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ORIGINAL ARTICLE

Impact of concurrent overexpression of cytosolic glutamine synthetase (GS_1) and sucrose phosphate synthase (SPS) on growth and development in transgenic tobacco

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Abstract

Main conclusion **The outcome of simultaneously increasing SPS and GS activities in transgenic tobacco, suggests that sucrose is the major determinant of growth and development, and is not affected by changes in N assimilation.**

Abstract Carbon (C) and nitrogen (N) are the major components required for plant growth and the metabolic pathways for C and N assimilation are very closely interlinked. Maintaining an appropriate balance or ratio of sugar to nitrogen metabolites in the cell, is important for the regulation of plant growth and development. To understand how C and N metabolism interact, we manipulated the expression of key genes in C and N metabolism individually and concurrently and checked for the repercussions. Transgenic tobacco plants with a cytosolic soybean glutamine synthetase (GS_1) gene and a sucrose phosphate synthase (SPS) gene from maize, both driven by the CaMV 35S promoter were produced. Co-transformants, with both the transgenes were produced by sexual crosses. While GS is the key enzyme in N assimilation, involved in the synthesis of glutamine, SPS plays a key role in C metabolism by catalyzing the synthesis of sucrose. Moreover, to check if nitrate has any role in this interaction, the plants were grown under both low and high nitrogen. The SPS enzyme activity in the SPS and SPS/GS_1 co-transformants were the same under both nitrogen regimens. However, the GS activity was lower in the co-transformants compared to the GS_1 transformants, specifically under low nitrogen conditions. The GS_1/SPS transformants showed a phenotype similar to the SPS transformants, suggesting that sucrose is the major determinant of growth and development in tobacco, and its effect is only marginally affected by increased N assimilation. Sucrose may be functioning in a metabolic capacity or as a signaling molecule.

Keywords Sucrose phosphate synthase (SPS) · Cytosolic glutamine synthetase (GS_1) · Co-transformants · C:N ratio · Sucrose:starch ratio

Abbreviations

SPS	Sucrose phosphate synthase
GS1	Cytosolic glutamine synthetase
Suc	Sucrose
Glc	Glucose
Gln	Glutamine
HN	High nitrogen
LN	Low nitrogen
C	Carbon
N	Nitrogen
NT	Non transformed
MzSPS	Maize SPS gene
Gmgln150	Soybean GS_1 gene without 3′UTR
GS_1/SPS	Co-transformant with both *GS_1* and *SPS* genes

Introduction

Carbon (C) and nitrogen (N) are the major components required for plant growth and the assimilation of C and N are very closely interlinked. Photosynthesis plays a key

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role in N assimilation, either directly or indirectly by providing C skeletons, reducing power and ATP. At the same time, photosynthesis and the partitioning of the assimilated C between synthesis of organic acids, starch and sucrose, are influenced by the availability of N (Foyer et al. 2006; Nunes-Nesi et al. 2010). While the origin of 2-oxoglutarate (2-OG) that is needed for glutamate (Glu) and glutamine (Gln) production, is not known, there is evidence suggesting that the TCA cycle in illuminated leaves is essential for providing 2-OG (Lemaitre et al. 2007). N assimilation takes place through the GS/GOGAT (Glutamine synthetase/Glutamate synthase) pathway and is essential, since it generates the primary amino donors for the biosynthesis of all nitrogenous compounds in plants. Besides 2-OG, the respiratory pathways must also generate oxaloacetate and pyruvate, which act as acceptors of ammonium from Gln to form asparagine (Asn). Plants possess an intricate regulatory mechanism that coordinates C and N metabolism under conditions of nutrient availability and environmental conditions (Coruzzi and Zhou 2001; Baena-Gonzalez 2010). This network regulates uptake and assimilation of inorganic nitrogen, allocation of nitrogen and many aspects of plant growth and development. There are several reports on the sensing and signaling for both C (Rolland et al. 2006; Paul 2007) and N availability (Scheible et al. 2004; Wang et al. 2003, 2004; Peng et al. 2007). Maintaining an appropriate ratio of sugars to nitrogen metabolites in the cell, which is referred to as the "carbon/nitrogen balance" (C/N ratio), is important for the regulation of plant growth and development (Palenchar et al. 2004; Commichau et al. 2006; Zheng 2009; Nunes-Nesi et al. 2010). There are distinct C and N sensory systems that monitor alterations in the levels of diverse metabolites like glucose, sucrose, nitrate (NO_3), ammonium (NH_4), Gln and Glu (Coruzzi and Bush 2001; Foyer et al. 2006; Forde and Lea 2007). Because the amounts of assimilated C and N largely influence plant growth, there have been many attempts to engineer C and N assimilation by the overexpression of key enzymes in either pathway, individually. However, only in a few cases have significant improvements in C and N assimilation been achieved, possibly because synchronous activation of a series of metabolic pathways might be necessary to influence assimilation. To understand how C and N metabolism interact, our goal here has been to manipulate key steps in C and N metabolism individually and concurrently in the same plant. The enzymes that we have focused on in this paper are glutamine synthetase (GS) and sucrose phosphate synthase (SPS). While GS is involved in the synthesis of Gln, SPS catalyzes the synthesis of sucrose (Suc).

Glutamine synthetase (GS; EC 6.3.1.2) is the key enzyme in N metabolism in plants, catalyzing the first step in the conversion of an inorganic form of N, ammonia, to an organic form, Gln, which is the starting compound for the synthesis of all other N containing molecules that make up a cell (Lea and Miflin 2011; Swarbreck et al. 2011). GS catalyzes the ATP dependent synthesis of Gln from NH_4 and Glu. Sucrose, a product of photosynthesis, is ultimately the source of Glu required for the GS/GOGAT pathway. There are two nuclear encoded isoforms of GS in plants, chloroplastic form (GS_2) and a cytoplasmic form (GS_1). While GS_2 is the isoform involved in the assimilation of NH_4^+ released during nitrate reduction and photorespiration, GS_1 assimilates ammonia produced by all other physiological activities. Cytosolic glutamine synthetase (GS_1) genes are part of a complex multi-gene family that range from at least two to five homologs, depending on the plant species (Lea and Miflin 2011; Swarbreck et al. 2011). The different GS_1 genes within any particular plant, exhibit differential expression pattern with regards to the tissue type and based on their location, they also perform different functions (Bernard and Habash 2009). Cytosolic GS_1, localized in the vasculature elements, is the predominant isoform found in non-photosynthetic tissues and its role is more complex due to the numerous isoforms found in plants (Bernard and Habash 2009; Lea and Miflin 2011).

Since GS_1 is considered to be the key component of efficiency of N use and yield (Hirel et al. 2001; Man et al. 2005; Martin et al. 2006; Tabuchi et al. 2005; El-Khatib et al. 2004), several attempts have been made to overexpress GS_1 with the goal of improving plant performance. However, the outcome has been rather inconsistent (Temple et al. 1993; Gallardo et al. 1999; Fuentes et al. 2001; Oliveira et al. 2002, Carvalho et al. 2003; Fu et al. 2003; Harrison et al. 2003; Ortega et al. 2001, 2004). The inconsistency has been attributed to the transgene, the plant in question and the conditions of growth.

Sucrose phosphate synthase (SPS; EC 2.3.1.14), the key enzyme in the synthesis of sucrose (Suc) in plants, catalyzes the first committed reaction in the pathway of Suc synthesis, producing sucrose-6-phosphate (Suc-6P) from fructose-6- phosphate and UDP-glucose. Sucrose phosphate phosphatase then dephosphorylates Suc-6P to Suc. 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 (Huber 2007; MacRae and Lunn 2006; Stitt et al. 2010; Sun et al. 2011). Besides its role as a substrate for metabolic requirements, Suc also acts as a signal molecule for regulation of gene expression (Loreti et al. 2001; Smeekens 2000; Wind et al. 2010), thus playing a role in both metabolism and development (Lunn and MacRae 2003; Eveland and Jackson 2012). Suc plays a crucial role in the functioning of different processes, which

are key to plant growth (Galtier et al. 1995; Grof et al. 2007; Ishimaru et al. 2004). There are several reports on the overexpression of SPS using transgenic approaches (Coleman et al. 2010; Haigler et al. 2007; Nguyen-Quoc and Foyer 2001; Tian et al. 2010; Zuñiga-Feest et al. 2005) with varied consequences, but in general, increased SPS activity is associated with the production of new sinks and increased sink strength, although the sinks may range from flowers, fruits, tubers, fiber, stem and more (Micallef et al. 1995; Park et al. 2008, 2009; Haigler et al. 2007; Nguyen-Quoc et al. 1999; Baxter et al. 2003; Ishimaru et al. 2008; Laporte et al. 2001).

The focus of this study is to analyze the repercussions of overexpressing GS_1 and SPS simultaneously at the physiological and biochemical level. Moreover, to check if availability of N has some kind of effect on the outcome of overexpressing these genes individually or concurrently, our study has also included growing the plants under low and high N regimens. We present data on the growth pattern, flowering time, fruit yield and sucrose/starch ratios in tobacco transformants concurrently expressing both a soybean GS_1 gene and the maize SPS gene driven by the CaMV35S promoter, along with the transformants expressing the individual genes.

Materials and methods

Gene manipulations

Standard procedures were used for all recombinant DNA manipulations (Sambrook et al. 2001). The *Zea mays SPS* gene (*SPS-1;* NCBI accession number NM 001112224) was kindly provided by Dr. Christine Foyer (University of Leeds, UK). To make the CaMV 35S/*Zea mays* SPS construct *(MzSPS)*, the *SPS1* 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 *SPS1* cloned fragment was subsequently released with *Bam*HI and partial digestion with *Sac*I and 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. The CaMV 35S/soybean GS_1 (NCBI accession # AF301590) (without its 3′UTR; *Gmgln150*) gene construct is as described by Ortega et al. (2006).

Plant transformation and growth conditions

The plasmids with the *MzSPS* and *Gmgln150* constructs were mobilized into *Agrobacterium tumefaciens* strain GV3101 by a freeze thaw method as described (Sambrook et al. 2001). *A. tumefaciens* mediated plant transformation of tobacco (*Nicotiana tabaccum*) was carried out using a standard protocol (Seger et al. 2009). A SPS transformant was sexually crossed with a GS_1 transformant and the seeds from these crosses were germinated on media containing 100 mg of kanamycin/L. Individual plants from the plates were analyzed by western blot analysis using the GS and SPS antibodies. Once identified as (SPS, GS_1 and GS_1/SPS), independent lines corresponding to each class along with NT plants were vegetatively propagated. They were then transferred to vermiculite and divided into two sets. While one set was watered with 0.5× Hoagland's nutrient solution supplemented with 1 mM NH_4NO_3, the other set was watered with 0.5× Hoagland's nutrient solution supplemented with 5 mM NH_4NO_3. The pots were placed under greenhouse conditions with no supplemental lights and with night time temperature of ∼15–20 °C and daytime temp of ∼30–35 °C. They were allowed to acclimate for 2 weeks before initiating the experiments. Plant growth was monitored through pod development.

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 (Bio-Rad, 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 and *Gmglnβ1* gene as used as probes to monitor expression of the *SPS* and *GS_1* transgene transcript. 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

SPS activity: Leaf tissue was ground in liquid N and homogenized with 5 volumes of extraction buffer [50 mM Hepes pH 7.5, 20 % (v/v) glycerol, 5 % (v/v) ethylene glycol, 5 mM EDTA, 10 mM magnesium chloride, 0.5 % Triton X-100, 10 mM DTT and a mixture of protease inhibitors (Roche, Indianapolis, IN)]. The homogenate was centrifuged for 10 min at 12,500*g* and desalted in Sephadex G25 columns against desalting buffer (25 mM Hepes pH, 20 % [v/v] glycerol, 5 % [v/v] ethylene glycol, 2.5 mM Magnesium chloride, 5 mM DTT and protease inhibitors). Protein concentration was measured by the Bradford protein assay (Bio-Rad, Hercules, CA) using bovine serum albumin as the protein standard. Total SPS activity was measured by quantifying the synthesis of Suc-

6P from UDP-Glc and Fru-6P as described (Aleman et al. 2010). Extracts equivalent to 63 μg of protein were incubated for 30 min at 30 °C in 125 μL reaction mixture containing 25 mM Hepes pH 7.5, 2.5 mM magnesium chloride, 10 mM Fru-6P, 12 mM UDP-Glc, and 40 mM of the allosteric activator Glc-6P. Reactions were stopped by incubation at 95 °C for 10 min and subsequently centrifuged at 4 °C at 12,500×*g* for 10 min. To destroy any unreacted hexose phosphates, 125 μL of 30 % KOH was added and then incubated for an additional 10 min at 95 °C. Samples were centrifuged again and the supernatant was transferred to new tubes. One mL of 0.14 % (w/v) Anthrone reagent in 85 % (v/v) H_2SO_4 was added into each reaction. After incubating at room temperature for 30 min the absorbance was measured at 625 nm. The amount of Suc-6P produced in each reaction was calculated against a standard curve of Suc.

GS activity: Leaf tissue was ground in liquid N and homogenized with 5 volumes of the extraction buffer containing 50 mM Tris–HCl pH 8.0, 20 % glycerol (v/v), 5 % ethylene glycol (v/v), 1 mM $MgCl_2$, 1 mM DTT, 1 mM EDTA and a protease inhibitor cocktail (Roche Applied Science, Indianapolis, IN, USA). The homogenate was centrifuged for 15 min at 20,000 g and desalted in Sephadex G25 columns against desalting buffer [62.5 mM Tris–Cl pH 6.8, 20 % glycerol (v/v), 5 % ethylene glycol (v/v), 1 mM DTT, 1 mM EDTA and protease inhibitors]. Protein concentration was measured as described above. The activity of GS was measured by the transferase assay (Ferguson and Sims 1971). Transferase units were calculated from a standard curve of γ-glutamyl hydroxamate. Activity is reported as l mol of γ-glutamyl hydroxamate produced min/1 mg of protein at 30 °C.

Western blot analysis: The protein extracts used for enzyme activity were used for western blot analysis. Proteins were fractionated by SDS-PAGE followed by immunoblotting using polyclonal antibodies raised against maize SPS (Agrisera, Sweden) or GS antibodies (Ortega et al. 2006). Immunoreactive bands were visualized with an alkaline phosphatase-linked secondary antibody using nitroblue tetrazolium and 5-bromo-4-chloro-3-indoyl-phosphate as substrates.

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 microlitre of the supernatant were 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. The supernatant was transferred to a new tube, 1 mL of Anthrone reagent was added to each reaction, and measured as described in the previous section.

Starch determination was performed in the pellets from the ethanol extracts following removal of the supernatant for Suc determination as previously described (Barsch et al. 2006; Aleman et al. 2010). The supernatant containing the Glc (C6 units) released by enzymatic hydrolysis was transferred to 1.5 ml tube and 50 μL of the supernatant was diluted to 250 μL with water, and 1 ml Anthrone reagent was added. The sample was boiled for five min, cooled on ice bath for additional five 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 leaf gas exchange (net photosynthetic rates)

Net photosynthetic rates (Pnet; μmol CO_2 m^{-2} s^{-1}), were determined at the same time of the day, generally between 8:00 and 11:00 am, on the first fully expanded leaf with an external light source attached to an infrared gas analyzer-based photosynthesis system (Li-Cor 6400, Lincoln, NE). A photo flux of ~1,000 μmol m^{-2} s^{-1} was maintained. The Pnet measurements were taken at ambient CO_2 (400 μmol CO_2 mol^{-1}) and (700 μmol CO_2 mol^{-1}) concentrations. Duplicate measurements were performed on each plant. Experiments were repeated three times and the results of one representative experiment of net photosynthetic rates per leaf area ± SE are presented.

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

Creation of tobacco co-transformants with both the *SPS* and GS_1 genes

Tobacco transformants containing both, a gene encoding for a maize *SPS* gene (Worrell et al. 1991) driven by the CaMV 35S promoter (*35S:MzSPS*) and a soybean GS_1 gene, *Gmglnβ1* (Ortega et al. 2006) also driven by the

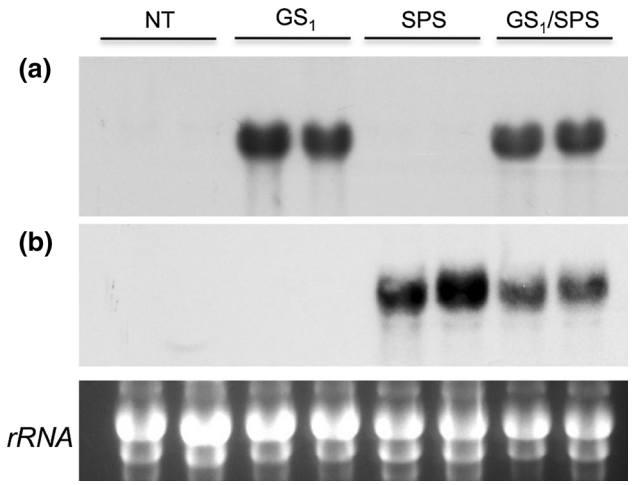

Fig. 1 Analysis of Gmglnβ1 and maize SPS transcript accumulation in the leaves of the three classes of transformants. After initial screening, four representative plants of control (NT) and the three classes of transformants (GS_1, SPS and GS_1/SPS) were selected and clonally propagated. Total RNA (18 μg) from the leaves of non-transformed and the three classes of transformants was subjected to northern blot hybridization using ^{32}P radioactive-labeled Gmglnb1 and *MzSPS* fragments, in a sequential manner. The gel was stained with SYBR gold and the rRNA bands are shown to indicate the RNA loads in each lane

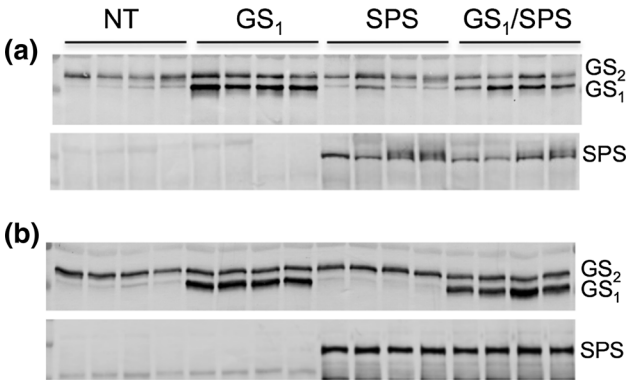

Fig. 2 Analysis of GS and SPS polypeptides in the leaves of the three classes of transformants. After initial screening, the same four representative plants of control (NT) and the three classes of transformants (GS_1, SPS and GS_1/SPS) used for transcript analysis were clonally propagated. The clones were grown under 1 mM (LN) or 5 mM (HN) high of ammonium nitrate. The samples were subjected to SDS PAGE in duplicate. The membranes were subjected to western blot analysis using the GS antibodies and SPS antibodies. The bands corresponding to GS_2, GS_1 and SPS are indicated. **a** Plants grown on LN; **b** plants grown on HN

CaMV 35S promoter (*GSβ150*) were produced by sexually crossing two transformants, each containing one of the two gene constructs. The progeny of these crosses were grown on kanamycin and the progeny were screened for the three classes of transformants (SPS, GS_1 and GS_1/SPS) by western blot analysis with the GS and SPS antibodies and out of these progeny plants, four randomly selected plants for each class of transformants were subjected to detailed analysis. In this paper, we present the data from the progeny of one representative cross. Progeny isolated from a cross ensures that the single gene transformants and transformants with both the gene constructs, do not differ with one another with regards to the insertion site in the genome. For further confirmation, we checked the transcript levels for the individual transgenes in the single gene transformants and the co-transformants, with the rationale that the transcript level for the individual genes should be the same in the two classes of transformants if the number of transgenes is the same in both single gene transformants and the co-transformants. As seen in Fig. 1, while the *Gmglnβ1* probe showed hybridizing bands in the lanes with RNA from GS_1 and GS_1/SPS transformants, the *MzSPS* probe hybridized to bands in the lanes with RNA from the SPS and GS_1/SPS transformants. Furthermore, relative to the rRNA loads, the hybridizing band for both the GS_1 and $MzSPS_1$ was approximated to be the same in the GS_1 and GS_1/SPS transformants and the SPS and GS_1/SPS transformants, respectively.

Accumulation of the GS_1 and SPS polypeptides in the transformants

The three classes of transformants along with non transformed (NT) plants were clonally propagated and the propagated plants were either grown on 1 mM NH_4NO_3 (low nitrogen; LN) or 5 mM NH_4NO_3 (high nitrogen; HN). The three classes of transformants growing under the two N regimens were analyzed for the steady state level of SPS and GS_1 proteins by western blot analysis. Total soluble protein from the fully expanded leaves of the same age, from NT plants and the three classes of transformants were extracted and subjected to SDS PAGE in duplicate followed by western blot analysis. While one blot was treated with the GS antibodies, the second blot was treated with the SPS antibodies. As seen in Fig. 2, while the GS antibodies showed the GS_2 immunoreactive band in all the samples, a strong immunoreactive GS_1 band was seen only in the lanes with samples from the GS_1 transformants and the co-transformants. The other lanes showed a lighter band with slower migration than the transgene product representing the endogenous GS_1 polypeptide. The intensity of the GS_1 band in the GS_1 transformants under low N regimen was higher than in the co-transformants (Fig. 2a) but was similar under high N conditions (Fig. 2b). The SPS antibodies showed immunoreactive bands in the SPS transformants and the co-transformants and the levels under both N regimens were similar (Fig. 2). The two panels representing low N conditions and high N conditions (Fig. 2a, b) cannot be compared with each other because the western blot analysis was done separately.

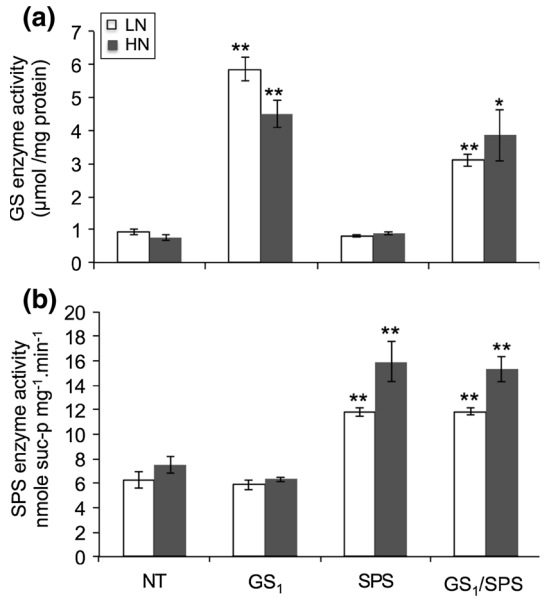

Fig. 3 Analysis of GS and SPS activity in the leaves of the three classes of transformants. Representative samples of leaf extract from the control (NT) and the three classes of transformants (GS_1, SPS and GS_1/SPS) growing on 1 mM (LN) or 5 mM (HN) ammonium nitrate, were subjected to: **a** GS transferase activity; activity values are plotted as µmol γ-glutamyl hydroxamate produced per min mg^{-1} of protein at 30 °C. **b** SPS enzyme activity; activity values are plotted as nmol Sucrose-P min^{-1} mg^{-1}. Values are the mean ± standard error ($n = 4$) of fully expanded leaves of NT, GS_1, SPS and GS_1/SPS. Significant differences from NT were evaluated by *t* test and shown by *asterisks* (*$P < 0.05$ or **$P < 0.01$)

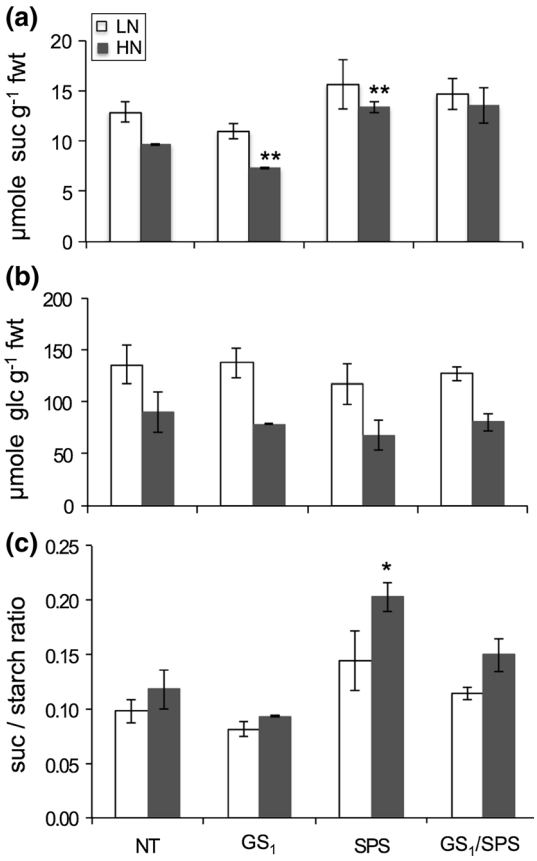

Fig. 4 Leaf carbohydrate analysis in NT and the three sets of transformed plants (GS_1, SPS GS_1/SPS). The leaves of plants growing under the two N conditions (LN and HN), used for SPS and GS activity analysis, were used for sucrose and starch measurement using the protocols described in "Materials and methods". **a** Sucrose content plotted as µmol of Suc g^{-1} fwt. **b** Starch content plotted as µmol of Glc g^{-1} fwt. **c** The values obtained in *panels* **a** and **b**, were used for the measurement of sucrose to starch (Suc/starch) and the ratios are plotted. Values are the mean ± standard error ($n = 4$) of fully expanded leaves of NT, GS, SPS and GS/SPS. Significant differences from NT were evaluated by *t* test and shown by *asterisks* (*$P < 0.05$ or **$P < 0.01$)

GS and SPS activity in the three classes of transformants

To check if the steady state level of the protein (GS_1 and SPS) in the 4 classes of plants corresponded with the respective enzyme activity, the fully expanded leaves in triplicate from the same representative plants used for western analysis, was used for GS and SPS enzyme activity measurements. As seen in Fig. 3a, GS activity was the highest in the GS_1 transformants followed by the co-transformants, while the NT and the SPS transformants exhibited lower levels, under both N conditions. The GS activity showed an almost 50 % reduction in the GS_1/SPS transformants compared to the levels in the GS_1 transformants, when grown under low nitrogen conditions but only a 10 % drop when grown under high N conditions. SPS activity in the SPS and GS_1/SPS transformants showed higher activity when grown under high N conditions and essentially showed no difference between the SPS transformants and the co-transformants under both N regimens (Fig. 3b). The enzyme activity data appears to follow the same trend as the accumulation of the corresponding protein.

Sucrose and starch content in the leaves of the transformants

The pathways of Suc and starch formation are interdependent as they compete for the pool of triose phosphates produced by the Calvin cycle. A consequence of this complex interdependence is that Suc formation and starch formation are reciprocally related. Thus increasing SPS activity will not only impact Suc levels but also affect starch levels in photosynthetic tissues (Worrell et al. 1991). To check if increased SPS activity in the SPS transformants and the GS_1/SPS transformants translates into higher Suc and starch content in the plants, total Suc and starch content was measured in the leaves of the three classes of transformants along with the NT plants grown under LN and HN conditions. As shown in Fig. 4a, all the different

classes of plants showed higher Suc accumulation under LN conditions compared to when grown under HN conditions. Both the SPS transformants and the co-transformants showed higher Suc accumulation compared to the GS_1 transformants and the NT plants under both N regimens. Very little difference in Suc level was seen between the SPS transformants and the co-transformants. It is interesting to note that the GS_1 transformants showed lower level of Suc accumulation compared to the NT plants. As is the case for Suc accumulation, starch levels were also higher in the plants grown under LN conditions. Though not statistically significant, the starch level was consistently the lowest in the SPS transformants followed by the co-transformants (Fig. 4b). The calculated Suc:starch ratio under both LN and HN conditions, was the highest in the SPS transformants followed by the co-transformants and the GS_1 transformants showed a ratio lower than in the NT plants (Fig. 4c). In all cases the ratio was higher when plants were grown under HN regimen.

Net photosynthetic rates

To check if the increased SPS activity and GS activity has any effect on photosynthetic rates (Pnet), all the classes of transformants and the NT plants growing under high nitrogen were subjected to Pnet measurements under ambient (400 μmol mol^{-1}) and elevated (700 μmol mol^{-1}) CO_2 concentration. As seen in Table 1, while all the transformants showed increase in Pnet over NT plants, the increase was significant only for the GS and SPS transformants under ambient and elevated CO_2 concentration. Both the NT plants and all the classes of transformants showed higher Pnet under elevated CO_2 conditions. The transformants grown in low N, showed very little difference in photosynthetic rates when compared to NT plants (data not shown).

Table 1 Photosynthetic rates in NT and the three sets of transformed plants growing on HN

	P_{NET} μmol $CO_2 m^{-2}$ s^{-1}	
	400 μmol CO_2	700 μmol CO_2
NT	13.38 ± 0.67	19.20 ± 0.71
GS	15.93 ± 0.55**	22.21 ± 0.72**
SPS	15.59 ± 0.54*	21.90 ± 0.77*
GS/SPS	14.28 ± 0.59	20.68 ± 0.75

Net photosynthetic rates (P_{net}) was measured as CO_2 μmol m^{-2} s^{-2}, was measured using the LiCor photosynthesis system under ambient (400 μmol) and elevated (700 μmol) CO_2 concentrations. Values are the mean ± standard error ($n = 8$) of NT, GS, SPS and GS/SPS growing under LN and HN conditions

Significant differences from NT were evaluated by *t* test and shown by asterisks (*$P < 0.05$ or **$P < 0.01$)

Growth profile and flowering time

To check if manipulating the C and/or N metabolic pathways has any effect on the growth, we monitored the heights of the plants from each class growing under the two different N conditions from the time they were moved from tissue culture into the greenhouse till the time of pod set (Fig. 5). The phenotype of the plants was also tracked over a period of 30 weeks (Fig. 7). As seen in Fig. 5a, under LN conditions, all the transformants and NT plants showed the same growth pattern for the first 3–4 weeks at which time the SPS, GS_1/SPS transformants and the NT plants transitioned to higher growth rate compared to the GS_1 transformants and continued to grow till the onset of flowering. The SPS transformants flowered ~2 weeks earlier than the NT plants while the co-transformants flowered around the same time as the NT (Figs. 6a, 7). The GS_1 transformants grew slower than the other classes and continued to grow till about 30 weeks when they started flowering (Figs. 5, 6, 7). Under HN conditions, all the different classes started with the same growth pattern till about 5 weeks past planting in pots, after which time the SPS and GS_1/SPS transformants and NT plants showed a sharper increase in growth and grew till the onset of flowering, around the 6th week for the SPS and GS_1/SPS transformants and 8th week for the NT plants. The SPS

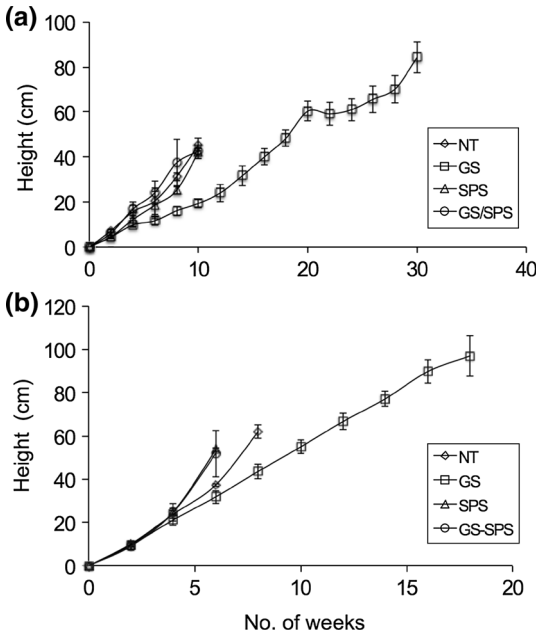

Fig. 5 Growth pattern of the NT and the three sets of transformants. The heights of the plants were measured in cm every 2 weeks till the time of onset of flowering. The value for plant height was then plotted against the number of weeks of growth in the green house. **a** Growth times of plants under LN conditions. **b** Growth times of plants under HN conditions. The heights of four individual plants for each class was measured at each time point and the average is represented here

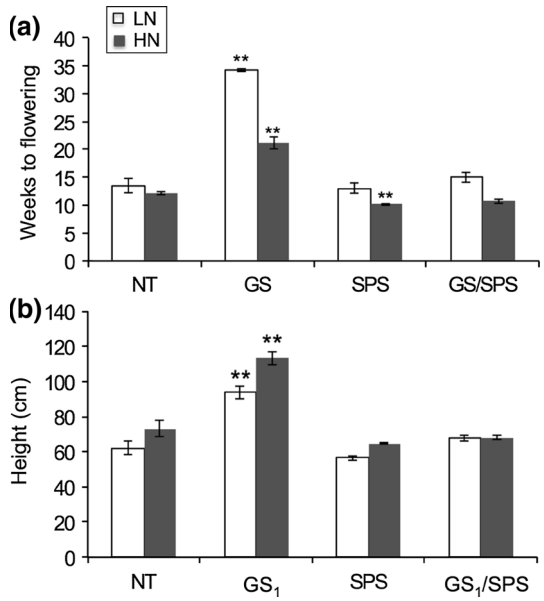

Fig. 6 Growth times and the flowering time of NT and the three sets of transformed plants (GS_1, SPS and GS_1/SPS) growing under LN and HN conditions. **a** The first time point of the onset of flowering. **b** Height of plants at the time of onset of flowering. Values are the mean ± standard error ($n = 4$) of NT, GS_1, SPS and GS_1/SPS growing under LN and HN conditions. The end point of each plot indicates the time flowering. Significant differences from NT were evaluated by *t* test and shown by *asterisks* (*$P < 0.05$ or **$P < 0.01$)

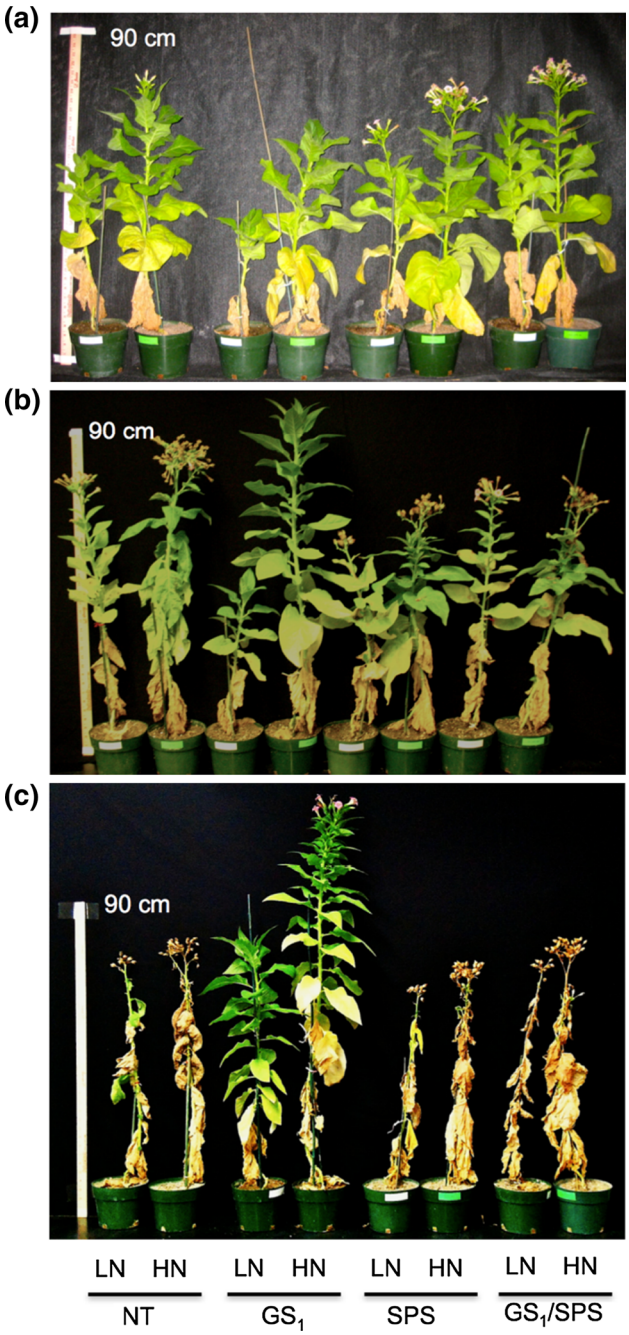

Fig. 7 Visual representation of the NT and the three sets of transformed plants growing under LN and HN. Plants for each class of transformants and NT were observed for phenotypic changes over a period of 14 weeks following establishment of the plants in the particular N regimens (LN and HN) and greenhouse conditions. *Pictures* of the NT and the three classes of transformants were taken and picture of one representative plant from each class is shown here. **a** 6 weeks **b** 10 weeks and **c** 14 weeks following establishment of the plants

transformants flowered 2 weeks before the NT plants and preceded the GS_1/SPS transformants in flowering by a few days. The GS_1 transformants continued to grow at the same growth rate till the onset of flowering around 20 weeks (Figs. 5, 6). The final height attained by each set of plants, in general was higher under HN. The GS_1 transformants at the time of flowering far exceeded all the other classes in height. The flowering time showed an inverse correlation with the final height attained by the different classes (Fig. 6). With regards to flowering, all classes of plants in general, flowered earlier under high N compared to under low N regimens (Figs. 6, 7).

Pod weight

Overexpression of SPS is accompanied by an increase in sink strength and the nature of the sinks varied among the different plants. Since the seed pods in tobacco are strong C and N sinks, the weight of individual pods and total pods for each plant was determined to address how overexpression of the transgenes would affect sink strength (Fig. 8). While the number of pods per plant was the highest in the NT plants, the weight of all the pods per plant and the weight of each pod was the highest in the SPS transformants, followed by the GS_1/SPS co-expressers and then the NT. The GS_1 transformants showed the lowest weight for individual pods and also for pods per plant. In all cases, the pod weight was higher under high nitrogen compared to low nitrogen.

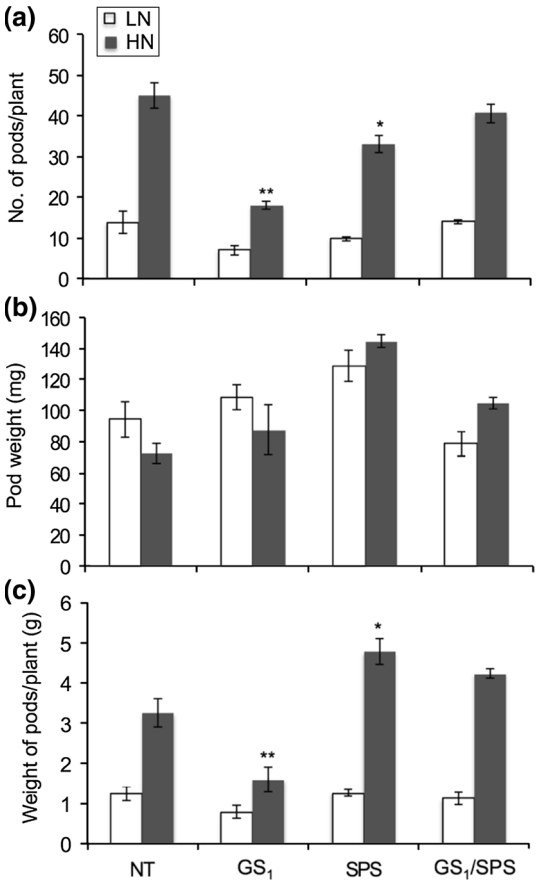

Fig. 8 Analysis of pod number and weight at maturity in the NT and the three sets of transformed plants growing under LN and HN. **a** The total number of pods per plant. **b** The weight of individual pod. **c** The weight of all the pods per plant. Values are the mean ± standard error ($n = 4$) of NT, GS, SPS and GS/SPS growing under LN and HN conditions. Significant differences from NT were evaluated by *t* test and shown by *asterisks* ($*P < 0.05$ or $**P < 0.01$)

Discussion

While there are several reports in the literature deliberating the consequences of overexpressing *GS1* and *SPS* individually, the present study, to the best of our knowledge, is the first attempt to simultaneously overexpress *GS1* and *SPS*. The motivation behind this study was to analyze the interaction between Suc synthesis and GS activity, the two key functions associated with primary metabolism.

Western blot analysis showed similar level of GS_1 and SPS protein in the co-transformants and the corresponding single gene transformant under HN regimen. However, under LN conditions, the co-transformants showed almost 50 % lower level of GS protein compared to the GS_1 transformants (Fig. 2). The enzyme activity for GS followed the same pattern as the western blot analysis. With regards to SPS, the protein level and enzyme activity level showed only minor differences between the co-expressers and the SPS transformants under both LN and HN conditions (Figs. 2, 3). Since the transformants used in the two N treatments are clonal, the decreased level of GS_1 protein and activity in the co-transformants compared to the GS_1 transformants under LN but not under HN, would imply that there is interaction between C and N metabolism only under LN conditions.

SPS overexpressing tobacco transformants in Baxter et al. (2003), showed many of the same traits seen in our study in that the source leaves had a higher (~2-fold) Suc:starch ratio and flowered earlier than the NT plants. Moreover, the rates of photosynthesis, in the fully expanded source leaves showed an increase only under optimal N conditions, as reported by Baxter et al. (2003). The SPS transformants in this study produced larger fruits (pods) and the weight of pods per plant was also higher, a trait that was not reported by Baxter et al. (2003). Compared to the NT plants, the GS_1 transformants grew for extended periods and exhibited delayed flowering when grown under both LN and HN. While there are many reports of overexpression of *GS1* in different plants in the literature, the outcomes are very varied (Eckes et al. 1989; Temple et al. 1993; Oliveira et al. 2002; Ortega et al. 2004; Kirby et al. 2006; Pascual et al. 2008). Poplar plants transformed with a pine *GS1* gene, showed increased growth in height (Gallardo et al. 1999). Enhanced expression of cytosolic *GS1* in *Lotus corniculatus* and *L. japonicus*, lead to early flowering and plant senescence (Vincent et al. 1997; Ortega et al. 2004). Fuentes et al. (2001) showed that tobacco transformants with an alfalfa *GS1* gene driven by the CaMV 35S promoter exhibited improved performance over NT plants only when grown under nitrogen deficient conditions, but not under HN conditions, even though the transformants exhibited increased GS activity under both N regimens. The GS_1 transformants in our present study, while exhibiting slower growth rate in the early stages of development compared to the NT plants and the other transformants, showed an extended life span and delayed senescence, more so, under LN. The inconsistency that has been observed in the literature with regards to the outcome of overexpressing *GS1* has been attributed to the transgene, the recipient of the transgene, and the growth conditions, specifically the availability and nature of N nutrients (Eckes et al. 1989; Temple et al. 1993; Oliveira et al. 2002; Ortega et al. 2004; Kirby et al. 2006). GS besides being regulated transcriptionally, is also subject to post-transcriptional (Ortega et al. 2006) and post-translational regulation (Ortega et al. 1999; Lima et al. 2006; Seabra et al. 2013), but how these regulatory steps cause prolonged life span, delayed senescence and late flowering, is not known.

The GS_1/SPS transformants displayed phenotypic and physiological attributes like the growth profile, flowering time and pod weight that is more similar to the SPS transformants than to the GS_1 transformants. At the

enzymatic level, the co-transformants under both N conditions showed similar SPS activity as the SPS transformants. GS activity on the other hand, was not consistent under the two N regimens. The activity in the GS_1 transformants was almost twice the level of activity in the GS_1/SPS transformants under LN but under HN regimen, the difference was significantly smaller. Since the plants grown under both LN and HN regimens are clonal, we could propose that under low nitrogen conditions, excess Suc or a downstream product of Suc metabolism, degrades GS and/or represses GS activity, possibly to maintain the C/N ratio (Zheng 2009). The lower GS activity in the co-transformants is contrary to what was expected, since Suc has been shown to activate GS_1, but the activation most likely is at the transcriptional level (Oliveira and Coruzzi 1999) and is thus not applicable to our studies since the *GS1* transgene is being driven by the CaMV 35S promoter.

SPS activity was consistently higher under the HN regimen, particularly in the SPS transformants. This is probably attributable to post-translational modification of the enzyme, a direct or indirect outcome of NO_3^- assimilation (Huber 2007). The Suc and starch content, however, was significantly higher under LN conditions. Under HN conditions, more of the Suc is probably diverted to the synthesis of C skeletons for the assimilation of N, thus accounting for lower Suc content. There are reports showing that in the presence of nitrate, starch levels go down (Paul and Stitt 1993). It is possible that under N deficient conditions, the photosynthate cannot be completely utilized and thus accumulates as starch. Furthermore, it has been shown that the level of mRNA for ADP-glucose pyrophosphorylase, a key enzyme in starch synthesis is decreased under high nitrogen (Scheible et al. 1997). An intriguing observation that we have made in this study is that while the Suc concentration is fairly similar in the SPS and GS_1/SPS transformants under both N conditions, the starch content is lower in the SPS transformants especially under HN regimen. The Suc in the co-transformants probably is used primarily to accommodate for the high energy consuming reaction catalyzed by GS instead of being stored as starch. This could also be the explanation for the low Suc:starch ratio in the GS_1 transformants ratio compared to the NT.

When comparing the growth pattern, all the transformants and the NT plants grew faster and flowered earlier under the HN regimen compared to the LN regimen. The fact that the GS_1/SPS transformants exhibited the same growth pattern as the SPS transformants and the onset of flowering was just a few days behind the SPS transformants (Figs. 5, 6, 7), would suggest that increased concentration of Suc represses the effects of increased GS activity with regards to growth and flowering time. This could be just a metabolic response, an attribute of the change in C/N ratio (Corbesier et al. 2002) or a consequence of Suc acting as a signaling molecule (Rolland et al. 2002; Palenchar et al. 2004; Price et al. 2004; Wind et al. 2010). The transition to flowering has been associated with leaf carbohydrate content, the degradation of starch and the transport of Suc to the shoot and root meristem (Corbesier et al. 1998). There is evidence suggesting that Suc promotes flowering (Moghaddam and Ende 2013) and the concept that has emerged is that high C:N ratio promotes flowering while a low C:N ratio promotes vegetative growth (Corbesier et al. 2002). The early flowering in the SPS transformants followed by the GS_1/SPS transformants can thus be attributed to the higher Suc content relative to organic N, when compared to the NT plants. On the same note, delayed flowering in the GS_1 transformants could be due to an increase in the relative availability of organic N resulting from increased assimilation and re-assimilation of ammonia.

While Fuentes et al. (2001), showed increased growth of tobacco transformants with the alfalfa GS_1 (*GS100*) gene, only under N deprived conditions, no mention was made as to their flowering time or their lifespan, our studies have clearly demonstrated delayed flowering and extended growth period under both N regimens. Increased and prolonged growth of the GS_1 transformants in this study, could be due to increased GS activity, that not only allows for primary assimilation of N but also the re-assimilation of ammonia released by the turnover of proteins during the natural process of senescence and the photorespiratory ammonia (Bernard and Habash 2009; Liu et al. 2008). We still do not have an explanation for the slow growth rate of the GS_1 transformants relative to the other classes of transformants and the NT plants, under low N conditions.

Enhancement of SPS activity would not only have a positive effect on the overall capacity to assimilate N but also for the availability of C for growth and development. The pods in the SPS over-expressers have significantly higher biomass individually and as a collection per plant followed by the co-expressers. There are several reports on the association of increased SPS activity with the production of new sinks and increased sink strength, although the sinks may range from flowers, fruits, tubers, fiber, stem and more (Micallef et al. 1995; Park et al. 2008, 2009; Haigler et al. 2007; Nguyen-Quoc et al. 1999; Baxter et al. 2003; Ishimaru et al. 2008; Laporte et al. 1997, 2001). An increase in pod size and overall pod weight per plant showed an inverse correlation with vegetative growth and height, in that the SPS transformants with its biggest pods and overall highest pod weight per plant, has the shortest stature compared to all the other classes of transformants. The source to sink interactions must be tightly coordinated because factors such as changes in the number of sink

tissues or changes in sink activity can result in the over consumption of photosynthate, which can negatively impact the plant at many levels, particularly plant growth and development (Engels et al. 2012). The low pod number in the GS_1 transformants can be a consequence of more photosynthate being used for vegetative growth.

Based on the results obtained in this study, it appears that increased SPS function is the major determinant of growth and development in tobacco, and it is only marginally affected by increased GS activity. Increased SPS activity in the SPS transformants and the GS_1/SPS transformants is accompanied by an increase in Suc level, which could then be rationalized to be the molecule with the central role. Sucrose, besides its contribution as a metabolite, is also a signaling molecule and it has been shown that many mRNAs and enzymes are synthesized de novo when the level of sucrose exceeds a certain threshold (Eveland and Jackson 2012; Wind et al. 2010). A wide array of plant developmental and metabolic processes is controlled by Suc, including nitrogen assimilation and transport but also carbon:nitrogen balance (Tognetti et al. 2013). KIN10/KIN11, which are members of the SnRK1 subfamily and closely related to the SNF1 and AMPK of yeast and mammals, respectively, are regulated by sucrose (Baena-Gonzalez et al. 2007). Suc functions as a signaling molecule also by increasing the levels of trehalose-6P, which plays a signaling role in carbon metabolism and growth (O'Hara et al. 2012). Suc, also affects expression of genes with roles in metabolism through the regulation of transcription factors, such as bZIP11 and other WRKYs. The regulation of these TFs is at the level of translation (Rahmani et al. 2009). There are other transcription factors involved in the expression of genes associated with many plant processes that are also known to be regulated by Suc (Teng et al. 2005). Suc also regulates the expression of the gene *Glb1* that encodes PII, a protein that coordinates the regulation of nitrogen assimilation in response to nitrogen, carbon and energy (Uhrig et al. 2009).

While our major intent in this study was to understand the interaction between C and N assimilation at the metabolic level, the analysis has been made more complex, considering that Suc is a multifaceted molecule acting both as a metabolite and a metabolic signal. To just understand how availability of C metabolites affects GS mediated N assimilation and vice versa, it would, be more appropriate to manipulate the expression of a gene in the C metabolic pathway whose function is more closely linked to the N metabolic pathway and has no signaling role.

Author contribution CSG, MS and SG conceived and designed the research. MS, SG, AP and JT conducted the experiments. CSG and SG analyzed the data. CSG and SG wrote the manuscript.

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