Sucrose synthase activity in the *sus1/sus2/sus3/sus4 Arabidopsis* mutant is sufficient to support normal cellulose and starch production

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Sucrose synthase (SUS) catalyzes the reversible conversion of sucrose and a nucleoside diphosphate into the corresponding nucleoside diphosphate-glucose and fructose. In *Arabidopsis*, a multigene family encodes six SUS (SUS1-6) isoforms. The involvement of SUS in the synthesis of UDP-glucose and ADP-glucose linked to *Arabidopsis* cellulose and starch biosynthesis, respectively, has been questioned by Barratt et al. [(2009) *Proc Natl Acad Sci USA* 106:13124–13129], who showed that (*i*) SUS activity in wild type (WT) leaves is too low to account for normal rate of starch accumulation in *Arabidopsis*, and (*ii*) different organs of the *sus1/sus2/sus3/sus4* SUS mutant impaired in SUS activity accumulate WT levels of ADP-glucose, UDP-glucose, cellulose and starch. However, these authors assayed SUS activity under unfavorable pH conditions for the reaction. By using favorable pH conditions for assaying SUS activity, in this work we show that SUS activity in the cleavage direction is sufficient to support normal rate of starch accumulation in WT leaves. We also demonstrate that *sus1/sus2/sus3/sus4* leaves display WT *SUS5* and *SUS6* expression levels, whereas leaves of the *sus5/sus6* mutant display WT *SUS1–4* expression levels. Furthermore, we show that SUS activity in leaves and stems of the *sus1/sus2/sus3/sus4* and *sus5/sus6* plants is ∼85% of that of WT leaves, which can support normal cellulose and starch biosynthesis. The overall data disprove Barratt et al. (2009) claims, and are consistent with the possible involvement of SUS in cellulose and starch biosynthesis in *Arabidopsis*.

carbohydrate metabolism | sink strength

Cellulose, and to a much lesser extent starch, are the world's most abundant biosynthesized compounds, serving as major sinks for carbon in plants. Taking into account the very basic role these polymers play in modern societies, a thorough understanding of the mechanisms involved in their biosynthetic processes will be critically important for the rational design of new experimental traits aimed at improving yields in agriculture, and producing more and better polymers that fit industrial needs and social demands (1–4).

Sucrose synthase (SUS) catalyzes the following reversible reaction

$$\text{Sucrose} + \text{NDP} \leftrightarrow \text{NDP-glucose} + \text{fructose},$$

where *N* stands for uridine, adenosine, guanosine, cytidine, thymidine or inosine. Although UDP is generally considered to be the preferred nucleoside diphosphate for SUS, numerous studies have shown that ADP serves as an effective acceptor molecule to produce ADP-glucose (ADPG) (5–14). SUS is highly regulated both at transcriptional and posttranslational levels (15–21), and plays a predominant role in the entry of carbon into metabolism in nonphotosynthetic cells, and in determining both sink strength and phloem loading (22–25). Individual SUS isoforms are needed for normal development in some plant organs, including carrot roots, pea and maize seeds, tomato fruit and cotton fibers (24, 26–29). Although the presence of SUS at the plasma membrane plays a role in directing the carbon flow to cell wall biosynthesis (30–33) a major role commonly attributed to this enzyme in sink organs is to convert the imported sucrose into UDP-glucose (UDPG), which is then transformed to hexose-phosphates and ADPG necessary for starch biosynthesis. In addition, SUS has been suggested to be involved, at least in part, in the direct conversion of sucrose into ADPG linked to starch biosynthesis in both autotrophic and heterotrophic cells (12, 14, 34–39).

SUS isoforms in the many plant species examined to date are encoded by a small multigene family. Studies of the predicted amino acid sequences and gene structure have shown that the *Arabidopsis* SUS family consists of six *SUS* genes displaying different developmental expression patterns (40, 41). The involvement of SUS in starch and cellulose biosynthesis in *Arabidopsis* has been recently questioned by Bieniawska et al. (42) and Barratt et al. (43), who showed that (*i*) leaves, siliques, stems, and roots of the *sus1/sus2/sus3/sus4* quadruple SUS mutant impaired in SUS activity accumulate WT content of ADPG, UDPG, cellulose and starch, and (*ii*) SUS activity in WT leaves is too low to account for the rate of starch accumulation in illuminated leaves. However, these authors assayed SUS activity in the sucrose synthetic direction under conditions that are far from optimal, which inadvertently lead to their conclusion. Several assay conditions that departed from optimal were: First, the SUS reaction assay mixture used by the authors contained 6 mM fructose, a concentration comparable to or lower than the reported K_m values for fructose in SUS from many species (10, 11, 44). Second, the pH of the SUS reaction assay mixture used by Bieniawska et al. (42) and Barratt et al. (43) was 9.4, which is far too basic with respect to both cytosolic pH and to previously reported optimum pH values for SUS activity in the synthetic direction (10, 45–48). Third, SUS assay reactions were stopped after 20 min of incubation, a condition that does not allow the attainment of reliable data under SUS initial velocity conditions.

Considering these circumstances we decided to explore the optimum pH conditions for both *Arabidopsis* SUS activity and stability of molecules (particularly UDPG) involved in the SUS reaction. We then carried out kinetic analyses of recombinantly

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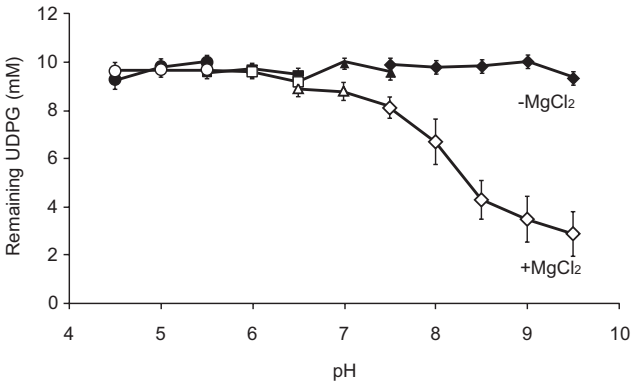

Fig. 1. UDPG is highly unstable at basic pH in the presence of $MgCl_2$. pH-stability curves of UDPG in the absence and presence of 3 mM $MgCl_2$. Ten mM UDPG was incubated at 25 °C for 5 min at different pH regimes, and the remaining UDPG was immediately analyzed by HPLC as described in *Materials and Methods*. The buffers used were 50 mM sodium acetate–acetic acid (pH 4.5–5.5, circles), 50 mM Mes/NaOH (pH 5.5–6.5, squares), 50 mM Hepes/NaOH (pH 6.5–7.5, triangles), and 50 mM Tris·HCl (pH 7.5–9.5, diamonds) (*Materials and Methods*). The results are the mean ± SD of three independent experiments.

produced SUS of *Arabidopsis* in the sucrose breakdown direction (UDPG and ADPG synthesis). Finally, we measured SUS activity in the sucrose cleavage direction in leaves of WT, *sus1*/*sus2*/*sus3*/*sus4* and *sus5*/*sus6* plants. We found that SUS activity in WT leaves is ~10-fold higher than that reported in ref. 42, which is sufficient to account for normal rates of starch accumulation during illumination. Most importantly, we found that SUS activity in the leaves and stems of *sus1*/*sus2*/*sus3*/*sus4* and *sus5*/*sus6* mutants was ~85% of that occurring in WT leaves. The overall data refute the claims of Barratt et al. (43) and are consistent with the possible involvement of SUS in the production of UDPG and ADPG linked to cellulose and leaf starch biosynthesis in *Arabidopsis*, respectively.

Results and Discussion

UDPG Is Highly Unstable at Basic pH in the Presence of $MgCl_2$. Using UDPG and fructose as substrates, Bieniawska et al. (42) and Barratt et al. (43, 44) measured SUS activity at pH 9.4 in the sucrose synthetic direction. The SUS reaction assay mixture used by these authors contained 3 mM $MgCl_2$. We must emphasize that under these conditions some nucleoside diphosphate-glucoses are highly unstable, being spontaneously converted into glucose-1,2-monophosphate and the corresponding nucleoside monophosphate. We investigated whether or not UDPG was unstable under the experimental conditions used in refs. 42–44. Toward this end, 10 mM UDPG was incubated at 25 °C for 5 min at different pH conditions in the presence or absence of 3 mM $MgCl_2$, and immediately subjected to HPLC analysis as indicated in *Materials and Methods*. Importantly, whereas UDPG was shown to be highly stable at any pH in the absence of $MgCl_2$, this nucleotide-sugar was highly unstable in the presence of $MgCl_2$ at pH values higher than 7.5 (Fig. 1). The overall data thus show that (*i*) SUS activity measurement analyses should not be carried out at basic pH when $MgCl_2$ is included in the SUS reaction assay mixture and (*ii*) SUS activity analyses in refs. 42–44 were carried out under conditions of limiting and undefined concentration of UDPG due to the high instability of this nucleotide sugar.

Production and Kinetic Characterization of Recombinant SUS. Using a modified version of the one-step continuous method for SUS assay of Huang et al. (49), Bieniawska et al. (42) and Barratt et al. (43, 44) concluded that *Arabidopsis* SUS has a maximum activity at pH 9.5 in the synthetic direction. Based on this conclusion, these authors measured SUS activity at pH 9.4 in different organs of *Arabidopsis* and pea, and in purified recombinant SUS preparations (42–44). We must emphasize, however, that the SUS assay system used in refs. 42–44 and 49 is based on coupling UDP produced by SUS to NADH oxidation by lactate dehydrogenase from *Leuconostoc mesenteroides*, which exhibits an optimum pH of 9.5 (50). It is thus highly conceivable that the determination of 9.5 as the SUS optimum pH was an artifact due to the inclusion in the assay mixture of a coupling enzyme whose optimum pH is 9.5. We thus produced and characterized recombinant SUS1 and SUS3, and carried out kinetic characterization studies in the sucrose breakdown direction (UDPG and ADPG synthesis) using the two-step assay method for SUS activity described in *Materials and Methods*. The reliability of the chromatographic methods of ADPG and UDPG identification and measurement was verified by adding known amounts of commercially available ADPG and UDPG to the SUS reaction assay mixture after the reaction was terminated. We also added purified adenosine diphosphate sugar pyrophosphatase (ASPP) (51) or uridine diphosphate sugar pyrophosphatase (USPP) (52) to the SUS reaction assay mixture after the reaction was completed. Fig. S1 shows that ASPP and USPP digestions totally removed substances that eluted at the position of pure, commercially available ADPG and UDPG, respectively, confirming the correct identification of the two nucleotide sugars. As shown in Fig. 2, the optimum pH of recombinant SUS1 and SUS3 activity was ~7. Furthermore, activities of the two SUS isoforms at pH 9.5 were ~10–15% of that observed at their optimum pH. Accordingly, kinetic characterization of SUS1 and SUS3 was undertaken at their optimum pH (pH 7). As summarized in Table 1, apparent K_m values of ~0.15 mM for ADP were approximately twofold lower than those of UDP in the presence of saturating sucrose, whereas apparent V_{max} with UDP was approximately twofold higher than with ADP. The kinetic studies of the sucrose cleavage reaction in the presence of saturating nucleoside diphosphate revealed a two- to threefold higher affinity for sucrose in the presence of saturating UDP than in the presence of saturating ADP (Table 1).

SUS Activity in WT Leaves Greatly Exceeds the Minimum Needed to Support Normal Rate of Starch Accumulation During Illumination. Previously reported values for starch accumulation rates in *Arabidopsis* leaves ranged between 30 and 110 nmol of glucose

Table 1. Kinetic parameters of SUS1 and SUS3

		SUS1		SUS3	
Variable substrate	Fixed substrate (mM)	K_m (mM)	V_{max} (U/mg protein)	K_m (mM)	V_{max} (U/mg protein)
ADP	Sucrose (500)	0.17 ± 0.01	290 ± 27	0.15 ± 0.01	470 ± 31
UDP	Sucrose (500)	0.39 ± 0.03	585 ± 48	0.25 ± 0.02	950 ± 78
Sucrose	ADP (5)	185 ± 25	290 ± 32	145 ± 16	470 ± 29
Sucrose	UDP (5)	53 ± 6.2	585 ± 53	48 ± 3.3	950 ± 74

Results are the mean ± SD of three independent experiments.

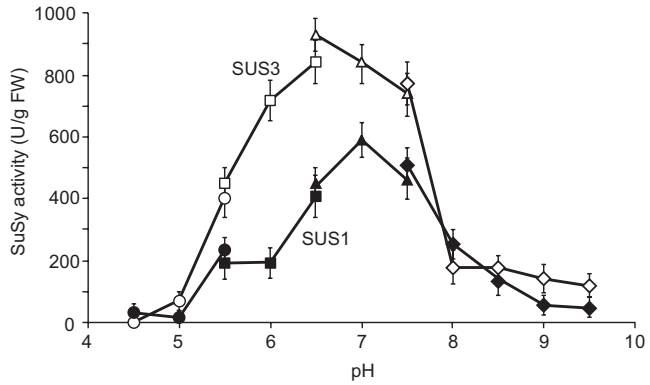

Fig. 2. Optimum pH of recombinant SUS1 and SUS3 is ~7. Optimum pH for the activity of recombinant SUS1 and -3 was determined using the two-step method for SUS activity assay described in *Materials and Methods*. The buffers used in the SUS reaction were those described in the legend of Fig. 1. Each buffer contained 200 mM sucrose, 2 mM UDP, and recombinant SUS. The results are the mean ± SD of three independent experiments.

transferred to starch min^{-1} g fresh $weight^{-1}$ (FW^{-1}) (53–55). Barrat et al. (43) thus concluded that SUS activity in the sucrose synthetic direction in leaves (23 nmol of sucrose produced from UDPG min^{-1} g FW^{-1}; ref. 42) is not enough to account for the rates of starch accumulation during illumination. In this work, we measured SUS activity in the sucrose cleavage direction (ADPG and UDPG synthesis) at pH 7 in crude extracts from leaves of WT plants cultured under a 16-h light/8-h dark regime, and at irradiance of 100 μmol photons sec^{-1} m^{-2} (*Materials and Methods*). Under these conditions, starch accumulates at a rate of ~42 ± 3.3 nmol of glucose transferred to starch min^{-1} g FW^{-1} (Fig. S2). Reactions were stopped at initial velocity conditions (after 3 min of incubation, see Fig. S3), and the resulting NDPG (ADPG or UDPG) was measured by HPLC. We checked the reliability of the chromatographic methods of UDPG and ADPG identification and measurement by adding purified USPP or ASPP and commercially available UDPG or ADPG to the SUS reaction assay mixture, respectively, after the reactions were stopped (Fig. S4). Using this two-step SUS assay method, we found that the optimum pH of SUS activity in leaf crude extracts was ~7 (Fig. 3). Furthermore, SUS activity in leaf crude extracts at pH 9.5 was ~10–15% of that measured at pH 7 (Fig. 3). Importantly, we

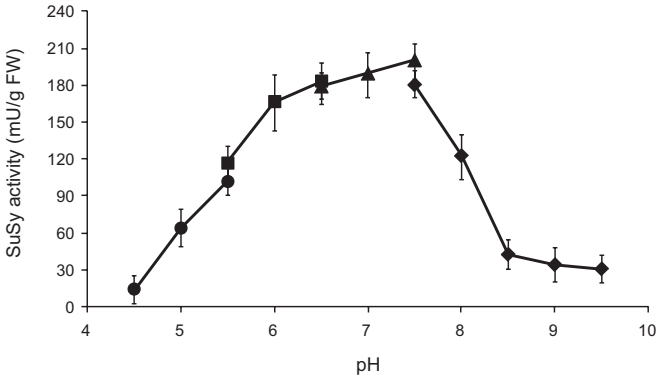

Fig. 3. Optimum pH of SUS in crude extracts of WT leaves is ~7. Optimum pH for the activity of SUS in crude extracts of WT leaves was determined using the two-step method for SUS activity assay described in *Materials and Methods*. The buffers used in the SUS reaction were those described in the legend of Fig. 1. Each buffer contained 200 mM sucrose, 2 mM UDP, and protein extract. The results are the mean ± SD of three independent experiments.

found that, when assayed at pH 7, total ADPG- and UDPG-producing SUS activity in leaves was 92.3 ± 10.5 mU g FW^{-1} and 182.5 ± 18.3 mU g FW^{-1}, respectively (Fig. 4). As illustrated in Fig. 4, most SUS activity was shown to be soluble. Therefore, we conclude that (*i*) Bieniawska et al. (42) largely underestimated SUS activity in WT *Arabidopsis* leaves, and (*ii*) SUS activity in WT *Arabidopsis* leaves is sufficient to support the rate of starch accumulation of 42 ± 3.3 nmol of glucose transferred to starch min^{-1} g FW^{-1} occurring during illumination (Fig. S2).

SUS Activity in *sus1/sus2/sus3/sus4* and *sus5/sus6* Mutants Is Sufficient to Support Normal Cellulose and Starch Biosynthesis. Barratt et al. (43) failed to detect any SUS activity in roots and stems of the quadruple *sus1/sus2/sus3/sus4* mutant when assayed at pH 9.5 in the sucrose synthetic direction. The same authors also showed that the *sus1/sus2/sus3/sus4* mutant and the double *sus5/sus6* mutant accumulate WT starch and cellulose content in different organs (also confirmed in our laboratory) and concluded that

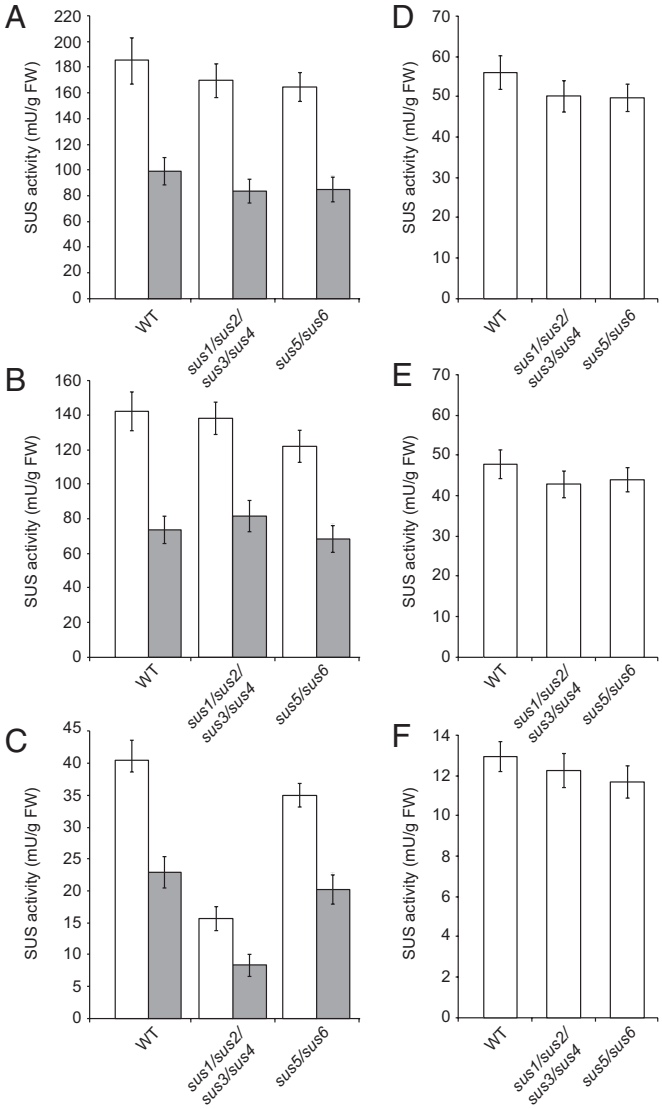

Fig. 4. UDPG- and ADPG-producing SUS activity (white and gray bars, respectively) in crude extracts (*A* and *D*), and 10,000 × *g* soluble (*B* and *E*) and pellet fractions (*C* and *F*) of leaves (*A*–*C*) and stems (*D*–*F*) of WT, *sus1/sus2/sus3/sus4* and *sus5/sus6* plants. The results are the mean ± SD of three independent experiments.

SUS is not required for cellulose and transitory starch biosynthesis (43). However, despite the fact that *SUS5* and *SUS6* expression represents ~50% of the total *SUS* expression in *Arabidopsis* leaves and stems (40, 41), these authors did not measure *SUS5* and *SUS6* expression in the leaves and stems of the quadruple *sus1/sus2/sus3/sus4* mutant. Furthermore, they did not measure SUS activity in the leaves of this mutant. We thus carried out real-time PCR analyses of *SUS5* and *SUS6*, and measured SUS activity in the sucrose cleavage direction at pH 7 in the leaves of the *sus1/sus2/sus3/sus4* mutant. We also carried out real-time PCR analyses of *SUS1–4,* and measured SUS activity in the leaves of the *sus5/sus6* mutant. These analyses revealed that *SUS5* and *SUS6* expression levels in *sus1/sus2/sus3/sus4* leaves, and *SUS1–4* expression levels in *sus5/sus6* leaves were comparable to those of WT leaves (Fig. 5). Most importantly, we found that total SUS activity in the leaves and stems of *sus1/sus2/sus3/sus4* and *sus5/sus6* mutants was ~85% of the WT, most of it being soluble (Fig. 4). We thus conclude that SUS activity in *sus1/sus2/sus3/sus4* and *sus5/sus6* mutants exceeds the minimum required to support normal cellulose and starch biosynthesis in stems, and for the rate of starch accumulation of ~42 nmol of glucose transferred to starch min^{-1} g FW^{-1} occurring in the leaves of these mutants during illumination (Fig. S2).

Using *SUS* antisensed potato tubers, Zrenner et al. (23) showed that a dramatic reduction in the amount of *SUS* mRNA resulted in only a moderate reduction of SUS activity. This finding indicates that (*i*) *SUS* transcription rate largely exceeds the translation capacity of the cell and/or (*ii*) posttranscriptional factors, such as translation efficiency and protein turnover and stability, may act as major determinants of SUS accumulation, a view that agrees with previous works showing that changes in transcript levels during environmental inputs are not accompanied by concomitant changes in the amount of their encoded proteins (56). Taking this into account, and considering that *SUS5* and *SUS6* expression represents ~50% of the total *SUS* expression in *Arabidopsis* leaves and stems (40, 41), it is not surprising that SUS activity in the *sus1/sus2/sus3/sus4* and *sus5/sus6* mutants is ~85% of the WT SUS activity.

Concluding Remarks. Data presented in this work refute the assertion made by Barratt et al. (43) that SUS is not a determinant factor in the biosynthesis of cellulose and starch in *Arabidopsis*. Their claim was primarily based on the low SUS activity in the synthetic direction observed in the *sus1/sus2/sus3/sus4* mutant, which appeared insufficient to account for the levels of starch and cellulose accumulated in different plant organs. Needless to say, further endeavors based on the production and characterization of *sus1/sus2/sus3/sus4/sus5/sus6* mutants will be necessary to confirm (or refute) the involvement of SUS in the sucrose–starch and sucrose–cellulose conversion processes in *Arabidopsis*.

There are several reasons explaining why the values of SUS activity reported in refs. 42 and 43 were a gross underestimation and differ greatly from those presented here. First, Bieniawska et al. (42) and Barratt et al. (43) measured SUS activity in the sucrose synthetic direction, whereas we have measured SUS activity in the sucrose breakdown (UDPG and ADPG synthesis) direction. Second, we measured SUS activity after 3 min incubation, whereas Bieniawska et al. (42) and Barratt et al. (43) measured SUS activity after 20 min of incubation, which is far from initial velocity conditions (Fig. S3). Third, the SUS reaction assay mixture used in refs. 42 and 43 contained 3 mM $MgCl_2$, and its pH was 9.0–9.5. Under these conditions, UDPG (the substrate for SUS reaction in the sucrose synthetic direction) is highly unstable (Fig. 1). Furthermore, the SUS reaction assay mixture used in refs. 42 and 43 contained 6 mM fructose, a concentration that is comparable or even lower than the reported K_m values for fructose in SUS from many species (10, 11, 44). Therefore, Bieniawska et al. (42) and Barratt et al. (43) measured SUS activity under conditions of substrate (fructose and UDPG) deficiency and/or instability. Fourth, we measured SUS activity under optimum pH conditions for the enzyme (Fig. 2), whereas Bieniawska et al. (42) and Barratt et al. (43) measured SUS activity at pH 9.4, which is far too basic with respect to previously reported SUS activity optimum pH values of 7.0–8.2 (10, 45–48).

Materials and Methods

Plants, Growth Conditions, and Sampling. The work was carried out using plants of *Arabidopsis thaliana* (cv. Columbia) and the homozygous *sus1/sus2/sus3/sus4* and *sus5/sus6* mutants (43) cultured in soil for 4 wk in growth chambers under a 16-h light (100 μmol photons sec^{-1} m^{-2}) 22 °C /8-h dark 18 °C regime. Homozygous *sus1/sus2/sus3/sus4* and *sus5/sus6* mutants were confirmed by PCR using the primers listed in Table S1 (see also Fig. S5). Harvested leaves and stems were immediately freeze-clamped and ground to a fine powder in liquid nitrogen with a pestle and mortar. To assay SUS activity (see below), 1 g of the frozen powder was resuspended at 4 °C in 5 mL of 100 mM Hepes (pH 7.5), 2 mM EDTA, and 5 mM DTT (extraction

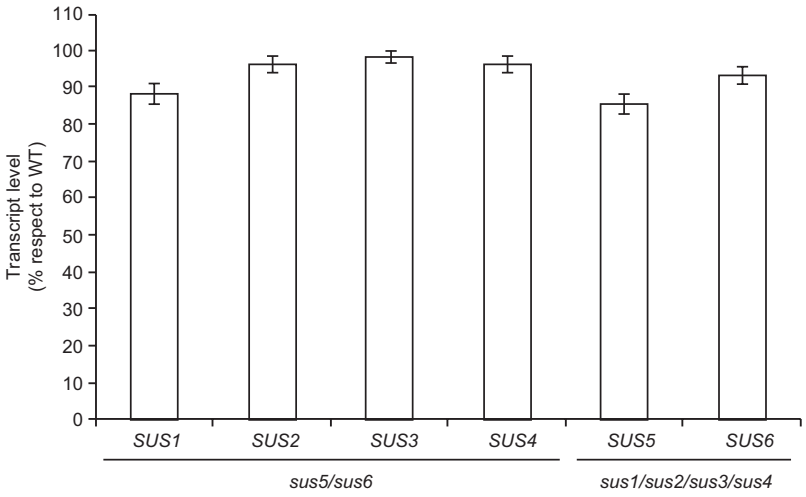

Fig. 5. Relative abundance of the indicated *SUS* transcripts in *sus1/sus2/sus3/sus4* and *sus5/sus6* leaves. Transcript levels were measured by quantitative RT-PCR using the primers listed in Table S3. Results are referred as percentage of transcript content with respect to that found in WT leaves. The results are the mean ± SD of three independent experiments.

medium). The homogenate was subjected to centrifugation for 10 min at 10,000 × *g* and 4 °C. The supernatant (referred to as the soluble fraction) was desalted by ultrafiltration on Centricon YM-10 (Amicon), and the retained material was resuspended in extraction medium.

Full-Length cDNA Synthesis. Total RNA was extracted from *Arabidopsis* leaves using the TRIzol method according to the manufacturer's procedure (Invitrogen). RNA was treated with RNase free DNase (Takara). A total of 1.5 μg of RNA was converted into first strand cDNA using poly(T) primers and the Expand Reverse Transcriptase kit (Roche) according to the manufacturer's instructions. Full-length cDNAs for each SUS-encoding gene were produced and isolated by PCR using the high-fidelity Accuzyme DNA polymerase (Bioline) and primers sets shown in Table S2 according to the manufacturer's instructions. The PCR products of each cDNA were cloned into pDONR vector (Invitrogen) and confirmed by sequencing.

Production and Purification of Recombinant SUS. Plasmid constructs for production of recombinant SUS1 and SUS3 (pDEST17-SUS1 and -3) were produced from the pDONR-SUS1 and pDONR-SUS3 vectors and pDEST17 (see above) using the Gateway technology (Fig. S6). pDEST17-SUS1 and pDONR-SUS3 were each transformed into *Escherichia coli* BLR(DE3) competent cells. Recombinant SUSs were then produced and purified essentially as described in ref. 42. The purified proteins were desalted by ultrafiltration and resuspended in extraction medium.

Real-Time Quantitative PCR. First-strand cDNA was produced as for full-length cDNA synthesis (see above). RT-PCR was performed using a 7900HT sequence detector system (Applied Biosystems) with the SYBR Green PCR Master Mix (Applied Biosystems) according to the manufacturer's protocol. Each reaction was performed in triplicate with 0.4 μL of the first-strand cDNA in a total volume of 20 μL. The specificity of the PCR amplification was checked with a heat dissociation curve (from 60 °C to 95 °C). Comparative threshold values were normalized to 18S RNA internal control and compared with obtain relative expression levels. The specificity of the obtained RT-PCR products was controlled on 1.8% agarose gels. Primers used for RT-PCRs are listed in Table S3.

Two-Step Assay Method for SUS Activity Assay. Measurements of SUS activity in both plant extracts and purified recombinant SUS preparations were performed in the direction of ADPG and UDPG synthesis in two steps: (*i*) SUS reaction and (*ii*) measurement of ADPG and UDPG produced during the reaction. Unless otherwise indicated, the SUS assay mixture contained 50 mM Hepes (pH 7.0), the indicated amounts of sucrose and nucleoside diphosphate (ADP or UDP), and the SUS preparation. The reaction was initiated by adding the SUS-containing protein extract. After 3 min at 37 °C (still under initial velocity conditions), reactions were stopped by boiling the assay mixture for 1 min. ADPG and UDPG were measured by HPLC on a Waters Associate's system fitted with a Partisil-10-SAX column, and by HPLC with pulsed amperometric detection on a DX-500 system (Dionex) fitted to a CarboPac PA10 column, as described in ref. 38. We define 1 unit (U) of enzyme activity as the amount of enzyme that catalyzes the production of 1 μmol of product per min. Kinetic parameters such as K_m and V_{max} were evaluated by Lineweaver-Burk plots.

Analysis of UDPG Stability. A total of 50 μL of buffered solutions (see below) containing 10 mM UDPG were incubated at 25 °C in the presence or absence of 3 mM $MgCl_2$. At the indicated incubation periods, 1 mL of 1 M $HClO_4$ was added to each solution. After neutralization with K_2CO_3, UDPG was chromatographically measured as indicated above. The buffers used were 50 mM sodium acetate-acetic acid (pH 4.5–5.5), 50 mM Mes/NaOH (pH 5.5–6.5), 50 mM Hepes/NaOH (pH 6.5–7.5), and 50 mM Tris·HCl (pH 7.5–9.5).

Analytical Procedures. Starch in plant extracts obtained by precipitation with 70% ethanol was measured by using an amyloglucosydase-based test kit (Boheringer Manheim). Protein content was determined by the Bradford method using Bio-Rad prepared reagent (Bio-Rad Laboratories).

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