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No evidence for the occurrence of substrate inhibition of *Arabidopsis thaliana* sucrose synthase-1 (AtSUS1) by fructose and UDP-glucose

Goizeder Almagro,[1] Edurne Baroja-Fernández,[1] Francisco José Muñoz,[1] Abdellatif Bahaji,[1] Ed Etxeberria,[2] Jun Li,[1] Manuel Montero,[1] Maite Hidalgo,[1] María Teresa Sesma[1] and Javier Pozueta-Romero[1,*]

[1]Instituto de Agrobiotecnología; Universidad Pública de Navarra; Consejo Superior de Investigaciones Científicas; Gobierno de Navarra; Mutiloako Etorbidea Zenbaki Gabe; Nafarroa, Spain; [2]IFAS; Citrus Research and Education Center; University of Florida; Lake Alfred, FL USA

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Abbreviations: ADPG, ADP-glucose; SuSy, sucrose synthase; U, unit of enzyme activity; UDPG, UDP-glucose, WT, wild type

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Sucrose synthase (SuSy) catalyzes the reversible conversion of sucrose and NDP into the corresponding nucleotide-sugars and fructose. The Arabidopsis genome possesses six *SUS* genes (*AtSUS1–6*) that code for proteins with SuSy activity. As a first step to investigate optimum fructose and UDP-glucose (UDPG) concentrations necessary to measure maximum sucrose-producing SuSy activity in crude extracts of Arabidopsis, in this work we performed kinetic analyses of recombinant AtSUS1 in two steps: (1) SuSy reaction at pH 7.5, and (2) chromatographic measurement of sucrose produced in step 1. These analyses revealed a typical Michaelis-Menten behavior with respect to both UDPG and fructose, with Km values of 50 μM and 25 mM, respectively. Unlike earlier studies showing the occurrence of substrate inhibition of UDP-producing AtSUS1 by fructose and UDP-glucose, these analyses also revealed no substrate inhibition of AtSUS1 at any UDPG and fructose concentration. By including 200 mM fructose and 1 mM UDPG in the SuSy reaction assay mixture, we found that sucrose-producing SuSy activity in leaves and stems of Arabidopsis were exceedingly higher than previously reported activities. Furthermore, we found that SuSy activities in organs of the *sus1/sus2/sus3/sus4* mutant were ca. 80–90% of those found in WT plants.

Sucrose synthase (SuSy) 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. This sucrolytic enzyme is highly regulated both at transcriptional and post-translational levels.[1-5] In many heterotrophic organs SuSy activity acts as a major determinant of sink strength that highly controls the conversion of sucrose into ADP-glucose (ADPG) and UDP-glucose (UDPG) linked to the biosynthesis of starch and cell wall polysaccharides, respectively.[6-12] Genetic evidence demonstrating the importance of SuSy in starch and cell wall polysaccharide production, and in sink strength determination in heterotrophic organs comes from QTL analyses of maize endosperms and cotton,[13,14] from the altered biomass and fiber yield in cotton plants with altered SuSy expression,[11,15] from the substantial (ca. 50–70%) reduction of starch levels in the seed endosperms of *sh1* maize mutants,[6] and from genetically engineered potato tubers and carrot roots exhibiting altered SuSy activity.[8,12,16] In addition, SuSy has been suggested to be involved, at least in part, in the direct conversion of sucrose into ADPG linked to starch biosynthesis in autotrophic organs.[17,18]

The Arabidopsis genome possesses six *SUS* genes (*AtSUS1–6*) displaying different developmental expression patterns that code for proteins with SuSy activity.[19] Earlier studies[20,21] have questioned the involvement of SuSy activity in starch and cellulose biosynthesis in Arabidopsis, showing that (a) leaves, siliques, seeds, stems and roots of the *sus1/sus2/sus3/sus4* mutant impaired in SuSy activity accumulate wild type (WT) content of ADPG, UDPG, cellulose and starch, and (b) SuSy activity in WT leaves is too low to account for the rate of starch accumulation in illuminated leaves. Most recently however, we[22] have shown that SuSy activity in the cleavage direction in WT Arabidopsis leaves is ca. 10-fold higher than UDP-producing SuSy activities shown in earlier studies,[20,21] greatly exceeding the minimum needed to support normal rate of starch accumulation during illumination. Furthermore, we found that SuSy activities in the insoluble and soluble fractions of *sus1/sus2/sus3/sus4* stems were ca. 10- and 100-fold higher, respectively, than previously reported activities.[21] Finally, we also found that SuSy activities in the leaves and stems of the *sus1/sus2/sus3/sus4* mutants were ca. 85% of those of WT plants, thus concluding that SuSy activity in *sus1/sus2/sus3/sus4* mutants is sufficient to support normal cellulose and starch

*Correspondence to: Javier Pozueta-Romero; Email: javier.pozueta@unavarra.es
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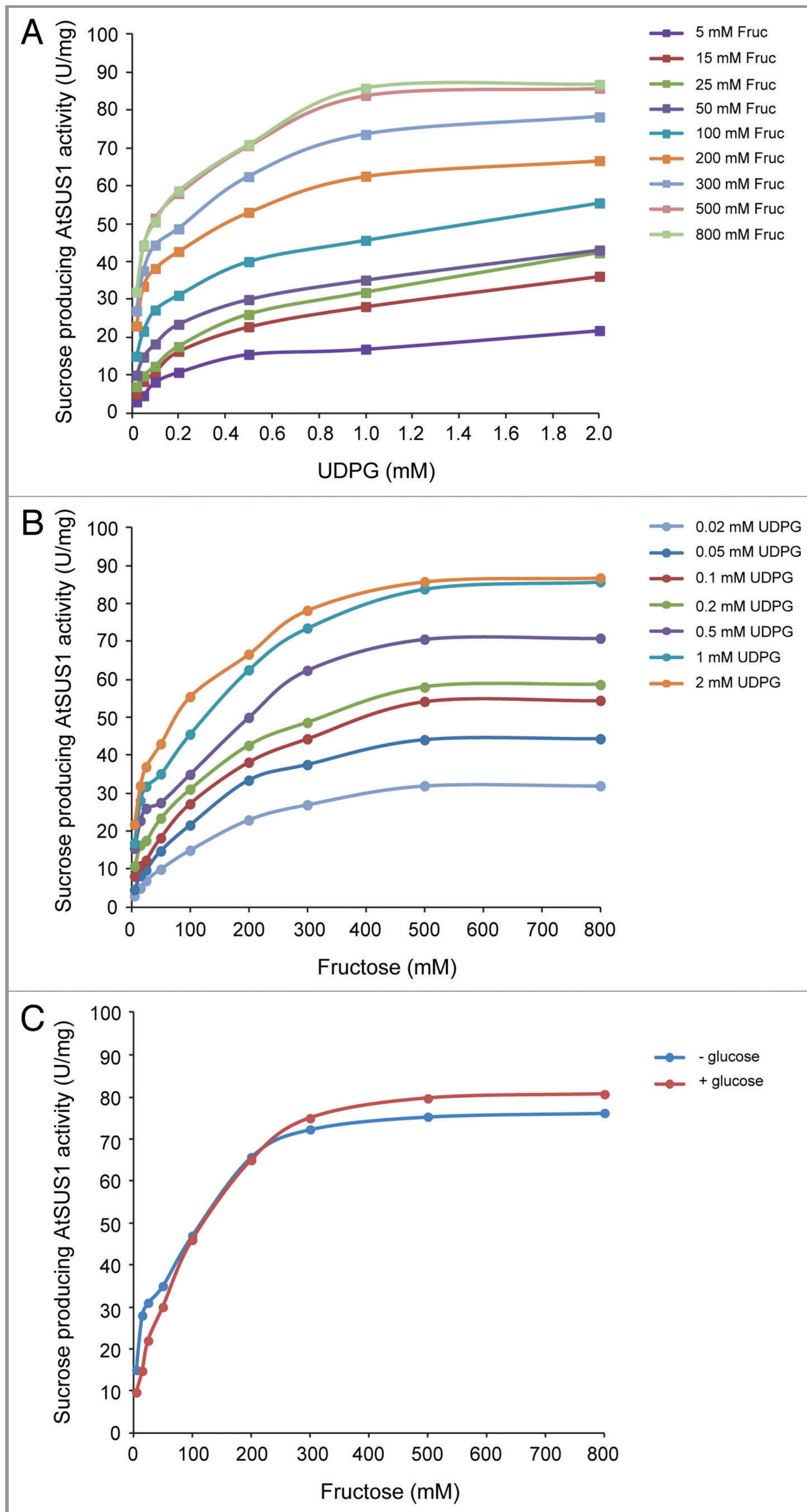

Figure 1. Kinetic studies of sucrose-producing activity of recombinant AtSUS1. (A) UDPG-dependent AtSUS1 activity was measured in the presence of different concentrations of fructose (5–800 mM). (B) Fructose-dependent AtSUS1 activity was measured in the presence of different concentrations of UDPG (0.02–2 mM). (C) AtSUS1 activity was measured in the presence or absence of 200 mM glucose, the indicated fructose concentrations and 1 mM UDPG. We define 1 unit (U) of enzyme activity as the amount of enzyme that catalyzes the production of 1 μmol of product per min.

biosynthesis in Arabidopsis.[22] Contrary to the claims of Barratt et al.,[21] Angeles-Núñez and Tiessen[23] showed that developing seeds of single *sus2* and *sus3* mutants accumulate considerably reduced levels of transitory starch. This and the fact that AtSUS2 is strongly associated with plastids[24] would support the idea that SUS is involved in a specific route for ADPG synthesis in Arabidopsis seeds.

There are several possible reasons that, individually or collectively, can explain why the values of SuSy activity shown in earlier reports[20,21] were a gross under-estimate and differed greatly from those presented by Baroja-Fernández et al.[22] First, Bieniawska et al.[20] and Barratt et al.[21] measured SuSy activity in the UDP synthetic direction, whereas Baroja-Fernández et al.[22] measured SuSy activity in the more physiological sucrose breakdown (UDPG and ADPG synthesis) direction. Second, the SuSy reaction assay mixture employed by Bieniawska et al.[20] and Barratt et al.[21] contained $MgCl_2$ and its pH was 9.0–9.5. Under these conditions, UDPG (the substrate for SuSy reaction in the sucrose synthetic direction) is highly unstable, being spontaneously converted into UMP and glucose-1,2-monophosphate.[22] Third, the SuSy reaction assay mixture employed by Bieniawska et al.[20] and Barratt et al.[21] contained 6 mM fructose, a concentration which is comparable, or even lower, than the reported K_m values for fructose in SuSy from many species.[25,26] Such low fructose concentration in the SuSy reaction assay mixture would prevent substrate inhibition by both fructose and UDPG, a

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phenomenon reported by several independent groups when measuring UDP-producing SuSy activity from UDPG and fructose.[20,26-29]

As a first step to investigate the optimum fructose and UDPG concentrations in the reaction mixture for assaying maximum SuSy activity in crude extracts of Arabidopsis, in this work we performed kinetic analyses of recombinant AtSUS1 in the sucrose synthetic direction in two steps: (1) SuSy reaction, and (2) measurement of sucrose produced during the reaction in step 1. The SuSy reaction assay mixture contained 50 mM HEPES, 3 mM $MgCl_2$, the indicated amounts of fructose and UDPG, and recombinant AtSUS1 obtained as previously described.[22] After 3 min at 25°C (still under initial velocity conditions), reactions were stopped by boiling the assay mixture for 1 min. Sucrose was then measured by HPLC with pulsed amperometric detection on a DX-500 system (Dionex) fitted to a CarboPac PA10 column. Preliminary analyses of optimum pH in the absence of $MgCl_2$ revealed that sucrose producing AtSUS1 activity has a broad pH optimum between 7.5 and 9.5, which is consistent with previous reports.[27,30,31] Because UDPG is highly unstable in the presence of $MgCl_2$ at pH values higher than 7.5,[22] the step one reaction in our kinetic analyses was conducted at pH 7.5.

Kinetic studies of sucrose producing AtSUS1 activity revealed a typical Michaelis-Menten behavior with respect to UDPG (**Fig. 1A**) and fructose (**Fig. 1B**), with Km values of 50 μM and 25 mM, respectively, as calculated using Lineweaver-Burk plots. These analyses revealed no substrate inhibition of SuSy by high concentrations of UDPG or fructose. Also, we found that glucose did not exert any inhibitory effect on AtSUS1 (**Fig. 1C**). Essentially the same results were obtained using SuSy purified from developing maize endosperm (not shown). Although consistent with previous reports showing the absence of substrate inhibition of SuSy by fructose and UDPG,[31-34] our results conflict with those of an earlier report[20] obtained using a one-step continuous method for the enzymatic assay of UDP-producing SuSy in the presence of 3 mM $MgCl_2$ at pH 9.5. Results included in this report[20] showed inhibitory effect of UDPG, fructose and glucose on UDP-producing AtSUS1 activity, and pointed to the occurrence of an ordered kinetic mechanism where (1) UDPG binds to AtSUS1 first and UDP dissociates last, (2) fructose binds to the AtSUS1-UDP complex forming a dead-end ternary complex, and (3) glucose binds to the AtSUS1-UDP complex, probably at the site vacated by sucrose.

Based on the kinetic properties obtained above, we measured maximum sucrose-producing SuSy activity in crude extracts from leaves and stems of WT and *sus1/sus2/sus3/sus4* mutants using the above described two-step assay method for SuSy activity. These analyses revealed that total sucrose-producing SuSy activities in leaves and stems of WT plants were 119 ± 13 mU g FW^{-1} and 68 ± 4 mU g FW^{-1}, respectively (**Fig. 2**), which are exceedingly higher than those shown in previous reports.[20,21] Furthermore, consistent with our earlier studies of SuSy activity in the cleavage direction,[22] we found that total sucrose-producing SuSy activities in the leaves and stems of *sus1/sus2/sus3/sus4* mutants were 80–90% of those of WT plants (**Fig. 2**).

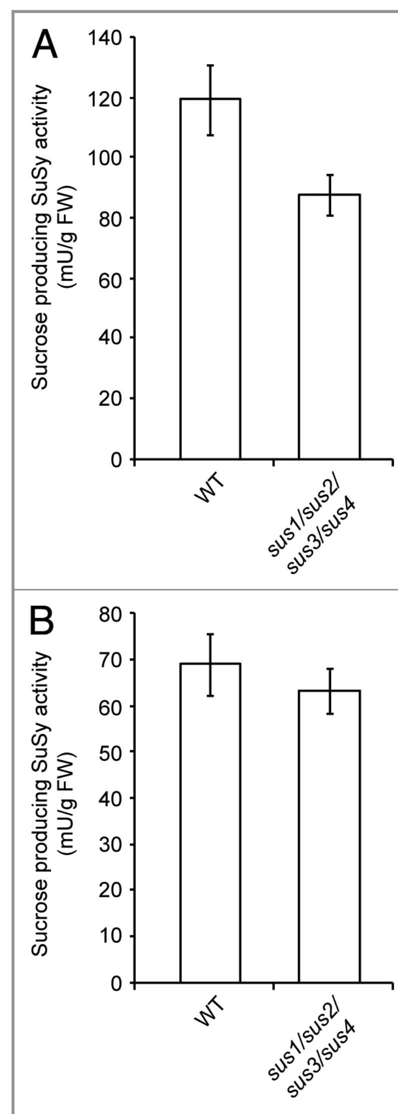

Figure 2. Sucrose-producing SuSy activity in crude extracts of (A) leaves and (B) stems of WT and *sus1/sus2/sus3/sus4 Arabidopsis* plants. SuSy activity was measured following the two-step assay method described in the main text. The SuSy reaction assay mixture of step 1 included 50 mM HEPES (pH 7.5), 3 mM $MgCl_2$, 1 mM UDPG and 200 mM fructose. After 3 min at 25°C (still under initial velocity conditions), reactions were stopped by boiling the assay mixture for 1 min. Sucrose was then measured by HPLC with pulsed amperometric detection on a DX-500 system (Dionex) fitted to a CarboPac PA10 column. The results are the mean ± SD of three independent experiments.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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