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Evidence for the sucrose-binding protein role in carbohydrate metabolism and transport at early developmental stage

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Despite the large amount of data regarding sucrose-binding proteins (SBP), their functions remain largely unknown and controversial. In this investigation we performed a detailed temporal and spatial characterization of the phenotypes related to photosynthesis, sucrose exudation and carbohydrate metabolism in *SBP* antisense plants to gain insights into the physiological role of SBP. Significant reductions in net photosynthesis and in stomatal conductance were observed in the *SBP* antisense lines but were restricted to the vegetative phase, and persisted during a daily time course at this phase. Photosynthesis was saturated at a substantially lower irradiance in source leaves of the antisense lines, suggesting that light utilization is decreased in these plants. A slight reduction in soluble sugars was observed throughout the development of source leaves, partially overlapping a decrease in sucrose synthase activity (EC 2.4.1.13); whereas a transient increase in starch and adinosine diphosphate (ADP)-glucose pyrophosphorylase activity (EC 2.7.7.27) as well as decreased leaf sucrose exudation were detected in the beginning of the vegetative phase. These changes in source leaves were accompanied by reductions in sucrose and starch in sink leaves, hexoses and sucrose in roots and hexoses in shoot apex, which were observed before the occurrence of a significant reduction in height and in leaf number in the transgenic lines. These alterations in growth parameters did not persist throughout the development, but were associated with a delay in flowering time and leaf senescence in the SBP antisense lines. A likely involvement of SBP in sink strength is discussed.

Introduction

The sucrose-binding protein (SBP) was first identified in soybean by its capacity to bind to a sucrose analog, 6′-deoxy-6′-(4-azido-2-hydroxy)-benzamido-sucrose, which competitively inhibited the influx of radiolabeled sucrose into protoplasts from developing soybean cotyledons (Ripp et al. 1988). Despite the lack of structural similarity between SBP and other known membrane transport proteins, subsequent progresses in characterizing SBP have implicated the protein as the low-affinity component of sucrose uptake system in plants. Lines of evidence for such a role include (1) the immunolocalization

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Abbreviations – A, net photosynthetic rate; AGPase, ADP-glucose pyrophosphorylase; Ca, ambient CO_2 partial pressure; CaMV-35S, cauliflower mosaic virus 35S; Ci, intercellular CO_2 partial pressure; FW, fresh weight; gs, stomatal conductance; lsd, least statistic difference; NPTII, neomycin phosphotransferase II; Pi, orthophosphate; PPFD, photosynthetic photon flux density; RuBP, ribulose-1,5′-bisphosphate; SBP, sucrose-binding protein; Susy, sucrose synthase; SUT, sucrose transporter; Triose-P, triose-phosphate; WT, wild-type.

of the protein to the plasma membrane of cells that are actively engaged in sucrose transport (Grimes et al. 1992, Harrington et al. 1997, Tegeder et al. 1999, Warmbrodt et al. 1989, 1991); (2) the partitioning and solubilization properties of SBP indicating that a proportion of the protein is tightly associated with the external surface of the plasma membrane (Overvoorde and Grimes 1994); (3) the demonstration that SBP from *Glycine max* (GmSBP1) mediates proton-independent sucrose transport across the yeast plasma membrane (Grimes and Overvoorde 1996, Overvoorde et al. 1996, 1997) and (4) with biochemical features that closely resemble the kinetics properties of the previously characterized linear component of sucrose uptake in higher plants (Grimes and Overvoorde 1996, Lin et al. 1984, Maynard and Lucas 1982a, 1982b). However, the biological relevance of these findings has been questioned by recent work, in which a direct comparison between the sucrose transporter- (SUT1-) and SBP-mediated sucrose uptake in yeast was performed (Elmer et al. 2003). The rate of sucrose transport mediated by GmSBP1 was 16-fold lower than that mediated by SUT1, which has been described as the proton-motive-force-driven sucrose symporter that mediates phloem loading and long-distance sucrose transport (Bürkle et al. 1998, Riesmeier et al. 1994). This low effectiveness of SBP transport activity is not consistent with the interpretation that GmSBP directly mediates sucrose transport in *planta*. In support of this argument, the *Vicia faba* SBP homolog failed to mediate sucrose uptake in the yeast system (Heim et al. 2001). Therefore, a SBP-mediated sucrose transport activity is unlikely to operate in higher plants and a scenario for SBP function remains elusive.

SBP shares similar structural characteristics with members of the vicilin family, which belongs to the cupin superfamily of proteins. Very likely SBPs and vicilins originated from the same precursor of the vicilin family (Contim et al. 2003, Heim et al. 2001). The similarities between vicilin-like seed storage proteins and SBP extend to include subcellular compartmentalization and expression, as a large proportion of SBP is colocalized in seed protein bodies, and exhibits cotyledon-specific expression (Elmer et al. 2003, Heim et al. 2001). Although these observations could implicate functional analogy to seed storage proteins, compelling evidence in the literature indicates that members of the GmSBP family may perform distinct functions. In fact, soybean SBP has been shown to alter plant growth and carbohydrate partitioning in leaves of tobacco (*Nicotiana tabacum* L. cv. Havana) and to enhance the rate of sucrose uptake in transgenic tobacco suspension cells (Delú-Filho et al. 2000, Pedra et al. 2000).

SBP2 repression studies in tobacco have shown some of the typical phenotypes caused by impairment of sucrose translocation, such as accumulation of carbohydrates within source leaves, inhibition of photosynthesis and stunted growth (Pedra et al. 2000). However, in those studies the physiological measurements were conducted only at a discrete time point in the vegetative phase and, therefore, a complete evaluation of SBP function throughout development was not performed. In addition, whether the antisense growth-related phenotypes resulted from impairment of a SBP-mediated sucrose transport or from activation of an alternative SBP-regulated sucrose uptake system was unsolved. This latter hypothesis has been raised because expression of a soybean SBP complementary DNA in tobacco cell lines altered sucrose-cleaving activities, and the increase in SBP2 levels had a stronger effect on sucrose synthase (Susy) activity than on sucrose uptake (Delú-Filho et al. 2000). The pleiotropic effects from manipulation of SBP levels would explain the higher efficiency of sucrose uptake by the transgenic tobacco cells and suggest that SBP may function in the sucrose translocation pathway by regulating the expression or activity of alternative carbohydrate uptake systems (Pirovani et al. 2002). However, these observations have not been extended to the whole plant level. Here we further characterized the homozygous SBP antisense T2 plants by measuring growth-rate parameters, carbohydrate partitioning in source and sink organs, sucrose export from detached leaves and carbohydrate-metabolizing activities throughout development. The overall altered plant development in the antisense lines supports the notion that sucrose translocation was impaired, but the biochemical and physiological phenotypes were restricted to the vegetative phase.

Materials and methods

Plant transformation and T2 lines generation

Transgenic tobacco (*N. tabacum* L. cv. Havana) plants expressing the *GmSBP2* gene under the control of the cauliflower mosaic virus 35S (CaMV-35S) promoter, either in the sense or antisense orientation, were derived from several independent transformants as described (Pedra et al. 2000). For these studies, we used in vitro propagated T2 transgenic plants expressing the *GmSBP2* in the sense or antisense direction. T2 seeds were germinated on Murashige-Skoog medium containing 100 mg L^{-1} kanamycin sulfate, and homozygous T2 lines with respect to the T-DNA loci were selected by determining the frequency of their antibiotic-resistant T3 seeds after self-pollination. The incorporation of the transgenes was further confirmed by PCR with neomycin phosphotransferase II (*NPTII*)– and *GmSBP2*-specific primers (Delú-Filho et al. 2000, Pedra et al. 2000), and accumulation of SBP2 was monitored in each generation by immunoblotting analysis as described by Pedra et al. (2000). The sense and antisense constructs are designated here as 35S-sbp2-S and 35S-sbp2-AS, respectively.

Plant growth conditions

The selected plants were maintained under in vitro conditions (Alvim et al. 2001). After acclimatization, plants were grown in a mixture of soil, sand and cattle manure (3:1:1) in greenhouse conditions under natural conditions of light, relative humidity (65–85%) and temperature (18–30°C) or in a controlled environment at 25°C and 250 μmol m^{-2} s^{-1} of photosynthetic photon flux density (PPFD). All the experiments were conducted with at least five clones from at least three independently transformed lines for each DNA construct.

Leaf gas-exchange measurements

For the measurements of gas exchange, we used an infrared gas (CO_2/H_2O) analyzer (LI-6400, LICOR, Lincoln, NE) in an open system. Measurements of leaf gas exchange included net CO_2 assimilation rate (A, μmol m^{-2} s^{-1}), stomatal conductance (gs, mol m^{-2} s^{-1}) and intercellular CO_2 partial pressure (Ci, μmol mol^{-1}). The measurements were done using a 6-cm^2 clamp-on leaf cuvette and in the same attached, mature and youngest fully expanded leaf. In the gas-exchange chamber, the conditions were preadjusted for flow rate (500 μmol s^{-1}), leaf temperature (25°C) and PPFD (1000 μmol m^{-2} s^{-1}). Reference CO_2 (approximately 360 μmol mol^{-1}) and H_2O (approximately 65 at 85% relative humidity) concentration were uncontrolled (ambient conditions). For A × Ci curves, reference CO_2 (50, 100, 200, 400, 600 and 800 μmol mol^{-1}) was provided by small CO_2 pressurized gas cylinders. For A × PPFD curves (0, 50, 100, 200, 400, 600, 800, 1000, 1500 and 2000 μmol photons m^{-2} s^{-1}) and the other experiments (1000 μmol photons m^{-2} s^{-1}) the PPFD during the measurements was obtained using an artificial quartz halide light source (red-80%/blue-20%, LI-6400-02B LED light source, LICOR, Lincoln, NE) controlled with a quantum sensor located inside the leaf cuvette. All measurements were carried out between 07:30 and 11:30 h except for day course experiments (7:00–18:00 h).

Carbohydrate determinations from tissues and exudation

Carbohydrates were extracted according to the method of Trethewey et al. (1998) from 50 mg of leaf disks. Harvested leaves were ground to a fine powder in liquid nitrogen and carbohydrates were extracted three times by boiling with 80% (v/v) ethanol for 20 min. Supernatants were collected by centrifugation (14 000 *g* for 5 min) and stored at −20°C until soluble sugar determination. The starch was extracted from the pellet with 0.2 *M* KOH at 95°C for 60 min and then neutralized with acetic acid. The supernatant was separated by centrifugation (14 000 *g* for 10 min) and stored at −20°C until starch determination. Sucrose, glucose and fructose were determined spectrophotometrically (Stitt et al. 1989). Starch content was analyzed using enzymatic hydrolysis and spectrophotometric determination of glucose (Trethewey et al. 1998). Phloem exudates were collected using the ethylenediaminetetraacetate method (King and Zeevaart 1974) according to Zhao et al. (2000) and sugars were determined as described above.

Enzyme assays

Enzymes were extracted according to the method of Geigenberger and Stitt (1993) from 200 mg of leaf disks. Cell-wall invertase activity (EC 3.2.1.26) was assayed by measuring the amount of glucose hydrolyzed from sucrose (Lowell et al. 1989). Susy (EC 2.4.1.13) was assayed by measuring the amount of uridine 5′-diphosphate–glucose hydrolyzed from sucrose (Geigenberger and Stitt 1993). Adinosine diphosphate (ADP)-glucose pyrophosphorylase (AGPase) activity (EC 2.7.7.27) was assayed by measuring the amount of glucose-1-phosphate formed from ADP-glucose (Sweetlove et al. 1996).

Protein and chlorophyll determination

Protein concentration was determined according to the method of Bradford (1976). Total chlorophyll content was determined spectrophotometrically after quantitative extraction with 80% (v/v) acetone in the presence of approximately 1 mg of $NaHCO_3$ as described by Lichtenthaler (1987).

Data analyses

Statistical analysis of data was performed using a one-way analysis of variance and the least statistic difference test for multiple pairwise comparisons at $P < 0.05$ using the Software Scientific (SOC) package (EMBRAPA, Brazil).

Results

The *SBP* antisense plants display transient growth-related phenotypes during the early vegetative phase and delayed flowering time

We have previously generated transgenic tobacco plants expressing the *SBP2* gene in either sense or antisense orientation (Pedra et al. 2000). The accumulation of an endogenous tobacco *SBP* homolog in untransformed plants as well as the extent of antisense repression and overexpression in T0 primary antisense and sense transformants have been already characterized in detail in previous reports (Delú-Filho et al. 2000, Pedra et al. 2000). The gene copy number of the construct in the transformed plants was further confirmed by segregation analysis of the *NPTII* gene in the T2 plants. These analyses suggested that 35S-sbp2-AS5-1, 35S-sbp2-AS5-2, 35S-sbp2-AS6-5, 35S-sbp2-AS6-6 and 35S-sbp2-AS6-9 T2 plants appeared to have an integrated T-DNA locus on a single chromosome, because 75% of their T3 segregating seedlings were resistant to kanamycin (Fig. S1). The plants 35S-sbp2-AS5-8 and 35S-sbp2-AS6-4 with at least a T-DNA locus were homozygous antisense lines. The lines that did not show Mendelian segregation were not considered further.

In general, the growth of the antisense transgenic lines was retarded when compared with control plants (Fig. S2A). This led to a delay in the induction of flowering in the antisense lines in comparison to the control plants [wild-type (WT) and pBI] and sense plants that bloomed at the same time (Figs. S2A and B). The intensity of the growth-related phenotypes exhibited by the antisense transgenic lines was very similar, regardless of their different copy number and gene dosage (Figs. S1 and S2B). Therefore, in

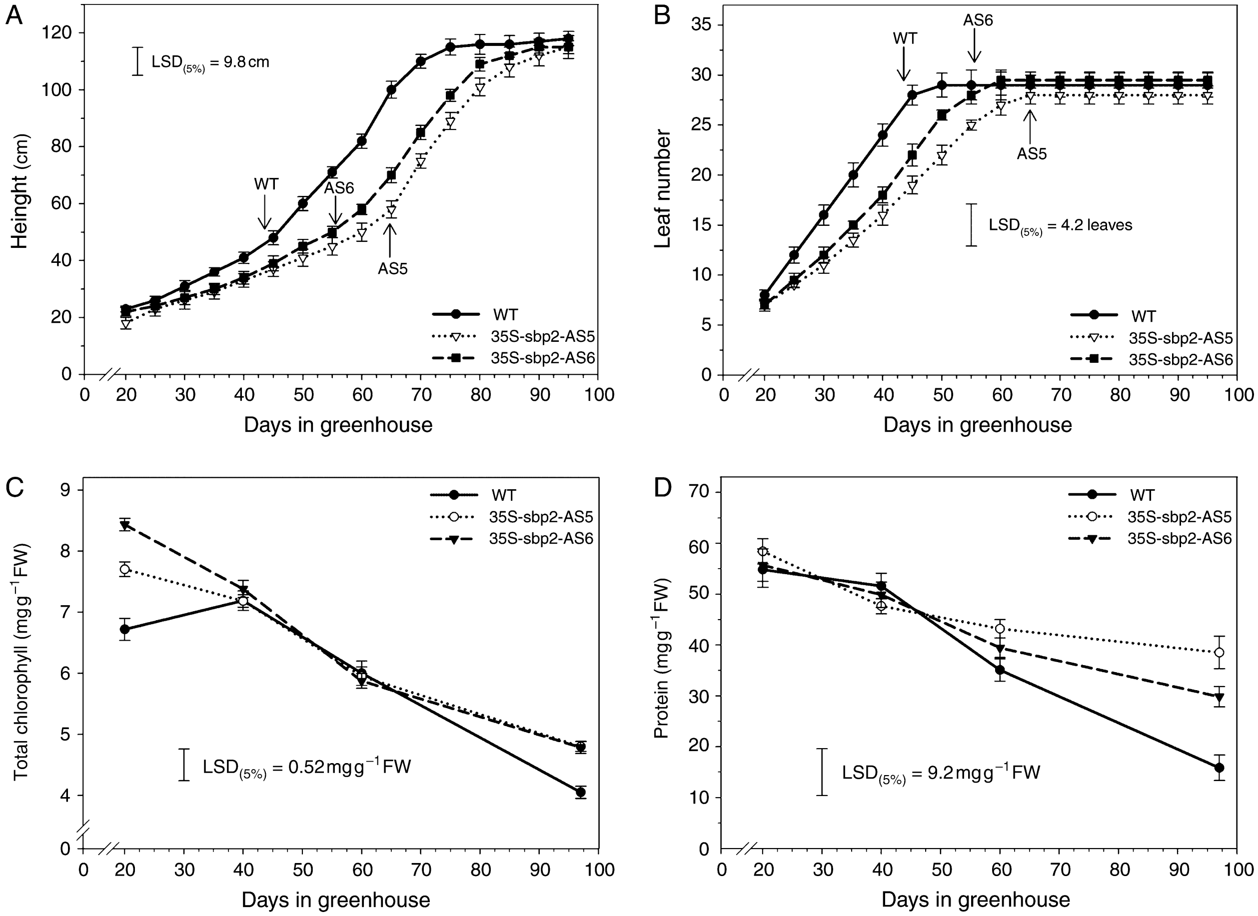

Fig. 1. Comparison of developmental performance and senescence between control (wild-type) and antisense (35S-sbp2-AS) T2 transgenic plants grown under greenhouse conditions. Plant height (A), number of leaves per plant (B), contents of total chlorophyll (C) and protein (D) on the fifth leaf (mature) from the plant top. Each point represents an average (±standard error) of at least six replicates. The data were analyzed by one-way analysis of variance followed by least statistic difference (LSD) (Fisher's LSD) test. Any difference between the points larger than lsd value is significant at $P < 0.05$. Arrows indicate the flowering time. FW, fresh weight.

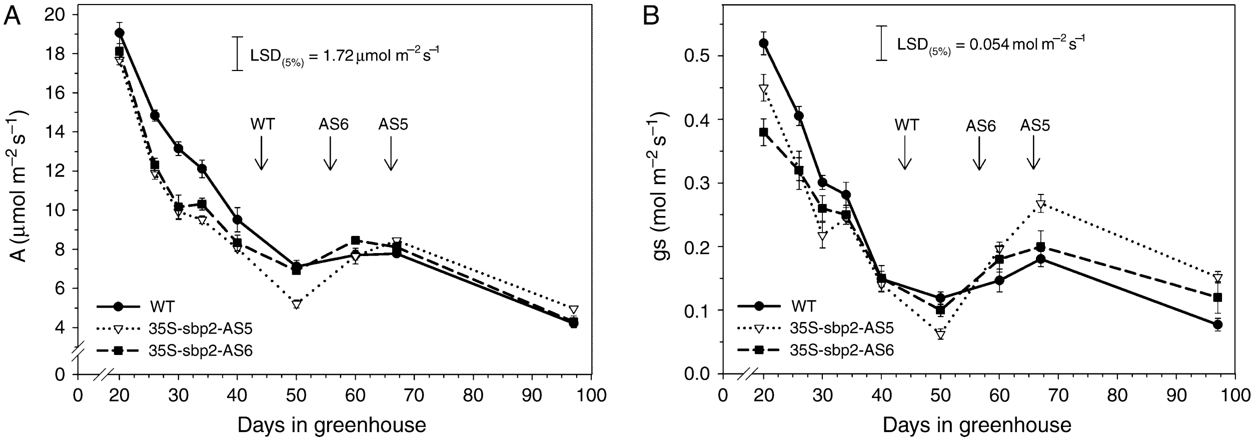

Fig. 2. Photosynthetic rate (A) and stomatal conductance (B) of the fifth leaf (mature) from the top of control (wild-type) and antisense (35S-sbp2-AS) T2 transgenic plants during the growth in greenhouse conditions. Analyses were performed at photosynthetic photon flux density of 1000 µmol m^{-2} s^{-1} and 25°C. Each point represents an average (±standard error) of at least six replicates. The data were analyzed by one-way analysis of variance followed by least statistic difference (LSD) (Fisher's LSD) test. Any difference between the points larger than LSD value is significant at $P < 0.05$. Arrows indicate the flowering time.

the further analyses, the data of 35S-sbp2-AS5-1, 35S-sbp2-AS5-2 and 35S-sbp2-AS5-8 lines were integrated and presented as 35S-sbp2-AS5 and the data of 35S-sbp2-AS6-4, 35S-sbp2-AS6-5, 35S-sbp2-AS6-6 and 35S-sbp2-AS6-9 lines were integrated as 35S-sbp2-AS6.

In the transgenic lines, a significant reduction in height was observed after 50 days in the greenhouse (Fig. 1A), and in leaf number, after 40 days (Fig. 1B). These differences did not persist throughout development, because they reached a similar final height as WT after 85 days and their total number of leaves equaled to that of control plants after 55–60 days under greenhouse conditions. These growth-related phenotypes were more accentuated in the antisense line 35S-sbp2-AS5 than in 35S-sbp2-AS6. These changes in vegetative growth led to a delay in the onset of flowering, which was also more pronounced in the antisense 35S-sbp2-AS5 line that flowered later (after 70 days in greenhouse) as compared with the 35S-sbp2-AS6 antisense (57 days), the controls WT (43 days) and pBI vector-transformed plants (42 days) (Fig. S2B). The developmental phenotypes were evaluated in 10 progenies of each line and, in the case of the antisense lines, they were found to be linked to the expression of the transgene. These results confirmed our previous observation regarding the primary transformant phenotypes of the antisense lines (Pedra et al. 2000).

Antisense expression of *SBP* gene delays leaf senescence

An increase in total chlorophyll content at day 20 (Fig. 1C) was observed in the transgenic lines, despite the fact that they displayed no differences in the other growth parameters analyzed at this developmental period as compared with control plants (Fig. 1A and B). The meaning of such change is not known, because after the induction of flowering in WT plants (around 40 days), a similar decrease in chlorophyll and leaf total protein content was observed in control and transgenic plants, although the onset of flowering in antisense lines was delayed. Nevertheless, late in the reproductive phase, during seed set, both leaf senescence parameters were significantly higher in all antisense lines analyzed, indicating delayed leaf senescence. This occurred after the recovery from the reduced vegetative growth (Fig. 1A and B) and probably paralleled a concomitant delay in leaf maturation of source leaves in transgenic plants.

The reduced net photosynthetic rate in antisense lines was transient and restricted to the vegetative phase

The reductions in A and gs in transgenic lines under saturating light (1000 µmol m^{-2} s^{-1}) were limited to the discrete periods within 25–35 days and 20–30 days, respectively (Fig. 2A and B). To further address the significance of these differences and the temporal restriction of this phenotype, a detailed daily time course of A was performed at this early developmental period (20 days in greenhouse), and late in the reproductive phase (at day 97). Although early in development (at day 20), the significant reduction in A persisted almost during all day for both antisense lines (Fig. 3A), in the reproductive phase, the diurnal photosynthesis of transgenic

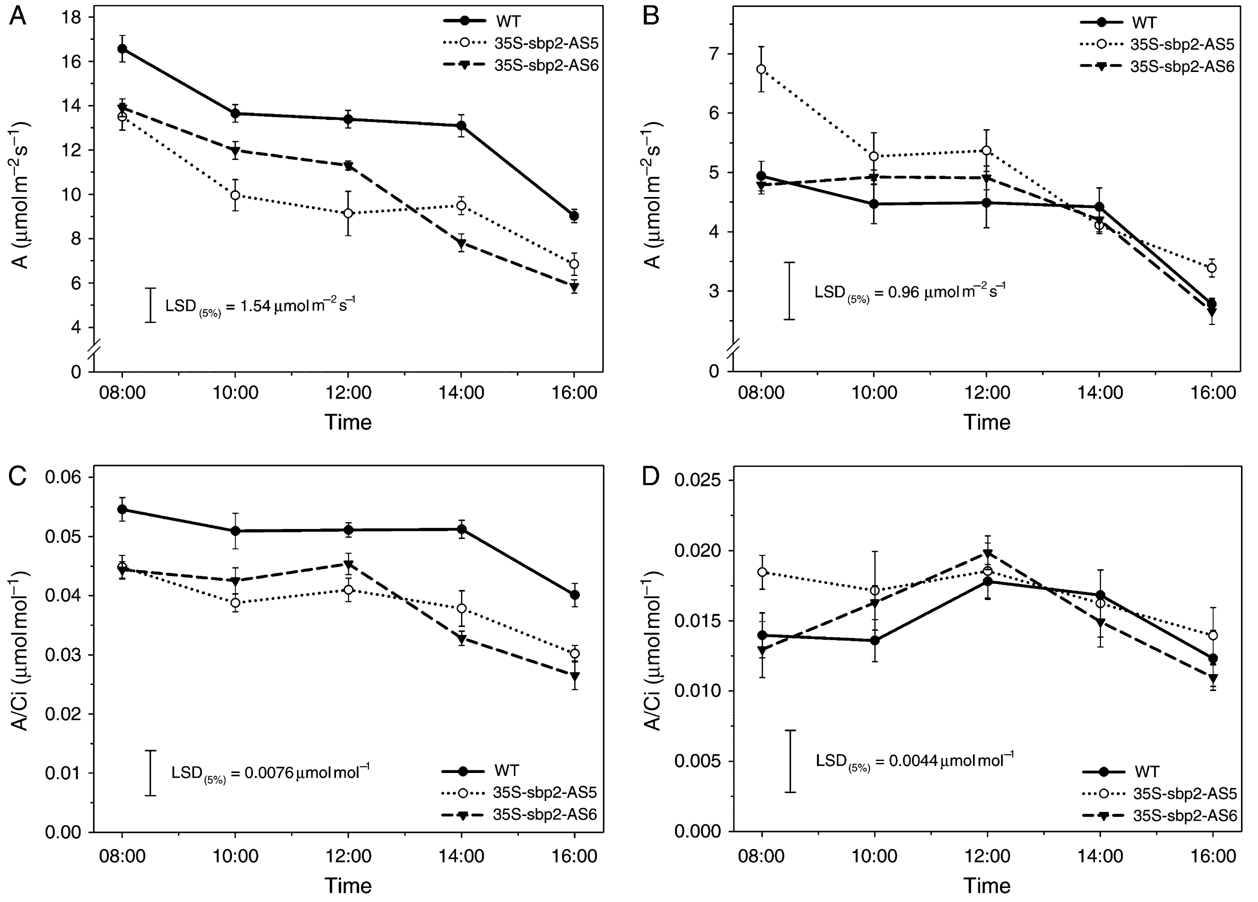

Fig. 3. Diurnal photosynthetic rate (A) (A and B) and A/Ci (Ci, intercellular CO_2 partial pressure) ratio (C and D). The measurements were conducted on the fifth leaf (mature) from the top of control (wild-type) and antisense (35S-sbp2-AS) T2 transgenic plants during the vegetative (20 days in greenhouse) (A and C) and reproductive phases (97 days in greenhouse) (B and D). Analyses were performed at photosynthetic photon flux density of 1000 μmol m^{-2} s^{-1} and 25°C. Each point represents an average (±standard error) of at least six replicates. The data were analyzed by one-way analysis of variance followed by least statistic difference (LSD) (Fisher's LSD) test. Any difference between the points larger than LSD value is significant at $P < 0.05$.

leaves was similar to that of WT leaves (Fig. 3B). No differences in Ci and Ci/Ca rate (Ca, ambient CO_2 partial pressure) among plants were observed, which varied in the range of 250–350 μmol mol^{-1} and 0.6–0.8, during the experiment, respectively (data not shown). Thus, the reduced diurnal photosynthesis of the transgenic plants during the vegetative phase was further reflected by a decline in A/Ci ratio as compared with control plants (Fig. 3C), which was not detected late in development (Fig. 3D). Collectively, these results suggest that the concomitant decline in gs could not account for the significant reduction of photosynthesis early in development.

To further examine whether the inhibition of photosynthesis followed a biochemical inhibition by feedback regulation we analyzed the CO_2 response curve of net photosynthesis (A) under saturating light in the vegetative phase (Fig. 4). The slopes of the A × Ci curves among plants were similar (Fig. 4A), although a discrete inflection in the curve of the AS5 transgenic line could be detected at Ci value of 800 μmol CO_2 ($P > 0.05$). Decrease of A with the progressive increase of Ci values may indicate that ribulose-1,5′-bisphosphate (RuBP)-regeneration and triose-phosphate (triose-P) utilization are the limiting factors in photosynthesis (Long and Bernacchi 2003, Walters et al. 2004). However, the changes observed were quite modest to support the interpretation that the restrictions in Calvin Cycle contributed significantly to the reduction in net photosynthesis in antisense leaves.

To determine whether some alteration in the photochemical reactions occurred in the transgenic plants, the photosynthetic response to light intensity was analyzed (Fig. 4B). As typical of a C_3 plant, tobacco control plants produced a light saturation of photosynthesis at 800 μmol m^{-2} s^{-1}. However, in antisense lines, the photosynthesis under saturating light conditions was

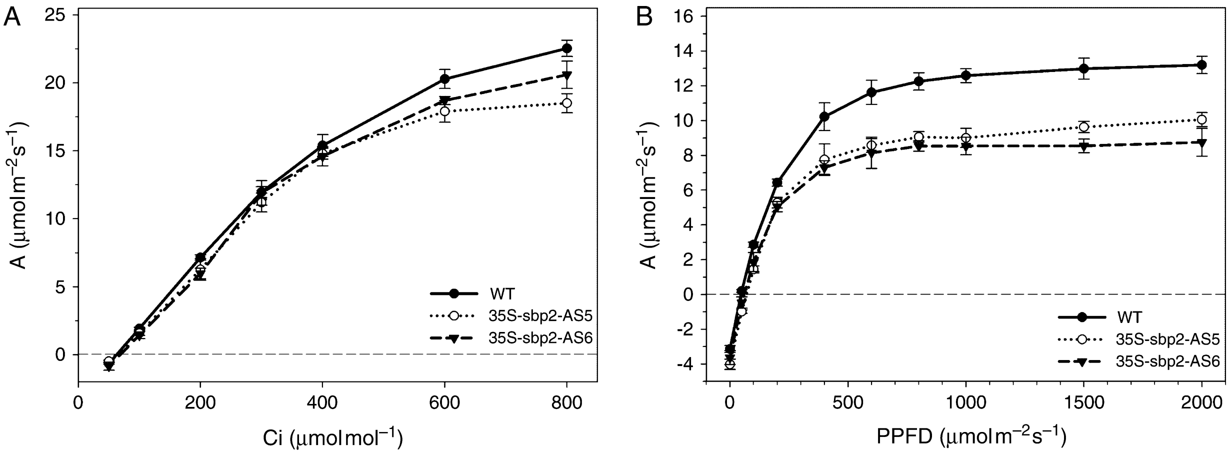

Fig. 4. CO_2 response curve, A × Ci (Ci, intercellular CO_2 partial pressure) (A) and light response curve, A × PPFD (PPFD, photosynthetic photon flux density) (B) of net photosynthetic rate (A) from fifth leaf (mature) from the top of control (wild-type) and antisense (35S-sbp2-AS) T2 transgenic plants in the vegetative phase (20 days in greenhouse). Analyses were performed at 25°C and for A × Ci curves at PPFD of 1000 μmol m^{-2} s^{-1}. Each point represents an average (±standard error) of at least eight replicates.

lower than in control leaves (600 μmol m^{-2} s^{-1}). Therefore, antisense transgenic plants exhibited reduced light-energy utilization.

Antisense expression of the *SBP* gene is associated with changes in carbohydrate metabolism in source leaves and sink tissues

To investigate whether reductions in photosynthesis and light utilization observed in transgenic lines were associated with changes in carbon metabolism, we characterized the carbohydrate accumulation during developmental and diurnal time course (20 days in greenhouse) in fully expanded leaves. Hexoses and sucrose content were reduced in the antisense source leaves during the course of the experiment (Fig. 5A, C and E). Likewise, during a diurnal cycle (at 20 days), the soluble sugar content as well as the rate of sugar accumulation were significantly reduced in the antisense lines when compared with WT plants (Fig. 5B, D and F). In contrast to soluble sugars, the starch content in the source leaves of antisense transgenic lines was significantly higher than in control plants 20 days after transplanting (Fig. 5G). This was accompanied by a transient increase in acid invertase, AGPase activities and a decrease in Susy activity (Fig. 6A, B and C). This temporary increase in starch accumulation persisted during a diurnal cycle, in which a significant increase both in the starch content and in the rate of starch accumulation was observed in the antisense lines as compared with WT plants (Fig. 5H).

To investigate whether sucrose transport was affected in *SBP* antisense lines, we determined the sucrose exudation rate at the beginning of the vegetative phase (20 days in greenhouse) and at the transition to the reproductive phase (60 days). The rate of sucrose exudation from detached mature leaves of antisense lines was decreased (40% reduction) when compared with the WT (Fig. 7). However, the reduced sucrose content in exudates from antisense leaves was only evident when it was measured from detached source leaves at early developmental stages. Sucrose exudation rates from control and antisense source leaves of plants at 60 days in greenhouse were similar. Further evidence for reduced sucrose transport through phloem was obtained by the analysis of carbohydrate content in sink tissues, such as young leaves, roots and shoot apex (Figs 8 and 9). Significant reductions in sucrose and starch in sink leaves (Figs 8A and 9), hexoses in shoot apex (Fig. 8B), and in hexoses and sucrose in roots (Fig. 8C) were observed in transgenic plants at 20 days in greenhouse. These results are consistent with impairment of sucrose translocation between the source and sink tissues in the antisense lines.

Discussion

SBP from soybean is an intriguing and enigmatic protein because of the apparent biochemical inconsistency between its assigned biological function as a sucrose transporter and its predicted topological structure. Although biochemical analyses indicated that a SBP from soybean was capable of transporting sucrose in a heterologous system (yeast), the protein is not structurally related

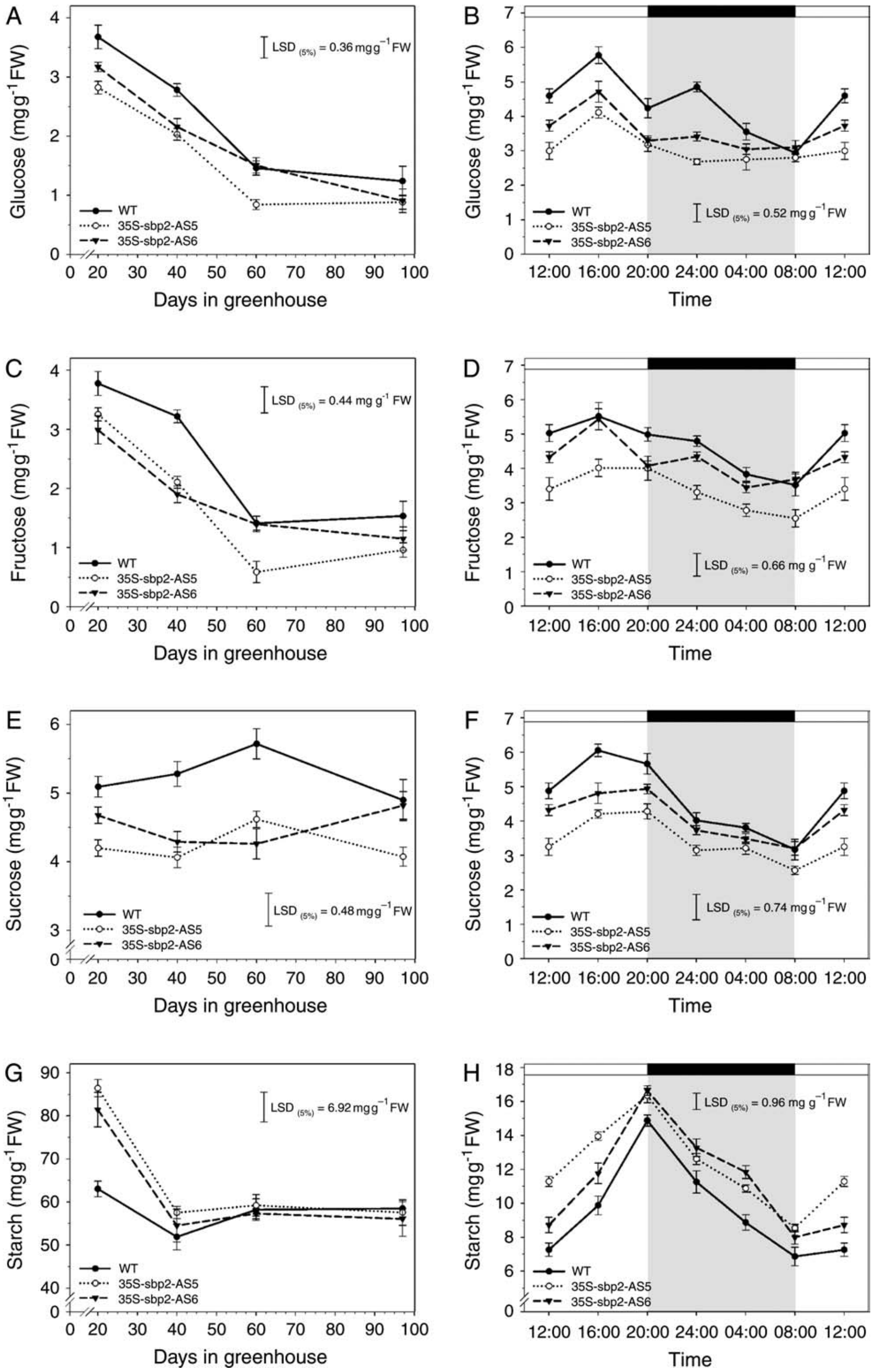

Fig. 5. Developmental (A, C, E and G) and diurnal changes (B, D, F and H) on carbohydrate content of fifth leaf (source leaf) from the top of control (wild-type) and antisense (35S-sbp2-AS) T2 transgenic plants. For the developmental analyses, the samples were collected after 5 h of natural illumination in greenhouse conditions. Diurnal analyses were performed at 20 days in greenhouse, photosynthetic photon flux density of 250 μmol photons m^{-2} s^{-1} and 25°C. Each point represents an average (±standard error) of at least five replicates. The data were analyzed by one-way analysis of variance followed by least statistic difference (*lsd*) (Fisher's *lsd*) test. Any difference between the points larger than *lsd* value is significant at $P < 0.05$. FW, fresh weight.

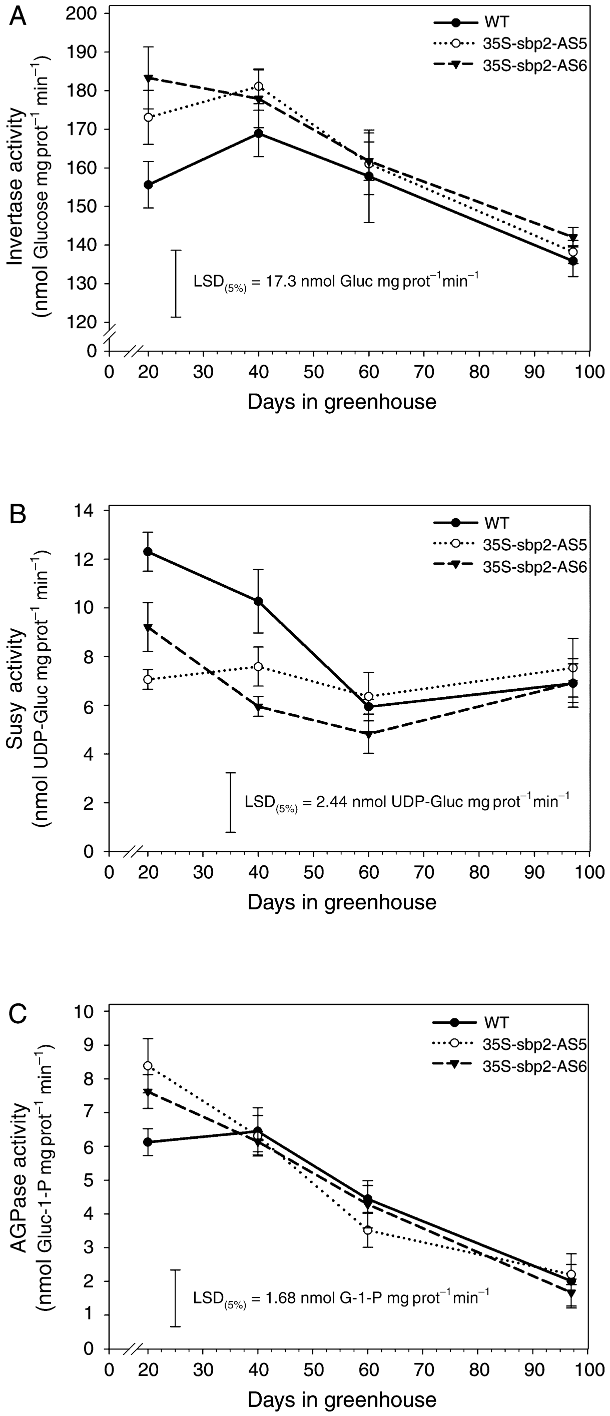

Fig. 6. Cell-wall invertase (A), sucrose synthase (B) and AGPase (C) activities of the fifth leaf (source leaf) from the top of control (wild-type) and antisense (35S-sbp2-AS) T2 transgenic plants during the growth in greenhouse conditions. Samples were collected after 5 h of natural illumination in greenhouse conditions. Each point represents an average (±standard error) of at least five replicates. The data were analyzed by one-way analysis of variance followed by least statistic difference (LSD) (Fisher's LSD) test. Any difference between the points larger than LSD value is significant at $P < 0.05$.

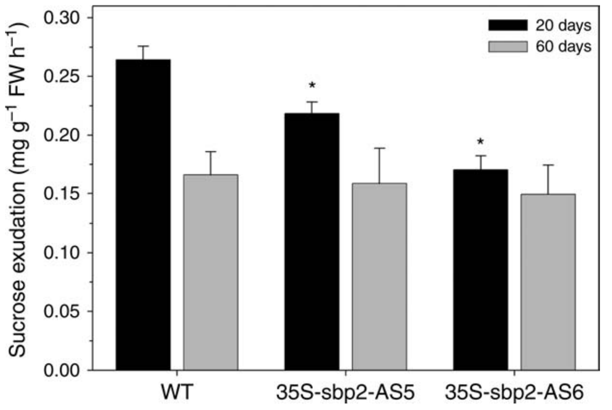

Fig. 7. Sucrose exudation from detached source leaves of control (wild-type) and antisense (35S-sbp2-AS) T2 transgenic plants. Exudates were collected from fully expanded leaves from plants in the vegetative (20 days in greenhouse conditions) and reproductive phases (60 days in greenhouse conditions) and the sucrose concentrations were determined. Each bar represents an average (±standard error) of at least five replicates. The data were analyzed by one-way analysis of variance followed by least statistic difference (LSD) (Fisher's LSD) test. *Denotes significant differences from control with $P < 0.05$. FW, fresh weight.

to any other membrane transport protein (Grimes and Overvoorde 1996, Overvoorde et al. 1996, 1997). Contrasting results have been reported for the *V. faba* SBP (VfSBP), which failed to transport sucrose in the yeast system (Heim et al. 2001). Nevertheless, functional analyses have demonstrated that *SBP* repression in tobacco promoted phenotypes consistent with impairment of sucrose translocation (Pedra et al. 2000). Here we further characterized these *SBP* antisense plants by analyzing growth, photosynthetic parameters and carbohydrate metabolism in different developmental periods and organs.

Consistent with inhibition of long-distance sucrose translocation, antisense repression of *SBP* homolog gene led to inhibition of photosynthesis and delay in plant growth and development. Nevertheless, the reduction in A and gs was restricted to the vegetative phase, representing a general trend for this developmental period. The significance of these changes was strengthened by the comparison between the daily time courses of A and A/Ci ratio at the beginning of the vegetative phase (20 days; Fig. 3A and C), when significant differences were observed during distinct diurnal times in the antisense lines, as opposed to the reproductive phase that in general did not exhibit significant alterations (Fig. 3B and D). These results are in agreement with our previous findings (Pedra et al. 2000), but restrict the significance of these changes to the vegetative phase. Because the alterations in gs were not accompanied by the reduction in Ci (data not shown), as it could be inferred from the absence of corresponding changes in A/Ci ratio (Fig. 3C),

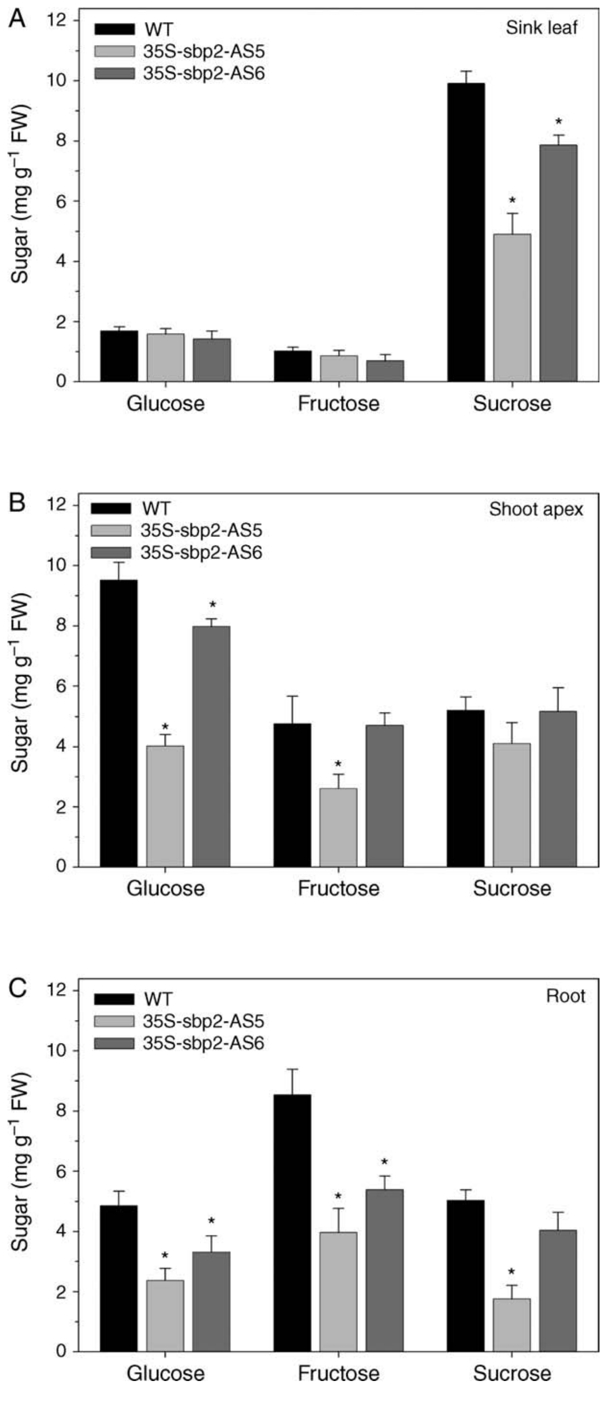

Fig. 8. Soluble sugar content of sink tissues (A, sink leaf; B, shoot apex and C, root) from control (wild-type) and antisense (35S-sbp2-AS) T2 transgenic plants in the vegetative phase (20 days in greenhouse). Samples were collected after 5 h at photosynthetic photon flux density of 250 μmol photons m^{-2} s^{-1} and 25°C. Each bar represents an average (±standard error) of at least five replicates. The data were analyzed by one-way analysis of variance followed by least statistic difference (LSD) (Fisher's LSD) test. *Denotes significant differences from control with $P < 0.05$. FW, fresh weight.

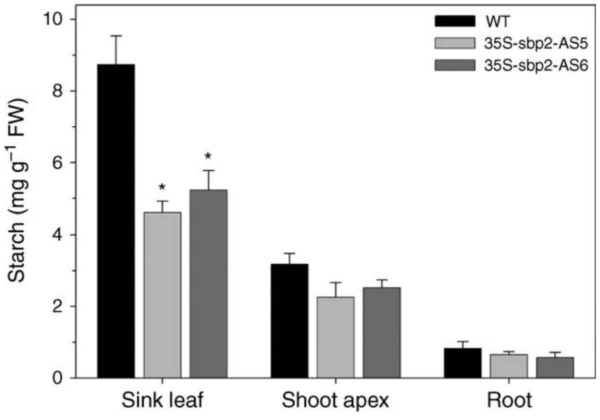

Fig. 9. Starch content of sink tissues from control (wild-type) and antisense (35S-sbp2-AS) T2 transgenic plants in the vegetative phase (20 days in greenhouse). Samples were collected after 5 h at photosynthetic photon flux density of 250 μmol photons m^{-2} s^{-1} and 25°C. Each bar represents an average (±standard error) of at least five replicates. The data were analyzed by one-way analysis of variance followed by least statistic difference (LSD) (Fisher's LSD) test. *Denotes significant differences from control with $P < 0.05$. FW, fresh weight.

it is unlikely that a reduction in stomatal aperture is the basis for the reduction in photosynthetic activity of the SBP antisense leaf. Furthermore, the transgenic lines and WT differed little in the photosynthetic response to Ci (Fig. 4A), suggesting that the ribulose bisphosphate carboxylase/oxygenase maximum activity and the limitation in RuBP regeneration of these plants were similar. However, significant differences were detected in the photochemical reactions, as light saturation of photosynthesis occurred at a lower irradiance in SBP antisense plants (600 μmol m^{-2} s^{-1}) than in control leaves (800 μmol m^{-2} s^{-1}). Therefore, antisense transgenic plants exhibited a reduced light utilization. Because the transgenic plants have shown a significant decrease in A, it is possible that light fluxes could exceed their photosynthetic capacity, a situation that could generate highly reactive intermediates causing oxidative damage to the photosynthetic apparatus (Scheibe et al. 2005). This photo-oxidative damage, if not repaired, decreases the efficiency and/or the maximum rate of photosynthesis. Several mechanisms of photoprotection could be induced in response, e.g. adjustments in light-harvesting antenna size, which could contribute to the decreased light utilization described here.

Photophosphorylation is the main biochemical event in the photochemical phase of photosynthesis, it is very sensitive to orthophosphate (Pi) concentration (Quick and Mills 1988), and it plays a major role in sustaining photosynthetic rate. As the rate of end-product (sucrose, starch, amino acids) synthesis determines the rate at which Pi is recycled back to the reactions of photosynthesis, we have analyzed the carbohydrate accumulation in source leaves during the development, and the daily time course at day 20 (Fig. 5). Although a transient increase in starch content was observed in the vegetative phase (at day 20), a parallel general reduction in hexoses and sucrose occurred. All these differences persisted during the diurnal course at day 20, at least in one transgenic line, and in the case of hexoses and sucrose, during the development. The decreased sucrose content and enhanced starch levels displayed by transgenic source leaves confirmed our previous results (Pedra et al. 2000), but also limit the significance of the increase in starch levels to the vegetative phase. The simultaneous increase in starch and decrease in sucrose observed at day 20 might indicate that a transient change in carbon allocation toward starch occurred in source leaves. Starch accumulation in leaves could be a result of reduction in sink demand (reviewed by Paul and Foyer 2001), or impaired sucrose export (Bürkle et al. 1998, Krapp and Stitt 1995, Riesmeier et al. 1994) or synthesis (Geigenberger and Stitt 2000). In *N. tabacum* source leaves, a decrease in Pi availability, as for example, because of reduced sucrose synthesis, affects the activity of the triose-P translocator. As a result, carbon is retained in the chloroplast where starch synthesis is facilitated through the activation of AGPase by low Pi/3-PGA ratio, resulting also in increased AGPase gene expression. Likewise, AGPase activity in SBP antisense plants was higher exactly when starch accumulation was greater than control plants (day 20; Figs 5G and 6C). Although starch accumulation in leaves may be associated with reduced photosynthetic rates (Cave et al. 1981, Grub and Mächler 1990, Nafziger and Koller 1976), the increase in starch content that was observed in source leaves was too modest to explain the reduction in net photosynthesis in the SBP antisense lines.

In addition to promote the accumulation of higher amount of starch in source leaves, antisense repression of the *SBP* homolog gene reduced the sucrose translocation from source leaves and lowered the sugar content in sink organs early in development. Nevertheless, the pattern of sucrose and hexose accumulation in antisense mature leaves was in contrast with the elevated levels of soluble sugars observed in plants in which sucrose translocation has been reduced by repression of the H^+:symporter SUT (Bürkle et al. 1998, Riesmeier et al. 1994). This contrast suggests that SBP and SUT exhibit distinct functions in sucrose translocation and hence the decrease in sucrose export in *SBP* antisense lines (Fig. 7) may not be functionally related to impairment in sucrose transport from source leaves. Accordingly, a tobacco SBP homolog has not been detected in source leaves (Pedra et al. 2000). As an alternative explanation, the decrease in the soluble sugar levels may be a direct result of the long-term decrease in photosynthesis during the vegetative phase, and the transient change in allocation of carbohydrates to starch synthesis. Change in C allocation requires coordinate changes in metabolism. The fact that reduction in Susy activity occurs only in the vegetative phase and partially overlaps the increase in AGPase may suggest that Susy and ADPGase are being coordinately regulated. These alterations in Susy activity are consistent with our previous results obtained with cell suspensions (Delú-Filho et al. 2000). The coregulation of both enzymes was firstly postulated based on metabolic analysis in transgenic plants with decreased SPS activity (Geigenberger and Stitt 2000). Collectively, our data suggest that the flux through sucrose synthesis was impaired in source leaves, probably because of reduced net photosynthetic rates.

If impairment in sucrose export from source leaves or increase in starch accumulation could not account for the reduction in photosynthesis and hence in sugar supply what would explain the SBP antisense phenotypes? The transient nature of the decrease in phloem transport and photosynthesis was associated with a reduction in vegetative growth of antisense lines. This strict correlation supports the explanation that photosynthesis rate in antisense leaves might have been feedback inhibited by a reduced sink demand at early stages of development. In fact, we provided several lines of evidence indicating that a reduced sink demand may be the primary determinant of the antisense *SBP* phenotypes. First, as compared with WT, the antisense lines accumulated significantly lower levels of carbohydrate in several sink organs during the vegetative period, whereas the changes in sugar content in the antisense source leaves occurred to a much less extent. This may reflect a more accentuated effect of SBP repression on sink strength than on sugar export from source leaves. Consistent with this interpretation, the SBP tobacco homolog has only been detected in young sink leaves (Pedra et al. 2000) and the SBP promoter has been shown to direct the expression of a linked gene to sink organs (Contim et al. 2003, Waclawovsky et al. 2006). Second, although we have previously demonstrated that the guanosine triphosphate-binding activity of SBP does not play a role in the SBP-mediated sucrose transport in yeast (Pirovani et al. 2002), the possibility that this biochemical activity of SBP is linked somehow to a biochemical signal in metabolism regulation still remains. We have been able to show that manipulation of SBP levels promotes alterations in carbohydrate metabolism in cell suspension cultures independently from photosynthesis and sugar supply (Delú-Filho et al. 2000). Finally, the height increase rate and leaf emergence were delayed and were associated with decreased carbon assimilation during the vegetative phase in the antisense lines. Conversely, when sink demand increased (flowering begins around 60 days in SBP lines; leaf number and height normalize around 60 and 80 days, respectively) and source strength in transgenic plants became similar to that in control plants (as inferred from an increase in leaf number), no differences were observed for net photosynthesis and sucrose exudation between WT and antisense plants.

Sucrose synthesis in source leaves and its transport via phloem represent major determinants for growth and development of a sink organ. In the antisense lines, the decrease in sucrose exudation paralleled significant reductions in sucrose and starch in sink leaves, hexoses in shoot apex, and in hexoses and sucrose in roots (Figs 8 and 9). These limitations in carbohydrate content in sink organs, particularly in sink leaves, were reflected by the transient delay in vegetative development (Fig. 1A and B) and were associated with an average of 20-day retardation in the flowering time in antisense lines. After the induction of flowering in WT (around 40 days), a sharp decline in chlorophyll and protein levels was observed in source leaves. However, this decline was less pronounced in antisense lines, which showed higher protein and total chlorophyll levels in their leaves after 97 days in greenhouse (Fig. 1C and D), indicating a delayed leaf senescence in these plants. These differences occurred when the control and transgenic plants exhibited similar photosynthetic rate. A transient increase in sugar content in leaves may be linked to decreased chlorophyll content, which functions as leaf senescence signal (Wingler et al. 2006). Because the sugar levels were lower in transgenic plants at the reproductive phase, and their leaf chlorophyll levels and protein content were higher than in WT leaves, we propose that decreased sugar content may contribute for the delay in senescence of the antisense leaves.

In summary, this investigation shows that changes in photosynthetic metabolism, sucrose export and growth in tobacco plants with reduced expression of SBP are transient and restricted to the vegetative phase of development. The overall altered plant development and carbohydrate allocation in the antisense lines support the notion that sucrose translocation was indeed impaired. In a general context, these pleiotropic physiological alterations were similar to those described by antisense repression of the H^+:symporter (sucrose exportation) (Bürkle et al. 1998, Riesmeier et al. 1994) and overexpression of apoplastic invertase (sink strength) (Sonnewald et al. 1997, von Schaewen et al. 1990). However, focusing on individual components of these multifactorial effects, such as sugar accumulation in the transgenic lines and temporal restriction of the phenotypes, the *SBP* antisense plants exhibited contrasting and unique phenotypic patterns that may underline a distinct and unique role of SBP in sucrose translocation and metabolism. This detailed temporal characterization of the metabolic and growth changes described here suggests that the metabolic effects from the reduction in *SBP* expression may be related to a temporally restricted change in sink strength and development, instead of being directly associated with sucrose transport from source leaves. As a member of the vicilin storage protein family, it is possible that tobacco SBP homolog is a vegetative storage protein. The transient nature of the vegetative storage protein accumulation could be associated with the transient phenotypes described here. Experiments to test this hypothesis are underway in our laboratory.

Supplementary material

Fig. S1. Segregation of the antisense transgene (T-locus, 35S-sbp2-AS) based on kanamycin resistance.

Fig. S2. Comparison of developmental performance between control (wild-type and pBI), sense (35S-sbp2-S) and antisense (35S-sbp2-AS) T2 transgenic plants. (A) Transgenic tobacco plants after 8 weeks in the greenhouse conditions. (B) Time of flowering induction in sense and antisense plants. Each bar represents an average (±standard error) of at least 10 replicates. The data were analyzed by one-way analysis of variance followed by least statistic difference (LSD) (Fisher's LSD) test. Significant differences between lines are indicated by different letters with $P < 0.05$.

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