fujii@bio.sci.osaka-u.ac.jp; Fax, +81-6-6850-6765
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Regular Paper

Cellulose synthesis in plants is believed to be carried out by the plasma membrane-associated rosette structure which can be observed by electron microscopy. Despite decade-long speculation, it had not been demonstrated whether the rosette is the site of catalytic activity of cellulose synthesis. To determine the relationship between this structure and cellulose synthesis, we successfully isolated detergent-insoluble rosettes from the plasma membrane of bean epicotyls. However, the purified rosettes did not possess cellulose synthesis activity in vitro. Conversely, detergent-soluble granular particles of ~9.5–10 nm diameter were also isolated and exhibited UDP-glucose binding activity and possessed β-1,4-glucan (cellulose) synthesis activity in vitro. The particle, referred to as the catalytic unit of cellulose synthesis, was enriched with a 78 kDa polypeptide which was verified as sucrose synthase like by mass spectrometry and immunoblotting. The catalytic units were able to bind to the rosettes and retained the cellulose synthesis activity in the presence of UDP-glucose or sucrose plus UDP when supplemented with magnesium. The incorporation of the catalytic unit into the rosette structure was confirmed by immunogold labeling with anti-sucrose synthase antibodies under an electron microscope. Our results suggest that the plasma membrane-associated rosette anchors the catalytic unit of cellulose synthesis to form the functional cellulose synthesis machinery.

Keywords: Azuki bean (*Vigna angularis*) • Catalytic unit • Cellulose synthesis • Rosette • Sucrose synthase.

Abbreviations: CBH, cellobiohydrolase; CESA, cellulose synthase A; CHAPS, 3-[(3-cholamidopropyl) dimethylammonio] propanesulfonic acid; DMP, dimethyl pimelimidate; DTT, dithiothreitol; EGTA, ethylene glycol-bis(2-aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; MALDI-TOF-MS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; *p*ABSF, 4-(2-aminoethyl)-benzenesulfonyl fluoride hydrochloride; PEG, polyethylene glycol; PIPES, piperazine-*N*, *N*′-bis (2-ethanesulfonic acid); TFA, trifluoroacetic acid.

Introduction

Organized biosynthesis and deposition of cellulose microfibrils are essential for anisotropic expansion of plant cells. The cellulose synthesis machinery was initially correlated to rosette-like structures associated with the plasma membrane in cells of both the green alga *Micrasterias denticulata* and higher plants (Kiermayer and Sleytr 1979, Giddings et al. 1980, Mueller and Brown 1980). Because of the repeated appearance of rosettes on the plasma membranes of cellulose-synthesizing organisms, there is little if any doubt about their function in cellulose biosynthesis. It has been hypothesized that the hexagonal rosette is assembled from 36 catalytic subunits for the synthesis of 36 β-1,4-glucan chains, to form a cellulose microfibril (Doblin et al. 2002). However, to date there is no direct experimental evidence demonstrating cellulose synthesis by the rosette.

In studies with a different approach, plant genes homologous to bacterial glucosyltransferase were first identified in a cDNA library prepared from developing cotton fibrils carrying out vigorous cellulose synthesis (Pear et al. 1996). Such plant genes, collectively called cellulose synthase A (*CESA*), are in a large gene family found in various plants (Richmond and Somerville 2000). Direct evidence showing the role of CESA in cellulose biosynthesis comes from a genetic analysis of the *Arabidopsis thaliana* mutant *rsw1*/*cesA1* (Arioli et al. 1998). In this mutant, the irregular appearance of plasma membrane-associated rosettes is concomitant with a reduction in crystallized cellulose and accumulation of non-crystallized β-1,4-glucan. To date, numerous mutations in *CESA* genes have been isolated and exhibit various degrees of defects in cellulose synthesis and cellulose microfibril deposition in the cell wall, as reviewed by Somerville (2006). Biochemical experiments have also demonstrated that, in cotton fibrils, membranes harboring CESA1 synthesize sitosterol-cellodextrins using sitosterol-β-glucoside as a primer (Peng et al. 2002). Using antibodies prepared against CESA proteins, immunogold labeling showed that CESA appeared near the rosette structures (Kimura et al. 1999). Thus, it is believed now that CESA polypeptides form

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large complexes, in various developmentally important combinations, to fulfill their roles in producing cellulose microfibrils with the guidance of cortical microtubules during cell growth (Mutwil et al. 2008).

Besides CESA polypeptides, several lines of evidence support the proposition that the cellulose synthesis complex contains additional components. An earlier attempt to isolate the cellulose synthase complex resulted in a prediction that the complex might contain at least 15 polypeptides, suggesting that proteins other than CESA are required for cellulose biosynthesis and deposition (Kudlicka and Brown 1997, Saxena and Brown 2005). Recent studies suggest that the endo-1,4-β-glucanase-like protein, KOR1, is present in various endomembranes and the developing cell plate, and is required for cellulose deposition and crystallinity in the cell wall (Zuo et al. 2000, Robert et al. 2005, Takahashi et al. 2009). Unfortunately, to date the identities of most non-CESA proteins in the complex are unknown.

One of the requirements for robust cellulose synthesis is the supply of its substrate UDP-glucose. Plasma membrane-associated sucrose synthase fulfills this function by catalyzing the formation of UDP-glucose from sucrose (Haigler et al. 2001). Although no direct interaction has been found between sucrose synthase and CESA, at the cell cortex sucrose synthase exhibits a localization pattern similar to that observed for CESA (Amor et al. 1995, Paredez et al. 2006). It was unclear whether sucrose synthase is part of the cellulose synthesis complex or the rosette.

To understand how the rosette functions in cellulose biosynthesis, the ultimate goal is to purify the complex to homogeneity. Technically, it is challenging to solubilize this gigantic structure from the plasma membrane. Using epicotyls of the Azuki bean *Vigna angularis*, we have purified protein fractions from the plasma membrane with the aim of maintaining cellulose synthesis activities. A detergent-soluble fraction contained granular particles and was able to carry out β-1,4-glucan synthesis in vitro. Conversely, a detergent-insoluble preparation of the plasma membrane exhibited rosette-like structures by electron microscopy but did not possess cellulose synthesis activity in vitro. We suggest the granular particles represent the catalytic unit of the cellulose synthesis machinery, and the detergent-insoluble, rosette-like structures form the scaffold or structural unit. The assembly of this catalytic subunit with the scaffold allows the formation of the functional cellulose synthesis complex, with the rosette appearance, on the plasma membrane.

Results

Identification of the soluble catalytic unit of cellulose biosynthesis

We used epicotyls of Azuki beans to obtain plasma membrane-associated proteins bearing cellulose synthesis activities. We aimed to obtain fractions containing proteins exhibiting in vitro UDP-glucose binding activities. NP-40-extracted proteins were separated by a linear sucrose density gradient of 17–7.5% (w/w) following DEAE–Sephacel column chromatography (see Materials and Methods). Protein fractions 7–11 contained a prominent band of molecular mass ~78 kDa (**Fig. 1A**), together with a few less prominent bands (**Fig. 1B**). This polypeptide was detected by photoaffinity labeling with [^{32}P]UDP-glucose and this result indicated that the 78 kDa polypeptide bound UDP-glucose in vitro.

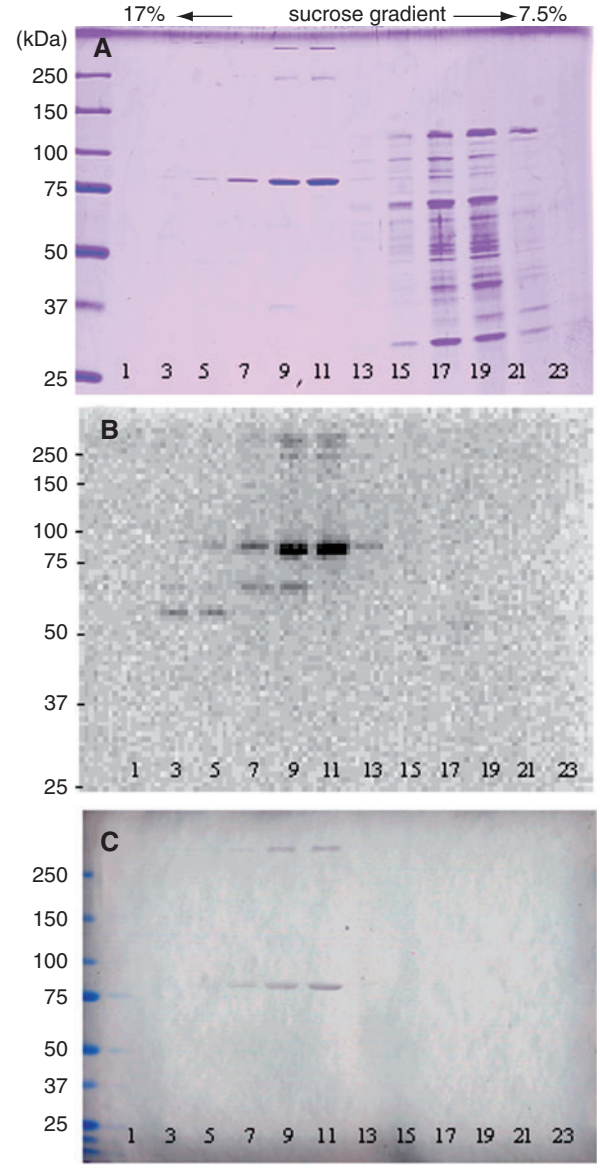

Fig. 1 Isolation and identification of the catalytic units. (A) Proteins from each fraction separated by sucrose gradient on an SDS–polyacrylamide gel as revealed by Coomassie Brilliant Blue staining. A major band at 78 kDa was detected by both photoaffinity labeling with [^{32}P]UDP-glucose (B) and immune blotting using antibodies raised against mung bean sucrose synthase (C) in fractions 7–11. Molecular weight markers are shown on the left. Fraction numbers are shown on the bottom of the gel corresponding to the sucrose gradient shown at the top.

We used preparations from these fractions to analyze further whether they possess cellulose biosynthesis activity. Under the electron microscope, abundant granules with a diameter of 9.5–10 nm were observed using these fractions (**Fig. 2A**). To determine whether these granules were able to carry out β-1,4-glucan synthesis in vitro, they were incubated with UDP-glucose in the presence of magnesium. Fibrils were detected near the granules by electron microscopy, and some of them appeared to terminate at the granules (**Fig. 2B**). When other fractions were subject to identical treatments, no fibril-like structures were observed (data not shown). To test whether these fibrils were made of β-1,4-glucans, the preparations were treated with β-1,4-glucanase (*Trichoderma viride*; Wako, Osaka, Japan), which resulted in their disappearance (data not shown). These results suggested that the fibrils were probably cellulose, and proteins in these fractions were carrying out cellulose biosynthesis activities. We propose that these soluble granules represent the catalytic unit of cellulose biosynthesis.

Identification of sucrose synthase as a prominent component of the catalytic unit

To reveal the identity of this 78 kDa polypeptide, peptides were analyzed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) and compared with existing proteins in GenBank. Multiple peptides matched those derived from sucrose synthase polypeptides in mung bean and soybean. This result was further confirmed by the amino acid sequence of sucrose synthase in Azuki bean deduced from its cDNA sequence (**Fig.e 3A)** (accession No. AB495095), which is 99% identical to the mung bean ortholog. Indeed, the 78 kDa band cross-reacted with antibodies raised against mung bean sucrose synthase (**Fig. 1C**).

To test further whether sucrose synthase is an integral component of the catalytic unit of cellulose biosynthesis, proteins were purified by immunoprecipitation using anti-sucrose synthase antibodies from the fractions of sucrose gradient centrifugation. The 78 kDa band was enriched in the precipitate (**Fig. 3B**). As seen in earlier fractions, similar granules were detected by electron microscopy, suggesting that these granules were enriched with sucrose synthase (**Fig. 3C**). Again, these granules were able to catalyze the formation of fibrils when UDP-glucose was supplied (**Fig. 3C**). To assess quantitatively the quality of the fibrils synthesized in vitro, fibrils formed with UDP-[^{14}C]glucose were treated with β-1,4- or β-1,3-glucanase and the mixture was passed through an Ultracel YM-3 membrane. The filtration would retain polysaccharide chains of >18 glucan subunits. After digestion with β-1,4-glucanase, but not β-1,3-glucanase, a significant drop was detected for polysaccharides with labeled substrates incorporated (**Fig. 3D**). When UDP-[^{14}C]glucose was replaced by [^{14}C]sucrose plus UDP, robust incorporation of glucan subunits was detected (**Fig. 3E**). Collectively, these results suggest that sucrose synthase is an integral component of the catalytic unit of cellulose biosynthesis as granular particles revealed here.

Isolation of detergent-insoluble rosette-like scaffold of the structural unit

When the plasma membrane preparation of epicotyls of Azuki beans was subject to aqueous two-phase partitioning, a detergent-insoluble fraction was obtained after treatment with NP-40 and separation by low speed centrifugation. This detergent-insoluble fraction contained structures resembling rosettes when examined by electron microscopy (**Fig. 4A**). They were assembled from ring-like subunits, and many of them formed hexagonal structures of 30–40 nm in diameter with six such subunits (**Fig. 4B**). These structures resembled the rosettes found on the plasma membrane observed by the freeze-fracture technique (Giddings et al. 1980, Mueller and Brown 1980). When they were tested for cellulose biosynthesis as described above, no β-1,4-glucan synthesis activity was detected in vitro (data not shown).

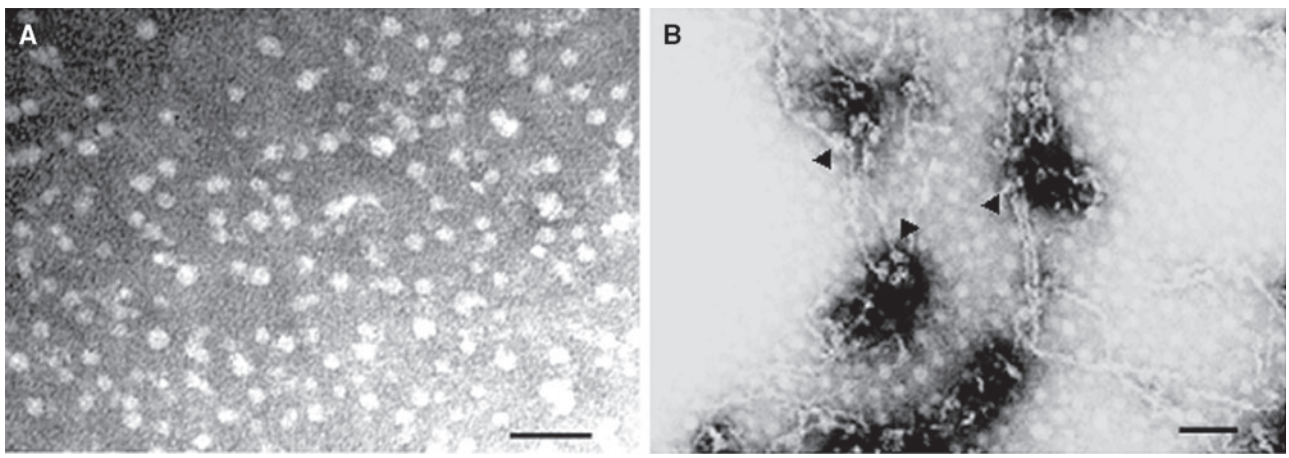

Fig. 2 Catalytic units before and after incubation with UDP-glucose. (A) Granules of 10 nm in diameter were detected in fractions 9–11. (B) Fibrils were associated with 10 nm granules (arrowheads) after incubation with UDP-glucose. Bars = 50 nm.

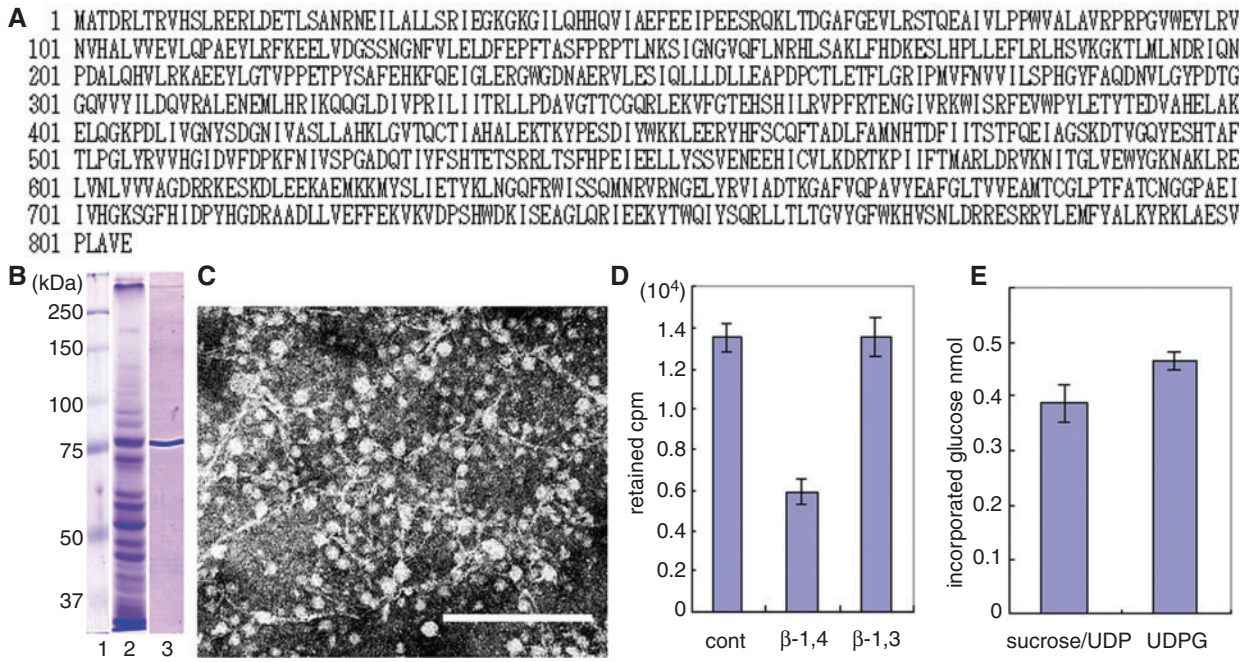

Fig. 3 Identification of sucrose synthase as the major component in the catalytic units. (A) The deduced amino acid sequence of sucrose synthase in Azuki bean. (B) Plasma membrane protein eluted with 0.1 M NaCl from a DEAE–Sephacel column (lane 2) and then precipitated by anti-sucrose synthase antibodies (lane 3) using SDS–PAGE analysis. Molecular weight markers are shown in lane 1. (C) Cellulose synthesis by immunoprecipitate preparation after incubation with UDP-glucose. Bar = 100 nm. (D) Ultracel YM-3 membrane-retained products in (C) after treatment with β-1,4 or β-1,3-glucanase. (E) Membrane-retained cellulose using either UDP-[^{14}C]glucose (UDPG) or [^{14}C]sucrose plus UDP (sucrose/UDP) as substrates determined by incorporated glucose.

Reconstitution of functional rosettes for cellulose biosynthesis

Because these hexagonal structures were almost morphologically identical to rosettes, we tested whether they interacted with granular particles of the catalytic unit. After incubation with the preparation containing the granular catalytic unit in the presence of magnesium, the detergent-insoluble, rosette-like structures were again isolated by centrifugation. We detected sucrose synthase by immunoblotting in the insoluble fraction, indicating that the soluble catalytic unit was probably associated with the insoluble rosette-like structures (**Fig. 5A**). To test further whether they indeed interacted in vitro, immuno-gold labeling was carried out using the sucrose synthase antibodies. Indeed, gold particles were detected in the rosette-like structures (**Fig. 5B**). Such labeling was not observed when detergent-insoluble rosettes alone were used in the control experiment (**Fig. 5C**). We have detected multiple labeling of sucrose synthase in one rosette-like structure, suggesting that multiple catalytic units were associated with each rosette-like structure (**Fig. 5B**).

To test whether the reconstituted preparations were functional in cellulose biosynthesis, similar experiments were carried out as described above. When associated with the catalytic units, the rosette-like structures were able to synthesize fibrils robustly (**Fig. 5D**). To verify that they were cellulose, cellobiohydrolase I (CBH I)–gold was also used for specific detection. The fibrils synthesized by these reconstituted rosettes were decorated by CBH I–gold as observed by electron microscopy (**Fig. 5E**).

Taken together, we suggest that the detergent-insoluble structures lacking the catalytic activity represent the scaffold or the structural unit for the cellulose biosynthesis machinery. The association of the catalytic unit with this scaffold allows the formation of rosettes on the plasma membrane which are functional in cellulose biosynthesis.

Discussion

Our results show that the plasma membrane-associated rosettes consist of the detergent-insoluble scaffold or structural unit plus soluble granules of the catalytic unit. We also report that sucrose synthase is a major integral component of the catalytic unit. This report also suggests that a single catalytic unit is competent for cellulose biosynthesis. The association of the catalytic unit with the scaffold allows cellulose to be synthesized by the reconstituted rosettes.

In cells undergoing rapid elongation, such as those of bamboo shoots, sucrose synthase constitutes up to ~1% of the total soluble proteins (Su et al. 1977). The function of sucrose synthase in cellulose biosynthesis has long been expected, based on both biochemical and cell biological evidence. Membrane-associated forms of sucrose synthase have been detected in the plasma membrane and implicated

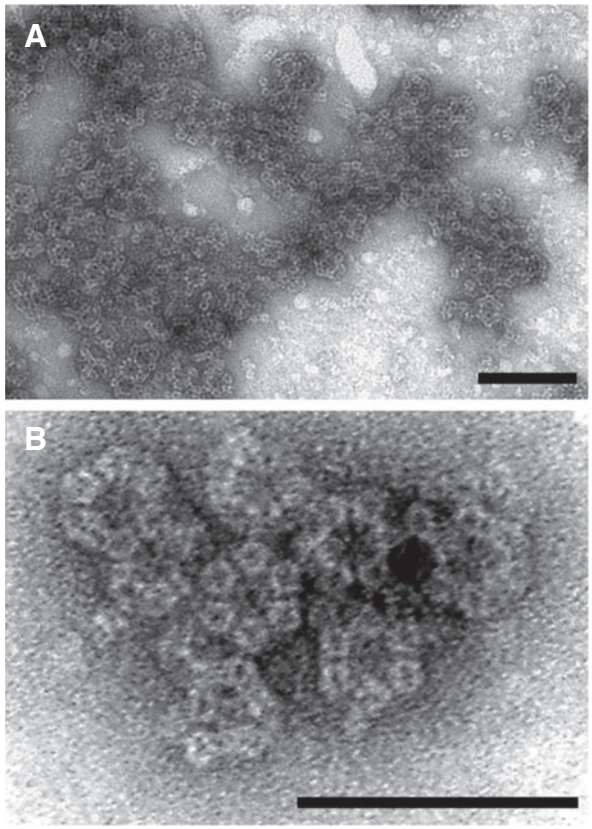

Fig. 4 Isolation of the structural unit/scaffold of cellulose synthesis. (A) Isolated structural units of the rosette by negative staining. Aggregates of hexagonal structures are observed. (B) Enlarged view of the hexagonal structural unit resembling the rosette. Bars = 100 nm.

in both cellulose and callose biosynthesis (Amor et al. 1995). Another line of evidence indicates that sucrose synthase is highly enriched near the plasma membrane in tracheary elements undergoing vigorous secondary cell wall thickenings (Salnikov et al. 2001, Salnikov et al. 2003). In addition, cells expressing plant-specific sucrose synthase exhibit enhanced cellulose production in the bacterium *Gluconacetobacter/Acetobacter xylinum* (Nakai et al. 1999). Conversely, down-regulation of sucrose synthase expression by antisense mRNA causes a reduction of both cellulose and starch biosynthesis in transgenic carrot plants (Tang and Sturm 1999). Similarly, initiation and elongation of cotton fiber cells were inhibited when sucrose synthase expression was repressed (Ruan et al. 2003). However, an *A. thaliana* quadruple mutant of *sucrose synthase* genes, in which sucrose synthase activity is down-regulated in soluble protein, shows no difference in cellulose content in stems when compared with wild-type *A. thaliana* plants (Barratt et al. 2009). In this mutant, sucrose synthase activity in centrifuged pellets showed almost the same activity as that in the wild type. Other sucrose synthases probably account for the normal activity in the mutant. Because rosettes are most probably present in the insoluble fraction, this activity may explain normal cellulose synthesis activity by efficiently supplying substrates.

In the present report, the cellulose synthesis catalytic activities are estimated to be 3.1 or 2.6 nmol glucose min^{-1} mg^{-1}, using UDP-glucose or sucrose plus UDP as the respective substrate. Using cotton fiber membranes, cellulose synthesis catalytic activities were calculated to be ~4.05 nmol glucose min^{-1} mg^{-1} from UDP-glucose and ~69.3 nmol glucose min^{-1} mg^{-1} from

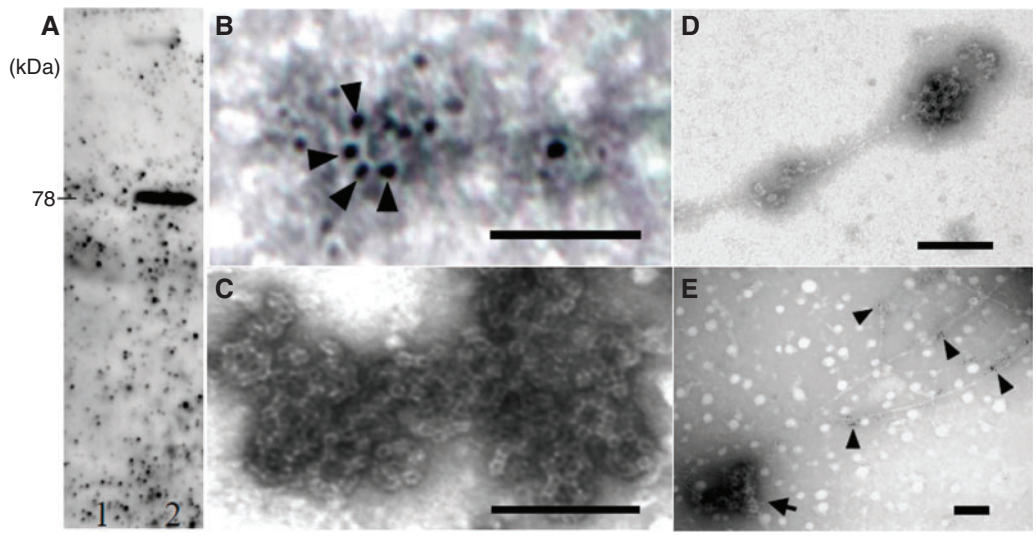

Fig. 5 Reconstitution of rosettes functional in cellulose biosynthesis. (A) Association of the catalytic unit with the structural unit. Analysis of the precipitated structural unit with buffer (lane 1) or the catalytic unit (lane 2) after immunoblotting with anti-sucrose synthase. (B) Immunogold labeling of sucrose synthase on reconstituted rosettes. Arrowheads point to the gold particles associated with rosettes. (C) Control experiment shows that sucrose synthase does not appear on the structural unit prior to incubation with the catalytic unit. (D) Fibrils synthesized by the reconstituted rosettes. (E) Arrow indicates a cluster of functional rosettes. Fibrils synthesized by reconstituted rosettes are detected by CBH I–gold (arrowheads). Bars = 100 nm.

sucrose (Amor et al. 1995). Compared with these activities in crude plasma membrane proteins, the rates in our study are relatively low. This may be due to differences in the source material or the loss of some unknown factors during the purification.

In this study, sucrose synthase was detected as a 78 kDa band. However, the cDNA sequence (accession No. AB495095) predicts a 91 kDa polypeptide for the sucrose synthase protein in Azuki bean. There are two possibilities for this discrepancy. The native sucrose synthase in this plant might migrate at 78 kDa on SDS–PAGE. It is also possible that proteolysis might have caused a cleavage of the polypeptide before loading.

We have also detected binding of UDP-glucose to a band of >700 kDa, which was detected by sucrose synthase antibodies in immunoblotting (**Fig. 2**). By MALDI-TOF-MS analysis, this band included sucrose synthase, suggesting that it contained an SDS-resistant aggregate of sucrose synthase.

We had anticipated that our purification would include CESA polypeptide(s). However, photoaffinity labeling with UDP-glucose did not detect polypeptides near 100 kDa, which is close to the typical molecular mass of CESA proteins. However, this result does not rule out the possibility that our preparation contains CESA. Unfortunately, antibodies against Azuki bean CESA were not available to us. However, CESA proteins have been immunoprecipitated from cell extracts in *A. thaliana* (Taylor et al. 2003). It was unclear whether sucrose synthase co-precipitated with CESA.

While the catalytic unit was initially purified from the plasma membrane fraction, it was also present in the cytosolic fraction (data not shown). Previous reports suggested that the cytoplasmic domain of the cellulose synthase complex bears the catalytic activity (Delmer 1999, Doblin et al. 2002, Saxena and Brown 2005, Somerville 2006, Bowling and Brown 2008). Our results suggest that the catalytic unit facing the cytosol interacts directly with the structural unit or scaffold. This interaction could be destroyed by detergent-based protein isolation. Whether such a model is common among all cellulose synthase complexes remains to be rigorously tested.

Our results support the proposition that sucrose synthase is part of the catalytic unit for cellulose synthesis, but the catalytic unit probably contains other proteins as well. As suggested by a published report, the functional cellulose synthase complex, as an intact rosette seen on the plasma membrane, is expected to contain >15 different polypeptides (Kudlicka and Brown 1997). Most models suggest that CESA polypeptides themselves jointly form the hexagonal rosette structure. It is possible that some structural proteins may oligomerize into the hexagonal scaffold so that the catalytic unit can be docked onto it.

Materials and Methods

Plant material

Azuki bean (*V. angularis*) seedlings were grown for 7 d at 26°C under continuous white light (FL20SS EX-D/18-H, Toshiba, Japan) at 90 μmol photons m^{-2} s^{-1}. Segments of the elongating part of epicotyls of light-grown seedlings were used for preparation of plasma membrane.

Preparation of the catalytic and the structural units of cellulose synthase

Plasma membrane was isolated and purified according to the method of aqueous two-phase partitioning, as described previously (Yoshida et al. 1983). All procedures were performed at 0–4°C. A 100 g aliquot of epicotyl segments was homogenized in 200 ml of homogenizing buffer of 50 mM PIPES [piperazine-*N*,*N*-bis (2-ethanesulfonic acid)]–KOH (pH 7.6) containing 300 mM sorbitol, 5 mM ethylene glycol-bis(2-aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), 5 mM EDTA, 50 μg ml^{-1} leupeptin, 10 μg ml^{-1} 4-(2-aminoethyl)-benzenesulfonyl fluoride hydrochloride (*p*-ABSF) and 100 μg ml^{-1} dithiothreitol (DTT) with an ultra-turrax homogenizer T25 (Janke and Kunkel type, Ika, Staufen, Germany). Homogenate was squeezed through four layers of gauze and the crude extract was centrifuged at 1,800×*g* for 10 min. The resulting supernatant was centrifuged at 100,000×*g* for 10 min to collect the microsomes. The microsomal pellet was suspended in partitioning mixture that contained 5.6% (w/w) dextran (cat. # 31392; Fluka, New York, USA), 5.6% (w/w) polyethylene glycol (PEG) (cat. # P3640; Sigma, St Louis, MO, USA) and 0.176% (w/w) NaCl. After mixing, the mixture was centrifuged at 1,800×*g* for 10 min. The resulting upper phase (PEG layer) was suspended in washing buffer of 50 mM PIPES–KOH (pH 7.6) containing 250 mM sorbitol, 10 μg ml^{-1} leupeptin, 10 μg ml^{-1} *p*-ABSF, 50 μg ml^{-1} DTT. The plasma membrane was collected by centrifugation at 100,000×*g* for 10 min. The plasma membrane precipitate was suspended in 10 ml of lysis buffer of 50 mM PIPES–KOH (pH 7.6) with 5% (v/v) NP-40, 50 μg ml^{-1} leupeptin, 10 μg ml^{-1} *p*-ABSF, 100 μg ml^{-1} DTT, and the mixture was centrifuged at 100,000×*g* for 10 min. The resulting supernatant was used as a source of the catalytic units of cellulose synthesis.

Structural units were extracted from the precipitate of the centrifugation. The precipitate was suspended in lysis buffer and centrifuged at 10,000×*g* for 10 min to precipitate the structural units. The precipitate was washed again, after which the pellet was suspended in column buffer and subjected to a differential sucrose density partitioning with 50% (w/w) and 10% (w/w) sucrose. After centrifugation at 1,800×*g* for 15 min, the 10% sucrose layer was obtained and centrifuged at 10,000×*g* for 5 min. The precipitate was suspended with column buffer and used as the structural units.

Catalytic units were extracted from the supernatant of the centrifugation at 100,000×*g* for 10 min after lysis of the plasma membrane. Supernatant was applied to a DEAE–Sephacel column. After thorough washes with column buffer of 50 mM PIPES–KOH (pH 7.6) with 0.2% (w/v) CHAPS {3-[(3-cholamidopropyl) dimethylammonio] propanesulfonic acid}, 50 μg ml^{-1} leupeptin, 10 μg ml^{-1} p-ABSF, 100 μg ml^{-1} DTT, proteins were eluted with the column buffer supplemented with 100 mM NaCl. Then the eluent was laid on the top of a linear sucrose gradient of 7.5–17% (w/v) and centrifuged at 100,000×*g*

for 12 h. Fractions of 1.2 ml were collected from the bottom of the tube.

Reconstitution was performed by mixing the catalytic units and structural units, rotated to mix at room temperature for 30 min in the presence of 1 mM magnesium. Free catalytic units were removed by centrifugation at 15,000×*g* for 10 min on the cushion of 10% (w/w) sucrose solution in the column buffer. The precipitate was used as the mixture of structural units and reconstituted rosettes.

UDP-glucose photoaffinity labeling

Synthesis of [α-^{32}P]UDP-glucose was carried out as published (Delmer et al. 1991). Proteins were incubated at 27°C for 5 min with 1 mM magnesium, 100 μM UDP-glucose including 0.5 μCi of [α-^{32}P]UDP-glucose. The sample was irradiated with short-wave UV (253.6 nm) from 2 cm above for 20 min on ice. Then samples were suspended in SDS sample buffer and subjected to SDS–PAGE. Gels were fixed and stained with Coomassie Brilliant Blue R-250, and destained gels were dried before being loaded on a Fuji imaging plate. Images of ^{32}P labeling were obtained by a BAS-2500 imaging analyzer (Fujifilm, Tokyo, Japan).

Cellulose synthesis assay

A 100 μM concentration of 0.2 μCi of [^{14}C]sucrose with 10 μM UDP or 100 μM of 0.1 μCi of UDP-[^{14}C]glucose were used for assaying cellulose synthesis. The reaction mixture contained these substrates and 5 μg of catalytic units of protein in a reaction buffer of 50 mM PIPES–KOH (pH 7.6) containing 10 μg ml^{-1} leupeptin, 10 μg ml^{-1} p-ABSF and 1 mM magnesium. An equal volume of 8% (w/v) KOH was added to terminate the reaction after incubation at 27°C for 30 min. The samples were filtered through Ultracel YM-3 membranes (Millipore, Bedford, MA, USA) to exclude the materials of <3 kDa and to retain the polysaccharides over approximately 18 glucan chains. The membrane was washed three times with water, and retained radioactivity was measured by a liquid scintillation counter.

To determine the glucan chain linkage, two units of β-1,4-glucanase (*T. viride*; Wako) or β-1,3-glucanase (yeast lytic type recombinant; Wako) were used. The product was incubated with either β-1,4-glucanase in 50 mM MES/KOH (pH 5.0) or β-1,3-glucanase in 50 mM Tris–HCl (pH 7.0) for 72 h at 40°C or room temperature, respectively. Then the reactions were filtered through Ultracel YM-3 membranes, and retained radioactivity was measured by a liquid scintillation counter.

Immunoblotting and immunoprecipitation

Proteins were separated by SDS–PAGE and transferred to a nitrocellulose filter. Samples were probed with polyclonal antibodies raised against recombinant protein of mung bean sucrose synthase (Nakai et al. 1997) followed by horseradish peroxidase-conjugated goat anti-rabbit IgG antibodies (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The detection was carried out using either 4-chloro-1-naphthol or a super signal femto trial kit (Thermo Scientific, Waltham, MA, USA) as the substrate.

For immunoprecipitation of sucrose synthase, anti-sucrose synthase antibodies were mixed with protein A–Sepharose beads (GE Healthcare UK Ltd., Buckinghamshire, UK) at room temperature for 30 min and antibody–protein A–Sepharose beads were prepared according to the manufacturer's instruction, followed by treatment with the cross-linker dimethyl pimelimidate (DMP). Proteins were mixed with beads at room temperature for 1 h and washed by brief centrifugation with column buffer. Proteins were eluted using 100 mM glycine–HCl buffer (pH 2.6) and separated from the beads by brief centrifugation, and the solution was immediately neutralized to pH to 7.6 with 2 M Tris.

MALDI-TOF/MS analysis

The 78 kDa band on the SDS–polyacrylamide gel was excised and washed three times each with 100 mM ammonium bicarbonate buffer, pH 8.0 and 100% acetonitrile. Gels were dehydrated with 100% acetonitrile followed by the addition of 3 μl of 3 μg μl^{-1} trypsin. After trypsin was absorbed by the gel at 0°C for >30 min, 10 μl of digestion buffer of 40 mM ammonium carbonate (pH 8.0) with 10% (v/v) acetonitrile were added and incubated overnight at 37°C. An aliquot of 1 μl of mixture was mixed with an equal volume of matrix solution containing 0.2 mg ml^{-1} α-cyano-4-hydroxycinnamic acid (CHCA), 0.1% (v/v) trifluoroacetic acid (TFA), 40% (v/v) acetonitrile. After mixing, 0.5 μl of mixed solution was put on a 600 nm Anchor Chip (Bruker Daltonics, Billerica, MA, USA) and dried. Then, it was washed with 0.1% (v/v) TFA and re-dried for analysis. The samples were analyzed with an Ultraflex TOF/TOF mass spectrometer (Bruker Daltonics) under reflector mode to collect peptide mass spectra. Mascot (Matrix Science, USA) in-house version software was employed to identify the spots from the Viridiplantae (Green Plants) database in a local Mascot server by PMF (peptide mass fingerprinting) analysis.

Electron microscopy

Samples were observed by negative staining with 1% (w/v) uranyl acetate. For immunogold labeling, anti-sucrose synthase antibodies were conjugated with colloidal gold according to Slot and Geuze (1981). The conjugates were applied on samples after a centrifugation at 5,000×*g* for 10 min to remove denatured IgG proteins and aggregated gold particles. Cellulose labeling was carried out using CBH I–gold complex prepared according to a published protocol (Chanzy et al. 1984). Reconstituted rosettes were incubated with 1 mM UDP-glucose in the presence of 1 mM magnesium for 15 min at room temperature prior to CBH I–gold incubation as reported (Okuda et al. 1993). Observation by transmission electron microscopy was achieved using JEOL 1200 EX (JEOL, Tokyo, Japan) at 80 kV for imaging.

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