Planta (2015) 242:1009–1024
DOI 10.1007/s00425-015-2342-0

ORIGINAL ARTICLE

Transgenic alfalfa (*Medicago sativa*) with increased sucrose phosphate synthase activity shows enhanced growth when grown under N_2-fixing conditions

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Received: 12 May 2015 / Accepted: 31 May 2015 / Published online: 9 June 2015

Abstract

Main conclusion **Overexpression of SPS in alfalfa is accompanied by early flowering, increased plant growth and an increase in elemental N and protein content when grown under N_2-fixing conditions.**

Sucrose phosphate synthase (SPS; EC 2.3.1.14) is the key enzyme in the synthesis of sucrose in plants. The outcome of overexpression of *SPS* in different plants using transgenic approaches has been quite varied, but the general consensus is that increased SPS activity is associated with the production of new sinks and increased sink strength. In legumes, the root nodule is a strong C sink and in this study our objective was to see how increasing SPS activity in a legume would affect nodule number and function. Here we have transformed alfalfa (*Medicago sativa*, cv. Regen SY), with a maize *SPS* gene driven by the constitutive *CaMV35S* promoter. Our results showed that overexpression of *SPS* in alfalfa, is accompanied by an increase in nodule number and mass and an overall increase in nitrogenase activity at the whole plant level. The nodules exhibited an increase in the level of key enzymes contributing to N assimilation including glutamine synthetase and asparagine synthetase. Moreover, the stems of the transformants showed higher level of the transport amino acids, Asx, indicating increased export of N from the nodules. The transformants exhibited a dramatic increase in growth both of the shoots and roots, and earlier flowering time, leading to increased yields. Moreover, the transformants showed an increase in elemental N and protein content. The overall conclusion is that increased SPS activity improves the N status and plant performance, suggesting that the availability of more C in the form of sucrose enhances N acquisition and assimilation in the nodules.

Keywords Carbon metabolism · Nitrogen assimilation · Sucrose phosphate synthase · *SPS* transgenic alfalfa · Plant growth · Nodule number · Flowering time

Abbreviations

SPS	Sucrose phosphate synthase
GS_1	Cytosolic glutamine synthetase
Suc	Sucrose
Glc	Glucose
Gln	Glutamine
NT	Non-transformed
SPS	Transformants alfalfa expressing *MzSPS*
3PG	Glyceride 3-phosphate
G6P	Glucose 6-phosphate
UDPGlc	Uridine diphosphate glucose
SucSy	Sucrose synthase
PEP	Phosphoenol pyruvate
α-KG	α Ketoglutarate
Asx	Asn/Asp
F6P	Fructose 6-phosphate
MDH	Malate dehydrogenase

Introduction

Sucrose phosphate synthase (SPS; EC 2.3.1.14) is the key enzyme in the synthesis of sucrose (Suc) in plants. It catalyzes the first committed reaction in the pathway of Suc

Electronic supplementary material The online version of this article (doi:10.1007/s00425-015-2342-0) contains supplementary material, which is available to authorized users.

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synthesis, producing sucrose-6-phosphate (Suc-6P) from fructose-6-phosphate and UDP-glucose. The immediate hydrolysis of Suc-6P to Suc and Pi, catalyzed by a specific Suc-6P phosphatase (SPP; EC 3.1.3.24), displaces the reversible SPS reaction from equilibrium in the direction of net Suc synthesis. Suc is the major stable product of photosynthesis that is transported from the photosynthetic tissues via the phloem into all heterotrophic tissues and is a source of energy and C metabolites (MacRae and Lunn 2006; Huber 2007; Stitt et al. 2010; Sun et al. 2011). Several groups have reported *SPS* expression in heterotophic sink tissues, such as flowers, potato tubers, cotton fibers and root nodules (Geigenberger et al. 1997; Babb and Haigler 2001; Chen et al. 2001; Im 2004; Aleman et al. 2010). It is likely that SPS activity modulates Suc cycling in heterotrophic cells to provide substrates for various metabolic functions while maintaining optimal Suc levels. Sucrose phosphate synthase is encoded by genes that are members of small multigene families. The gene members besides being regulated transcriptionally are also regulated at the posttranslational level through covalent modification and allosteric regulation via metabolites, glucose-6-phosphate (Glc-6P) and inorganic phosphate (Pi) (Huber and Huber 1996; Toroser and Huber 1997; Lunn and MacRae 2003; Huber 2007).

Besides its role as a substrate for metabolic requirements, Suc also acts as a signal molecule for regulation of gene expression (Smeekens 2000; Loreti et al. 2001; Weise et al. 2004; Rolland et al. 2006; Wind et al. 2010), and plays a role in both metabolism and development (Lunn and MacRae 2003; Eveland and Jackson 2012; Tognetti et al. 2013; Ruan 2014). Since Suc plays such a crucial role in the functioning of different processes that are key to plant growth (Grof et al. 2007; Tognetti et al. 2013; Ruan 2014), efforts have been made to increase SPS activity using transgenic approaches and the up-regulation of SPS activity has been shown to alter growth and development in many different plants (Micallef et al. 1995; Laporte et al. 1997; Nguyen-Quoc et al. 1999; Laporte et al. 1997, 2001; Nguyen-Quoc and Foyer 2001; Baxter et al. 2003; Haigler et al. 2007; Ishimaru et al. 2008; Park et al. 2008, 2009; Coleman et al. 2010; Tian et al. 2010; Seger et al. 2015; Maloney et al. 2015).

Alfalfa (*Medicago sativa*) is a perennial legume cultivated as an important forage crop that can establish a symbiotic N_2-fixing association with the bacteria *Sinorhizobium meliloti*, resulting in the formation of nodules (Ferguson et al. 2010; Oldroyd et al. 2011). The root nodule is the site where the differentiated bacteroids convert free N_2 into NH_4^+ that is then assimilated by host-encoded enzymes. Symbiotic N_2 fixation requires a complex metabolic interdependence between each symbiotic partner (Lodwig et al. 2003). The enzyme nitrogenase encoded by bacterial genes, catalyzes the ATP-dependent reduction of N_2 to NH_4^+ in the bacteroids harbored in the infected cells of the root nodules. The ammonium produced is assimilated in the cytosol of the infected cells via the host-encoded enzymes, glutamine synthetase (GS) and glutamate synthase (GOGAT) to produce glutamine (Gln) and glutamate (Glu) (Lea and Miflin 2010). The assimilation of NH_4^+ requires ATP, reducing power and empty C skeletons for loading up with the N groups derived from NH_4^+. Translocated Suc provides the C source that supports bacteroid growth and fuels N_2 fixation and NH_4^+ assimilation in the nodules (Baier et al. 2007). In fact, it is estimated that one-third of the total photosynthate in alfalfa is allocated for N_2-fixation and NH_4^+ assimilation (Maxwell et al. 1984).

There are no reports of overexpressing *SPS* in any leguminous plant but since the root nodule is a strong C sink, the question arises as to whether overexpression of *SPS* in a legume plant would affect nodulation and nodule function. Towards this goal, we transformed alfalfa with a *SPS* gene from maize (*Zea mays SPS1*; Worrell et al. 1991) driven by the *CaMV35S* promoter and several independent transformants were obtained. The transformants exhibited increased growth rate, an increase in biomass, early flowering and free soluble proteins. Furthermore, the transformants also showed an increase in nodulation, N_2-fixation with an overall increase in N content compared to non-transformed (NT) alfalfa plants.

Materials and methods

Gene manipulations

Standard procedures were used for all recombinant DNA manipulations. The *SPS* gene from *Z. mays* (*SPS-1*; NCBI accession number NM-001112224) was kindly provided by Dr. Christine Foyer (University of Leeds, UK). To make the *CaMV 35S*/Maize *SPS* construct (*MzSPS*), the *SPS* cDNA was amplified by PCR to include a *SacI* restriction site at the end of the coding region and cloned in the pCRBlunt vector (Life Science Technologies, Carlsbad, CA). The *SPS* cloned fragment was subsequently released with *Bam HI* and partial digestion with *Sac I*. It was then ligated between the *CaMV35S* promoter and *NOS* terminator of the binary plasmid pBI 121 (Jefferson et al. 1987) from which the *uidA* reporter gene had been removed.

Plant transformation and growth conditions

The plasmid with the *MzSPS* construct was mobilized into *Agrobacterium tumefaciens* strain GV3101 by a freeze thaw method as described (Sambrook et al. 2001). *A.*

tumefaciens-mediated plant transformation of alfalfa (*M. sativa* cv. Regen-SY) was carried out by the method developed by Bagga et al. (2004). All transformants and NT plants have an identical background, except for the presence and position of the transgene in the genome, since transformation was performed on the same clonal material. To ensure that the NT plants used in this study have faced the same tissue culture conditions as the transformants, they were obtained by regeneration and independent regenerants were used as the control NT sets. Independent transgenic lines were randomly selected, and along with NT plants, were transferred to pots containing vermiculite, and placed under greenhouse conditions. The primary sets of NT and transformed alfalfa plants were fed 0.5× Hoagland's nutrient solution. Since alfalfa is self incompatible, the plants were propagated by cuttings rather than seeds, Four independent transformants and 2–3 independent regenerants of NT plants, were used for all physiological and biochemical analysis. The transformants and NT plants were clonally propagated through cuttings. For each plant, 3–6 clonally propagated plants (as indicated for the different figures) were used for analysis. Cuttings were maintained with sterile 0.5× Hoagland's nutrient solution until the initiation of roots about 1–2 weeks after planting, at which point the plants were inoculated with *S. meliloti* strain 2011. Thirty-five to 50 days after inoculation, the plants were tested for physiological parameters and tissues were harvested for molecular and biochemical analysis. For diurnal studies, plant tissues were harvested at dusk (5:00 p.m.) and dawn (5:00 a.m.).

Measurement of growth parameters

The clonally propagated NT and transformed plants were grown in the greenhouse till the onset of flowering in the transformed plants and were cut down to the base and then allowed to grow again. This process was repeated twice and the measurements were then made at the 3rd cut. At the onset of flowering (~60 days for the transformants and ~85 days for the NT plants), the tops of the plants, for both the transformants and the NT plants were harvested for fresh and dry weight measurements. For dry weight the plant material was oven dried at 65 °C for 48 h.

RNA isolation and analysis

Total RNA was isolated from alfalfa tissues by LiCl precipitation (Ortega et al. 2006). RNA samples were fractionated on 1.3 % (w/v) agarose/formaldehyde gels, transferred to nylon membranes according to the manufacturer's instructions (BioRad, Hercules, CA, USA) and hybridized to ^{32}P labeled probes prepared from plasmid inserts labeled by random primer extension using the Prime-a-Gene system (Promega, Milwaukee, WI). A cDNA fragment of the *MzSPS* gene was used as a probe to monitor expression of the maize *SPS* transgene. To check for RNA loads, rRNA was stained with SYBR Gold (Molecular Probes, Eugene, OR, USA). Standard hybridization conditions were used (Ortega et al. 2006).

Protein analysis

Total soluble protein extraction for both western analysis and SPS activity determination were performed as described (Aleman et al. 2010; Seger et al. 2015). Proteins were analyzed by SDS-PAGE in 12 % acrylamide (w/v) gels using a Mini-PROTEAN electrophoresis apparatus (BioRad, Hercules, CA). The fractionated proteins from the gels were electroblotted onto Immobilon-P (Millipore, Bedford, MA). Detection of polypeptides was performed using polyclonal antibodies raised against maize SPS (Bioworld, Dublin, OH), sucrose phosphate phosphatase (SPP) (provided by Dr. Uwe Sonnewald; Institute für Biologie, Universität Erlangen-Nürnberg, Erlangen, Germany), Sucrose synthase (SucSy) (provided by Dr. Raymond Chollet; University of Nebraska, Lincon, NE), soybean GS_1 (Ortega et al. 2006), PEP carboxylase (PEPC), Asparagine synthetase (AS) and Malate dehydrogenase (MDH) (provided by Dr. Carol Vance, University of Minnesota, MN). Immunoreactive bands were visualized with an alkaline phosphatase-linked secondary antibody using nitroblue tetrazolium and 5-bromo-4-chloro-3-indoyl-phosphate as substrates. The immunoreactive bands were scanned by Canon scanner and stored as jpeg pictures. The pictures were individually imported to ImageJ software (Schneider et al. 2012) and the bands were quantified three times and averaged.

Enzyme activity

SPS activity was assayed by quantifying the fructosyl moiety of sucrose using the Anthrone test (Seger et al. 2015) and the activity is expressed as nmol Suc-P mg^{-1} protein min^{-1}. Total GS activity was measured using the transferase assay (Ferguson and Sims 1971) as described by Seger et al. (2015). Transferase units were calculated from a standard curve of γ-glutamyl hydroxamate. Activity is reported as μmole of γ-glutamy hydroxamate produced per mg of protein at 30 °C.

Carbohydrate analysis

To measure Suc content, tissue was ground in liquid N and homogenized in 25 volumes (w/v) of 80 % (v/v) ethanol. Samples were incubated at 70 °C for 90 min, followed by a

10 min centrifugation at 12,500 rpm. Five hundred μL of the supernatant was dried in a speed-vac concentrator, re-suspended in the same amount of deionized H_2O, and centrifuged again for 10 min at 12,500 rpm. A 250 μL aliquot of the solubilized fraction was diluted to 1 mL with water and 125 μL of the dilution was added to one volume of 30 % KOH, and incubated at 95 °C for 10 min, then centrifuged for 5 min. This step was included to destroy the hexoses. The supernatant was transferred to a new tube, 1 mL of Anthrone reagent was added to each reaction, and activity is expressed as nmol Suc-P mg^{-1} protein min^{-1}.

Starch determination was performed in the pellets from the ethanol extracts following removal of the supernatant for Suc determination as previously described (Aleman et al. 2010; Seger et al. 2015). The supernatant containing the Glc (C6 units) released by enzymatic hydrolysis was transferred to 1.5 mL tube and 50 mL of the supernatant was diluted to 250 μL with water, and 1 mL Anthrone reagent was added. The sample was boiled for 5 min, cooled on ice bath for additional 5 min, centrifuged briefly and the absorbance was measured at 625 nm. The starch content in each reaction was calculated against a standard curve of Glc.

Measurement of chlorophyll content

Chlorophyll was extracted from leaf tissues with 100 % acetone as described (Lichtenthaler 1987). Chlorophyll *a* and *b* content was determined spectrophotometrically at 661.8 and 644.8 nm. For spectrophotometric measurements, concentrations of total chlorophyll content were calculated using equations derived from predetermined extinction coefficients as follows:

$$\text{Chl } a = 11.24A_{661.6} - 2.04A_{644.8}$$

$$\text{Chl } b = 20.13A_{644.8} - 4.19A_{661.6}$$

$$\text{Chl } a + b = 7.05A_{661.6} + 18.09A_{644.8}$$

Measurement of leaf gas exchange (net photosynthetic rates)

Net photosynthetic rates (Pnet), measured as leaf gas exchange in μmol CO_2 m^{-2} s^{-1}, were determined at the same time of the day, generally between 8:00 and 11:00 a.m., on the most recent fully expanded alfalfa trifoliate using a conifer chamber with an external light source attached to an infrared gas analyzer-based photosynthesis system (Li-Cor 6400, Lincoln, NE). The plants were put under supplemental light provided by Sylvania metalarc lamps 1000 w (Orsam Sylvania, MA, USA) at least 30 min before measurements were taken. The Pnet measurements were taken at ambient CO_2 (400 μmol CO_2 mol^{-1}) concentration. Leaf areas of alfalfa trifoliates were quantified using a Li-Cor LI3000 portable area meter.

Amino acid analysis

Total amino acid analysis in leaf and stem tissues was performed at the Molecular Structure Facility, University of California, Davis. Tissue samples were soaked overnight in performic acid and the liquid-phase hydrolysis was performed in 6 N HC1/0.1 % phenol at 110 °C for 24 h; then the samples were dried and suspended in buffer containing norleucine.

Elemental carbon and nitrogen measurements

Total C and N content, as percent of dry weight, was determined at the Soil Genesis Laboratory in New Mexico State University. Oven dried at 65 °C leaf and root tissue harvested from alfalfa plants 50 days after inoculation with *S. meliloti* were ground to a fine powder. Approximately 0.060 and 1.5 mg of ground tissue were used for measuring C and N content, respectively, using an elemental analyzer (Eurovector, Milan, Italy) interfaced to an isotope ratio mass spectrometer (Isoprime, Manchester, UK).

Statistical analysis

The data were subjected to the unpaired student *t* test at levels of ($P \leq 0.01$ and $P \leq 0.05$). The values represent the mean ± SE and n represents the biological replicates.

Results

Alfalfa transformants with the *SPS* gene showed both the accumulation of the transgene product and increased SPS enzyme activity.

A maize *SPS* cDNA (Worrell et al. 1991) was engineered between the constitutive *CaMV35S* promoter and the *nopaline synthase* (NOS) terminator. The gene construct referred to as the *MzSPS* was introduced into alfalfa using *A. tumefaciens*-mediated transformation. Fifteen independent transformants were selected from individual explants and were tested positive by PCR selection (Data not shown). The transformants were also subjected to western blot analysis and as seen in Fig. S1, they showed accumulation of the transgene product. Four independent transformants were transferred to pots and grown in the greenhouse for further analysis.

To check for the functionality of the gene construct, RNA isolated from the leaves of the transformants and the nontransformed (NT) plants were subjected to northern blot hybridization using the *MzSPS* DNA fragment as a probe.

While all the transformants showed a hybridizing band, the NT plants did not, though the level of hybridization in the transformants showed plant-to-plant variation, most likely an attribute of position effect (Fig. 1a). For further analysis, protein extract from the leaves of the same set of plants was subjected to western blot analysis using the SPS antibodies. An immunoreactive band of ~130 kD was seen in all the lanes but the band intensity was much higher in the lanes with the extracts from the transformants compared to the NT plants (Fig. 1b). To check if increased accumulation of SPS protein translates to higher level of SPS enzyme activity, the same protein extract used for western blot analysis was used for measurement of SPS enzyme activity. The transformants showed higher SPS maximum activity rates compared to the NT plants. The activity level was in close correspondence with the accumulation of the transcript and the protein (Fig. 1c). Taken together, the results indicate that the *SPS* transformants express the transgene at both the transcript and protein level and they exhibit significantly higher SPS enzyme activity in their leaves compared to the NT plants.

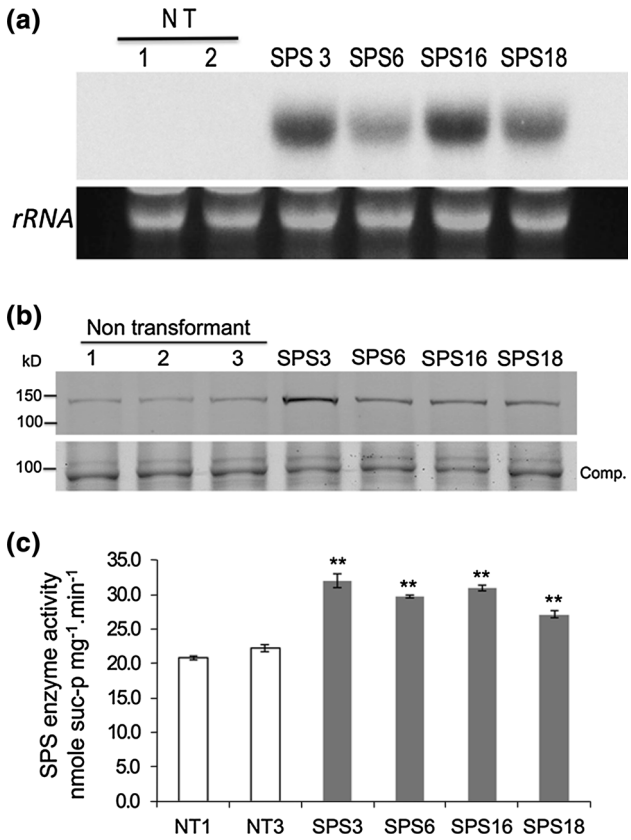

Fig. 1 Analysis of the functionality of the *SPS* gene construct in the SPS transformants. 4 randomly selected *SPS* transformants and nontransformed plants (NT) were clonally propagated and inoculated with *S. meliloti* and subjected to RNA, protein and enzyme activity analysis. **a** Northern blot analysis of RNA isolated from the leaves of the transformants and NT plants probed with a *MzSPS* DNA fragment. A measure of RNA loads is indicated by the EtBr stained lanes of rRNA. **b** Western blot analysis of proteins isolated from the leaves of the transformants and NT plants using MzSPS antibodies. Coomassie blue stained band for an endogenous protein is shown as standard for protein loads. Based on the migration of the MW markers, the position of the 150 kD and the 100 kD markers is indicated. **c** The leaf extract from the plants used for protein analysis was used for enzyme activity measurement by quantifying the synthesis of Suc-6P from UDP-Glc and Fru-6P. SPS enzyme activity values are plotted as nmol Suc-P mg^{-1} protein min^{-1}. Values are the mean ± SE of samples from three different plants ($n = 3$) for each class of transformants and NT plants. Significant differences from NT plants were evaluated by *t* test and shown by *asterisks* (*$P < 0.05$ or **$P < 0.01$)

The *SPS* transformants showed higher level of sucrose accumulation in the leaves

To check how increased SPS activity affects the diurnal accumulation of Suc and starch in the leaves, total Suc and starch content was measured in the leaves of the same *MzSPS* transformed and NT alfalfa plants as used for measurement of SPS activity, after light and dark periods. Clonally propagated plants were placed under greenhouse conditions and leaf tissues were harvested from one set of plants at dawn representing the dark period and from another clonal set at dusk, representing the light period on that same day.

In general, all the plants showed higher Suc and starch content in the leaves in the light compared to in the dark. The leaves of the transformants showed higher level of Suc compared to the NT plants. During the light period, the transformed plants exhibited approximately 55 % increase in leaf Suc content, while in the dark the increase was 40 % when compared with the NT plants (Fig. 2a). The starch content showed smaller difference between the transformants and the NT plants under both light and dark conditions (Fig. 2b). It is interesting to note that transformant #6 showed relatively higher Suc and starch content under both light and dark conditions. Clearly, the heterologous expression of the maize *SPS* transgene in alfalfa has resulted in enhanced carbon partitioning to Suc synthesis in the leaves.

The *SPS* transformants displayed higher chlorophyll content and higher photosynthetic rates

The leaves of the transformants showed a more bluish green color compared to the NT plants (Fig. 4b). The difference in leaf pigmentation could be accounted for by a change in the chlorophyll content and pigment analysis of the *SPS* transformants showed an increase in the levels of chlorophyll *a* and chlorophyll *b*, when compared to the NT plants (Fig. 3a). However, the change in chlorophyll content was not significant but nevertheless the total chlorophyll content showed an increase and the Chl *a*/Chl *b* ratio showed a drop in the transformants compared to the NT plants (Fig. 3b).

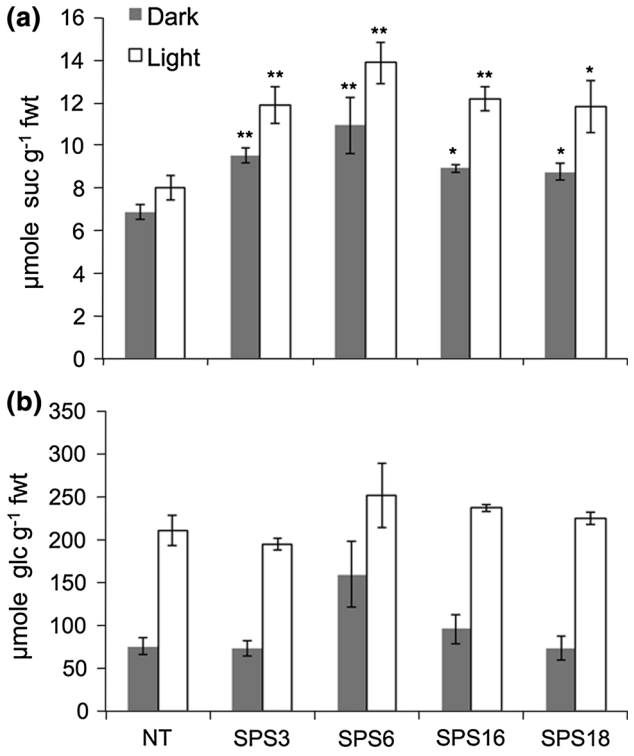

Fig. 2 Sucrose and starch determination in the leaves of the *SPS* transformants and NT plants. Leaf samples from the same set of NT and transformed plants as used for SPS activity were harvested at 5:00 a.m. (dark period) and 5:00 p.m. (light period) and were analyzed for sucrose **a** and starch content **b** as described in M&M. Sucrose content was plotted as µmole of Suc g^{-1} fwt and starch content was plotted as µmole of Glc g^{-1} fwt. Values are the mean ± SE ($n = 3$). Significant differences from NT were evaluated by *t* test and shown by *asterisks* (*$P < 0.05$ or **$P < 0.01$)

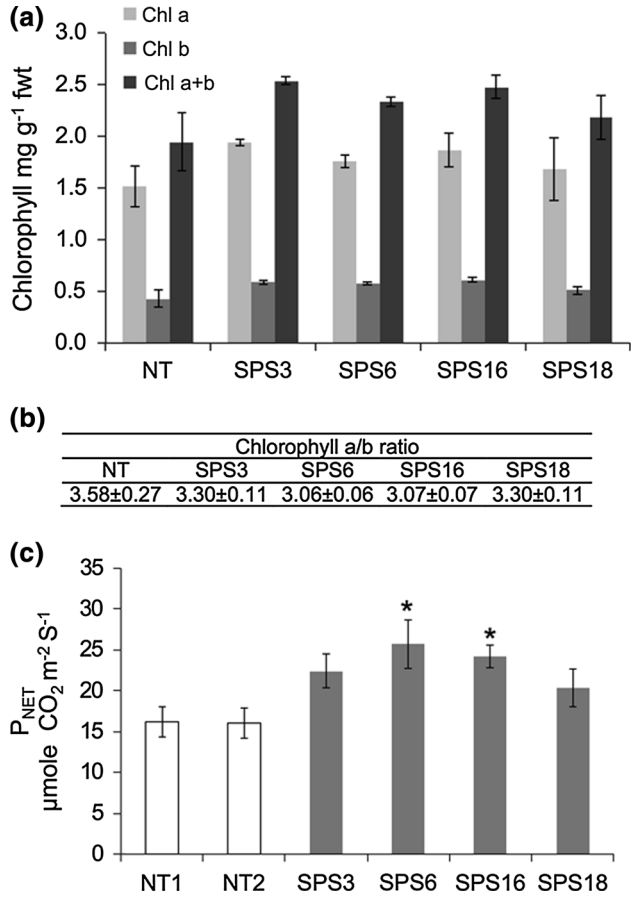

Fig. 3 Analysis of leaf chlorophyll content and photosynthetic rates in the *SPS* transformants and NT plants. **a** Chlorophyll was extracted in acetone from fresh leaf tissue harvested from greenhouse grown alfalfa plants. Chlorophyll *a* and *b* content was measured spectrophotometrically as described in M&M. Values of total chlorophyll, chlorophyll *a* and *b* are presented as mg g^{-1} fwt. Total chlorophyll represents the summation of chlorophyll *a* and *b*. Values are the mean ± SE ($n = 4$). **b** The chl *a*/*b* ratio was calculated from the data presented in panel (**a**). Values are the mean ± SE ($n = 4$). (**c**) Net photosynthetic rates (Pnet), measured as µmol CO_2 m^{-2} s^{-1}, was determined using a conifer chamber (6400-05) attached to a Li-Cor 6400 photosynthesis system. The chamber concentration was set to 400 µmol CO_2. Values are the mean ± SE ($n = 6$, two replicates of three plants). Significant differences from NT were evaluated by *t* test and shown by *asterisks* (*$P < 0.05$ or **$P < 0.01$)

The up-regulation of SPS activity in the source leaves is known to affect photosynthetic rates (Kaschuk et al. 2010). As such, net photosynthetic rates (Pnet) of greenhouse grown nodulated alfalfa (NT and *MzSPS* transformants) plants were measured under ambient (400 µmol mol^{-1}) CO_2 concentration. As seen in Fig. 3c, Pnet rates in the *MzSPS* transformed plants were higher than the rates in NT plants. It is important to point out that Chl *a*/*b* ratio showed an inverse correlation with the photosynthetic rates.

The *SPS* transformants exhibited increased growth rates and early flowering

Clonally propagated plants for each of the four transformants and the NT plants were inoculated with *S. meliloti*. Following growth for ~1 month, the plants were all cut down to the same height and then grown for a month (April to May), at which time they were photographed (Fig. 4a, b) and their heights measured (Fig. 4c). The transformants and the NT plants were then allowed to grow till the onset of flowering and the time of flowering was noted (Fig. 4d). For further confirmation of our premise that increased growth of the transformants is due to increased SPS level, additional transformants, were grown for a month and photographed (Fig. S2). These transformants were picked from those that were tested positive for the presence of SPS protein (Fig. S1). The transformants showed a dramatic increase in growth, and the plant height of the transformants at the time of onset of flowering was ~55 cm while the NT plants measured ~35 cm at the same time. Moreover, the transformants also showed increased branching, a big increase in the girth and an intense bluish green color (Fig. 4b). Onset of flowering for the transformants was between 30 and 35 days while the NT plants flowered around 50 days post-cutting. The

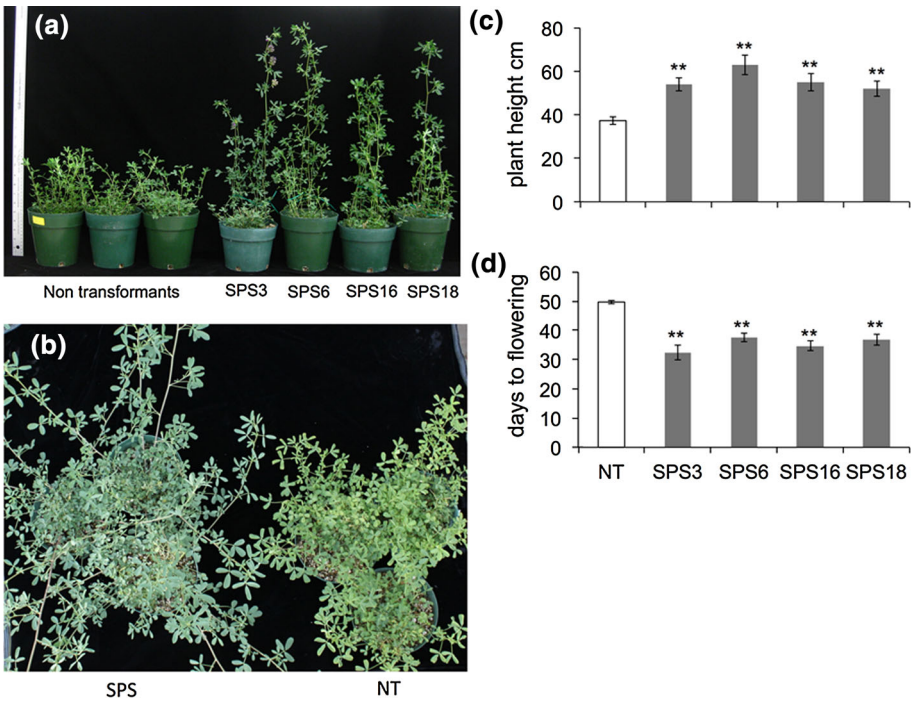

Fig. 4 Analysis of growth characteristics and flowering time in the transformants and NT plants. The inoculated plants were allowed to grow for ~1 month and then the plants were cut down to the base and were grown till the time of onset of flowering in the transformants. **a** Front view of the SPS and NT plants. **b** Top view of three transformants and three NT plants grouped together. **c** Height of the plants at the time of photographing was measured and plotted in centimeters. **d** The time for the onset of flowering was noted and plotted. Values are the mean ± standard error ($n = 6$). Significant differences from NT were evaluated by *t* test and shown by *asterisks* (*$P < 0.05$ or **$P < 0.01$)

process of cutting down the plants and letting them grow to the time of flowering was repeated four times and in each case the transformants exhibited faster growth and earlier flowering time,

For dry weight measurements, the plants were cut down to the base couple of times and all the measurements were done at the time of the third cut. As seen in Table S1, the transformants and the NT plants showed the same dry matter weight at the time of flowering, however, the transformants flowered 15–20 days (~65 days) earlier than the NT plants (~83 days). This would mean that over the same period of time, the biomass achieved by the transformants was ~30 % higher than the NT plants. The difference in the flowering time between the plants analyzed in Fig. 4 and Table S1, is that in the latter case, the plants were growing during the winter months (January to March) while the plants were growing during the warmer months (April to May). Low temperature and shortening of daylight are known to decrease top growth.

The *SPS* transformants showed an increase in root and shoot mass and higher nodule number and weight

50 days following inoculation, clonally propagated plants were uprooted and the roots and shoots were photographed and the fresh weight of both the shoot and root systems was measured (Fig. 5). The transformants when compared with the NT plants showed striking differences in growth (Fig. 5a) with an increase of between two- and threefold in the fresh weight of the entire plant (Fig. 5b) and of the shoots and roots separately (Fig. 5c, d). A similar trend in fresh weight of the plant parts was seen at 35 days post-inoculation (Data not shown). The plants used for the measurement of shoot and root weight, were also analyzed for the number of nodules per plant and their total weight at 35 days post-inoculation. Eight replicates, produced by cuttings, were used for nodule count and weight measurement (Fig. 5e, f). The transformants showed a ~twofold increase in both nodule number and weight.

The nodules of the *SPS* transformants exhibited higher SPS activity

Since the *MzSPS* gene is being driven by the constitutive *CaMV35S* promoter, we analyzed the nodules of the NT plants and the transformants (50 days post-inoculation) for SPS activity. The transformants exhibited much higher level (two to threefold) of activity compared to NT plants. Measurement of the carbohydrate content in the nodules, though not significant, showed an increase in both Suc and starch levels (Table 1).

The nodules of the *SPS* transformants showed changes in the levels of enzymes in C and N metabolism

To check if an increase in Suc levels in the leaves and nodules of the transformants has any effect on the expression of some key genes involved in C and N metabolic

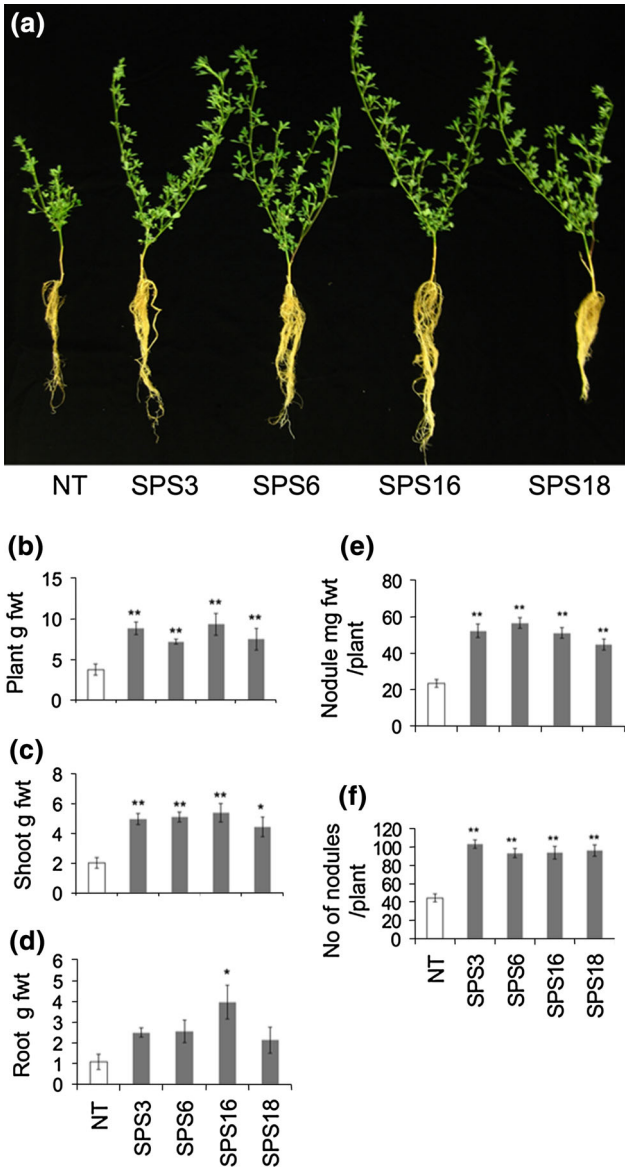

Fig. 5 Analysis of shoot, root and nodule characteristics in the *SPS* transformants and NT plants. Established transformants and NT plants were used to obtain shoots for propagation. Once established (~10 days after the start day), the cuttings were inoculated with *S. meliloti* and allowed to grow for a period of 50 days and then the plants were uprooted. The whole plant, the shoots and roots were weighed separately and the values were plotted as grams fresh weight. The nodules were harvested from the roots 35 days post-inoculation and counted and weighed. **a** Visual representation of SPS transformants and NT plants following uprooting. **b** Fresh weight of the whole plant. **c** Shoot fresh weight. **d** Root fresh weight. **e** Number of nodules per plant. **f** Fresh weight of nodules per plant. Values are the mean ± standard error ($n = 4$) for the shoots and roots and ($n = 8$) for the nodules. Significant differences from NT were evaluated by *t* test and shown by *asterisks* (*$P < 0.05$ or **$P < 0.01$)

pathways, we checked for steady state level of some of these enzymes using western blot analysis. These included sucrose synthase (SucSy), phosphoenolpyruvate carboxylase (PEPC), nodule-enhanced malate dehydrogenase (neMDH), cytosolic glutamine synthetase (GS_1) and asparagine synthetase (AS). Total soluble protein, extracted from the leaves and nodules of the two classes of plants, was subjected to SDS-PAGE and western blot analysis using antibodies to the aforementioned proteins. The leaf samples did not show any visible difference in the levels of the different proteins analyzed (Data not shown).

Protein extracts from the root nodules of the NT plants and the 4 different classes of transformants, harvested at 35 and 50 days post-inoculation were subjected to western blot analysis with the different antibodies listed above. The intensity of the immunoreactive bands for each antibody was scanned and the intensity was standardized against the Coomassie-stained bands used as a reference for protein loads (Fig. 6b). The values for the intensity of the immunoreactive bands for each protein in all the transformants taken together was compared with the values obtained for the NT plants grouped together. As seen in Fig. 6b, c, the nodules of the transformants, showed an appreciable increase in the level of all the proteins analyzed. The western profile was similar for the nodules at two stages, 35 and 50 days post-inoculation and here we are presenting the data obtained with the 35 days old nodules. The protein extracts were also analyzed for SPS protein using western blotting with the antibodies against MzSPS protein. While nodules from both the NT plants and the transformants showed a co-migrating immunoreactive band with the SPS antibodies, the level was substantially higher in the transformants. A higher MW immunoreactive band was seen in the lanes with the nodule extract from NT plants that was not very apparent in the transformants. We rule out the possibility of this band being an artifact, since this band was seen in repeated experiments with different nodule extracts.

An increase in the availability of C skeletons resulting from increased PEPC level and an increase in GS level, prompted us to measure GS enzyme activity in the protein extracts of nodules used for western blot analysis. As seen in Fig. 7, the transformants exhibited significantly higher GS activity in the nodules compared to the NT plants.

Taken together, the data here suggests that the level of key enzymes involved in N metabolism is higher in the nodules of the transformants.

The stems of the transformants showed a significant increase in the level of Asx

The stem and leaves of the transformants and the NT plants were analyzed for amino acid composition. As seen in Tables 2 and 3, the transformants showed a significant increase (20 %) in Asx in the stem and an overall increase in all the amino acids in the leaves.

Table 1 SPS activity and carbohydrate content in the nodules of NT plants and the transformants

Genotype	SPS activity (nmole suc-P mg^{-1} min^{-1})	Sucrose (μmole suc g^{-1} fwt)	Starch (μmole glc g^{-1} fwt)
NT	7.99 ± 1.75	14.68 ± 1.14	21.35 ± 7.37
SPS3	25.70 ± 2.61**	17.41 ± 4.27	31.61 ± 4.95
SPS6	28.74 ± 2.84**	24.12 ± 9.96	43.77 ± 7.11
SPS16	31.00 ± 5.63*	29.54 ± 2.49	43.31 ± 8.68
SPS18	21.39 ± 4.64	19.56 ± 4.92	39.18 ± 4.00

SPS enzyme activity and carbohydrate content (Suc and starch) were measured in the nodule extracts from the NT and transformed plants as described in M&M. Enzyme activity measurement was done by quantifying the synthesis of Suc-6P from UDP-Glc and Fru-6P and the activity values are plotted as nmol Suc-P mg^{-1} protein min^{-1}. Sucrose content was plotted as μmole of Suc g^{-1} fwt and starch content was plotted as μmole of Glc g^{-1} fwt. Values are the mean ± standard error ($n = 3$). Significant differences from NT were evaluated by *t* test and shown by asterisks (* $P < 0.05$ or ** $P < 0.01$)

The *SPS* transformants showed an increase in elemental N and protein content

For further analysis of the N status of the transformants, leaves and roots (without the nodules) of NT plants and the *SPS* transformants, were subjected to elemental N, C and protein (total and soluble) measurements. The total nitrogen and protein content is increased by 20 % in the leaves, while the increase was very slight in the roots. The C content showed no difference between the NT and transformed plants. The *SPS* transformants showed an overall higher level of protein based on estimation from elemental N content (6.25 × N) and measurement of total protein in the leaf extracts. Soluble protein content, measured as mg per unit fresh weight of tissue, also showed a 20 % increase, the same value obtained based on the elemental N content in the leaves of the transformants (Table 4).

Discussion

The data presented here shows that the constitutive expression of a maize *SPS* gene in alfalfa is accompanied by a dramatic increase in plant growth and biomass and early flowering, traits that would have a great impact on agriculture, considering alfalfa is a forage crop. While different plants have shown distinct responses to increased SPS activity in the leaves, the outcome in each case is associated with the generation of more sinks and increased sink strength (Laporte et al. 1997; Nguyen-Quoc and Foyer 2001; Haigler et al. 2007; Coleman et al. 2010; Tian et al. 2010; Seger et al. 2015). In this study, we see a substantial increase in both shoot and root biomass, along with an increase in the number and size of the root nodules in the *MzSPS* transformants, when compared to the NT plants (Fig. 5e, f). This could be an attribute of the increased synthesis of Suc in the photosynthetic tissues.

The effect of up-regulating SPS activity in alfalfa has a positive influence on photosynthesis, as the transformed plants consistently exhibited significantly higher photosynthetic rates when compared to the NT plants. These findings are consistent with other reports in the literature (Galtier et al. 1995; Signora et al. 1998; Ono et al. 1999, 2003; Baxter et al. 2003; Strand et al. 2003; Lundmark et al. 2006; Ramon et al. 2008; Stitt et al. 2010). The leaves of the transformants showed an intense bluish green color accompanied by an increase in chlorophyll (Chl) content with a more significant increase in Chl *b* level resulting in a significant drop in the Chl *a*:*b* ratio. The Chl *a*:*b* ratio serves as an indicator of N availability (Hikosaka and Terashima 1995; Kitajima and Hogan 2003) and a lower Chl *a*:*b* ratio in the leaves of the *MzSPS* transformants would imply a high LHCII to PSII ratio. Chloroplasts enriched with LHCII (higher Chl *b* to Chl *a*) have been suggested to better maintain the energy balance between PSII and PSI (Bjorkman and Demmig-Adams 1994), thus contributing to the enhancement of photosynthetic rates in the transformants.

The Suc and starch concentration were higher after the light period than after the dark period, with the starch levels being as much as threefold higher, supporting the idea that starch is typically degraded during the dark period to provide substrates for continued synthesis and export of Suc to sustain nocturnal growth and metabolism (Zeeman et al. 2010; Geigenberger 2011). This is in agreement with earlier reports (Micallef et al. 1995; Laporte et al. 1997, 2001; Nguyen-Quoc et al. 1999; Baxter et al. 2003; Haigler et al. 2007; Ishimaru et al. 2008; Park et al. 2008, 2009). The transformants, in comparison to the NT plants, showed significantly higher level of Suc in the leaves, both after light and dark periods. However, with regards to the starch content, the *SPS* transformants showed a small but not significant increase in the leaves compared to the NT plants under both light and dark conditions. An increase in the

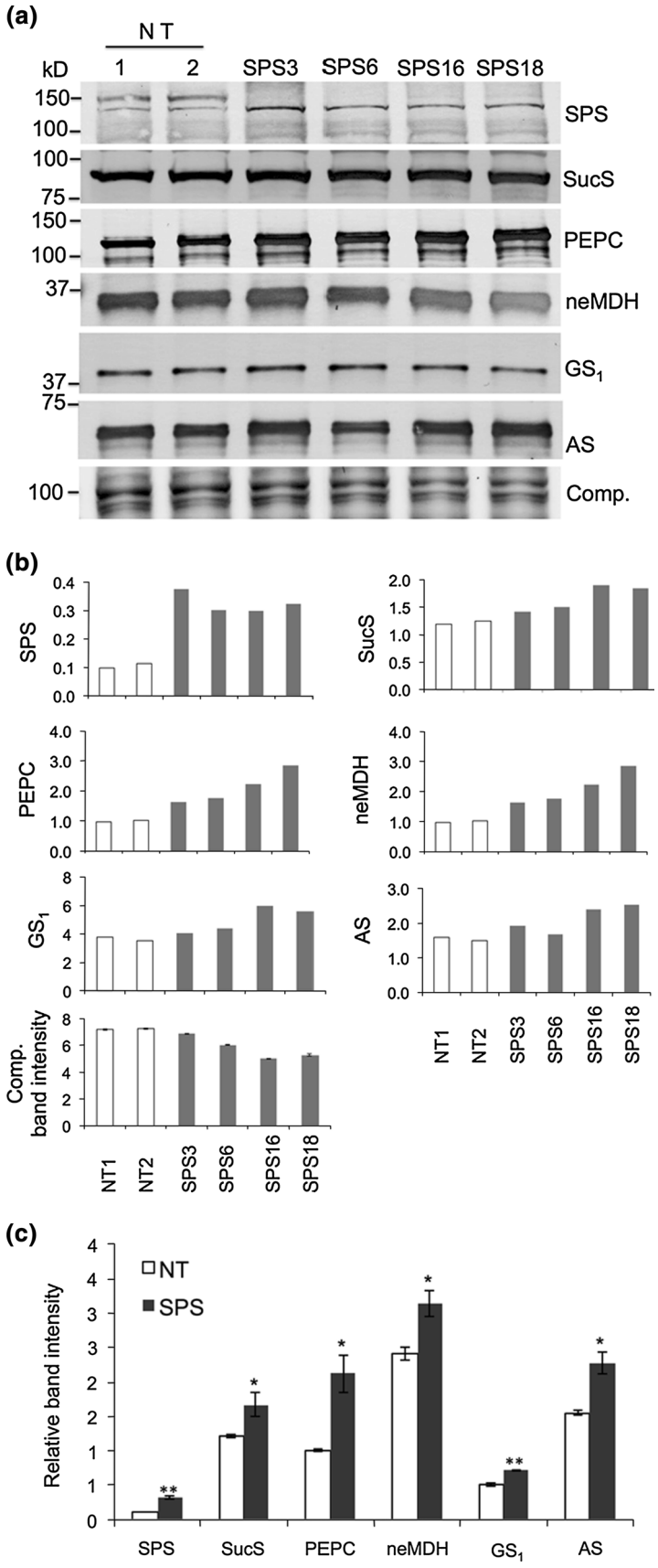

Fig. 6 Examination of the steady state level of key enzymes in C and N metabolic pathways in the nodules of SPS transformants and NT plants. **a** Protein extracts from the nodules were subjected to SDS-PAGE followed by western blot analysis using the different antibodies (as indicated). A Coomassie blue stained (Comp) region of the protein gel is shown here as a reference for protein loads. The MW in kD indicated for each panel were based on the migration of known molecular weight markers. **b** The immunoreactive bands were quantified and the band intensity was normalized to the Coomassie-stained endogenous protein band (Comp). Values are the mean ± standard error ($n = 3$). **c** Average relative band intensity of NT plants and the transformants. Values are the mean ± standard error, ($n = 2$) for NT and ($n = 4$) for the transformant. Significant differences from NT were evaluated by *t* test and shown by *asterisks* (*$P < 0.05$ or **$P < 0.01$)

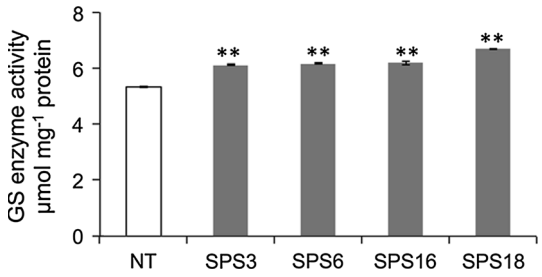

Fig. 7 Analysis of GS activity in the nodules of the transformants and NT plants. Nodule protein extract used for the western blot analysis (Fig. 6) was used to measure GS transferase activity; activity values are plotted as µmole γ-glutamyl hydroxamate produced per minute per mg of protein at 30C. Significant differences from NT plants were evaluated by *t* test and shown by *asterisks* (*$P < 0.05$ or **$P < 0.01$)

Suc level in the transformants compared to the NT plants can be attributed to higher SPS activity. These findings agree with previous observations in *MzSPS* expressing Arabidopsis, tomato and tobacco plants (Baxter et al. 2003; Galtier et al. 1995; Signora et al. 1998; Seger et al. 2015) and other transformed plants expressing different heterologous *SPS* genes (Micallef et al. 1995; Park et al. 2008, 2009; Haigler et al. 2007).

Sucrose is a dominant regulator of growth processes in plants (Lastdrager et al. 2014) and trehalose-6-phosphate (T6P) that is derived from Suc has been shown to be essential for plant growth (Yadav et al. 2014). Since auxin is central to plant growth and development, the rapid growth rates in the transformants would imply that the auxin metabolic pathway is activated. In fact, Suc is known to induce auxin levels (Lilley et al. 2012; Sairanen et al. 2012) and also induces auxin transport and signal transduction in *Arabidopsis* (Stokes et al. 2013).

Early flowering in the transformants could be in response to increased Suc levels in the phloem as suggested by Corbesier et al. (1998, 2002). Sucrose is the most extensively studied compound that might participate in long-distance signaling for flowering. Sucrose seems to interact in many ways with the flowering network. Again, as has been postulated for growth, T6P rather than Suc may act as the signal for flowering time (Wahl et al. 2013; Wingler et al. 2012). It can be speculated that the T6P signal is integrated into the miR156/SPL node of the floral induction pathway (Matsoukas et al. 2012). Since Suc has a role both in promoting growth and in the transition from the vegetative phase to the reproductive phase, we could propose that the pathways involved in both growth and flowering times are interconnected or that both share the same pathway controlled by Suc as a signal.

The *MzSPS* transformed alfalfa plants showed a substantial increase in the number of nodules. The regulatory pathway that controls the number of nodules in the roots has been shown to be mediated by signal molecules and phytohormones (Ferguson et al. 2010). The same signal molecules also interact or crosstalk with sugar pathways in the

Table 2 Total amino acid content in the stem of NT (NT1, NT2, NT3) and transgenic (SPS6, SPS16, SPS18) alfalfa plants

Amino acid	nmole mg^{-1} fwt Stem								% Increase
	Non-transformants			Ave	SPS transformants			Ave	
	NT1	NT2	NT3		SPS6	SPS16	SPS18		
Asx	40.30	37.60	33.06	36.99	41.53	46.40	44.58	44.17	19.42
Thr	14.97	15.39	12.67	14.34	13.63	15.26	16.35	15.08	5.14
Ser	18.00	18.61	15.31	17.30	16.13	18.23	19.68	18.01	4.10
Glx	25.21	25.05	21.24	23.83	23.45	25.60	27.33	25.46	6.83
Pro	16.61	17.70	13.32	15.87	14.13	16.17	19.80	16.70	5.21
Gly	24.53	25.02	21.06	23.54	22.34	24.86	28.14	25.11	6.70
Ala	22.95	23.64	19.68	22.09	21.24	23.37	25.83	23.48	6.28
Val	17.01	17.09	14.45	16.18	15.71	17.23	18.78	17.24	6.53
Ile	12.56	12.63	10.81	12.00	11.76	12.83	13.89	12.83	6.91
Leu	21.77	21.94	18.37	20.69	20.34	22.11	24.21	22.22	7.39
Tyr	7.36	6.30	5.97	6.54	6.55	7.31	6.93	6.93	5.93
Phe	11.81	11.67	10.10	11.20	11.63	12.29	13.05	12.32	10.07
His	5.44	5.75	4.93	5.37	5.11	5.77	6.24	5.71	6.16
Lys	17.54	18.36	15.55	17.15	16.24	18.06	19.26	17.85	4.10
Arg	11.04	10.21	9.15	10.14	10.08	11.34	10.95	10.79	6.47
Total	266.91	266.97	225.61	253.16	250.53	276.86	294.90	274.09	8.27

Data are the mean values in nmole mg^{-1} fwt ± SE ($n = 4$)

Asx a mixture of Asp/Asn, *Glx* a mixture of Glu/Gln

Table 3 Total amino acid content in the leaves of NT (NT1, NT2, NT3) and transgenic (SPS6, SPS16, SPS18) alfalfa plants

Amino acid	nmole mg^{-1} fwt Leaf								% Increase
	Non-transformants			Ave	SPS transformants			Ave	
	NT1	NT2	NT3		SPS6	SPS16	SPS18		
Asx	99.86	98.10	84.85	94.27	101.46	114.69	104.40	106.85	13.34
Thr	48.83	53.30	43.92	48.68	53.46	55.36	52.98	53.93	10.78
Ser	45.38	51.35	42.57	46.43	52.92	54.59	51.36	52.96	14.05
Glx	83.66	90.70	73.70	82.68	89.26	94.98	93.60	92.61	12.01
Pro	46.90	50.65	42.45	46.67	50.97	53.18	55.86	53.34	14.29
Gly	81.38	86.10	71.32	79.60	85.48	88.96	88.74	87.73	10.21
Ala	79.66	84.78	69.68	78.04	84.00	86.78	87.48	86.09	10.32
Val	55.72	59.38	48.45	54.52	58.99	61.50	60.48	60.32	10.65
Ile	41.03	43.40	36.57	40.33	43.75	45.50	45.42	44.89	11.30
Leu	76.14	80.34	66.91	74.46	80.83	83.52	84.00	82.78	11.18
Tyr	25.86	27.58	22.58	25.34	26.49	27.65	26.28	26.81	5.78
Phe	40.14	42.47	34.92	39.18	42.13	43.20	43.32	42.88	9.47
His	16.41	17.22	14.49	16.04	17.33	18.05	17.52	17.63	9.91
Lys	51.03	53.22	45.34	49.86	54.13	57.60	56.16	55.96	12.23
Arg	40.28	42.55	34.70	39.17	41.33	43.71	41.88	42.31	8.00
Total	832.41	881.30	732.45	815.39	882.47	929.28	909.60	907.12	11.25

Data are the mean values in nmole mg^{-1} fwt ± SE ($n = 4$)

Asx a mixture of Asp/Asn, *Glx* a mixture of Glu/Gln

regulation of many other pathways (Hammond and White 2008; Engels et al. 2011). Hence, we speculate that cross talk between sugar and phytohormone pathways may be responsible for the increase in nodule number in the *MzSPS* transformed alfalfa. These effects may, however, be attributed to the increased ability to modify sink capacity in response to higher C availability since the availability of photosynthate has been shown to play a role in the control of nodule number (Francisco and Harper 1995; Novak 2010).

The root nodule is not only a C sink, but is also the source of fixed nitrogen, making this a unique organ (White et al. 2007). Sucrose synthase (SucSy) activity is positively correlated with sink strength in several plant species (D'Aoust et al. 1999; Tang and Sturm 1999; Zrenner et al. 1995; Coleman et al. 2009) and the enzyme is vital for proper nodule function (Baier et al. 2007). The root nodules of the *SPS* transformants showed an increase in the level of SucSy suggesting an increase in the transport of Suc from the leaves. While the exact consequence of increased SPS activity in the nodules of the transformants is not known, one could postulate that increased SPS activity along with increased SucSy could enhance the cycling of the two processes, turnover and synthesis of Suc. This cycling process is needed to produce important metabolites for nitrogen metabolism and for the synthesis of other macromolecules like cellulose and starch and for regulating the expression of genes needed for proper functioning of the nodules.

The products of Suc hydrolysis are metabolized by glycolytic enzymes to generate phosphoenolpyruvate (PEP). Subsequently, phosphoenolpyruvate carboxylase (PEPC) catalyzes the conversion of PEP and bicarbonate to oxaloacetate (OAA) that is converted to malate by MDH. Malate is the primary source of C and energy for the bacteroids (White et al. 2007). These compounds are also used to produce various organic acids via the TCA cycle, particularly 2-oxoglutarate, which provides the C skeletons for the NH_4^+ assimilatory pathway (Lancien et al. 2000). The increase in the level of the key enzymes in C and N metabolism, like PEP carboxylase (PEPC) that we have observed in the nodules, would suggest increased availability of C skeletons. PEPC enzyme is also allosterically regulated by reversible phosphorylation and by metabolic effectors (O'Leary et al. 2011) and the level of accumulation and phosphorylation status of PEPC is influenced by the supply of sugars in a wide range of tissues, which include the root nodules (O'Leary et al. 2011). An increase in PEPC along with an increase in GS activity would imply increased assimilation of fixed N. Moreover, an increase in AS level would imply conversion of Gln to Asn. Because of its high N/C ratio, Asn would be a more efficient N transport compound. An increase in neMDH (nodule-enhanced MDH) in the nodules would mean that there is more malate for the functioning of the bacteroids that in turn can positively impact nitrogenase activity. While, we did not measure nitrogenase activity based on nodule

number or weight, nitrogenase activity at the whole plant level was significantly higher for the transformants compared to NT plants (Data not shown). The fact that the stems of the transformants showed significant increase in the level of Asp/Asn, would lend credence to an increase in NH_4^+ assimilation in the nodules of the transformants (Table 2). Further support for increased acquisition of N and its assimilation in the nodules comes from the fact that there is a 20 % increase in the level of elemental N in the leaves of the transformants (Table 4). The elemental C content, however, remained the same as in the NT plants, suggesting that most of the leaf C in the transformants is probably transported out into sink tissues. The increase in elemental N in the SPS transformants over the NT plants would suggest that C is limiting in the alfalfa plants for optimal levels of N_2-fixation and ammonia assimilation in the nodules.

Besides the nodules, ammonia assimilation also takes place in the leaves. Thus, higher levels of Suc produced in the leaves could also have a positive effect on the assimilation of ammonia in the leaves and could contribute to increased growth (Fig. 8). However, no increase in GS activity was seen in the leaves of the transformants.

Based on all the findings in this study, we propose a model to demonstrate how overexpression of *SPS* can improve nodule number and nodule function and thus the N status of the alfalfa plants (Fig. 8). An increase in SPS activity in the leaves of the *SPS* transformants leads to increased Suc synthesis in the leaves followed by its export into the nodules where it acts both as a signaling molecule and as a key C metabolite. SPS activity is increased in the nodules due to the constitutive expression of the *SPS* transgene and SucSy levels are elevated in response to increased Suc import. Increased SucSy and SPS activities in the nodules allows for more efficient cycling of synthesis and breakdown of Suc, thus increasing the level of the hexoses while maintaining Suc levels for the purpose of signaling. The hexoses function as the substrates for the synthesis of starch and cellulose, and for providing energy and C skeletons while Suc functions to up-regulate the expression of genes (*PEPC*, *MDH*, *GS* and *AS*) that have key roles for N_2-fixation and assimilation. A result of increased acquisition of N_2 and its assimilation in the nodules leads to increased export of assimilated N to the aerial parts. The overall outcome is an increase in the export of amides to the aerial parts of the plant that leads to increased growth.

Several key characteristics are exhibited by the *SPS* transformants, such as early flowering time, faster growth rates, increased leaf chlorophyll and protein content, increased biomass and highly developed root system. Faster growth could translate into additional cuttings and along with increased biomass would have a great economic impact. Moreover, greater concentration of Suc in the plant cells would increase the osmotic potential of the plants.

Table 4 Elemental nitrogen and carbon and protein content in non-transformants and SPS transformants

Genotype	N (%DM)		TP (%DM)		C (%DM)		C/N		TSP mg g^{-1} fwt leaf
	Root	Leaf	Root	Leaf	Root	Leaf	Root	Leaf	
NT	2.68 ± 0.07	3.96 ± 0.14	16.73 ± 0.53	24.75 ± 0.88	45.90 ± 2.10	44.51 ± 0.45	17.20 ± 1.04	11.29 ± 0.53	46.14 ± 5.33
SPS3	2.87 ± 0.10	4.71 ± 0.10*	17.91 ± 0.70	29.45 ± 0.75*	45.44 ± 0.62	47.09 ± 0.97	15.73 ± 0.35	9.79 ± 0.19	58.59 ± 1.85
SPS6	2.90 ± 0.08	4.68 ± 0.02*	18.10 ± 0.56	29.20 ± 0.14*	45.78 ± 0.81	45.82 ± 0.45	15.56 ± 0.51	9.77 ± 0.09	58.93 ± 1.30
SPS16	2.77 ± 0.06	4.71 ± 0.13*	17.30 ± 0.45	29.40 ± 0.95*	45.33 ± 1.10	45.99 ± 0.36	16.66 ± 0.50	9.65 ± 0.24	59.02 ± 1.24

Oven dried at 65 °C, leaf and root tissue harvested from alfalfa plants 50 days after inoculation with *S. meliloti* were ground to a fine powder. Approximately 0.060 and 1.5 mg of ground tissue were used for measuring C and N content, respectively, using an elemental analyzer. The values of N and C content are represented as a percent of dry matter. Total protein (TP) was calculated as following: N × 6.5. Total soluble protein (TSP) was extracted from leaf fresh tissues using Bradford method. Values are the mean ± standard error ($n = 3$). Significant differences from NT were evaluated by *t* test and shown by asterisks (* $P < 0.05$ or ** $P < 0.01$)

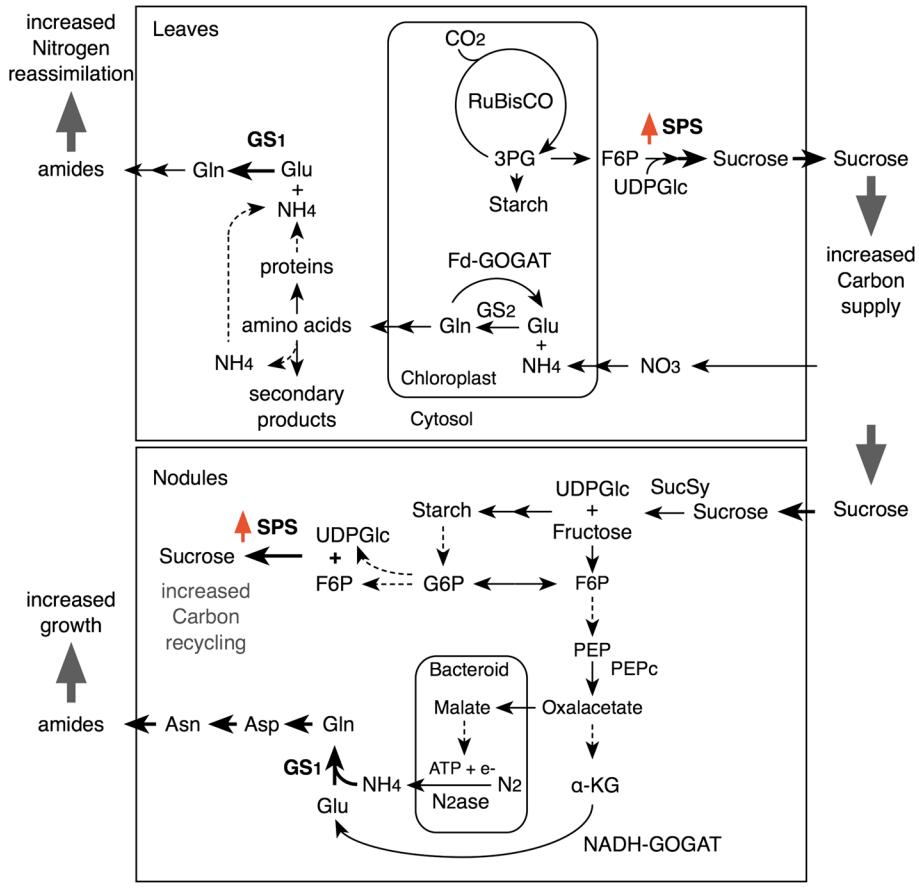

Fig. 8 Proposed model to explain how increased SPS activity improves the N status of nodulated alfalfa plants. The *arrows* in *red* next to the enzymes imply up-regulation and the *big arrows* in *bold* represent increases in the export of Suc and amides from the leaves and nodules, respectively. The abbreviated names of the enzymes and the components of the different reactions are mentioned in the text

Greater osmotic potential confers greater drought tolerance and improves water use efficiency and freeze tolerance. Therefore, up-regulation of SPS activity may be a possible target for the improvement of forage legumes. It would be interesting to check how increased SPS activity exclusively in the photosynthetic cells would affect the two C sinks, seeds and nodule, in a seed legume.

Author contribution statement CSG, SG and MS conceived and designed the research. SG, MS, FMV, SB and JLO conducted the experiments. CSG and SG analyzed the data. CSG and SG wrote the manuscript.

Acknowledgments This work was supported by the Agricultural Experiment Station at New Mexico State University, by the National Institutes of Health (Research Initiative for Scientific Research Enhancement Program), grant number R25GM061222 and by internal funds from the VPR's office at NMSU. SG was supported by a scholarship from the Ministry of Higher Education, Egypt. The authors want to thank Jacquelene Laredo and Amanda Peel for technical help.

References

Aleman L, Ortega JL, Martinez-Grimes M, Seger M, Holguin FO, Uribe DJ, Garcia-Ibilcieta D, Sengupta-Gopalan C (2010) Nodule-enhanced expression of a sucrose phosphate synthase gene member (*MsSPSA*) has a role in carbon and nitrogen metabolism in the nodules of alfalfa (*Medicago sativa*). Planta 231:233–244

Babb VM, Haigler CH (2001) Sucrose phosphate synthase activity rises in correlation with high-rate cellulose synthesis in three heterotrophic systems. Plant Physiol 127:1234–1242

Bagga S, Armendaris A, Klypina N, Ray I, Ghoshroy Endress M, Sutton D, Kemp JD, Sengupta-Gopalan C (2004) Genetic engineering ruminal stable high methionine protein in the foliage of alfalfa. Plant Sci 166:273–283

Baier MC, Barsch A, Kuster H, Hohnjec N (2007) Antisense repression of the *Medicago truncatula* nodule-enhanced sucrose synthase leads to a handicapped nitrogen fixation mirrored by specific alterations in the symbiotic transcriptome and metabolome. Plant Physiol 145:1600–1618

Baxter CJ, Foyer CH, Turner J, Rolfe SA, Quick WP (2003) Elevated sucrose-phosphate synthase activity in transgenic tobacco sustains photosynthesis in older leaves and alters development. J Exp Bot 54:1813–1820

Bjorkman O, Demmig-Adams B (1994) Regulation of photosynthetic light energy capture, conversion, and dissipation in leaves of higher plants. In: Schulze ED, Caldwell MM (eds) Ecophysiology of photosynthesis. Springer-Verlag, Berlin, pp 17–47

Chen WL, Huang DJ, Liu PH, Wang HL, Su JC, Lee PD (2001) Purification and characterization of sucrose phosphate synthase from sweet potato tuberous roots. Bot Bull Acad Sinica 42:123–129

Coleman HD, Yan J, Mansfield SD (2009) Sucrose synthase affects carbon partitioning to increase cellulose production and altered cell wall ultrastructure. Proc Natl Acad Sci USA 106:13118–13123

Coleman HD, Beamish L, Reid AM, Park JY, Mansfield SD (2010) Altered sucrose metabolism impacts plant biomass production and flower development. Transgenic Res 19:269–283

Corbesier L, Lejeune P, Bernier G (1998) The role of carbohydrates in the induction of flowering in *Arabidopsis thaliana*: comparison between the wild type and a starchless mutant. Planta 206:131–137

Corbesier L, Bernier G, Prilleux C (2002) C to N ratio increases in the phloem sap during floral transition of the long-day plants *Sinapis alba* and *Arabidopsis thaliana*. Plant Cell Physiol 43:684–688

D'Aoust MA, Yelle S, Nguyen-Quoc B (1999) Antisense inhibition of tomato fruit sucrose synthase decreases fruit setting and the sucrose unloading capacity of young fruit. Plant Cell 11:2407–2418

Engels C, Kirkby E, White P (2011) Mineral nutrition, yield and source-sink relationships. In: Marschner P (ed) Marschner's mineral nutrition of higher plants. Academic Press (Elsevier), London, pp 85–135

Eveland AL, Jackson DP (2012) Sugars, signaling, and plant development. J Exp Bot 63:3367–3377

Ferguson AR, Sims AP (1971) Inactivation in vivo of glutamine synthetase and NAD-specific glutamate dehydrogenase: its role in the regulation of glutamine synthesis in yeast. J Gen Microbiol 69:423–427

Ferguson BJ, Indrasumunar A, Hayashi S, Lin MH, Lin YH, Reid DE, Gresshoff PM (2010) Molecular analysis of legume nodule development and autoregulation. J Integr Plant Biol 52:61–76

Francisco PB, Harper JE (1995) Translocatable leaf signal autoregulates soybean nodulation. Plant Sci 107:167–176

Galtier N, Foyer C, Murchie E, Alred R, Quick P, Voelker T, Thepenier C, Lasceve G, Betsche T (1995) Effects of light and atmospheric carbon dioxide enrichment on photosynthesis and carbon partitioning in the leaves of tomato (*Lycopersicon esculentum* L.) plants over-expressing sucrose phosphate synthase. J Exp Bot 46:1335–1344

Geigenberger P (2011) Regulation of starch biosynthesis in response to a fluctuating environment. Plant Physiol 155:1566–1577

Geigenberger P, Reimholz R, Geiger M, Merlo L, Canale V, Stitt M (1997) Regulation of sucrose and starch metabolism in potato tubers in response to short-term water deficit. Planta 201:502–518

Grof CPL, Albertson PL, Bursle J, Perroux JM, Bonnett GD, Manners JM (2007) Sucrose-phosphate synthase, a biochemical marker of high sucrose accumulation in sugarcane. Crop Sci 47:1530–1539

Haigler C, Singh B, Zhang D, Hwang S, Wu C, Cai WX, Hozain M, Kang W, Kiedaisch B, Strauss RE, Hequet EF, Wyatt BG, Jividen GM, Holladay AS (2007) Transgenic cotton overproducing spinach sucrose phosphate synthase showed enhanced leaf sucrose synthesis and improved fiber quality under controlled environmental conditions. Plant Mol Biol 63:815–832

Hammond JP, White PJ (2008) Sucrose transport in the phloem: integrating root responses to phosphorus starvation. J Exp Bot 59:93–109

Hikosaka K, Terashima I (1995) A model of the acclimation of photosynthesis in the leaves of C3 plants to sun and shade with respect to nitrogen use. Plant Cell Environ 18:605–618

Huber SC (2007) Exploring the role of protein phosphorylation in plants: from signalling to metabolism. Biochem Soc Trans 35:28–35

Huber SC, Huber JL (1996) Role and regulation of sucrose-phosphate synthase in higher plants. Annu Rev Plant Physiol Plant Mol Biol 47:431–444

Im KH (2004) Expression of sucrose-phosphate synthase (SPS) in non-photosynthetic tissues of maize. Mol Cells 17:404–409

Ishimaru K, Hirotsu N, Kashiwagi T, Madoka Y, Nagasuga K, Ono K, Oshugi R (2008) Overexpression of a maize SPS gene improves yield characters of potato under field conditions. Plant Prod Sci 11:104–107

Jefferson RA, Kavanagh TA, Bevan MW (1987) GUS fusions: β-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. EMBO J 6:3901–3907

Kaschuk G, Hungria M, Leffelaar PA, Giller KE, Kuyper TW (2010) Differences in photosynthetic behavior and leaf senescence of soybean (*Glycine max* L.) dependent on N_2 fixation or nitrate supply. Plant Biol 12:60–69

Kitajima K, Hogan KP (2003) Increases of chlorophyll a/b ratios during acclimation of tropical woody seedlings to nitrogen limitation and high light. Plant Cell Environ 26:857–865

Lancien M, Gadal P, Hodges M (2000) Enzyme redundancy and the importance of 2-oxoglutarate in higher plant ammonium assimilation. Plant Physiol 123:817–824

Laporte MM, Galagan JA, Shapiro JA, Boersig MR, Shewmaker CK, Sharkey TD (1997) Sucrose-phosphate synthase activity and yield analysis of tomato plants transformed with maize sucrose-phosphate synthase. Planta 203:253–259

Laporte MM, Galagan JA, Prasch AL, Vanderveer PJ, Hanson DT, Shewmaker CK, Sharkey TD (2001) Promoter strength and tissue specificity effects on growth of tomato plants transformed with maize sucrose-phosphate synthase. Planta 212:817–822

Lastdrager J, Hanson J, Smeekens S (2014) Sugar signals and the control of plant growth and development. J Exp Bot 65:799–807

Lea PJ, Miflin BJ (2010) Nitrogen assimilation and its relevance to crop improvement. In Foyer C, Zhang H (eds) Ann Plant Rev, vol 42, Nitrogen metabolism in plants in the postgenomic era. Willey-Blackwell, pp 1–40

Lichtenthaler HK (1987) Chlorophylls and carotenoids: pigments of photosynthetic membranes. Methods Enzymol 148:350–382

Lilley JL, Gee CW, Sairanen I, Ljung K, Nembhauser JL (2012) An endogenous carbon-sensing pathway triggers increased auxin flux and hypocotyl elongation. Plant Physiol 160:2261–2270

Lodwig EM, Hosie AH, Bourdès A, Findlay K, Allaway D, Karunakaran R, Downie JA, Poole PS (2003) Amino-acid cycling drives nitrogen fixation in the legume-Rhizobium symbiosis. Nature 422:722–726

Loreti E, Bellis LD, Alp A, Perata P (2001) Why and how do plant cells sense sugars? Ann Bot 88:803–812

Lundmark M, Cavaco AM, Trevanion S, Hurry V (2006) Carbon partitioning and export in transgenic Arabidopsis thaliana with altered capacity for sucrose synthesis grown at low temperature: a role for metabolite transporters. Plant Cell Environ 29:1703–1714

Lunn JE, MacRae E (2003) New complexities in the synthesis of sucrose. Curr Opin Plant Biol 6:208–214

MacRae EA, Lunn JE (2006) Control of sucrose biosynthesis. In: Plaxton W, MacManus M (eds) Adv Plant Res—control of primary metabolism in plants. Blackwell, Oxford, pp 234–257

Maloney MJ, Park JY, Unda F, Mansfield SD (2015) Sucrose phosphate synthase and sucrose phosphate phosphatase interact in planta and promote plant growth and biomass accumulation. J Exp Bot. doi:10.1093/jxb/erv101

Matsoukas IG, Massiah AJ, Thomas B (2012) Florigenic and antiflorigenic signaling in plants. Plant Cell Physiol 53:1827–1842

Maxwell CA, Vance CP, Heichel GH, Stade S (1984) CO_2 fixation in alfalfa and birdsfoot trefoil root nodules and partitioning of ^{14}C to the plant. Crop Sci 24:257–264

Micallef BJ, Haskins KA, Vanderveer PJ, Roh K-S, Shewmaker CK, Sharkey TD (1995) Altered photosynthesis, flowering, and fruiting in transgenic tomato plants that have an increased capacity for sucrose synthesis. Planta 196:327–334

Nguyen-Quoc B, Foyer CH (2001) A role for 'futile cycles' involving invertase and sucrose synthase in sucrose metabolism of tomato fruit. J Exp Bot 52:881–889

Nguyen-Quoc B, N'Tchobo H, Foyer C, Yelle S (1999) Overexpression of sucrose phosphate synthase increases sucrose unloading in transformed tomato fruit. J Exp Bot 50:785–791

Novak K (2010) On the efficiency of legume supernodulating mutants. Ann Appl Biol 157:321–342

Oldroyd GED, Murray JD, Poole PS, Downie JA (2011) The rules of engagement in the legume-rhizobial symbiosis. Annu Rev Genet 45:119–144

O'Leary B, Park J, Plaxton WC (2011) The remarkable diversity of plant PEPC (phosphoenolpyruvate carboxylase): recent insights into the physiological functions and post-translational controls of non-photosynthetic PEPCs. Biochem J 436:15–34

Ono K, Ishimaru K, Aoki N, Takahashi S, Ozawa K, Ohkawa Y, Ohsugi R (1999) Characterization of a maize sucrose-phosphate synthase protein and its effect on carbon partitioning in transgenic rice plants. Plant Prod Sci 2:172–177

Ono K, Sasaki H, Hara T, Kobayashi K, Ishimaru K (2003) Changes in photosynthetic activity and export of carbon by overexpressing a maize sucrose-phosphate synthase gene under elevated CO_2 in transgenic rice. Plant Prod Sci 6:281–286

Ortega JL, Moguel-Esponda S, Potenza C, Conklin CF, Quintana A, Sengupta-Gopalan C (2006) The 3′ untranslated region of a soybean cytosolic glutamine synthetase (GS_1) affects transcript stability and protein accumulation in transgenic alfalfa. Plant J 45:832–846

Park JY, Canam T, Kang KY, Ellis DD, Mansfield S (2008) Overexpression of an arabidopsis family A sucrose phosphate synthase (SPS) gene alters plant growth and fibre development. Transgenic Res 17:181–192

Park JY, Canam T, Kang KY, Unda F, Mansfield SD (2009) Sucrose phosphate synthase expression influences poplar phenology. Tree Physiol 29:937–946

Ramon M, Rolland F, Sheen J (2008) Sugar sensing and signaling. In American society of plant biologists. Arabidopsis Book 6:e0117

Rolland F, Baena-Gonzalez E, Sheen J (2006) Sugar sensing and signaling in plants: conserved and novel mechanisms. Annu Rev Plant Biol 57:675–709

Ruan Y-L (2014) Sucrose metabolism: gateway to diverse carbon use and sugar signaling. Annu Rev Plant Biol 65:33–67

Sairanen I, Novak O, Pencik A, Ikeda Y, Jones B, Sandberg G, Ljung K (2012) Soluble carbohydrates regulate auxin biosynthesis via PIF proteins in Arabidopsis. Plant Cell 24:4907–4916

Sambrook J, Russell DW, Irwin N, Janssen KA (2001) Molecular cloning: a laboratory manual, 2nd edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY

Schneider CA, Rasband WS, Eliceiri KW (2012) NIH Image to ImageJ: 25 years of image analysis. Nat Methods 671-675

Seger M, Gebril S, Tabilona J, Peel A, Sengupta-Gopalan C (2015) Impact of concurrent overexpression of cytosolic glutamine synthetase (GS_1) and sucrose phosphate synthase (SPS) on growth and development in transgenic tobacco. Planta 241:69–81

Signora L, Galtier N, Skot L, Lucas H, Foyer C (1998) Overexpression of sucrose phosphate synthase in Ababidopsis thaliana results in increased foliar sucrose/starch ratios and favours decreased foliar carbohydrate accumulation in plants after prolonged growth with CO_2 enrichment. J Exp Bot 49:669–678

Smeekens S (2000) Sugar-induced signal transduction in plants. Annu Rev Plant Physiol Plant Mol Biol 5:49–81

Stitt M, Lunn J, Usadel B (2010) Arabidopsis and primary photosynthetic metabolism—more than the icing on the cake. Plant J 61:1067–1091

Stokes ME, Chattopadhyay A, Wilkins O, Nambara E, Campbell MM (2013) Interplay between sucrose and folate modulates auxin signaling in Arabidopsis. Plant Physiol 162:1552–1565

Strand A, Foyer CH, Gustafsson P, Gardestrm P, Hurry V (2003) Altering flux through the sucrose biosynthesis pathway in transgenic *Arabidopsis thaliana* modifies photosynthetic acclimation at low temperatures and the development of freezing tolerance. Plant Cell Environ 26:523–535

Sun J, Zhang J, Larue CT, Huber SC (2011) 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. Plant Cell Environ 34:592–604

Tang GQ, Sturm A (1999) Antisense repression of sucrose synthase in carrot (*Daucus carota* L.) affects growth rather than sucrose partitioning. Plant Mol Biol 41:465–479

Tian H, Ma L, Zhao C, Hao H, Gong B, Yu X, Wang X (2010) Antisense repression of sucrose phosphate synthase in transgenic muskmelon alters plant growth and fruit development. Biochem Biophys Res Commun 393:365–370

Tognetti JA, Pontis HG, Martinez-Noel MA (2013) Sucrose signaling in plants: a world yet to be explored. Plant Signal Behav 8:3

Toroser D, Huber SC (1997) Protein phosphorylation as a mechanism for osmotic-stress activation of sucrose-phosphate synthase in spinach leaves. Plant Physiol 114:947–955

Wahl V, Ponnu J, Schlereth A, Arrivault S, Langenecker T, Franke A, Feil R, Lunn JE, Stitt M, Schmid M (2013) Regulation of flowering by trehalose-6-phospahte signaling in *Arabidopsis thaliana*. Science 339:704–707

Weise A, Elzinga N, Wobbes B, Smeekens S (2004) A conserved upstream open reading frame mediates sucrose-induced repression of translation. Plant Cell 16:1717–1729

White J, Prell J, James EK, Poole P (2007) Nutrient sharing between symbionts. Plant Physiol 144:604–614

Wind J, Smeekens S, Hanson J (2010) Sucrose: metabolite and signaling molecule. Phytochemistry 7:1610–1614

Wingler A, Delatte TL, O'Hara LE, Primavesi LF, Jhurreea D, Paul MJ, Schluepmann H (2012) Trehalose 6-phosphate is required for the onset of leaf senescence associated with high carbon availability. Plant Physiol 158:1241–1251

Worrell AC, Bruneau JM, Summerfelt K, Boersig M, Voelker TA (1991) Expression of a maize sucrose phosphate synthase in tomato alters leaf carbohydrate partitioning. Plant Cell 3:1121–1130

Yadav UP, Ivakov A, Feil R, Duan GY, Walther D, Giavalisco P, Piques M, Carillo P, Hubberten H-M, Stitt M, Lunn JE (2014) The sucrose-trehalose 6-phosphate (Tre6P) nexus: specificity and mechanisms of sucrose signaling by T6P. J Exp Bot 65:1051–1068

Zeeman SC, Kossmann J, Smith AM (2010) Starch: its metabolism, evolution, and biotechnological modification in plants. Annu Rev Plant Biol 61:209–234

Zrenner R, Salanoubat M, Willmitzer L, Sonnewald U (1995) Evidence of the crucial role of sucrose synthase for sink strength using transgenic potato plants (*Solanum tuberosum* L.). Plant J 7:97–107