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ORIGINAL ARTICLE

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Decreased sucrose-6-phosphate phosphatase level in transgenic tobacco inhibits photosynthesis, alters carbohydrate partitioning, and reduces growth

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Abstract The aim of this work was to examine the role of sucrose-6-phosphate phosphatase (SPP; EC 3.1.3.24) in photosynthetic carbon partitioning. SPP catalyzes the final step in the pathway of sucrose synthesis; however, until now the importance of this enzyme in plants has not been studied by reversed-genetics approaches. With the intention of conducting such a study, transgenic tobacco plants with reduced SPP levels were produced using an RNA interference (RNAi) strategy. Transformants with less than 10% of wild-type SPP activity displayed a range of phenotypes, including those that showed inhibition of photosynthesis, chlorosis, and reduced growth rates. These plants had strongly reduced levels of sucrose and hexoses but contained 3–5 times more starch than the control specimens. The leaves were unable to export transient starch during extended periods of darkness and as consequence showed a starch- and maltose-excess phenotype. This indicates that no alternative mechanism for carbon export was activated. Inhibition of SPP resulted in an approximately 1,000-fold higher accumulation of sucrose-6-phosphate (Suc6P) compared to wild-type leaves, whereas the content of hexose-phosphates was reduced. Although the massive accumulation of Suc6P in the cytosol of transgenic leaves was assumed to impair phosphate-recycling into the chloroplast, no obvious signs of phosphate-limitation of photosynthesis became apparent. 3-Phosphoglycerate (3-PGA) levels dropped slightly and the ATP/ADP ratio was not reduced in the transgenic lines under investigation. It is proposed that in SPP-deficient plants, long-term compensatory responses give rise to the observed acceleration of starch synthesis, increase in total cellular Pi content, decrease in protein content, and related reduction in photosynthetic activity.

Keywords *Nicotiana* (sucrose) · Photosynthesis · Starch · Sucrose synthesis · Sucrose-6-phosphate phosphatase

Abbreviations AGPase: ADP-Glucose pyrophosphorylase · cFBPase: Cytosolic fructose-1,6-bisphosphatase · chl: Chlorophyll · Fru1,6bisP: Fructose-1,6-bisphosphate · Fru2,6bisP: Fructose-2,6-bisphosphate · Fru6P: Fructose-6-phosphate · Glc1P: Glucose-1-phosphate · Glc6P: Glucose-6-phosphate · IC-MS: Ion-exchange chromatography coupled to mass-spectrometry · ORF: Open reading frame · PEP: Phosphoenolpyruvate · 3-PGA: 3-Phosphoglycerate · Suc6P: Sucrose-6-phosphate · SPP: Sucrose-6-phosphate phosphatase · SPS: Sucrose-6-phosphate synthase · RNAi: RNA interference · T6P: Trehalose-6-phosphate · TPT: Triose-phosphate translocator · UDP-Gluc: Uridine 5′-diphosphoglucose

Introduction

Sucrose is the transport carbohydrate most widely distributed in the plant kingdom. It forms the interface between photosynthetically active source tissue and heterotrophic sink tissue, serving as an energy source for growth and development or supplying the accumulation of storage reserves such as starch. Beyond its role in energy metabolism, sucrose and its metabolites have been shown to affect the expression of genes involved in a number of metabolic pathways (Koch 1996). Given the central role played by sucrose in plant life, tight regulation of its synthesis and partitioning is vital for plant growth and development. Carbon that is fixed by photosynthesis is either exported to the cytosol via the triose phosphate–phosphate transporter and mainly converted

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to sucrose, or is transiently stored as starch within the chloroplast. The formation of sucrose occurs via a two-step reaction. First, sucrose-6-phosphate (Suc6P) is formed from UDP-glucose and fructose-6-phospate (Fru 6P) catalyzed by sucrose-6-phosphate synthase (SPS), subsequently Suc6P is hydrolyzed to form sucrose by a specific sucrose-6-phosphate phosphatase (SPP). The reaction catalyzed by SPP is essentially irreversible and displaces the reversible SPS reaction from equilibrium into the direction of net sucrose synthesis (Stitt et al. 1987). SPS has been identified as a major control point of the whole pathway. The enzyme is regulated by a hierarchy of mechanisms involving post-translational modification via phosphorylation, direct control by metabolic effectors (reviewed in Huber and Huber 1996), and protein–protein interactions involving 14-3-3 proteins (Toroser et al. 1998). This complex regulation ensures that sucrose synthesis is balanced with the momentary rate of photosynthesis. If sucrose synthesis is too fast, intermediates are withdrawn from the Calvin cycle and photosynthesis is inhibited (Stitt et al. 1987; Stitt 1986). If sucrose synthesis is too slow, P_i is sequestered from the chloroplast into phosphorylated intermediates, and ATP synthesis, regeneration of ribulose-1,5-bisphosphate, and photosynthesis are impaired (Stitt et al. 1987; Stitt 1986).

The role of SPP in the regulation of photosynthetic carbon partitioning has been relatively neglected. Earlier reports that in vivo SPP activity is substantially higher than SPS activity suggested that SPP is unlikely to play a regulatory role in sucrose synthesis (Hawker and Smith 1984). However, recent evidence that SPS and SPP might form a complex in vivo (Echeverria et al. 1997), opened the possibility that SPP could have a role in metabolite channeling between the two enzymes and could contribute to the control of flux through the pathway. It was not until recently that the enzyme has been purified to homogeneity from rice leaves, and the gene cloned (Lunn et al. 2000). Biochemical characterization revealed that the enzyme exists as a dimer of 50-kDa subunits and is highly specific for Suc6P (Lunn et al. 2000). Genes encoding SPP-like sequences have as yet been described from several plant species, including *Arabidopsis*, maize, tomato, rice, and wheat (Lunn 2003) and it appears that they encode small gene families with, e.g., four members in *Arabidopsis* and three in rice (Lunn 2003). Amino acid comparison revealed that the catalytic domain of SPP has some similarity (about 35%) to the carboxy-terminal region of SPS and it has been suggested that the SPP-like domain of SPS could be involved in SPS–SPP complex formation, comparable to the trehalose-synthesizing complex in yeast (Lunn et al. 2000). Remarkably, trehalose-6-phosphate (T6P) is not only an intermediate of trehalose metabolism, but has also been shown to posses important signaling functions in plants and yeast (Thevelein and Hohmann 1995; Schluepmann et al. 2003; Pellny et al. 2004). A similar function in signaling for Suc6P has also been proposed (Lunn et al. 2000; Paul et al. 2001; Lunn and MacRae 2003), although until now no experimental evidence exists for such a proposal.

To examine the *in planta* role of SPP and the potential impact of Suc6P on plant metabolism, we cloned SPP from *Nicotiana tabacum* and silenced its expression in transgenic tobacco plants using RNA interference (RNAi). Transformants were analyzed with respect to the effects on photosynthesis, sucrose synthesizing capacity, carbohydrate partitioning, and plant development.

Materials and methods

Transgenic plants, growth and maintenance

Tobacco plants (*Nicotiana tabacum* cv. Samsun NN) were obtained from Vereinigte Saatzuchten eG (Ebsdorf, Germany) and grown in tissue culture under a 16-h-light/8-h-dark regime (irradiance 150 μmol quanta m^{-2} s^{-1}) at 50% humidity on Murashige Skoog medium (Sigma, St. Louis, MO, USA) containing 2% (w/v) sucrose. Plants in the greenhouse were kept in soil under a light/dark regime of 16 h light (25°C) and 8 h (20°C) dark.

Cloning of SPP from tobacco and expression in *E. coli*

Standard cloning procedures were carried out as described (Sambrook et al. 1989). Putative SPP ORFs were amplified by PCR from a tobacco cDNA library derived from leaf material. Primers FB223 5′-ATGGATCAGC TAACCAGTCGCCGCAC-3′ and FB225 5′-CTAAA AGAACCAGGACGCGGAGTCACT-3′, respectively, were used in the reaction. The primers were deduced from EST sequences from tomato (GenBank Accessions BI933832 for FB223 and BG125217 for FB225, respectively) which were predicted to encode SPP-like sequences based on their similarity on protein level to SPP1 from *Arabidopsis thaliana* (GenBank Acc. No. AF283565). Resulting DNA fragments were cloned into pCR2.1 (Invitrogen, Karlsruhe, Germany) and sequenced. For expression in *E. coli* the coding region of the putative NtSPP2 was amplified by PCR using primers FB228 (5′- GGATCCATGGATCAGCTAAC-CAGTGCC-3′) and SPPr (5′- GTCGACCTAAAAGA-ACCAGGACGCGGAGTCACT-3′), which introduced *Bam*HI and *Sal*I recognition sites, respectively, into the sequence (underlined). The resulting fragment was cloned between the *Bam*HI and *Sal*I site of expression vector pMal-c2 (New England Biolabs, Beverly, MA), producing a maltose-binding protein (MBP)-NtSPP2 fusion protein. The resulting plasmid pMal-NtSPP2 was transformed into *E. coli* strain M15 (Qiagen, Hilden, Germany) and the protein expressed as outlined in the instructions accompanying the pMal-c2 vector (New England Biolabs, Beverly, MA). Bacteria were lysed by sonication. After centrifugation, the MBP-NtSPP2

fusion protein was purified from the extract using amylose-agarose resin and eluted using maltose. The eluted protein was analyzed by SDS-PAGE.

Plasmid construction and plant transformation

To generate the *NtSPP2* RNAi construct, a fragment of *NtSPP2* comprising nucleotides 1–660 (based on the translational start codon) was amplified by PCR, using primers FB228 and FB229 (5′- GTCGACTACCATTACACCATAACACATC-3′; a *Sal*I site is underlined). The resulting DNA fragment was digested with *Bam*HI and *Sal*I before it was ligated in sense orientation into pUC-RNAi, a vector containing the first intron of the gibberellin 20 oxidase gene from potato flanked by a short polylinker sequence (Chen et al. 2003). The same fragment was inserted in antisense orientation into the *Bgl*II/*Xho*I sites of pUC-RNAi already carrying the sense fragment. The resulting RNAi fragment was excised by *Pst*I and cloned between the 35S CaMV promoter and the OCS terminator of the binary vector pBinAR (Höfgen und Willmitzer 1990) using a compatible *Sbf*I site, producing the vector pBin-NtSPP-RNAi.

Transformation of tobacco plants by *Agrobacterium*-mediated gene transfer using *A. tumefaciens* strain C58C1:pGV2260 was carried out as described previously by Rosahl et al. (1987).

Antibody preparation and immunodetection

Antibodies against SPP from tobacco were manufactured by a commercial organization (Eurogentec, Seraing, Belgium) by immunizing rabbits with affinity purified MBP-NtSPP2 fusion protein. Protein extracts were prepared by homogenization of leaf material in a buffer containing 25 mM HEPES pH 7.0, 12 mM MgCl2, 0.5 mM EDTA, 8 mM DTT, 10 μM PMSF, 0.1% Triton and 10% glycerol. Protein content was determined according to Bradford (1976) with bovine γ-globulin as the standard. After heat denaturation, 50 μg total protein was subjected to electrophoresis on a 10% (w/v) SDS-polyacrylamide gel and subsequently transferred onto nitrocellulose membrane (Porablot, Macherey und Nagel, Düren, Germany). Immunodetection was carried out using the ECL kit (Amersham Pharmacia Biotech, Freiburg, Germany), according to the manufacturer, using the rabbit anti-SPP primary antibody and peroxidase-conjugated secondary antibody (Pierce, Rockford, IL, USA).

RNA isolation and northern blot

Total RNA was extracted from tobacco leaf material as described by Logemann et al. (1987), and 30 μg per sample were separated on a 1.5% (w/v) formaldehyde-agarose gel using conditions described by Sambrook et al. (1989). After electrophoresis, RNA was transferred to a nitrocellulose membrane (GeneScreen, NEN Lifescience Products, Boston, USA) and fixed by UV-crosslinking. Radioactive labeling of cDNA fragments was performed using the High Prime-kit (Roche, Mannheim, Germany) and [$\alpha-^{32}$P]-dCTP. Hybridization was carried out as described previously (Herbers et al., 1994) and signals were detected by exposure to Kodak X-ray films (Sigma).

Semi-quantitative RT-PCR

For RT-PCR experiments, 2.5 μg of DNAse-treated total RNA was reverse-transcribed into cDNA with oligo(dT) (30-mer) using M-MLV[H$^-$] reverse transcriptase (Promega). A fraction (about 1/20) of the first strand cDNAs was used as a template for PCR with gene-specific primers in a volume of 100 μl with 1 U of *Taq*-polymerase (Takara, Shouzo, Japan), 20 μM each dNTP, and 0.25 μM of each primer. An initial denaturation step for 5 min at 95°C was followed by 25–35 cycles of 5 s at 95°C; 45 s at 55°C and 1 min, 72°C. PCR products were separated on 1% (w/v) agarose gels containing ethidium bromide and visualized by UV light. Amplification of actin using primers 5′-ATGG CAGACGGTGAGGATATTCA-3′ and 5′-GCCTTT GCAATCCACATCTGTTG-3′ served as an internal control.

Enzyme activities

To determine enzyme activities, frozen leaf material was extracted as described above. SPP and fructose-6-phosphatase activities were assayed as described in Lunn et al. (2000). SPS activity was assayed by quantifying the fructosyl moiety of sucrose using the anthrone test exactly as described by Baxter et al. (2003). AGPase activity was measured as detailed by Müller-Röber et al. (1992) and cytosolic FBPase activity was assayed as in Zrenner et al. (1996).

Chlorophyll determination

Chlorophyll was measured in ethanol extracts and concentrations were determined as described by Lichtenthaler (1987).

CO_2 exchange and carbon partitioning

Net CO_2 uptake rates were measured with a portable photosynthesis system LI-6400 (LI-COR, Lincoln, NE). For determination of light response curves of CO_2 gas exchange, photosynthetic photon flux density (PPFD) was varied between 0 and 2000 μmol photons m^{-2} s^{-1}. Concentration of CO_2 in the air entering the chamber was adjusted to 400 μmol mol^{-1} and leaf temperature

was maintained at 25°C. The resulting data for the relationship between A and I (light response curves) were fitted by the equation of a non-rectangular hyperbola as described by Hajirezaei et al. (2002). A–c_i response curves at PPFD = 2000 µmol m^{-2} s^{-1} were obtained by measuring net CO_2 uptake rates at three different ambient CO_2 concentrations (approximately 200, 380, and 940 µmol mol^{-1}). The incorporation of $^{14}CO_2$ into tobacco leaf disks was performed as described by Quick et al. (1989). Leaf disks were incubated in an oxygen electrode (LD-2, Hansatech, Kings Lynn, UK) under CO_2-saturated atmosphere, which was generated through the addition of 400 µl of 2 M K_2CO_3/$KHCO_3$ (pH 9.3) solution enriched with $NaH^{14}CO_3$ (specific activity 0.14 MBq $mmol^{-1}$). Samples were illuminated for 20 min using the beam of a slide-projector and immediately frozen in liquid nitrogen.

Determination of chlorophyll fluorescence

Chlorophyll fluorescence was measured using a PAM-2000 portable fluorometer (Walz, Effeltrich, Germany) as described in Hajirezaei et al. (2002).

Starch, soluble sugars, and metabolites

Soluble sugar and starch levels were determined in leaf samples extracted with 80% (v/v) enthanol per 20 mM HEPES, pH 7.5 as described (Stitt et al. 1989). Maltose was measured from the same extracts with HPLC using the conditions exactly as described by Börnke et al. (2001). 3-PGA and hexose-phosphates were measured spectrophotometrically in neutralized trichloroacetic acid (TCA) as described (Stitt et al. 1989). From the same extracts fructose-1,6-bisphosphate (Fru1, 6bisP), UDP-glucose, sucrose-6-phosphate and adenylates were determined using ion chromatography coupled to mass spectrometry (IC-MS). Fructose-2,6-biphosphate (Fru2, 6bisP) was extracted from frozen leaf material using ice-cold 50 mM NaOH as described (van Schaftingen 1984) and measured using IC-MS.

The IC-MS instrumentation consisted of a Dionex HPLC system (Dionex, Idstein, Germany) with a MSQ mass detector (Dionex). Anionic compounds were separated on a 250×2 mm AS11-HC column (Dionex) connected to a 10×2 mm AG 11-HC guard column (Dionex) and an ATC-1 anion trap column which is placed between the eluents and separation columns to remove anions present in the solutions. The gradient was accomplished with H_2O (buffer A; HPLC-grade, Millipore) and 100 mM NaOH (buffer B). The column was equilibrated with a mixture of buffer A (96%) and buffer B (4%) at a flow rate of 0.3 ml min^{-1} and heated to 30°C during the whole measurement. The gradient was produced by changes of buffer B as follows: 0–8 min, 4%; 8–28 min, 30%; 28–38 min, 60%; 38–51 min, 100%; 51–64 min, 4%.

Detection of metabolites was achieved by atmospheric pressure ionization-electrospray mass detection in the negative ion mode. The drying/carrier gas was nitrogen heated to 350°C with a flow of 400 l h^{-1} at a pressure of 60 psi. The capillary was set to 3,500 V with a fragmentator voltage of 50 V and dwell time of 1 second. Ions in the range of m/z from 100 to 600 were scanned. Single ion mode (SIM) was performed in time windows of 5 min during total run time (64 min), i.e., up to five SIMs were run in parallel to enhance sensitivity. Sample injection volume varied from 5 to 10 µl.

Recovery experiments were undertaken in order to confirm the validity of the measurements. A defined portion of the metabolite of interest was added to the leaf disks before extraction and determined exactly as described above. The recovery rates were between 85 and 110% of the amount added.

Determination of total phosphate

Determination of inorganic phosphate on a total cell basis was carried out using the ascorbic acid–ammonium molybdate reagent as described (Ames 1966).

Visualization of starch in tobacco leaves

Following illumination for 12 h, plants were kept in complete darkness for 24 h. Subsequently, leaves were destained with 80% ethanol at 80°C and then stained with Lugol's solution to visualize the starch content.

Nucleotide sequences

The nucleotide sequences reported in this paper have been lodged with Genbank/DDBJ/EMBL under accession number AY729655 (*NtSPP1*) and AY729656 (*NtSPP2*), respectively.

Distribution of materials

Upon request, all novel materials described in this publication will be made available in a timely manner for non-commercial research purposes, subject to the requisite permission from any third-party owners of all or parts of the material. Obtaining any permissions will be the responsibility of the requestor.

Results

Cloning of SPP from *N. tabacum* and its expression in *E. coli*

The GenBank EST database was searched with the deduced amino acid sequence of the *Arabidopsis AtSPP1*

gene (AF283565) using the TBLASTN algorithm, to find cDNA clones from *Solanaceaous* species that might encode SPP. Several SPP-like ESTs were identified from potato and tomato, respectively. Two ESTs from tomato (BI933832 and BG125217) contained the putative translational start and stop site, respectively, and hence were used to deduce oligonucleotides flanking the potential SPP coding region. These were used to amplify SPP sequences from cDNA derived from tobacco source leaves by RT-PCR. Subsequent cloning and sequence analysis of the PCR fragments revealed that they fell into two different classes sharing 88% identity on the nucleotide level and 94% on protein level, respectively, with respect to each other, both showing high similarity to other SPP-like sequences from different species. The two different clones were designated as *NtSPP1* and *NtSPP2*. Both contained a contiguous open reading frame (ORF) of 1278 bp encoding a protein with a predicted molecular mass of 48 kDa. The *Arabidopsis* genome contains four *SPP*-like genes (Lunn 2003) and the total number of isoforms in tobacco is unknown. However, since we were not able to PCR amplify additional *SPP*-like sequences from tobacco leaf material (data not shown) it seems likely that *NtSPP1* and *NtSPP2*, respectively, represent the major leaf isoforms of the enzyme in this plant. To further confirm that these clones indeed were SPP genes, the putative *NtSPP2* was expressed in *E. coli* and shown to encode a functional SPP enzyme (Table 1). The tobacco SPP was shown to be specific for Suc6P and is inhibited by EDTA, as it has been shown for other SPPs (Lunn et al. 2000; Lunn 2003).

Generation of transgenic tobacco plants with decreased expression of SPP

In order to study the *in planta* role of SPP, the corresponding transcript was targeted through an RNA interference (RNAi) strategy. To this end, a fragment containing the first 660 bp of the SPP ORF was amplified by PCR and inserted first in the antisense orientation downstream of the CaMV 35S promoter, followed by a short intron, and by the same SPP fragment, but in sense orientation. The sequence identity of this region was more than 90% between *NtSPP1* and *NtSPP2* and thus assumed to be sufficiently high to target both transcripts for RNAi. The resulting construct was subsequently used to generate transgenic tobacco plants by *Agrobacterium*-mediated gene transfer. Of the 80 primary transformants which were transferred to the greenhouse, 10 exhibited various degrees of growth retardation, had jagged pale green leaves, and developed necrotic lesions in old leaves. One of the transformants was only moderately affected and showed similar growth behavior as the wild-type control plants. The other, more strongly affected, primary transformants grew much smaller than the wild-type and were delayed in setting seed by up to 3 weeks. All the phenotypically affected transformants contained less sucrose than the control specimen and displayed reduced SPP mRNA level, as revealed by RT-PCR (data not shown). At an early stage, a decision was taken to analyses the progeny of these transformants to avoid potential complication arising from regeneration in tissue culture. To this end, three strongly repressed lines (SPPi-17, SPPi-18, and SPPi-36) and one moderately affected line (SPPi-31) were chosen for a detailed analysis in the T1 generation. Seeds of the aforementioned SPPi lines were germinated on kanamycin-containing medium alongside with ME-1, a transgenic control line containing the GUS gene driven by a cytosolic FBPase (cFBPase) promoter from potato (Ebneth 1996), and resistant plants were analyzed 6–7 weeks after transfer to the greenhouse. Inhibition of SPP expression was not inherited uniformly and T1 plants of each line had a range of phenotypes. T1 plants of line SPPi-36 displayed a particular wide range of phenotypes, with individuals varying from those having wild-type-like appearance to plants that were strongly retarded in growth, indicating that multiple transgene copies inserted at different loci were segregating. This line was selected to estimate the flux control coefficient for SPP in the pathway for sucrose synthesis as described in the following. Offspring of the other three lines that retained the down-regulation of expression were selected on the basis of having phenotype development comparable to that of the parent plant and these lines were chosen for a detailed physiological analysis. Figure 1a illustrates the growth retardation of the selected transgenic lines versus control plants.

Table 1 Enzyme activity of recombinant NtSPP2

Substrate	Phosphatase activity, nmol min^{-1} mg^{-1} protein	
	SPP activity	Residual activity
Suc6P	260	n.d.
Fru6P	n.d.	12

Recombinant NtSPP2 was overexpressed in *E. coli* as a fusion to the maltose-binding protein and partially purified on amylose resin. Phosphatase activity was measured using Suc6P or Fru6P, respectively, as substrates either in the absence (SPP activity) or presence of 20 mM EDTA (residual activity). Data represent the mean of two measurements. *n.d.* Not detectable

Transcript level, Western blot, and enzyme activity measurements for the selected transgenic lines

Initial attempts to detect SPP mRNA by northern blotting failed, most likely owing to the low abundance of the transcript (data not shown). Therefore, RT-PCR with gene-specific primers targeting both isoforms was conducted using *actin* as an internal control. The results showed that SPP mRNA steady-state level was drastically reduced in all three lines (Fig. 1b). Using an antiserum raised against NtSPP2 but recognizing both highly identical isoforms, it was shown that SPP protein was

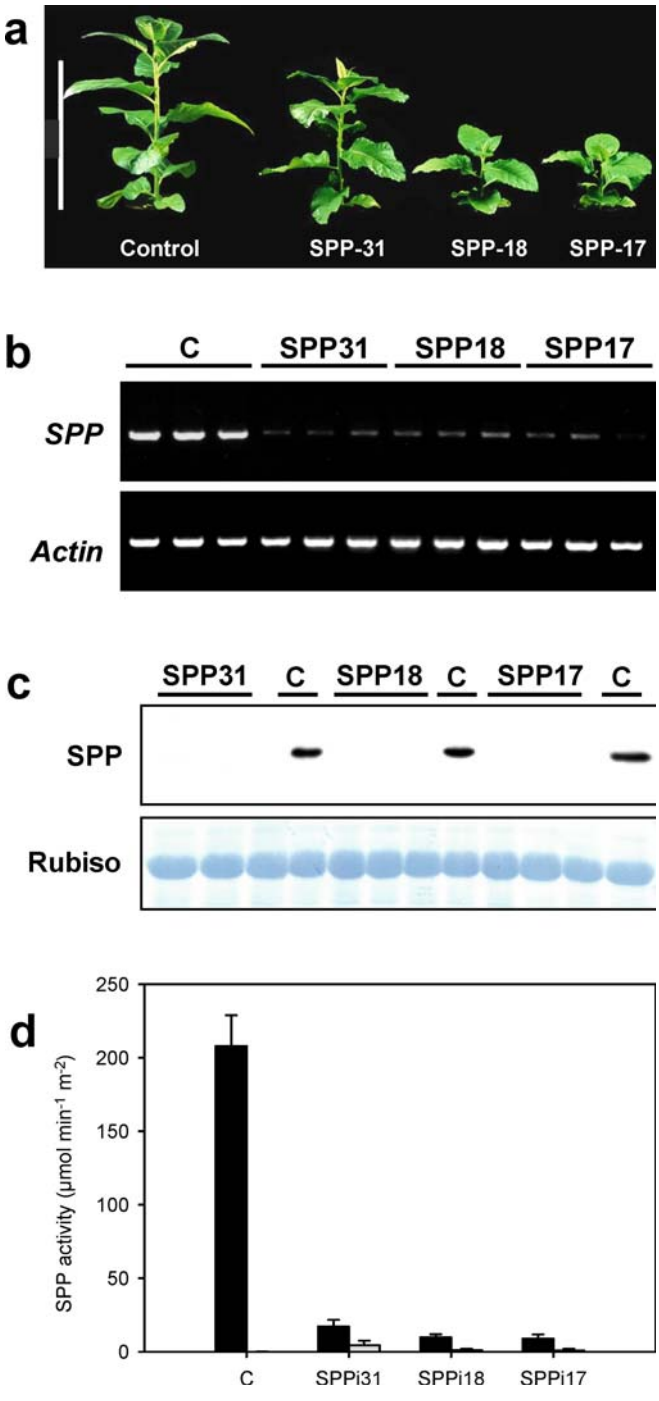

Fig. 1 Basic characterization of tobacco plants inhibited in SPP expression. **a** Phenotype of SPPi tobacco plants. Transgenic lines showed strong growth retardation with respect to the control plant. The picture was taken 6 weeks after transfer of the plants to the greenhouse. The white bar is equivalent to 40 cm. **b** RT-PCR analysis of SPP expression in SPPi tobacco plants. Total RNA was isolated from leaves and used for RT-PCR as described in Materials and methods applying 35 amplification cycles. Equal amounts of cDNA were controlled by amplification of a constitutively expressed *actin* gene (30 cycles). Samples from three independent plants per line were analyzed. **c** Western blot analysis of SPP expression. Fifty micrograms of protein was separated by SDS-PAGE and immunodecorated using a polyclonal SPP antibody. Amidoblack staining of the Rubisco band was taken as a control for equal loading. Samples from three independent plants per line were analyzed. **d** SPP activity in leaves. Enzyme activity was determined in leaves from six individual plants per line (±SD). *Black bars* indicate phosphatase activity in the absence of 20 mM EDTA whereas *gray bars* stand for the residual activity in the presence of 20 mM EDTA

virtually eliminated in the transgenics (Fig. 1c) and, accordingly, SPP activity was reduced to 4.3% (SPPi-17), 4.6% (SPPi-18), and 8.2% (SPPi-31), respectively, of that of control plants when calculated on a leaf area basis (Fig. 1d).

Basic characterization of SPPi plants

Shoot growth was reduced by 23–77% in the SPPi transformants (Table 2) compared to control plants. Fresh weight per leaf area was slightly increased while dry weight decreased by 14–22%, indicating that the transgenic plants contained more water (Table 2). Consistent with the pale green phenotype, chlorophyll (chl) content was decreased by 24–43% (Table 2). Protein content decreased by 17–50% on a leaf area basis (Table 2). When comparing transgenic plants to wild-type of the same age (6 weeks), leaf number in SPPi plants was reduced as compared to that of the control plants (Table 2). However, seeds of RNAi plants germinated at the same time after sowing as the control seeds, and the first true leaves of the transgenic plants emerged at the same time as in case of the wild-type plants (data not shown). Flowering was delayed by 16–25 days in SPPi plants; however, total leaf number at the time of flowering was increased by 6–13, which is indicative of a delayed senescence.

CO_2 gas exchange

Light-saturated photosynthesis at 400 µmol mol^{-1} CO_2 (Fig. 2a) was inhibited by 52, 62, and 67% in SPPi-31, SPPi-18, and SPPi-17, respectively. Maximum quantum yield (calculated as the initial slope of the light response curve) was not affected in the transformants (data not shown), indicating that the efficiency of the light reaction, including light harvesting and electron transport, is not influenced in SPPi plants. Other factors are involved in the reduction of maximum net CO_2 uptake. These could include limitation of the rate of diffusion of CO_2 to the site of carboxylation and/or the amount and the catalytic activity of Rubisco (Long et al. 1993). In an attempt to separate these possible co-limiting factors A/C_i response curves were measured for a PPFD value of 2,000 µmol quanta m^{-2} s^{-1} for two plants of SPPi line 17 (Fig. 2b). The initial slope of the A/C_i response curves, i.e., the carboxylation efficiency, was distinctly higher in the control plant than in the transgenic plants, indicating a correspondingly higher Rubisco activity in the control plants. This is in keeping with the reduced

Table 2 Basic characterization of SPPi transformants

Line	Shoot length (cm)	SLFW (mg FW.cm^{-2})	SLDW (mg DW.cm^{-2})	Chl (μg.cm^{-2})	Total protein (μg.cm^{-2})	Flowering time (days)	Leaf number 6-week-old plant	Leaf number flowering plant
Control	42.9 ± 4.1	16.2 ± 0.7	2.2 ± 0.08	45.2 ± 3.56	440 ± 37	69 ± 0.8	23 ± 0.5	30 ± 1.0
SPPi31	32.8 ± 3.1	17.2 ± 0.8	1.9 ± 0.08	34.5 ± 3.16	364 ± 24	85 ± 1.2	20 ± 0.4	36 ± 2.2
SPPi18	10.8 ± 1.2	20.8 ± 1.3	1.9 ± 0.11	31.2 ± 1.91	288 ± 29	92 ± 1.7	18 ± 0.8	38 ± 2.9
SPPi17	9.7 ± 1.5	18.4 ± 1.3	1.7 ± 0.23	25.6 ± 2.87	218 ± 33	94 ± 0.8	18 ± 0.8	43 ± 2.6

Shoot length, specific leaf fresh weight (SLFW) and dry weight (SLDW), respectively, chlorophyll, total protein and leaf number were determined 6 weeks after transfer of the plants into the greenhouse. Flowering time was recorded from the time the plants were soiled till the first blooming flower appeared. Total leaf number was counted again when the plants were flowering. Each value represents the mean (± SD) of six different plants

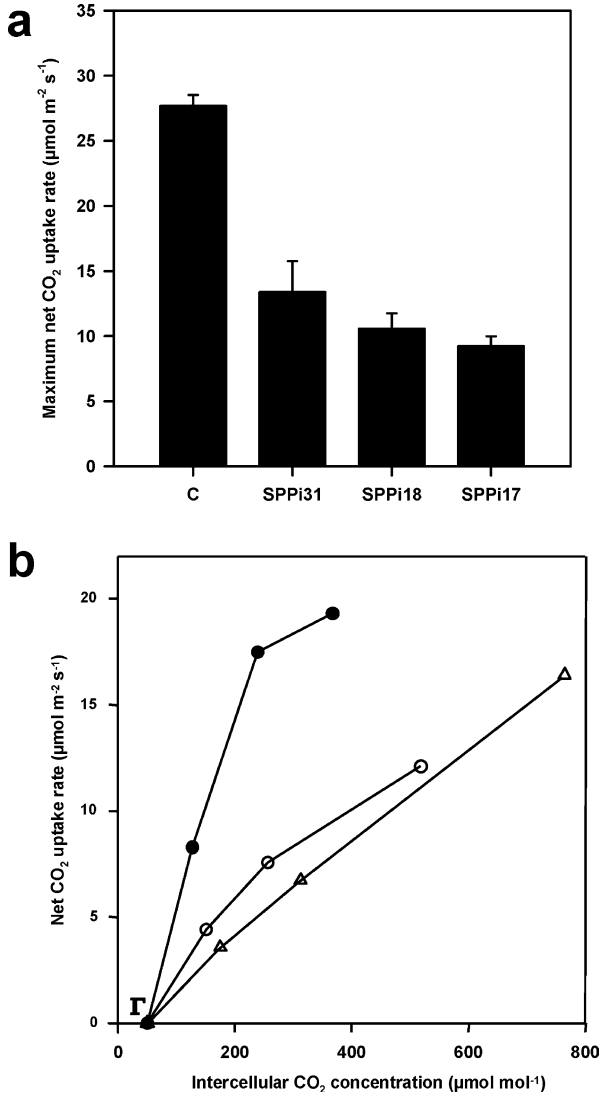

Fig. 2 CO_2 exchange in SPPi tobacco plants versus control plants. **a** Net CO_2 uptake rates of attached leaves were measured using a portable photosynthesis system LI-6400. Values represent the mean (± SD) of six measurements. **b** $A - c_i$ response curves at PPFD = 2,000 μmol m^{-2} s^{-1} were obtained by measuring net CO_2 uptake rates at three different ambient CO_2 concentrations (approximately 200, 380, and 940 μmol mol^{-1}) in source leaves of two transgenic tobacco plants from line SPPi17 (*open circles and triangles*) and a control plant (*closed circles*), respectively. Leaf 8 as counted from the top was used for the measurements

protein content of SPPi plants which also affects Rubisco content. The inhibition of photosynthesis in SPPi transformants was accompanied by a decrease of F_v/F_m (from 0.83 ± 0.004 in control plants to 0.74 ± 0.02, 0.76 ± 0.019 and 0.77 ± 0.008 in lines 17, 18, and 31, respectively), indicative of a damage to these leaves from photoinhibition.

Repression of SPP strongly impairs leaf carbohydrate metabolism

Analysis of assimilate accumulation provided strong evidence that the decreased SPP content compromised the ability of SPPi plants to synthesize sucrose. As shown in Table 3, sucrose levels determined in source leaves 5 h into the light period were reduced by 59–73% in the transgenics as compared to the control. Moreover, the levels of glucose and fructose were also decreased by 65–85% in those plants. In contrast, starch levels were drastically increased by a factor of 3 to 5 indicating that photosynthetic carbon partitioning was altered in favor of starch (Table 3).

To determine whether altered degradation rates or altered rates of starch synthesis might be responsible for the elevated starch accumulation, SPPi plants along with control plants were subjected to a prolonged dark period and, subsequently, leaves were stained for starch content with iodine. In leaves from control plants starch was totally degraded after 24 h of dark treatment, whereas leaves from the transgenic plants still contained enough starch to stain blue in the presence of iodine (Fig. 3), indicating they were unable to mobilize excess carbohydrates accumulated during the previous day.

Recent evidence suggests that maltose serves as a major metixotic intermediate in the conversion of starch to sucrose during the night (Niityllä et al. 2004; Chia et al. 2004). In order to investigate whether the impaired ability of SPPi plants to synthesize sucrose would lead to an accumulation of primary products of starch breakdown, the amounts of maltose in leaves of transgenic plants were determined in samples taken at the end of the dark period. It was found that the leaves of the transgenic lines contained considerably more maltose than the control plants (Table 3). This indicates that

Table 3 Metabolite levels in leaves of 6-week-old SPPi transformants compared to control plants

	Control	SPPi31	SPPi18	SPPi17
Carbohydrates				
Sucrose	1416 ± 200	575.8 ± 171	523.8 ± 109	387.5 ± 146.2
Glucose	1640 ± 10	510.6 ± 143	685.7 ± 154	230.6 ± 141.5
Fructose	1651 ± 354	584.9 ± 243	482.3 ± 167.3	256.8 ± 151.3
Maltose	15.25 ± 6	21.1 ± 15.7	76.3 ± 35.7	290.8 ± 67.7
Starch	3.5 ± 1	11.1 ± 2.2	15.8 ± 5.1	18.0 ± 4.6
Nucleotides and Nucleotide sugar				
ATP	17.1 ± 1.6	12.0 ± 3.72	8.92 ± 2.17	7.74 ± 2.24
ADP	17.5 ± 6.0	6.9 ± 1.27	8.60 ± 2.17	11.65 ± 4.61
AMP	2.1 ± 0.34	2.5 ± 0.47	3.36 ± 0.58	2.23 ± 1.13
ATP/ADP	0.98	1.74	1.04	0.66
UDP-Gluc	18.1 ± 2.9	16.7 ± 3.27	15.77 ± 3.14	12.62 ± 6.53
Phosphorylated intermediates				
Suc6P	0.203 ± 0.093	172.8 ± 50.1	233.5 ± 01.7	214.5 ± 59.0
3-PGA	8.69 ± 3.36	7.42 ± 2.19	7.37 ± 2.31	7.56 ± 2.24
Pyruvate	1.78 ± 0.89	1.37 ± 0.97	1.5 ± 0.17	1.26 ± 0.29
PEP	1.51 ± 0.67	1.12 ± 0.37	1.56 ± 0.27	0.81 ± 0.32
Fru-1,6-bisP	1.01 ± 0.18	0.68 ± 0.28	0.60 ± 0.32	0.48 ± 0.20
Glc6P	36.77 ± 6.61	26.40 ± 9.44	23.75 ± 4.28	22.13 ± 2.54
Fru6P	11.85 ± 2.13	9.59 ± 3.33	9.04 ± 1.69	8.47 ± 0.91
Glc1P	2.49 ± 0.55	2.01 ± 0.74	1.41 ± 0.18	1.41 ± 0.30
Pi	875.9 ± 188.1	3141.1 ± 523.1	2765.7 ± 386.6	2885.2 ± 558
3-PGA/Pi	1.028 ± 0.456	0.241 ± 0.073	0.268 ± 0.080	0.268 ± 0.085
Phosphoester/Pi	6.96	6.97	9.97	8.82

Leaves were harvested 5 h into the photoperiod (except for maltose where sampling took place at the end of the dark period). Concentrations are given in μmol m^{-2} except that of starch which is presented as mmol m^{-2} hexose equivalents. 3-PGA/Pi and phosphoeseter/Pi ratios are given as percentage. Values represent the mean (±SD) from at least five different plants per line

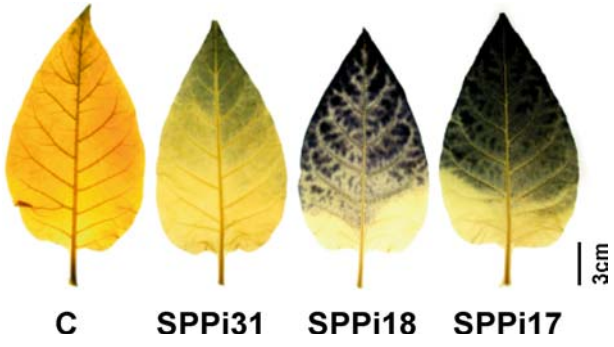

Fig. 3 Visualization of starch accumulation in leaves from SPPi plants as compared to the control. After 12 h of illumination, plants were kept in complete darkness for 24 h. Source leaf blades were de-colored with hot 80% ethanol and subsequently stained with Lugol's solution. The tenth leaf (as counted from the top) is shown in each case

starch breakdown per se is operating in SPPi plants but primary products of this process cannot be further metabolized.

To further investigate whether the observed starch-excess phenotype is accumulative and thus, is merely a result of reduced starch turn-over during the dark period, or whether it is additionally caused by alterations in carbon partitioning during the course of photosynthesis, we investigated the fate of recently assimilated CO_2. To this end, leaf disks of dark-adapted plants were incubated in a leaf-disk electrode under saturating light and saturating $^{14}CO_2$ for 20 min. Following ethanol extraction, radiolabel was determined in the soluble and insoluble fraction. The percentage of ^{14}C incorporated into starch increased from 48% in control plants to 62–66% of the total label incorporated in SPPi lines (Table 4). By ion exchange chromatography the soluble fraction was further subdivided into neutrals (mainly sucrose), anions (phosphorylated intermediates and organic acids), and cations (amino acids). Consistent with a block in sucrose synthesis, the incorporation of label into the neutral fraction decreased from 38% in the control to 9% and 6%, respectively, in the mutants. Labeling of the cationic fraction, predominantly consisting of amino acids, was unchanged. A massive fivefold to sixfold increase in the proportion of label incorporated into the anionic fraction was observed in the SPPi plants as compared to the control plants, indicating that the soluble fraction in the transgenics is predominantly composed of phosphorylated intermediates and organic acids, respectively, whereas these compounds account only for a small proportion of this fraction in wild-type plants.

Intermediates

The results of the above experiments indicate that profound changes occur in the SPPi plants in the content of metabolites undergoing high fluxes. In addition to conventional enzymatic assays for the determination of hexose-phosphates and 3-PGA, we used high-performance ion exchange chromatography coupled to mass spectrometry (IC-MS) for a range of other low abundance metabolites (for details see Materials and methods). This analytical technique offers the following advantages: high selectivity, high sensitivity (within the nanomolar range), the fact that no sample derivatization is required, separation at ambient temperature, and absolute quantification of individual compounds. These

Table 4 Incorporation of $^{14}CO_2$ into starch and soluble components under saturating light and CO_2

	^{14}C-incorporation			
	Control	SPPi31	SPPi18	SPPi17
Insoluble	48.46 ± 5.64	62.27 ± 6,46	67.37 ± 2.74	66.97 ± 1.38
Soluble	51.54 ± 5.64	37.73 ± 6.46	32.63 ± 2.74	33.03 ± 1.38
Insoluble/soluble	95.9 ± 21.8	171.7 ± 48.6	208.3 ± 27.5	203.2 ± 13.0
Neutral	38.12 ± 8.00	9.45 ± 4.19	6.20 ± 0.73	6.07 ± 0.27
Anionic	5.46 ± 1.59	18.82 ± 3.86	19.55 ± 2.41	20.73 ± 1.65
Cationic	7.95 ± 1.04	9.46 ± 1.83	6.88 ± 1.05	6.23 ± 0.38

Leaf disks were cut from six separate dark-adapted plants per line and illuminated in an oxygen electrode containing air saturated with $^{14}CO_2$. After 20 min the leaf disks were extracted and fractionated.
Data are presented as percentage (±SD) of total incorporation ($n = 6$)

advantages permit the accurate measurement of many phosphorylated intermediates, inorganic ions, nucleotides, and nucleotide sugars in a single HPLC run. The method proved to be particularly useful for measurement of Suc6P, whose levels in wild-type plants are only 0.2 ± 0.09 μmol m^{-2} and thus more than 100 times less the amounts of Glc6P (Table 3). However, Suc6P levels increase around 1000-fold in SPPi lines, which is in keeping with the reaction catalyzed by the enzyme. Unexpectedly, the amount of 3-PGA did not alter in SPPi lines indicating that no P_i-limitation of photosynthesis occurred under steady-state conditions, although recycling of P_i into the chloroplast should be severely impaired owing to a lower rate of sucrose synthesis and the massive accumulation of Suc6P. Pyruvate and PEP levels remained largely unchanged and thus glycolysis most likely does not substantially contribute to the 3-PGA pool. Decreased SPP expression left UDP-Gluc levels largely unaltered but caused a decrease of hexose-P. Although there was no change in 3-PGA, Fru1,6bisP decreased in all SPPi lines. Surprisingly, on a whole-cell level there was a substantial increase in inorganic phosphate in the transgenics, actually quenching the increase in phosphoester/phosphate ratio entailed by the strong increase in Suc6P, although it remains unclear if the compartmentation of the P_i-pool restricts its use in photosynthesis (Table 3).

The total adenylate pool in the SPPi lines was reduced to approximately 60% of wild-type level (Table 3) but the ATP:ADP ratio was not consistently changed.

Measurement of the activities of AGPase, cytosolic FBPase, and SPS

We next investigated whether decreased SPP expression affects the activities of other key enzymes of carbohydrate metabolism. AGPase activity, which was chosen as a representative for starch synthesis, decreased in all the SPPi lines (Table 5). The maximal catalytic activity (V_{max}) of SPS in SPPi plants increased slightly, by 10%, 13% and 23% in lines 17, 18 and 31, respectively, when calculated on the basis of leaf area (Table 5). This was more appreciable when calculated on a total protein basis (53%, 63%, and 54%, respectively; data not shown, compare Tables 2 and 5). This was unexpected, since the levels of Fru6P and Glc6P, which are the substrate and activator of SPS, respectively, were decreased in these lines, and, in turn, P_i, the allosteric inhibitor of the enzyme, was increased. This argues for a regulation of the enzyme at the point of transcription eventually causing SPS protein amounts to increase. In strong support of this hypothesis, Northern blot analyses revealed an up-regulation of SPS mRNA steady-state level in the SPPi lines (Fig. 4). SPS activity was also assayed under limiting, V_{sel}, conditions in order to reveal changes in the phosphorylation state of the protein, which is known to influence its in vivo activity (Huber et al. 1989). Under these assay conditions, activity increased by 127, 148, and 154% in SPPi-17, −18, and 31, respectively. The activation state of SPS, reflected by the ratio between V_{max} and V_{sel}, increased by approximately

Table 5 Enzyme activities in leaves of tobacco transformants expressing the SPP-RNAi construct

	Enzyme activity μmol min^{-1} m^{-2}			
	Control	SPPi31	SPPi18	SPPi17
SPS				
V_{max}	101.72 ± 17.23	125.21 ± 11.80	114.61 ± 17.43	112.25 ± 15.8
V_{sel}	19.65 ± 3.59	40.14 ± 5.67	34.04 ± 7.47	32.51 ± 4.37
V_{sel}/V_{max} (%)	19.64 ± 3.66	31.96 ± 1.71	29.44 ± 2.26	28.99 ± 0.89
AGPase	74.61 ± 4.41	30.10 ± 4.02	31.47 ± 8.67	39.91 ± 5.18
cFBPase	50.5 ± 6.45	41.41 ± 4.09	40.29 ± 2.73	38.34 ± 3.06

Enzyme activities were determined in leaf samples of 6-week-old plants. Samples were taken 5 h into the light period. SPS activity was measured under both optimal (V_{max}) and limiting (V_{sel}) assay conditions.
Data are means (±SD) of determinations on six individual plants per line

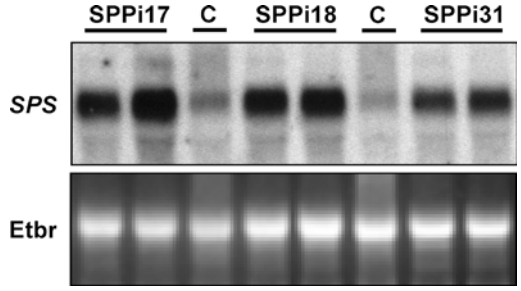

Fig. 4 Northern blot analysis of SPS mRNA steady-state level in control and SPPi plants. Total RNA (30 µg) was subject to gel blot hybridization using ^{32}P-labeled SPS cDNA from tobacco (GenBank Acc. No. AF 194022). Ethidium bromide staining of 25S rRNA serves as control for equal loading. Results of two independent plants per line are shown

one third, from 20% in control plants to 30% in SPPi lines, indicative of a lower phosphorylation state of the enzyme pool.

Control of sucrose synthesis is assumed to be shared by SPS and cFBPase, and both enzymes are supposed to be coordinately regulated (Stitt 1989). The activity of cFBPase is subject to allosteric control by the signal metabolite Fru2,6bisP (Stitt 1990). It strongly inhibits cFBPase, which converts Fru1,6bisP to Frc6P as a starting point for sucrose biosynthesis. Upon illumination Fru2,6bisP levels are diminished, and thus causing feed-forward stimulation of sucrose biosynthesis. Fru2,6bisP levels determined 5 h into the light period revealed no differences in the level of this metabolite between SPPi plants and wild-type (data not shown). Activity of cFBPase dropped by approximately 20% in SPPi lines when calculated on the basis of leaf area (Table 5), which is in the range of the reduction in total protein content observed in these lines. Taken together, the data clearly demonstrate that in SPPi plants sucrose biosynthesis was not up-regulated at the cFBPase step.

Estimation of the flux control coefficient for SPP in the pathway for sucrose synthesis

The relative contribution of an enzyme to the control of flux in a pathway can be experimentally estimated from transgenic plants with a stepwise reduction in the activity of the enzyme. The change in enzyme activity and the resulting change in the flux through the pathway are quantified and plotted against each other to estimate the control coefficient for that enzyme (Kacser and Burns 1973). If the enzyme under investigation is markedly reduced in its activity over other enzymes in the pathway, the control coefficient will tend to rise and thus will be overestimated. It is, therefore, important to include plants with a relative small change in activity and to estimate the control coefficients from the slope of the flux-enzyme amount plot in the vicinity of the wild-type value (Stitt 1994). T1 plants from the segregating SPPi line 36 were chosen for this experiment, since a considerable proportion of those plants were indistinguishable from the wild-type. Nevertheless, residual SPP activity in these plants ranged from 80% to 20% as compared to the wild-type (Fig. 5). The control coefficient for SPP over sucrose synthesis estimated from the slope of the plot in that range of residual enzyme activity was found to be close to zero (-0.081 ± 0.095).

Discussion

SPP is not rate limiting for sucrose biosynthesis in tobacco source leaves

SPP catalyses the final step of sucrose biosynthesis, which is the hydrolysis of Suc6P into sucrose and inorganic phosphate. The latter is transported back into the chloroplast where it sustains ATP synthesis and regeneration of ribulose-1,5-bisphosphate. While the regulatory role of other enzymes in sucrose biosynthesis, such as SPS or cFBPase, is well established, little is known about the contribution of SPP to control of flux through the pathway. In order to obtain a better insight into the in vivo role of this enzyme, transgenic tobacco plants with a reduced level of SPP expression have been created using an RNAi approach. Removal of up to 80% of SPP activity had only minor effects on leaf sucrose content and plant growth behavior. Using transgenic plants showing only a moderate reduction in enzyme activity, the control coefficient for SPP in the pathway of sucrose biosynthesis was estimated to be close to zero. These data are in agreement with earlier reports that SPP activity by far exceeds that of SPS and thus SPP is likely to exert no major control over flux through the pathway (Hawker and Smith 1984).

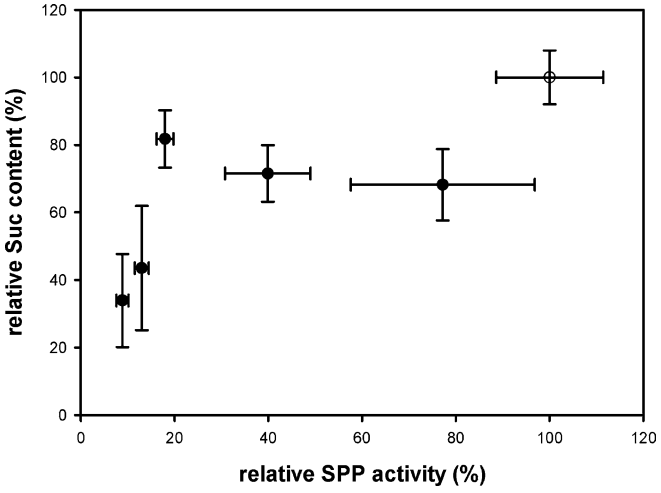

Fig. 5 Relation of SPP enzyme activity and sucrose content in a segregating T1 population of line SPP-36 (*closed circles*) normalized to the wild-type control (*open circle*). The control coefficient for SPP for the pathway of sucrose biosynthesis was estimated from plants with 20–100% residual SPP activity. Each value represents the mean of at least 4 independent plants ± SD

SPP repression alters photosynthetic carbon partitioning in favor of starch and leads to a starch excess phenotype

The shift in carbon partitioning from sucrose in favor of starch observed in SPPi plants with less than 10% residual enzyme activity is reminiscent of the changes observed in potato plants with reduced levels of TPT or cFBPase, respectively (Riesmeier et al. 1993; Zrenner et al. 1996). Starch levels in these plants were increased by a factor of three to four at the end of the light period as compared to wild-type plants. The transgenic plants lacked a clear phenotype mainly because they were able to mobilize starch at a much higher rate during the night and, thereby, compensating for the limitation in the upper part of the pathway leading to sucrose (Heineke et al. 1994; Riesmeier et al. 1993; Zrenner et al. 1996). Recent evidence suggests that maltose is the primary product of starch breakdown and is the major metabolite exported from the chloroplast at night (Niittylä et al. 2004). Maltose is further metabolized by a specific glucosyltransferase eventually providing the substrates for sucrose synthesis (Chia et al. 2004), thereby bypassing the reactions catalyzed by TPT and cFBPase, respectively. Although SPPi plants show a similar response with respect to the shift in carbon partitioning in favor of starch, they lack the option of compensating for the block in sucrose synthesis by using an alternative pathways, because the lesion is downstream of the point where sucrose synthesis during the day and during the night converge. The accumulation of maltose in leaves of SPPi plants at the end of the dark period is indicative of a reduction of starch breakdown. The accumulation of maltose may further indicate that the glucosyltransferase catalyzing the transfer of one glucosyl unit of maltose to glycogen and releasing the other (Chia et al. 2004) is prone to feedback inhibition if its products cannot be efficiently further metabolized. A starch-excess phenotype has also been observed in plants restricted in sucrose transport capacity due to antisense inhibition of SUT1, a sucrose-transporter involved in phloem loading (Bürkle et al. 1998). However, in contrast to SPPi plants, the SUT1 antisense plants were not only impaired in their ability to export transient starch during prolonged periods of darkness but also accumulated large amounts of soluble sugars in their leaves (Bürkle et al. 1998). On the other hand, *Arabidopsis* plants with antisense repression of SPS displayed no redistribution of assimilated carbon towards starch, and leaf starch content was actually decreased in these plants (Strand et al. 2000). Seemingly, the metabolic changes brought about by a block of sucrose synthesis at the point of the SPP reaction can trigger markedly different responses with respect to carbon partitioning as compared to a block in the step catalyzed by SPS. The partitioning of assimilated CO_2 in favor of starch in SPPi lines occurred irrespective of a reduction in AGPase activity of approximately 50%. Previous studies with AGPase antisense potato plants indicated that a reduction of this enzyme to less than 40% residual activity was necessary before it became limiting for starch synthesis (Müller-Röber et al. 1992).

Feedback regulation and P_i limitation of photosynthesis

P_i in addition to light and CO_2 represents a prime substrate for photosynthesis in higher plants. During photosynthesis, triose-phosphates are exported to the cytosol and converted to sucrose, releasing P_i which than returns to the chloroplast to support photosynthesis. This mutual dependence of chloroplast and cytosolic metabolism requires a high degree of regulation. If sucrose synthesis outpaces photosynthesis, stromal metabolites would be depleted and the regeneration of Ru1,5 bp would be inhibited. On the other hand, if sucrose synthesis is too slow, accumulation of phosphorylated metabolites would lead to a P_i limitation of photosynthesis (Stitt 1997; Stitt et al. 1987). The organic phosphoester-pool in SPPi plants is at least doubled as compared to the control plants, owing to the massive accumulation of Suc6P in these plants. This could impose similar consequences on photosynthesis as observed after feeding of phosphate-sequestering agents to leaf tissue. Incubation of detached leaves in solutions containing mannose or 2-deoxyglucose strongly affect photosynthesis, since these compounds are readily phosphorylated in vivo but cannot be further metabolized. As a result cytosolic P_i is sequestered into organic compounds and becomes limiting (Klein and Stitt 1998). However, upon analysis of the steady-state levels of metabolites, this explanation seems unlikely to account for the limitation of photosynthesis. First, there is no increase in 3-PGA or decrease in the ATP/ADP ratio as would be expected when photosynthesis is P_i-limited (Stitt 1997). Second, although the sum of phosphoesters measured in SPPi plants is higher there is a concomitant increase of total P_i content, eventually resulting in a strong decrease of the 3-PGA/P_i ratio. In plants, the cytosolic P_i pool is relatively constant and excess P_i accumulates in the vacuole (Foyer and Spencer 1986), from which it is gradually released if cytosolic P_i is depleted, allowing for short-term adjustment of P_i available in the cytoplasm. Although it is difficult to estimate which proportion of the extra P_i found in SPPi plants is metabolically accessible, elevated P_i levels observed in these plants can be interpreted as a long-term adaptation preventing Pi limitation of photosynthesis, despite the tremendous build-up of Suc6P as a consequence of SPP deficiency. This response is comparable to what is observed when warm grown plants are transferred to chilling temperatures. As a short-term effect, large pools of phosphorylated intermediates accumulate owing to the inhibition of sucrose synthesis under low temperatures (Hurry et al. 1993), eventually leading to P_i limitation of photosynthesis. In the long term, however, acclimation to low temperatures involves redistribution of P_i increasing the amount available in the cytosol and thus relieving P_i limitation despite high levels of

phosphorylated intermediates (Hurry et al. 1993). It is conceivable that a similar mechanism operates in SPPi plants to prevent long-term P_i limitation of photosynthesis.

Another well-defined mechanism exerting feedback control of photosynthesis is sugar-mediated repression of the Calvin cycle, which has been observed frequently in plants accumulating starch and soluble sugars as a consequence of impaired sucrose loading into the phloem (Bürkle et al. 1998; von Schaewen et al. 1990; Krapp und Stitt 1995). Since the levels of soluble sugars in SPPi plants are lower than in the control plants it can be ruled out that carbon-catabolite repression of photosynthesis involves hexose-induced repression of photosynthetic genes via, e.g., a hexokinase-mediated signaling step, as has been shown in other systems (Jang et al. 1997). Another possibility involves impaired chloroplast function as a consequence of excessive starch accumulation interfering with CO_2 diffusion or rupturing chloroplasts (Grub and Mächler 1990).

Taking into account the results of CO_2 gas exchange measurements it seems likely that the decrease in photosynthetic activity (Fig. 2) is largely attributable to an induced reduction of protein content and a concomitant decrease of Rubisco content. In this context, the reduction of chlorophyll content is of minor importance.

SPS activity is increased in SPPi plants

SPS activity in SPPi plants is increased in terms of both V_{max} and V_{sel} which probably reflects a response to counteract the decrease in sucrose biosynthetic capacity in these plants. It has previously been demonstrated that SPS transcript abundance is responsive to sugars. Provision of glucose to excised sugar beet leaves strongly increased the steady-state level of SPS mRNA, whereas exogenous sucrose slightly repressed expression (Hesse et al. 1995). SPPi plants show an increase in SPS expression in the absence of an increase of soluble sugars, which makes it tempting to speculate that in this case it is the low sucrose content acting as the signal for induction. Alternatively, the ratio between glucose and sucrose could serve as a measure to determine the cell's demand for SPS activity, however, this is unlikely since both glucose and sucrose are decreased to a similar extent in SPPi plants.

Usually, SPS is regulated in concert with cFBPase, the enzyme catalyzing the second major regulatory step of sucrose biosynthesis (Stitt 1989). Cytosolic FBPase is subject to allosteric control by the signal metabolite Fru2,6bisP which acts as a potent inhibitor of enzymatic activity (Stitt 1990). With the onset of photosynthesis, rising 3-PGA and decreasing Pi inhibit fructose-6-phosphate,2-kinase. This results in declining Fru2,6bisP levels, eventually leading to a stimulation of cFBPase activity. Increasing levels of hexose-P then stimulate SPS. In turn, accumulating sucrose leads to a partial inactivation of SPS and rising hexose-P leads to an activation of fructose-2,6-bisphosphatase. The resulting increase in Fru2,6bisP then inactivates cFBPase. Thus, the measurement of Fru2,6bisP provides indirect evidence about cFBPase activity. Given the stimulation of SPS activity, unaltered Fru2,6bisP content as well as the decrease in cFBPase activity in the SPPi transformants is unexpected, but is fully consistent with the observation that Fru6P does not accumulate. This indicates that SPS and cFBPase are regulated independently in SPPi plants. This resembles the situation observed during phases of starch mobilization at night when cFBPase but not SPS is dispensable for sucrose synthesis. In the transformants, however, these changes do not achieve a net stimulation of sucrose because the initial problem (low SPP activity) is still present.

The idea has been put forward that Suc6P might posses a signaling function in analogy to that described for T6P (Lunn et al. 2000; Paul et al. 2001; Lunn and MacRae 2003). Transgenic tobacco plants engineered to contain twofold to threefold more T6P than the wild-type displayed higher photosynthetic capacity whereas plants with reduced T6P content showed the opposite effect (Pellny et al. 2004). The change in Suc6P content in SPPi plants is much more dramatic (approximately 1000-fold increase as compared to the control plants) and the interpretation of the metabolic phenotype is complicated by the far-reaching effects of sucrose deprivation on the whole plant level. However, the changes observed can well be explained otherwise than by a specific signaling function of Suc6P. Apparently, the high Suc6P content is rather part of the resulting chain of events than a signal responsible for the changes observed in photosynthetic carbohydrate metabolism in SPPi transformants.

Conclusions

This is the first investigation using an RNAi approach to study the role and significance of SPP in regulating sucrose biosynthesis, carbohydrate partitioning, and photosynthesis. The key findings of our work can be summarized as follows: (i) SPP is abundant and does not exert significant control of sucrose synthesis under normal conditions; (ii) the fact that ca. 90% of SPP activity can be "lost" before any effect on sucrose synthesis can be observed suggests that the enzyme is unlikely to be tightly coupled to the SPS complex in vivo, certainly there does not seem to be a requirement for a 1:1 stoichiometry between SPS and SPP as has been suggested before (Lunn et al. 2000); (iii) the loss of SPP activity results in a change in carbon partitioning in favor of starch, something that is not observed in SPS antisense plants but does occur in cFBPase antisense *Arabidopsis* plants (Strand et al. 2000).

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