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Decrease in leaf sucrose synthesis leads to increased leaf starch turnover and decreased RuBP regeneration-limited photosynthesis but not Rubisco-limited photosynthesis in *Arabidopsis* null mutants of *SPSA1*

JINDONG SUN[1,2*†], JISEN ZHANG[2,*‡], CLAYTON T. LARUE[2,3§] & STEVEN C. HUBER[2,3]

[1]*Institute for Genomic Biology and* [2]*Department of Plant Biology and Crop Science, University of Illinois at Urbana-Champaign, Urbana, IL 61801, USA and* [3]*USDA ARS, Urbana, IL 61801, USA*

ABSTRACT

We investigated the individual effect of null mutations of each of the four sucrose-phosphate synthase (SPS) genes in *Arabidopsis* (*SPSA1*, *SPSA2*, *SPSB* and *SPSC*) on photosynthesis and carbon partitioning. Null mutants *spsa1* and *spsc* led to decreases in maximum SPS activity in leaves by 80 and 13%, respectively, whereas null mutants *spsa2* and *spsb* had no significant effect. Consistently, isoform-specific antibodies detected only the SPSA1 and SPSC proteins in leaf extracts. Leaf photosynthesis at ambient [CO_2] was not different among the genotypes but was 20% lower in *spsa1* mutants when measured under saturating [CO_2] levels. Carbon partitioning at ambient [CO_2] was altered only in the *spsa1* null mutant. Cold treatment of plants (4 °C for 96 h) increased leaf soluble sugars and starch and increased the leaf content of SPSA1 and SPSC proteins twofold to threefold, and of the four null mutants, only *spsa1* reduced leaf non-structural carbohydrate accumulation in response to cold treatment. It is concluded that SPSA1 plays a major role in photosynthetic sucrose synthesis in *Arabidopsis* leaves, and decreases in leaf SPS activity lead to increased starch synthesis and starch turnover and decreased Ribulose 1,5-bisphosphate regeneration-limited photosynthesis but not ribulose 1·5-bisphosphate carboxylase/oxygenase (Rubisco)-limited photosynthesis, indicating a limitation of triose-phosphate utilization (TPU).

Key-words: *Arabidopsis thaliana*; carbon partitioning; cold stress; diurnal growth; gene expression; null mutant; photosynthesis, starch synthesis; sucrose-phosphate synthase (SPS); sucrose synthesis.

Correspondence: S. Huber. Fax: +217 244 4419; e-mail: schuber1@illinois.edu

*These authors contributed equally to the article.
†Current address: Pioneer Hi-bred International, 7300 NW 62nd Ave., Johnston, IA 50131-1004.
‡Permanent address: College of Life Sciences, Fujian Normal University, Fuzhou 350108, China.
§Current address: Monsanto Company, 800 N. Lindbergh Boulevard, St. Louis, MO 63167.

INTRODUCTION

Sucrose-phosphate synthase (SPS) is a key enzyme of sucrose synthesis that uses uridine diphosphate (UDP)-glucose and fructose 6-phosphate as substrates and is a key control point of carbon flux into sucrose that is regulated by protein phosphorylation and metabolic effectors (Huber & Huber 1996; Stitt *et al*. 1988; Winter & Huber 2000). In many species, SPS activity is regulated by day and night diurnal cycles (Ohsugi & Huber 1987) and is induced by cold treatment (Guy, Huber & Huber 1992; Hurry *et al*. 1994; Reimholz *et al*. 1997), osmotic stress (Quick *et al*. 1989) and elevated [CO_2] (Hurry *et al*. 1994; Seneweera *et al*. 1995).

There are several SPS genes in terrestrial plants (Castleden *et al*. 2004; Lunn & MacRae 2003; Lutfiyya *et al*. 2007). In dicots, SPS genes can be grouped into three families, that is A, B and C (Langenkamper *et al*. 2002; Lunn & MacRae 2003), and generally, there is at least one gene in each of the three families. Another phylogenetic tree of plant and cyanobacterial SPS proteins based on alignment of the glycosyl transferase domain grouped families 1a, 1b, 2d, 2m, 3 and 4 (Lutfiyya *et al*. 2007). In *Arabidopsis*, there are four SPS isoforms, referred to as SPSA1 (AT5G20280), SPSA2 (AT5G11110), SPSB (AT1G04920) and SPSC (AT4G10120). Monocots have an additional family that is called the D family (Castleden *et al*. 2004; Grof *et al*. 2006).

Studies on the individual roles of each of the SPS isoforms in plant photosynthesis are generally lacking. In tobacco and sugar cane, each of the SPS genes exhibits distinct expression patterns in various tissues (Chen, Hajirezaei & Bornke 2005; Grof *et al*. 2006), but the functional significance is not known. However, a previous study on SPS isoforms in tobacco showed that silencing *SPSC* led to a several-fold increase in leaf starch content, which was attributed to impaired starch mobilization at night (Chen *et al*. 2005). It is not known whether SPSC also plays a major role in photosynthetic sucrose synthesis in other dicots such as the model plant *Arabidopsis*.

Photosynthesis is limited either by ribulose 1·5-bisphosphate carboxylase/oxygenase (Rubisco) or Ribulose 1,5-bisphosphate (RuBP) regeneration depending on

environmental conditions. In general, photosynthesis is limited by light harvesting and assimilatory power under low light and by the carboxylation and oxygenation of Rubisco under low [CO_2] and high light. Typically, photosynthesis is limited by light harvesting and assimilatory power under low light. If light is not limiting, photosynthesis (A) is limited by the carboxylation and oxygenation of Rubisco under low intercellular [CO_2] (C_i). With a further increase in C_i, there is an inflection (dA/dC_i decreases and approaches zero) where RuBP regeneration is limiting. In some instances, a further increase in C_i may result in another transition to a plateau or a slight decrease in A with an additional increase in C_i ($dA/dC_i < 0$) if triose-phosphate utilization (TPU) becomes limiting, which mainly reflects the synthesis of starch and sucrose (Farquhar, Caemmerer & Berry 1980; Long & Bernacchi 2003; Sage 1994; Sharkey 1985; Sun *et al*. 2009). However, the third phase is often not obvious because the RuBP regeneration limitation and TPU limitation can be difficult to separate.

The majority of the photosynthetically fixed carbon in leaves is partitioned between sucrose and starch which are synthesized in the cytoplasm and chloroplasts, respectively. A portion of photosynthetic carbon flux is allocated to starch synthesis to support plant growth and maintenance during the night (Huber 1983; Smith & Stitt 2007; Stitt, Lunn & Usadel 2010). ADP-glucose pyrophosphorylase, an important enzyme for starch synthesis, is inhibited by phosphate (P_i) and stimulated by 3-phosphoglycerate (Smith & Stitt 2007). Carbon flux is preferentially directed to sucrose at lower rates of triose-phosphate production under low CO_2 levels, with increased partitioning to starch synthesis occurring as sucrose synthesis reaches saturation (Smith & Stitt 2007; Stitt *et al*. 2010; Sun, Okita & Edwards 1999).

Modification of SPS activity by overexpressing or suppressing SPS genes in plants generally alters the ratio of sucrose to starch and carbon partitioning into the sink (Chen *et al*. 2005; Galtier *et al*. 1993; Lundmark *et al*. 2006; Park *et al*. 2008; Signora *et al*. 1998; Strand *et al*. 2000) and crop yield (Laporte *et al*. 2001; Ono *et al*. 2003). However, how changes in SPS expression affect starch levels and turnover, and plant growth, is not as consistent. Transgenic tomato plants expressing high activities of maize SPS accumulated more sucrose, glucose and fructose and less starch and showed no difference in total dry matter production, even though shoot-to-root ratios were increased compared to wild-type plants (Galtier *et al*. 1993, 1995). Suppression of *SPSC* in tobacco caused little change in SPS activity but several-fold increase in starch levels (Chen *et al*. 2005). Antisense suppression of SPS activity in *Arabidopsis* decreased synthesis of both sucrose and starch and resulted in a ~50% reduction in plant growth (Strand *et al*. 2000).

The effects of SPS manipulation on leaf photosynthesis are also variable. Overexpression of maize SPS under an ribulose 1,5-bisphosphate carboxylase small subunit promoter in *Arabidopsis* had no effect on photosynthesis measured under saturating [CO_2] and saturating light levels in plants grown under ambient [CO_2] but plants grown at elevated [CO_2] showed less down-regulation of photosynthesis compared with wild-type controls (Signora *et al*. 1998). Suppression of SPS activity in *Arabidopsis* caused decreased photosynthesis under both ambient and saturating [CO_2] and high light (Strand *et al*. 2000). In contrast, overexpression of maize SPS in tomato plants resulted in increased photosynthesis measured under elevated [CO_2] (Galtier *et al*. 1993, 1995). It has also been reported that photosynthesis is not different between SPS overexpressers and wild-type plants in transgenic tomato (Laporte *et al*. 2001) and potato (Ishimaru *et al*. 2008) plants.

In this study, we characterized the *Arabidopsis* null mutants lacking each of the four SPS genes. The overall objective was to determine which of the SPS genes affect leaf carbon partitioning, photosynthesis and plant growth. Specifically, we sought to determine whether SPSC plays a major role in nocturnal sucrose synthesis in *Arabidopsis* similar to its reported role in tobacco (Chen *et al*. 2005). In addition, we wanted to determine whether null mutants of any of the SPS genes affected the leaf carbohydrate accumulation that occurs following several days of cold acclimation (Stitt & Hurry 2002; Kaplan *et al*. 2007). To facilitate these studies, we also produced isoform-specific anti-peptide antibodies to monitor relative expression of individual SPS isoforms at the protein level.

MATERIALS AND METHODS

Plant growth conditions

Arabidopsis thaliana ecotype Columbia null mutants *spsa1* (salk_148643C; AT5G20280), *spsa2* (salk_064922C; AT5G11110), *spsb* (salk_152217C; AT1G04920) and *spsc* (salk_020179; AT4G10120) were obtained from the *Arabidopsis* Biological Resource Center (Ohio State University, Columbus). The sites of T-DNA insert are at positions 2219, 1252, 2402 and 830 from the translation start sites, respectively, for *spsa1*, *spsa2*, *spsb* and *spsc* mutants. Plants were grown in controlled environmental growth chambers with a 10 h photoperiod (short day) and photosynthetic photon flux density (PPFD) of 200 μmol m^{-2} s^{-1} provided by fluorescence lamps to obtain larger rosette leaves and delay flowering. Day and night temperatures were 23 ± 1 °C and 18 ± 1 °C, respectively. Relative humidity in the growth chamber was 70%. The *Arabidopsis* plants were planted in Sunshine LC1 soil mixture in 38 mm Cone-tainers™ (Li-Cor, Lincoln, NE, USA). After 1 to 2 weeks, plants were thinned to one plant per tube. Plants were irrigated with modified Hoagland solution (Hoagland & Arnon 1950), which consisted of 0.625 mM K_2SO_4, 0.5 mM $MgSO_4$, 0.25 mM KH_2PO_4, 3 mM Ca, 20 μM Fe- ethylenediaminetetraacetic acid (EDTA), 35 μm 330 Fe [Sequestrene 330; Ciba-Geigy, Greensboro, NC, USA], 46 μM H_3BO_3, 9 μM $MnCl_2$, 0.76 μM $ZnSO_4$, 0.32 μM $CuSO_4$, 0.12 μM $NaMoO_4$ and 12 mM N. Uniform, healthy 6- to 9-week-old plants before bolting were used for experiments.

Cold treatment

Six- to 9-week-old plants were transferred to 4 °C for 96 h prior to sampling for various analyses. Irradiance was 130 μmol m^{-2} s^{-1} provided by fluorescence lamps and the photoperiod was 10 h.

Plate assay

Seeds were sterilized with 70% (v/v) ETOH for 5 min, then with 50% (v/v) bleach with 0.1% (v/v) Triton X-100 for 5 min, and finally washed four times with distilled and deionized H_2O. Seeds were sown on half-strength Murashige and Skoog (MS) agar plates with or without 0.5% (w/v) sucrose and kept at 4 °C for 4 d before transfer to a growth chamber with an 8 h photoperiod and PPFD of 200 μmol m^{-2} s^{-1} provided by fluorescence lamps. The temperature was kept at 21 ± 1 °C and relative humidity in the growth chamber was 60%. The seedlings were either grown on horizontal plates to visualize rosette size or vertical plates to visualize root growth.

Plant growth analysis

Arabidopsis plants were imaged a few times a week with a digital camera. The projected areas of the plants were estimated from the images of the plants with WinRHIZO Pro 2007d software (Regent Instruments Inc, Canada).

Assay of SPS activity

SPS activity was assayed as described previously (Huber *et al*. 1992) in extracts of leaves harvested about 1 h into light period. Leaf samples were harvested into liquid nitrogen in pre-weighed foil envelopes and extracted and assayed the same day. The samples were ground under liquid nitrogen and then homogenized with extraction buffer (1 g tissue 2 mL^{-1} buffer) consisting of 50 mM Mops-NaOH (pH 7.5), 10 mM $MgCl_2$, 1 mM EDTA, 2.5 mM dithiothreitol (DTT) and 0.1% (v/v) Triton X-100. Homogenates were centrifuged at 15 000 *g* at 4 °C for 5 min, and supernatants were desalted on G-25 Sephadex spin columns that had been pre-equilibrated in extraction buffer minus Triton X-100. SPS activity was determined *in vitro* by measuring the formation of sucrose under limiting (V_{sel}) or saturating (V_{max}) substrate conditions. The desalted tissue extract (50 μL) was added to 20 μL of reaction buffer, resulting in the following final concentrations for the limiting reaction: 10 mM UDP-glucose, 3 mM fructose 6-phosphate 12 mM glucose 6-phosphate, 10 mM P_i, 50 mM Mops-NaOH (pH 7.5), 15 mM $MgCl_2$ and 2.5 mM DTT. Phosphate was removed for the V_{max} assay and the concentration of fructose 6-phosphate was increased to 10 mM and that of glucose 6-phosphate to 40 mM. Samples were incubated at 30 °C for 20 min and the reaction was stopped by the addition of 70 μL of 30% KOH. To remove any residual fructose 6-phosphate from the samples that would otherwise react with the sucrose detection reagent, samples were heated for 10 min at 105 °C and then cooled on ice prior to the addition of 1 mL of 0.14% anthrone in 12.8 M sulphuric acid. For full colorimetric development, samples were incubated at 40 °C for 30 min and the A_{620} was recorded. Protein content was determined using the Bradford procedure with bovine serum albumin as the standard (Bradford 1976).

Immunodetection of SPS isoforms

Protein extracts were prepared by homogenization of leaf material in a buffer containing 100 mM Tris–HCl (pH 8.0), 2% (v/v) β-mercaptoethanol, 5 mM EGTA, 10 mM EDTA, 2% sodium dodecyl sulphate (SDS), 1 mM 4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride, 10 μg mL^{-1} leupeptin, 1 mM benzamidine, 5 mM caproic acid, 2 μM (L-transepoxysuccinyl-leucylamido-[4-guanidino]butane) (E64), 10 μM Z-Leu-Leu-Leu-CHO (MG132), 0.5 μM microcystin-LR and 10 mM NaF. Protein content was determined according to Bradford (1976) with bovine serum albumin as the standard. After heat denaturation at 95 °C for 3 min, 20 μg total protein was subjected to electrophoresis on a 7% (w/v) SDS-polyacrylamide gel and subsequently transferred onto a nitrocellulose membrane. Anti-SPSA1 and anti-SPSC were used at a 1:3000 dilution. Anti-SPSA2 and SPSB antibodies were used at a 1:1000 dilution, and all primary antibodies were detected with 1:10 000 dilution of Alexa Fluor® 680 goat anti-rabbit IgG secondary antibody (Invitrogen, Carlsbad, CA, USA). Anti-peptide polyclonal antibodies were produced against the following sequences: SPSA1, CDSDVRDALKKLE; SPSA2, HDVDA DGDDENPQTC; SPSB, RTPEIKSKPELQGKC; and SPSC, CEKLLRSEENFKRED. The terminal Cys in each sequence was added to facilitate conjugation of the antigen peptide. The antibodies were produced in rabbits and affinity purified by GenScript USA (Piscataway, NJ, USA). Each of the SPS antibodies is able to react specifically with the respective antigen sequence on a dot-blot assay (Supporting Information Fig. S1).

RT-PCR for transcripts of SPS isoforms

Leaf total RNA was extracted with RNeasy kit (Qiagen, Valencia, CA, USA). First-strand cDNA was synthesized by reverse transcription from deoxyribonuclease-treated total RNA with Superscript II RT (Invitrogen). A fraction of the first-strand cDNAs was used as a template for PCR with gene-specific primers that span the T-DNA insert sites and PCR super mix (Invitrogen). An initial denaturation step for 5 min at 94 °C was followed by 35 cycles of 30 s at 94 °C, 45 s at 55 °C and 45 s at 70 °C. PCR products were mixed with 6x loading dye (Promega, Madison, WI, USA) and separated on 1% (w/v) agarose gels containing ethidium bromide and visualized by ultraviolet light. The 1 Kb Plus DNA ladder was used as the reference (Invitrogen). The primer pairs used were: SPSA1, 5′-ATCTTTGATCGT CCCACCAG-3′ (forward) and 5′-CAACCTTGGTTTGG TGCTTT-3′ (reverse); SPSA2, 5′-CCGATTCCTGATT CACCAGT-3′ (forward) and 5′-CCGAGACATTCTTCCT

TTGG-3′ (reverse); SPSB, 5′-CCAGACCCAAAGAAGA ACATA-3′ (forward) and 5′-CTCCCTCATTGTCATAG CAATCAAC-3′ (reverse); and SPSC, 5′-CCCGTATCC GCTCTGAAATGC-3′ (forward) and 5′-AGCGACCTT GCTATGCTCACTA-3′ (reverse). The PCR products were 461, 549, 883 and 427 nt, respectively, for the *spsa1*, *spsa2*, *spsb* and *spsc* mutants.

Expression data for SPS genes in various tissues and various developmental stages in *Arabidopsis* were extracted from high-quality ATH1 22 k microarrays using meta-profile analysis [Source: 2010 Genevestigator V3; (Hruz *et al*. 2008)]. Each signal value in a meta-profile corresponds to the average expression level of one gene over a set of samples sharing the same biological context and has proven to be quite accurate for gene expressions.

Phylogenetic analysis

Eighty-four SPS amino acid sequences of various species were extracted from phytozome (http://www.phytozome.net) and National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov). All SPS amino acid sequences were aligned using ClustalX [version 2.0; (Thompson *et al*. 1997)]. The aligned file was then edited using the BioEdit program (version 7.0.5.3, Hall 1999) and the glucosyltransferase domain (TIGR02468) (Castleden *et al*. 2004) was extracted from each sequence. The edited sequences containing the glucosyltransferase domain were reimported into ClustalX and aligned. Phylogenetic trees were built based on both full-length sequences and domain-only sequences using neighbour-joining tree algorithms with 100 bootstrap replicates using PHYLIP package (Phylogeny Inference Package, version 3.6, Joe Felsenstein; http://evolution.genetics.washington.edu/phylip.html). Phylogenetic trees were viewed in TreeView [version 1.6.6; (Page 1996)].

Gas exchange

Gas exchange was measured using portable gas exchange systems (Li-Cor 6400LCF). The CO_2 sensors and water vapour sensors of the gas exchange systems were calibrated using gas of known [CO_2] with 21% oxygen and nitrogen as a balance, and known water vapour concentrations generated with a controlled humidification system, respectively (Li-610 Portable Dew Point Generator; Li-Cor). Leaf temperatures were set at 25 °C, and PPFD levels were maintained at 1500 μmol m^{-2} s^{-1} using a chamber-integrated red-blue light source with 10% blue light for all of the measurements. The relative humidity was maintained between 60 and 70% in the leaf chamber. After steady-state CO_2 and water vapour exchange were achieved, A_{sat} (light-saturated CO_2 assimilation rates) and the responses of A to C_i and A to PPFD were measured. A_{sat} was measured at 400 μbar [CO_2] at PPFD of 1500 μmol m^{-2} s^{-1}. The responses of A to C_i were measured at PPFD 1500 μmol m^{-2} s^{-1} with [CO_2] starting at 400 μbar surrounding the leaf, and [CO_2] was decreased stepwise to 50 μbar [CO_2]. The [CO_2] was then set again to 400 μbar and increased stepwise to 1500 μbar. Each individual A/C_i curve consisted of 10 individual measurements at various [CO_2] levels (400, 200, 100, 50, 400, 600, 800, 1000, 1200 and 1500 μbar) and took approximately 40 min to complete. The *Arabidopsis* chamber (Li-6400-17, Li-Cor) was used to measure whole-plant gas exchange. A/C_i curves were analysed using the equations of Caemmerer & Farquhar (1981) with a linear two-segment model as described previously (Long & Bernacchi 2003), and using the temperature-dependent kinetic parameters of Rubisco described by Bernacchi *et al*. (2002). In this study, the third phase for A/C_i was not obvious, and thus, addition of TPU to the model did not affect the V_{cmax} and J_{max} estimates; that is, calculations with a two- or three-segment fitting model gave similar results (Sun, Feng, Leakey, Zhu, Bernacchi & Ort, unpublished).

Chlorophyll fluorescence

Chlorophyll fluorescence was measured simultaneously with gas exchange using portable gas exchange systems with the integrated chlorophyll fluorescence chamber (Li-6400LCF, Li-Cor). The quenching of photochemical efficiency of photosystem II (PSII) (q_P) was calculated as $(F_m' - F_s)/(F_m' - F_o')$, and the quantum yield of non-cyclic electron transport (Φ_{PSII}) was calculated as $(F_m' - F_s)/F_m'$ and electron transport rate (ETR) was calculated as $(\Phi_{PSII} \times PPFD \times \alpha \times \beta)$, where α, the light absorbed by the leaf, was assumed to be 0.87, and β, the factor for the partitioning of photons between incident PSII and PSI, was assumed to be 0.5 (Sun *et al*. 1999).

Determination of starch, sucrose, glucose and fructose by coupled enzymes

Leaf starch, sucrose and hexoses were extracted and determined as previously described (Sun *et al*. 2002). Leaf discs (2 cm^2) were extracted with 80% (v/v) ethanol several times until the leaf discs were colourless. The ethanol soluble fractions from each sample were pooled and frozen at –20 °C until analysed for sugars. The leaf residue was homogenized in 1.5 or 2 mL microcentrifuge tubes with two tungsten beads inside for 2 min with a tissue lyser at maximum frequency (30; TissueLyser, Qiagen). The homogenate was treated with 0.2 mL of 0.5 M KOH and heated for 30 min at 95 °C with a heating block. The pH was adjusted to approximately 5.5 by the addition of 0.2 mL of 1 M acetic acid. Each of the samples was incubated with amyloglucosidase (10 units in a sample volume of 0.4 mL) at 55 °C for 2 h; tests showed that no additional sugars were released beyond 2 h. Free sugars were determined spectrophotometrically in each extract by the coupled enzyme methods as previously described (Sun *et al*. 2002; Winder *et al*. 1998).

RESULTS

Expression of leaf SPS genes

Eighty-four SPS isoforms (Supporting Information Table S1) were clustered into four families, that is, A, B, C

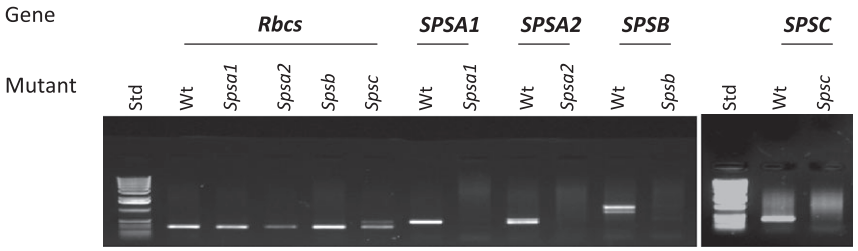

Figure 1. RT-PCR analysis for the transcripts of sucrose-phosphate synthase (SPS) null mutants. Ribulose 1·5-bisphosphate carboxylase/oxygenase small subunit (*rbcs*) was used as the positive control. Lanes 1 to 5, transcripts of *rbcs* for wild-type, *spsa1*, *spsa2*, *spsb* and *spsc* null mutants, respectively. Lanes 6–7, transcript of *SPSA1* in wild-type and *spsa1* mutant, respectively. Lanes 8–9, transcript of *SPSA2* in wild-type and *spsa2* mutant, respectively. Lanes 10–11, transcript of *SPSB* in wild-type and *spsb* mutant, respectively. Lanes 12–13, transcript of *SPSC* in wild-type and *spsc* mutant, respectively. Molecular weight standard (Std), 1 Kb Plus DNA ladder.

and D (Supporting Information Fig. S2). SPS isoforms from *Arabidopsis* as well as all other dicotyledonous plants were grouped into families A, B and C. Monocotyledonous plants have an additional SPS family, family D. SPS isoforms in families A, B and C were well separated between dicotyledonous plants and monocotyledonous plants. In *Arabidopsis*, there are two genes in family A (*SPSA1, SPSA2*), one gene in family B (*SPSB*) and onw gene in family C (*SPSC*).

Mutations of SPS genes were confirmed with RT-PCR for *SPS* transcripts with specific primers that spanned the T-DNA insert site of each gene (Fig. 1). Transcripts of *SPSA1*, *SPSA2*, *SPSB* and *SPSC* were detected in wild-type plants but were undetectable in the null mutants of *spsa1*, *spsa2*, *spsb* and *spsc*.

Expression of *SPS* genes varied across developmental stages (Supporting Information Fig. S3). In general, expression decreased in the order *SPSA1* > *SPSC* > *SPSA2* > *SPSB* across all developmental stages with the exception of germinated seeds. Overall, expression of *SPSA1* increased with plant development, and was higher in reproductive stages compared to vegetative stages. Expression of *SPSB* was low across all developmental stages except the stages of germinated seeds and mature siliques. Expression of *SPSC* changed little across developmental stages except the stage of germinated seeds in which SPSC expression was lowest. Expression of *SPSA2* was higher in early developmental stages.

SPS genes were also differentially expressed in various organs (Supporting Information Fig. S4). Gene expression of *SPSA1* was found in all tissues and was the highest among SPS genes in all tissues except roots. The highest expression of *SPSA1* was in green tissues such as mature leaves and stems. Gene expression of *SPSA2* was the highest among SPS genes in roots. The highest expression of *SPSA2* was in roots, stems and flowers. The highest expression of *SPSB* was in seeds and the highest expression of *SPSC* was in mature leaves. In mature leaves, expression of *SPSA1* was higher than *SPSC*, which in turn was higher than *SPSA2*. Expression of *SPSB* was lowest in mature leaves. SPS genes varied in their diurnal variation in expression (Supporting Information Fig. S5). Expression of *SPSA1* exhibited strong diurnal changes with highest expression at the end of the light period and lowest expression at the end of night period. Expression of *SPSC* also appeared to be rhythmic with highest values observed during the first 2 h of the photoperiod, whereas expression of *SPSA2* and *SPSB* was generally quite low and did not change diurnally. Generally, similar results were reported previously for targeted expression analysis of transcripts for *SPSA1*, *SPSA2* and *SPSC* (Gibon *et al*. 2004). Interestingly, while transcripts for *SPSA1* and *SPSC* are generally high and fluctuate diurnally, upon transfer to extended darkness, *SPSC* transcripts drop very quickly, while *SPSA1* transcripts remain high (Gibon *et al*. 2004). It should be noted that expression data presented in Supporting Information Figs S3–S5 are derived from a meta-analysis of published microarrays rather than from targeted expression analysis. The advantage of the meta-analysis is that differences in expression that are consistent are documented, but the disadvantage is that subtle differences in expression that might be quite important are lost in the analysis. Nevertheless, the important point to note is that *SPSA1* and *SPSC* have unique expression patterns that distinguish them from the other SPS genes.

Leaf SPS isoform proteins

Null mutations of individual SPS genes were confirmed with Western blots using isoform-specific polypeptide antibodies against SPSA1, SPSA2, SPSB and SPSC (Fig. 2). The proteins of SPSA1 and SPSC were detected in wild-type plants, but not in their respective null mutants *spsa1* and *spsc*. Proteins of SPSA2 and SPSB were undetectable in wild-type plants and all SPS mutants (data not shown). All of the anti-peptide antibodies readily recognized their antigen polypeptides on dot blots (Supporting Information Fig. S1), and consequently, we conclude that the leaf contents of SPSA2 and SPSB proteins are below our detection levels. Interestingly, SPSA1 protein levels were slightly higher in the *spsc* and *spsb* null mutants compared to wild-type plants (Fig. 2).

Leaf SPS activities

Null mutants of individual SPS genes had differential effects on the total activity of SPS measured in leaf extracts (Fig. 3). Loss of SPSA1 protein had the greatest effect on

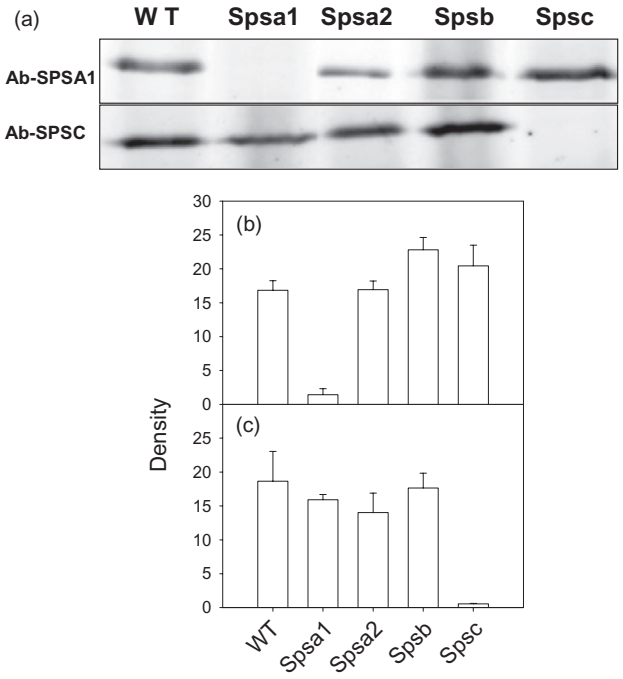

Figure 2. Western blot analysis for leaf SPSA1, SPSA2, SPSB and SPSC protein levels in wild-type and sucrose-phosphate synthase (SPS) null mutants using isoform-specific anti-peptide antibodies. Twenty micrograms of total protein was applied to each lane. (a) Typical Western blots. Antibodies specific for SPSA2 and SPSB did not cross-react with any proteins in leaf extracts and therefore are not shown. Densitometric analysis of relative levels of (b) SPSA1 protein and (c) SPSC protein. Values in (b) and (c) are means ± SE of three independent experiments.

SPS activity. In *spsa1* mutants, SPS V_{max} and V_{sel} decreased by 80 and 70%, respectively, and SPS activation increased by 50%. In contrast, mutation of *SPSC* only slightly affected SPS activity. In *spsc* mutants, SPS V_{max} and V_{sel} decreased by 13 and 6%, respectively, and SPS activation increased by 8%. Loss of SPSA2 and SPSB had no significant effect on SPS activity or activation. A second set of SPS mutants including *spsa1* (salk_099817c), *spsa2* (salk_017821c), *spsb* (salk_009524c) and *spsc* (salk_037958c) with different T-DNA insert sites were also determined for SPS maximum and limiting activities and showed similar results (Supporting Information Table S2) to those presented in Fig. 3.

Leaf non-structural carbohydrates under normal conditions and after cold stress

Leaf starch content and starch turnover were measured under normal growth conditions and after cold treatment of plants for 4 d (Fig. 4). Leaf starch levels and starch turnover were similar in wild-type and SPS mutants with the exception of *spsa1*. With the *spsa1* mutant, leaf starch levels at the end of the light period were increased by 67 and 48% and starch turnover was increased by 54 and 61% compared with wild-type plants under normal and cold-treated conditions, respectively. Leaf starch levels at the end of the night period were also slightly higher in *spsa1* than in wild type under normal growth conditions. Cold stress decreased starch turnover in all genotypes, and as a result, the starch content in leaves at the beginning of the photoperiod was clearly increased (Fig. 4).

Leaf sucrose, glucose and fructose were determined under normal growth conditions and after cold treatment for 4 d (Fig. 5). Leaf sucrose levels in the wild-type and SPS null mutants were generally similar under normal growth conditions. With all genotypes tested, there was some sucrose accumulation during the photoperiod, whereas glucose and fructose levels were much lower and did not change diurnally. After 4 d of cold acclimation, leaf sucrose contents were increased five- to 10-fold (Fig. 5d), and leaf hexose contents were increased 30- to 50-fold (Fig. 5e,f). In general, sucrose was the most abundant soluble sugar under normal growth conditions, whereas glucose was the most

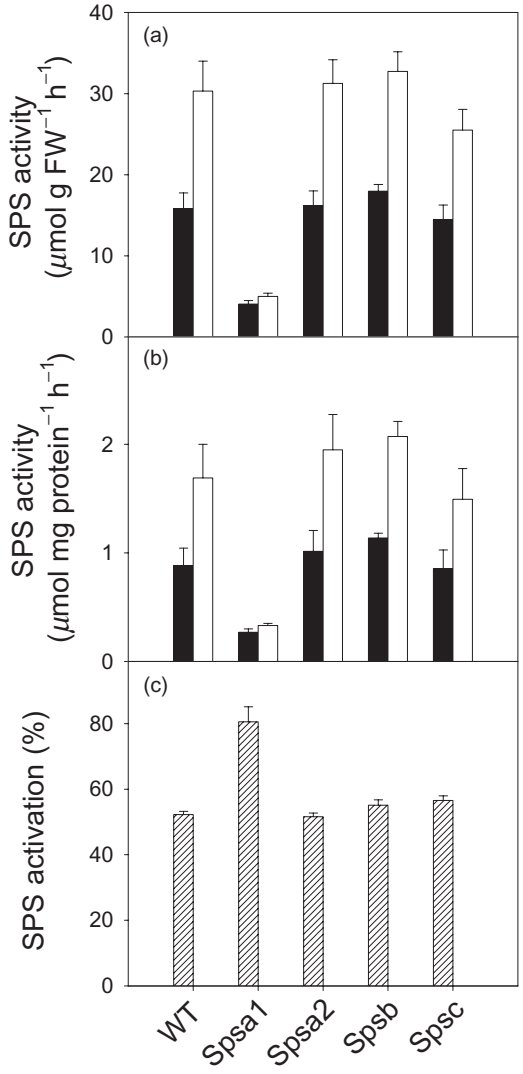

Figure 3. Leaf sucrose-phosphate synthase (SPS) activity measured with limiting (V_{sel}, black bar) and saturating substrates (V_{max}, white bar) expressed on a (a) fresh weight and (b) protein basis. (c) SPS activation is V_{sel} expressed as a percentage of V_{max} activity. Values are means ± SE of five independent experiments.

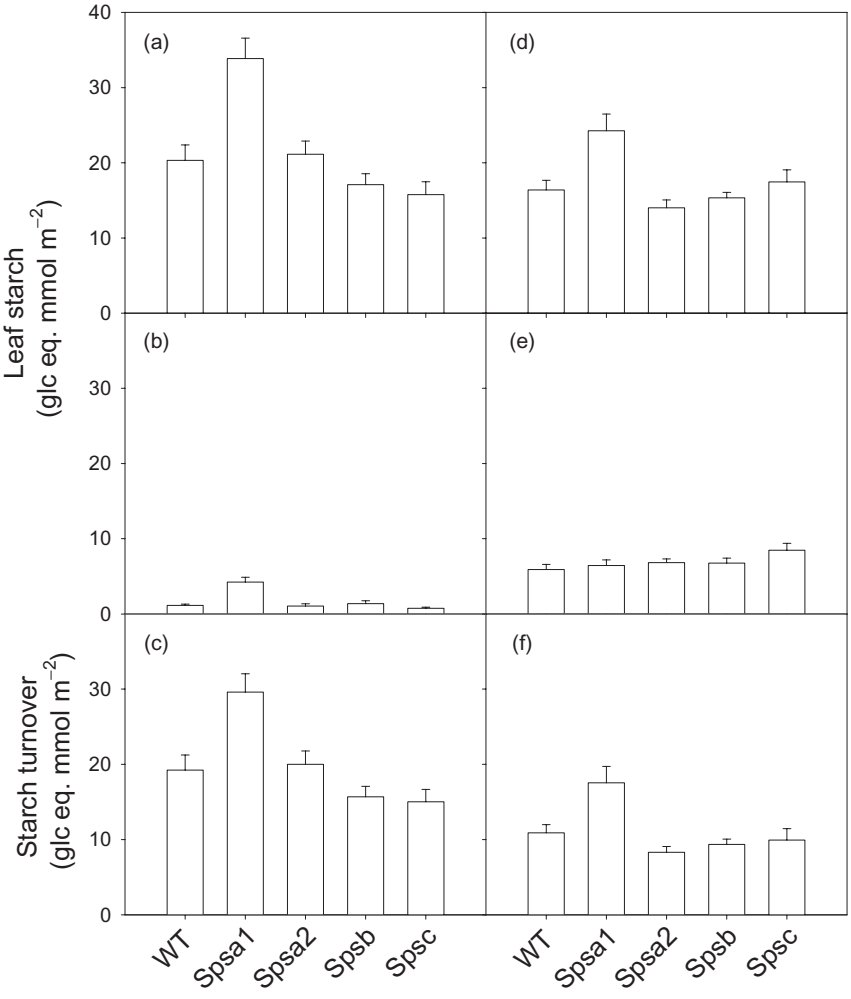

Figure 4. Leaf starch levels at the end of light period (a,d), at the end of night period (b,e) and calculated starch mobilization (c,f) under normal growth conditions (a–c) and after cold treatment (d–f). Values are means ± SE of eight independent experiments.

abundant form under cold-treated conditions. However, while there was variation among the genotypes tested, there were no other patterns to emerge for the soluble sugars.

It is well known that SPS protein and enzymatic activity are increased after exposure of plants to several days of cold temperature (Guy *et al*. 1992; Hurry *et al*. 1994). In the present study, cold treatment of wild-type plants increased the content of SPSA1 and SPSC protein two- to threefold relative to normal temperature control plants (data not shown). Similar increases were observed in all of the *sps* null mutants that contained *SPSA1* and *SPSC*. Importantly, SPSA2 and SPSB proteins were not detected by immunoblotting in any of the genotypes following cold treatment (data not shown).

Plant growth

Plant rosette growth of *spsa1* mutant plants in soil was indistinguishable from wild-type plants (Fig. 6). To investigate if sucrose availability affects plant growth, *spsa1* plants were grown on MS agar plates either with sucrose or without sucrose (Fig. 7). The rosette sizes of the *spsa1* null mutants were indistinguishable from wild-type plants either with or without sucrose (Fig. 7b). Similarly, the projected root areas of *spsa1* mutants in agar plates were indistinguishable from wild-type plants with sucrose but were slightly lower compared to wild-type plants in the absence of sucrose (Fig. 7a). Day and night growth rates of *spsa1* mutants were also investigated (Fig. 8). Root growth rates were generally higher during the day than during the night on agar plates either with or without sucrose. Growth rates of *spsa1* roots were indistinguishable from wild-type plants on agar plates with sucrose (Fig. 8a) but were slightly lower on agar plates without sucrose (Fig. 8b).

Gas exchange and Chl fluorescence

Light-saturated CO_2 assimilation rates (A_{sat}) were measured under ambient or saturating [CO_2] levels (Fig. 9a). A_{sat} values were similar among wild-type and SPS mutants under ambient [CO_2] levels. However, A_{sat} values were 22% lower in *spsa1* compared to wild-type plants under saturating [CO_2] levels. The change in A_{sat} was not due to stomatal limitation, and this was confirmed by measurement of stomatal conductances (g_s), which were similar among wild-type and SPS mutants (data not shown).

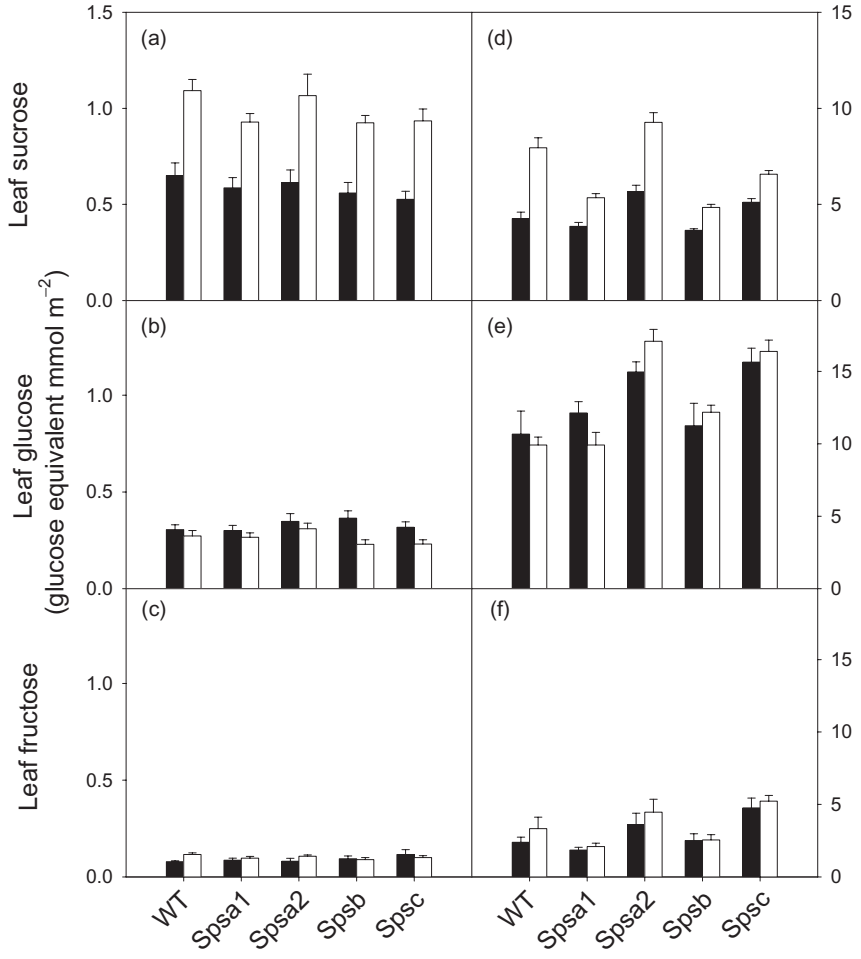

Figure 5. Leaf sucrose, glucose and fructose levels at the end of the night period (black bars) and at the end of light period (white bars) under normal growth conditions (a–c) and after cold treatment (d–f). Values are means ± SE of eight independent experiments.

Concurrent with gas exchange measurements, we also examined modulated Chl fluorescence and found that photochemical quenching (q_P; data not shown) and *ETR* values were similar among wild-type and the SPS null mutants under ambient [CO_2] levels (Fig. 9b). However, both parameters were significantly lower in *spsa1* than in wild-type plants under saturating [CO_2] (Fig. 9b). The q_P and *ETR* values were 20 and 21% lower in *spsa1*, respectively, compared to wild type under saturating CO_2 levels. As a result, q_P (not shown) and *ETR* (Fig. 9b) in *spsa1* were essentially unchanged under saturating versus ambient [CO_2].

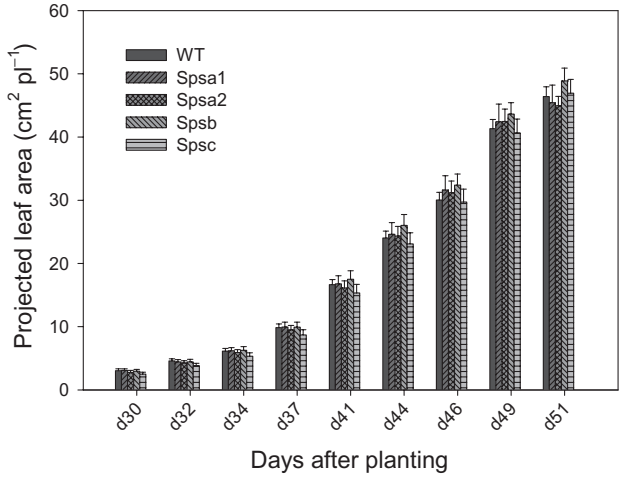

Figure 6. Plant growth in soil at ambient [CO_2] in wild-type (WT), *spsa1*, *spsa2*, *spsb* and *spsc* plants. Values are means ± SE of 10 independent experiments.

Light-saturated A/C_i curves for wild-type and *spsa1* null mutant plants are shown in Supporting Information Fig. S6. Based on the A/C_i curves, the values of maximum Rubisco carboxylation rates (V_{cmax}) and maximum *ETRs* (J_{max}) were obtained (see Materials and Methods). V_{cmax} values were similar among the wild-type and the SPS mutants (Fig. 9c), whereas J_{max} values were 18% lower in *spsa1* compared to wild type (Fig. 9d). J_{max} values in *spsa2*, *spsb* and *spsc* were similar to the wild type.

DISCUSSION

SPSA1 plays a major role in photosynthetic sucrose synthesis in *Arabidopsis*

Different from the previous study in tobacco (Chen *et al.* 2005), of the genes encoding SPS in *Arabidopsis*, *SPSA1* appears to play the major role in leaf sucrose synthesis. This

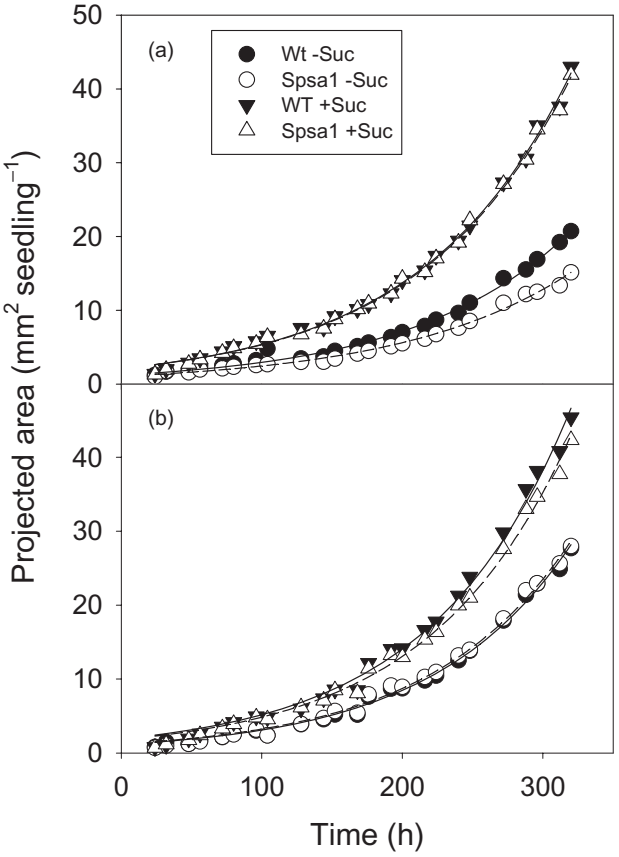

Figure 7. Plant growth on MS plate at ambient [CO_2] in wild-type (solid symbols and solid lines) and *spsa1* (open symbols and dashed lines) without sucrose (circle) and with sucrose (triangle). (a) Side view of vertical plates. (b) Top view of horizontal plates. Each point represents the average of 54 seedlings on three plates. Values are means ± SE.

is suggested by several lines of evidence. Firstly, leaf SPS activities were significantly lower in the *spsa1* mutant compared to the other null mutants (Fig. 3). The null mutation of *spsa1* led to an 80% reduction in SPS maximum activity, whereas the *spsc* null only resulted in a 13% reduction in SPS activity, and the other null mutations had no effect. Secondly, RuBP regeneration-limited photosynthesis was significantly lower only in the *spsa1* mutant, as J_{max} as well as A_{sat} and *ETR* under saturating [CO_2] and light levels were all lower in *spsa1* mutant compared to wild-type and the other SPS null mutants (Fig. 9 and Supporting Information Fig. S6). Thirdly, carbon partitioning into starch and sucrose was changed significantly only in *spsa1* and not in the other SPS null mutants. The ratio of starch to sucrose was twofold higher in *spsa1* compared to wild-type plants, and was basically unchanged in the other SPS null mutants (Figs 4 & 5). Fourthly, expression of *SPSA1* is higher than *SPSC*, which in turn is higher than *SPSA2* and *SPSB* in leaves as well as in many other tissues (Supporting Information Figs S2 & S3). Thus, our results suggest that while all four *SPS* genes are expressed in leaves, only SPSA1 and SPSC are expressed at the protein level to a significant extent. The leaf contents of SPSA2 and SPSB proteins were below detection by immunoblot analysis, despite the fact that the isoform-specific antibodies detected their respective antigen peptides with similar avidity (Supporting Information Fig. S1).

In general, gene expression was qualitatively correlated with relative protein content in leaves. *SPSA1* was expressed at the highest level among organs (Supporting Information Fig. S3) and across developmental stages (Supporting Information Fig. S2), and the SPSA1 protein

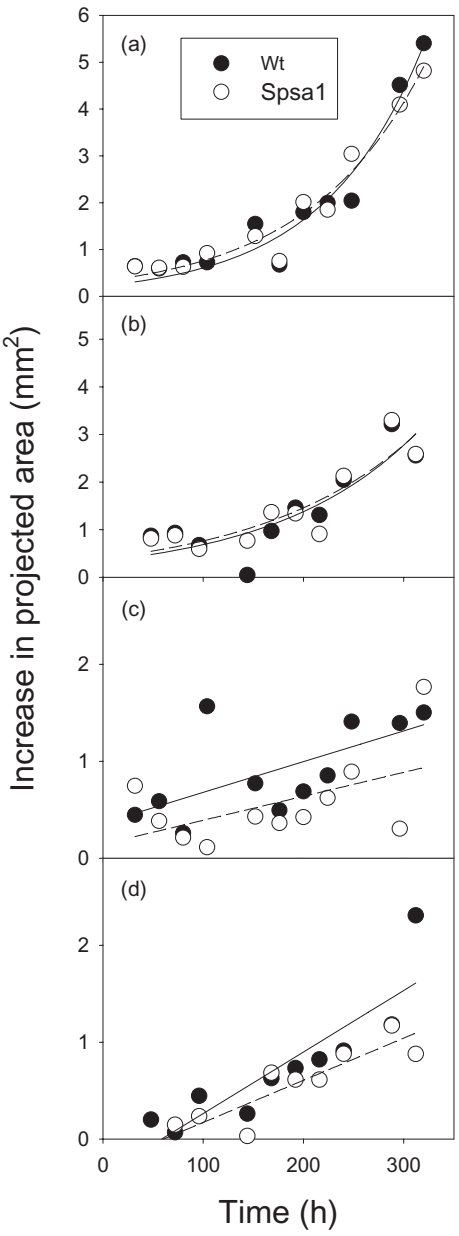

Figure 8. Plant root growth on MS agar plates at ambient [CO_2] in wild-type (solid circle and solid lines) and *spsa1* (open circle and dashed lines) in the presence of sucrose (a,b) and absence of sucrose (c,d) during the day (a,c) and during the night (b,d). Each point represents the average of 54 seedlings on three plates. Values are means ± SE.

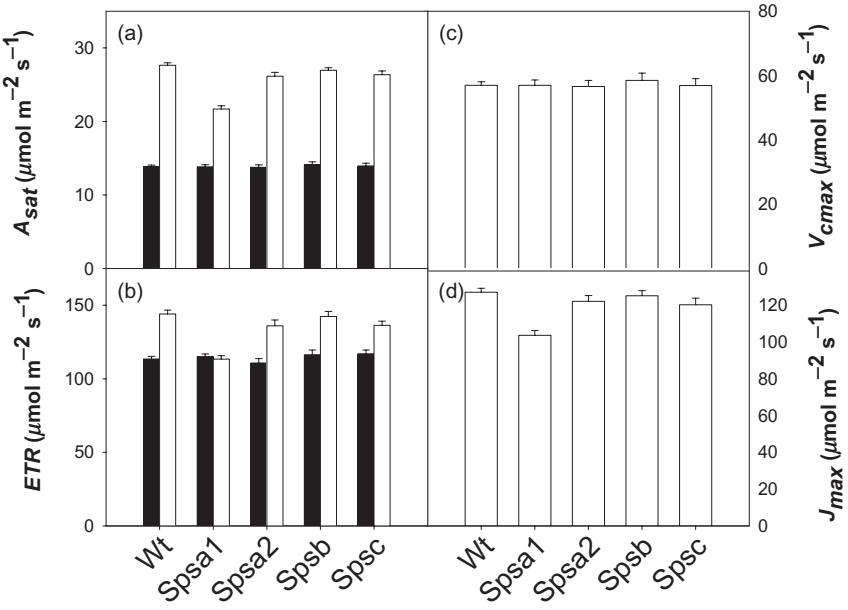

Figure 9. Leaf light saturated (a) CO_2 assimilation rate (A_{sat}) and (b) electron transport rate (*ETR*) measured at 400 ppm [CO_2] (black bar) and 800 ppm [CO_2] (white bar). (c) Leaf maximum Rubisco carboxylation rate (V_{cmax}). (d) Leaf maximum *ETR* (J_{max}). Replicates were 15.

was by far the major contributor to total SPS activity and sucrose biosynthesis in leaves. Interestingly, expression of *SPSA1* is also strongly diurnally regulated compared with other SPS genes (Supporting Information Fig. S4). This indicates that SPS, especially *SPSA1*, is regulated transcriptionally in addition to post-translationally (protein phosphorylation) as reported previously (Huber & Huber 1991, 1996; McMichael *et al*. 1993). The relative levels of SPS isoforms in the present study differ substantially from those reported previously for *Arabidopsis* Col-0 using mass spectrometry to identify and quantify isoforms (Lehmann *et al*. 2008). In their report, SPS4 and SPS5a (corresponding to SPSC and SPSA2, respectively, in our study) were the dominant isoforms in leaves in terms of protein amounts, and SPS5a (our SPSA2) was the isoform that increased most dramatically when plants were exposed to low temperature for 5 d. The reasons for these differences are not clear and will be the basis for future studies. In our studies, SPSA1 and SPSC both contributed to total leaf SPS activity but not equally, with SPSA1 being the major component. Future studies will be required to determine whether this reflects isoform abundance, inherent catalytic activity or isoform-specific post-translational modification. SPS isoforms are highly homologous and perform the same function, that is, sucrose synthesis, that is regulated by allosteric effectors (Doehlert & Huber 1983) and regulatory phosphorylation (McMichael *et al*. 1993), and while it is likely that isoform differences exist, they have not been explored.

There is one thing in common between *Arabidopsis* (this study) and tobacco (Chen *et al*. 2005), and that is that the most important SPS isoform for photosynthetic sucrose synthesis is the one that is expressed at highest levels in green tissues and is transcriptionally, strongly regulated diurnally. However, in contrast to our results with SPS null mutants, only minor differences were found among antisense *SPSA* and *SPSC* transgenics and the wild-type plants in terms of total SPS activities in tobacco (Chen *et al*. 2005), perhaps indicating that specific SPS genes were not silenced sufficiently. It is also possible that there are additional SPS family members that were not silenced and also contributed to sucrose synthesis in tobacco. Nonetheless, silencing of nicotiana tabacum SPS isoform C leads to a strong increase in tobacco leaf starch content, not because of increased partitioning of carbon into starch but, rather, as a result of reduced starch breakdown at night (Chen *et al*. 2005). Such an effect was not observed in the present study, where the spsc null mutant had leaf starch contents at the end of the day and night periods that were very similar to wild-type plants (Fig. 4). Thus, there is no evidence that SPSC in Arabidopsis plays a special role in sucrose synthesis during starch breakdown at night.

Decrease in leaf SPS activity leads to a decrease in TPU and RuBP regeneration-limited photosynthesis but not in Rubisco-limited photosynthesis

Rubisco-limited photosynthesis of the SPS null mutants was essentially unchanged relative to wild-type plants. Maximum rates of Rubisco carboxylation (V_{cmax}) were similar among SPS mutants and wild-type plants (Fig. 9). Light-saturated A_{sat} and *ETR* measured under ambient [CO_2] were also similar among SPS mutants and wild-type plants (Fig. 9). However, RuBP regeneration-limited photosynthesis was lower in the *spsa1* null mutant that had the lowest leaf SPS activity compared to wild-type plants. Correspondingly, maximum rates of electron transport (J_{max}) were significantly lower in *spsa1* mutants than in wild-type plants (Fig. 9). A_{sat} and *ETR* measured under elevated CO2 levels were significantly lower in *spsa1* mutants compared with wild-type plants (Fig. 9). Conceivably, the effects of SPS on leaf photosynthesis reported in the literature may

vary since photosynthesis alternates between Rubisco-limited and RuBP regeneration-limited conditions depending on the environmental parameters.

Sucrose synthesis is nearly saturated at ambient [CO_2] and high light, but starch synthesis continues to increase until CO_2 levels are saturating (Sun *et al*. 1999). Accordingly, low sucrose synthesis capacity cannot be fully compensated by increased starch synthesis under saturating [CO_2] and light. Under saturating CO_2 and light, the key enzymes SPS and ADP-glucose pyrophosphorylase for sucrose synthesis and starch synthesis, respectively, are maximally activated and sucrose synthesis can become the limiting factor for carbon assimilation. Triose-P is directed either into starch in the chloroplast or exported into the cytosol with exchange of P_i (Sharkey 1985; Stitt *et al*. 2010). Low sucrose synthesis limits triose-phosphate export out of chloroplasts and import of P_i into the chloroplast, thereby limiting RuBP regeneration and RuBP-controlled photosynthesis. The decreased RuBP regeneration-limited photosynthesis is clearly resulted from limited TPU due to insufficient sucrose synthesis.

Plant growth is plastic and can accommodate changes in carbon allocation between sucrose and starch

Shoot growth of the *spsa1* null mutant was indistinguishable from wild-type plants in either soil (Fig. 6) or on MS agar plates with or without sucrose (Fig. 7b) despite the fact that more carbon was being allocated to starch during the day and remobilized at night. These results are consistent with the notion that changes in carbon allocation can affect diurnal growth patterns without necessarily reducing the rate of growth (Kehr *et al*. 1998). Interestingly, the rate of root growth was reduced in the *spsa1* null mutant when grown on agar plates in the absence of sucrose and the effect was most pronounced for growth during the 8 h photoperiod (Fig. 8c) relative to the 16 h dark period (Fig. 8d). These responses are generally consistent with the notion that root growth of many species tends to occur during the photoperiod (Huber 1983).

Our results are also generally consistent with the previous report that overall vegetative growth at ambient [CO_2] was not enhanced with increased expression of SPS in tomato plants (Galtier *et al*. 1993), but differs from the report that plant growth was reduced by about 50% in antisense SPS *Arabidopsis* plants (Strand *et al*. 2000). The reduction in SPS activity in the antisense strategy was about 75%, which is roughly similar to the *spsa1* null mutant used in the present study. However, shoot growth was substantially reduced in one approach (i.e. antisense inhibition of SPS) but not the other (T-DNA insertional mutagenesis used in the present study). The molecular bases for these differences depending on the mechanism used to reduce SPS expression are not entirely apparent and will be interesting to explore in the future.

The consistent feature is that plant growth paralleled the rate of carbon assimilation under growth conditions [i.e. ambient (CO_2)], and was reduced in the antisense approach but not in the *spsa1* null mutants. In the antisense plants, the reduction in sucrose synthesis was not sufficiently compensated by increased starch synthesis, whereas that compensation occurred in the *spsa1* null mutants that allocated additional carbon into starch so that rates of CO_2 assimilation were similar to wild-type plants [at ambient (CO_2)].

In conclusion, analysis of individual null mutants (*spsa1*, *a2*, *b* and *c*) suggests that SPSA1 plays the major role in photosynthetic sucrose synthesis in *Arabidopsis* leaves, accounting for about 85% of the total leaf SPS activity. In the *spsa1* null mutant at ambient [CO_2], photosynthetic rate was unaffected but increased carbon was partitioned into starch during the day and was remobilized at night, so that plant growth was relatively unaffected. At saturating [CO_2], photosynthetic rate was reduced by ~20% as a result of decreased RuBP regeneration capacity due to limitation of TPU.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Figure S1. Isoform-specific antibodies detect their peptide antigens with similar reactivity on dot blots. These results suggest that the failure of the anti-SPSA2 and anti-SPSB antibodies to cross-react with their protein targets in leaf extracts is not caused by lack of recognition of the sequences by these antibodies.

Figure S2. Phylogenetic tree of SPS amino acid sequences from *Arabidopsis* and other plants (see Supporting Information Table S1 for the sequences for each numbers). The unrooted neighbourhood-joining tree was constructed from an alignment of glucosyltransferase domain regions of SPS genes using the Phylogeny Inference Package. The families are circled with solid lines and labelled as A, B, C or D. 'm' and 'd' designations indicate monocot and dicot subgroups that are circled with dash lines. Bootstrap values are underlined and in italics with 100 replicates.

Figure S3. Expression of SPS genes across developmental stages in wild-type *Arabidopsis* plants by microarray meta-profile analysis. Gene expression is set relative to the highest expression point of the whole dataset, and numbers in brackets are the numbers of microarrays used in the analysis. Source: Genevestigator V3 (Hruz *et al*. 2008).

Figure S4. Tissue-specific expression of SPS genes in *Arabidopsis* wild-type plants using microarray meta-profile analysis. The numbers in the brackets are the numbers of arrays. Source: Genevestigator V3 (Hruz *et al*. 2008).

Figure S5. Diurnal expression of SPS genes in *Arabidopsis* wild-type plants using microarray meta-profile analysis. Source: Genevestigator V3 (Hruz *et al*. 2008). 0 h, light off. 12 h, light on.

Figure S6. A/C_i curves for (a) wild type plants and (b) spsa1 null mutants.

Table S1. SPS sequences* and groups**. (*Sequence source: http://www.arabidopsis.org; http://www.phytozome.net; **See Fig. S2 for clusters)

Table S2. Second set of *sps* null mutants confirms that *SPSA1* is the major contributor to total leaf SPS activity.

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