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Characterization of multiple *SPS* knockout mutants reveal redundant functions of the four Arabidopsis sucrose phosphate synthase isoforms in plant viability, and strongly indicates that enhanced respiration and accelerated starch turnover can alleviate the blockage of sucrose biosynthesis

Q1 Abdellatif Bahaji[a,1], Edurne Baroja-Fernández[a,1], Adriana Ricarte-Bermejo[a], Ángela María Sánchez-López[a], Francisco José Muñoz[a], Jose M. Romero[b], María Teresa Ruiz[b], Marouane Baslam[a], Goizeder Almagro[a], María Teresa Sesma[a], Javier Pozueta-Romero[a,*]

[a] *Instituto de Agrobiotecnología (CSIC/UPNA/Gobierno de Navarra), Iruñako etorbidea 123, 31192 Mutiloabeti, Nafarroa, Spain*
[b] *Instituto de Bioquímica Vegetal y Fotosíntesis, Universidad de Sevilla-CSIC, Avenida Américo Vespucio 49, Isla de la Cartuja, 41092 Sevilla, Spain*

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ABSTRACT

We characterized multiple knock-out mutants of the four Arabidopsis sucrose phosphate synthase (SPSA1, SPSA2, SPSB and SPSC) isoforms. Despite their reduced SPS activity, *spsa1/spsa2*, *spsa1/spsb*, *spsa2/spsb*, *spsa2/spsc*, *spsb/spsc*, *spsa1/spsa2/spsb* and *spsa2/spsb/spsc* mutants displayed wild type (WT) vegetative and reproductive morphology, and showed WT photosynthetic capacity and respiration. In contrast, growth of rosettes, flowers and siliques of the *spsa1/spsc* and *spsa1/spsa2/spsc* mutants was reduced compared with WT plants. Furthermore, these plants displayed a high dark respiration phenotype. *spsa1/spsb/spsc* and *spsa1/spsa2/spsb/spsc* seeds poorly germinated and produced aberrant and sterile plants. Leaves of all viable *sps* mutants, except *spsa1/spsc* and *spsa1/spsa2/spsc*, accumulated WT levels of nonstructural carbohydrates. *spsa1/spsc* leaves possessed high levels of metabolic intermediates and activities of enzymes of the glycolytic and tricarboxylic acid cycle pathways, and accumulated high levels of metabolic intermediates of the nocturnal starch-to-sucrose conversion process, even under continuous light conditions. Results presented in this work show that SPS is essential for plant viability, reveal redundant functions of the four SPS isoforms in processes that are important for plant growth and nonstructural carbohydrate metabolism, and strongly indicate that accelerated starch turnover and enhanced respiration can alleviate the blockage of sucrose biosynthesis in *spsa1/spsc* leaves.

Abbreviations: A_n, net photosynthetic CO_2 fixation rate; ADPG, ADP-glucose; AGP, ADPG pyrophosphorylase; Ci, intercellular CO_2 concentrations; CL, continuous light; DAG, days after germination; F1, 6P2, fructose-1,6-bisphosphate; F6P, fructose-6-phosphate; G1P, glucose-1-phosphate; G6P, glucose-6-phosphate; G6PDH, G6P dehydrogenase; g_s, stomatal conductance; G3PDH, glyceraldehyde-3-phosphate dehydrogenase; HK, hexokinase; IDH, isocitrate dehydrogenase; MDH, malate dehydrogenase; OPPP, oxidative pentose phosphate pathway; PEP, phospho-enol-pyruvate; 3-PGA, 3-phosphoglycerate; PGI, phosphoglucose isomerase; 6PGDH, 6-phosphogluconate dehydrogenase; PGM, phosphoglucomutase; Pi, inorganic orthophosphate; PK, pyruvate kinase; PPase, alkaline pyrophosphatase; S6P, sucrose-6-phosphate; SDH, succinate dehydrogenase; SPP, sucrose-phosphate phosphatase; SPS, sucrose-phosphate synthase; SS, starch synthase; SuSy, sucrose synthase; TCA, tricarboxylic acid cycle; TPT, triose-phosphate/phosphate translocator; U, unit of enzyme activity; UDPG, UDP-glucose; UGP, UDPG pyrophosphorylase; WT, wild type.

* Corresponding author. Tel.: +34 948168009; fax: +34 948232191.
E-mail address: javier.pozueta@unavarra.es (J. Pozueta-Romero).
[1] A.B. and E.B.-F. have equally contributed to this work.

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1. Introduction

In the majority of higher plants sucrose is the main end-product of photosynthesis. It serves as the mobile form of photoassimilate that is transported from leaves to sink organs. This disaccharide plays a major role in growth, and also acts as a signaling molecule in the control of the expression of genes involved in multiple processes such as central carbon and nitrogen metabolisms [1], storage of proteins [2], cell cycle and differentiation [3], flowering [4] and seed development [5]. During the day, photosynthetically fixed carbon is either retained within the chloroplast of leaf mesophyll cells to fuel the synthesis of transitory starch, or exported to the cytosol as triose phosphates by means of the triose-phosphate/phosphate translocator (TPT) to be converted into activated forms of hexoses and sucrose (Fig. S1A). Sucrose is synthesized by the action of two enzymes: sucrose-phosphate synthase (SPS, which catalyzes the conversion of fructose-6-phosphate (F6P) and UDP-glucose (UDPG) into sucrose-6-phosphate (S6P)), and sucrose-phosphate phosphatase (SPP, which hydrolyzes S6P to produce sucrose) (Fig. S1A). During the night, starch is mobilized to produce maltose that is transported to the cytosol by means of the MEX1 translocator (Fig. S1B). Once in the cytosol maltose is converted into heteroglycans, glucose-1-P (G1P), UDPG, S6P and sucrose by the stepwise reactions of the cytosolic disproportionating enzyme DPE2, glucan phosphorylase PHS2, UDPG pyrophosphorylase (UGP), SPS and SPP [6–8] (Fig. S1B). Glucose, another starch breakdown product, can be transported to the cytosol by means of the pGlcT transporter [9]. Once in the cytosol, glucose can be converted into glucose-6-P (G6P), G1P, UDPG, S6P and sucrose by the stepwise reactions of hexokinase (HK), phosphoglucomutase (PGM), UGP, SPS and SPP [9] (Fig. S1B).

SPS is a key control point of carbon flux into sucrose that is regulated by a hierarchy of mechanisms including posttranslational modification via protein phosphorylation, activation by G6P and inhibition by inorganic orthophosphate (Pi) [10,11], and transcriptional regulation of *SPS* gene expression [12,13]. SPS isoforms in the many plant species examined to date are encoded by a small *SPS* multigene family. Studies of the predicted amino acid sequences and gene structure have shown that the *Arabidopsis* SPS family consists of four *SPS* genes, referred to as *AtSPSA1* (At5g20280), *AtSPSA2* (At5g11110), *AtSPSB* (At1g04920) and *AtSPSC* (At4g10120) [14,15]. Genome-wide expression analyses (https://www.geneinvestigator.ethz.ch) and comparative studies of *SPS* gene expression in *Arabidopsis* [14,15] provided evidence for distinct, but partially overlapping spatial and temporal expression patterns for the four *SPS* genes. Metabolic studies of an *spsa1/spsc* double knockout *Arabidopsis* mutant revealed effects on growth and leaf nonstructural carbohydrate metabolism in this mutant [15]. Thus *spsa1/spsc* plants cultured under 8 h light/16 h dark photoregime displayed a dwarf phenotype. Also, these plants accumulated low levels of sucrose and moderately high levels of both starch and maltose when compared with wild type (WT) plants, strongly indicating that SPSA1 and SPSC have overlapping functions in aspects related with growth and leaf nonstructural carbohydrate metabolism. According to Volkert et al. [15], the increase in starch was probably not due to an increased partitioning of carbon into starch, but was rather caused by an impaired starch mobilization during the night due to impairment in downstream metabolization of maltose.

Mutants impaired in TPT and cytosolic fructose 1,6-bisphosphatase display a nearly WT growth phenotype [16–19], strongly indicating the operation in these mutants of mechanism(s) of diurnal sucrose biosynthesis additional/alternative to that illustrated in Fig. S1A, and showing that TPT and cytosolic fructose 1,6-bisphosphatase are not essential for plant viability. While SPS catalyzes an undoubtedly crucial step in sucrose biosynthesis, the challenge still remains to determine if SPS is an essential function for plant viability and if, in addition to the functional overlapping occurring between SPSA1 and SPSC *in planta*, there are other functional interactions between the four SPS isoforms. Towards the end of exploring possible interactions between the four SPS isoforms *in planta*, and between sucrose biosynthesis and other metabolic pathways when SPS-mediated sucrose production is limited, in this work we conducted a comprehensive study of different multiple *SPS* knock-out mutants. Results presented in this work show that, in Arabidopsis, (a) SPS is essential for plant viability, implying that sucrose is mainly synthesized through the SPS-SPP pathway and (b) the four SPS isoforms are functionally redundant in processes that are important for plant growth, vegetative and reproductive development, and nonstructural carbohydrate metabolism. Furthermore, the results provide strong evidence supporting the occurrence in illuminated *spsa1/spsc* leaves of mechanisms alleviating the blockage of the starch-to-S6P conversion process such as accelerated starch turnover, and channeling of starch breakdown products towards the glycolytic, oxidative pentose phosphate (OPP) and tricarboxylic acid cycle (TCA) pathways.

2. Materials and methods

2.1. Plants, growth conditions and sampling

Unless otherwise indicated WT *Arabidopsis thaliana* L. (Heynh) ecotype Columbia and T-DNA insertion lines in this background were cultured in soil in growth chambers under the indicated photoperiod conditions (light intensity of 90 μmol photons s^{-1} m^{-2}) (22 °C during the light period and 18 °C during the dark period). Harvested source leaves were immediately freeze-clamped and ground to a fine powder in liquid nitrogen with a pestle and mortar.

2.2. Production of multiple T-DNA knock-out lines

The T-DNA insertion mutants *spsa1* (SALK_119162), *spsa2* (SALK_064922), *spsb* (GABI_368F01) and *spsc* (SAIL_31_H05) were obtained from the European *Arabidopsis* Stock Center (NASC) (Fig. S2). The T-DNA insertion in *spsa1* mutant is in the third intron, whereas the T-DNA insertions in *spsa2*, *spsb* and *spsc* are in the fifth, ninth and fifth exon of *SPSA2*, *SPSB* and *SPSC*, respectively (Fig. S2). By crossing these mutants, self-pollinating the resulting heterozygous mutants, and PCR screening for homozygous progeny using the oligonucleotide primers listed in Table S2 we produced the *spsa1/spsa2*, *spsa1/spsb*, *spsa1/spsc*, *spsa2/spsb*, *spsa2/spsc*, *spsb/spsc*, *spsa1/spsb/spsc*, *spsa1/spsa2/spsb*, *spsa1/spsa2/spsc*, *spsa2/spsb/spsc*, and *spsa1/spsa2/spsb/spsc* mutants (Table S1, Fig. S3).

The knock-out status of the T-DNA mutants was confirmed by RT-PCR for *SPS* transcripts using specific primers that spanned the T-DNA insert site of each gene (Table S3). To this end, total RNA was extracted from leaves using the trizol method according to the manufacturer's procedure (Invitrogen). RNA was treated with RNAase free DNAase (Takara). RT-PCR was conducted with SuperScript III one-step RT-PCR with Platinum *Taq* DNA polymerase kit (12574-018; Invitrogen) using 100 ng of RNA and the *SPSA1*, *SPSA2*, *SPSB* and *SPSC* specific primers listed in Table S3. 18S RNA was used as the positive control. PCR products were separated on 1% (w/v) agarose gels containing ethidium bromide and visualized by ultraviolet light. *SPSA1*, *SPSA2*, *SPSB* and *SPSC* PCR products were detected in WT plants, but were undetectable in *spsa1*, *spsa2*, *spsb* and *spsc* mutants, respectively (Fig. S4).

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