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Antisense and sense expression of a sucrose binding protein homologue gene from soybean in transgenic tobacco affects plant growth and carbohydrate partitioning in leaves

João Helbert F. Pedra [a], Nelson Delú-Filho [b], Carlos P. Pirovani [b], Luis A.S. Contim [a], Ralph E. Dewey [c], Wagner C. Otoni [b], Elizabeth P.B. Fontes [a,*]

[a] *Departamento de Bioquímica e Biologia Molecular, Universidade Federal de Viçosa 36571.000, Viçosa-MG, Brazil*
[b] *Departamento de Biologia Vegetal/BIOAGRO, Universidade Federal de Viçosa 36571.000, Viçosa-MG, Brazil*
[c] *North Carolina State University, Campus Box 7620, 1244 Williams Hall, Raleigh, NC 27695-7620, USA*

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Abstract

We isolated a cDNA from a soybean library, which encodes sucrose binding protein (SBP) homologue, designated S-64. To analyze the function of the SBP homologue, transgenic tobacco plants were obtained by introducing chimeric genes containing the *s*-64 coding region linked to the 35S CaMV promoter, either in the sense or antisense orientation, via *Agrobacterium tumefaciens*-mediated transformation. The accumulation of the SBP homologue was increased in transgenic plants expressing the heterologous *sbp* gene, whereas those expressing the antisense construct had reduced levels of the protein. The antisense transgenic plants developed symptoms characteristic of an inhibition of sucrose translocation and displayed a reduction in plant growth and development. In contrast, overexpression of the protein accelerated plant growth and the onset of flowering induction. The overall developmental performance of the transgenic plants was correlated with their photosynthetic rate under normal conditions. While photosynthesis in the antisense lines was decreased, in the sense lines photosynthetic rates were increased. Furthermore, both antisense repression and overexpression of the SBP homologue in transgenic lines altered carbohydrate partitioning in mature leaves. Taken together, these results indicate that S-64 protein is functionally analogous to SBP, representing an important component of the sucrose translocation pathway in plants.

Keywords: *Nicotiana*; Soybean; Sucrose binding protein; Sucrose transport

1. Introduction

In higher plants, carbon autotrophy is a central feature of photosynthetically active tissues such as mature leaves (source tissues) which export photoassimilates to heterotrophic tissues, such as seeds, stems and roots (sink tissues). Sucrose is the major transported form of assimilated carbon that is translocated in the vascular system of many higher plants. The translocation of sucrose from its site of synthesis to sink tissues involves its export from the mesophyll cells, phloem loading and sucrose unloading from the phloem cells to the sink tissues (reviewed in ref. [1]). Both symplastic and apoplastic loading steps are believed to coordinate the phloem loading and unloading mechanisms. Symplastic transport is mediated by the plasmodesmata interconnecting adjacent cells [2,3] and apoplastic mechanisms involve the transport of photoassimilates across the plasma membrane and intervening cell wall spaces, a process mediated by membrane transport proteins (reviewed in ref. [1]).

The importance of an apoplastic step for phloem loading is demonstrated by strong plant

Abbreviations: CaMV, cauliflower mosaic virus; SBP, sucrose binding protein; SUT, sucrose transporter.

* Corresponding author. Tel.: +55-31-899-2949; fax: +55-31-899-2864.

E-mail address: bbfontes@mail.ufv.br (E.P.B. Fontes)

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growth-related phenotypes caused by the expression of a yeast invertase in the apoplast of transgenic solanaceous species, such as tobacco, tomato and potato [4–7]. Such phenotypic effects include leaf curling and local bleaching, reduced root growth and tuber yield. Furthermore, expression of the heterologous apoplastic invertase dramatically affected assimilate partitioning. Because hexose sugars do not appear to be translocated efficiently in the phloem, these results support a carrier-mediated sucrose transport from the apoplastic compartment as a predominant pathway for phloem loading in many plants. In support of these conclusions, biochemical studies with isolated cells and plasma membrane vesicles have identified sucrose transport activities in several plant species [8–10]. Based on kinetic studies, sucrose uptake in isolated cells is catalyzed by a saturable high affinity system and a linear low affinity system [11]. The high affinity component is sensitive to sulphydryl-reactive reagents and to diethylpyrocarbomate, and has been described as a sucrose/proton cotransporter.

Complementation of mutant yeast strains has been successfully used to isolate cDNA's encoding putative H^+-ATPase/sucrose cotransporters from spinach and potato, designated SoSUT1 and StSUT1, respectively [12,13]. More recently, homologous cDNA's have been isolated from *Plantago major*, *Arabidopsis thaliana*, tomato and tobacco [14–17]. The cDNA-encoded products have structural features of metabolite transporters. They are highly hydrophobic proteins and possess two sets of six membrane-spanning domain structures, separated by a large cytoplasmic loop. The biochemical characterization of the cDNA-encoded products revealed that they resemble the high affinity component of sucrose uptake system in plants [18]. Their activity is pH dependent and inhibited by protonophores, thiol-group modifying agents and diethylpyrocarbonate.

Previous reports demonstrate that the SUT gene products represent an important component of the carrier-mediated apoplastic transport process. These acid transporters are localized in the sieve element-companion cell complex and probably mediate sucrose uptake from the apoplast [12,13,19,20]. In potato, the pattern of SUT mRNA expression follows the sink-to-source transition and is coordinated with accumulation of sucrose transport activity [13]. Furthermore, antisense repression of SUT1 in transgenic plants inhibits sucrose export from leaves, which alters leaf morphology and plant development [19–21].

Using a photoaffinity labeling technique a sucrose binding protein (SBP) has been purified from soybean cotyledons [22] and its cDNA isolated from a soybean seed expression library [23]. Several lines of evidence suggest that SBP is involved in sucrose transport and may represent the linear, low affinity component of sucrose uptake in plants. The SBP is a membrane-associated protein, localized in cells that are actively engaged in sucrose transport, such as mesophyll cells of young sink leaves, the companion cells of mature phloem and the cells of cotyledons undergoing differentiation [23,24]. In addition, expression of the *sbp* gene and accumulation of the encoded protein are temporally coordinated with the rate of sucrose uptake in cotyledons [23]. Direct evidence implicating the SBP in sucrose transport has been obtained with complementation studies using a secreted-invertase deficient mutant yeast, incapable of growth on medium containing sucrose as the only carbon source [25,26]. Ectopic expression of the *SBP* cDNA alone reverses the mutant yeast phenotype and SBP-mediated sucrose uptake in yeast displays linear, non-saturable kinetics [25,27]. However, the biological relevance of this transport mechanism has not been addressed in plants. In this investigation, we isolated a SBP homologue cDNA from soybean and analyzed its function by expressing the sense and antisense genes in tobacco. The resulting phenotypes of the antisense plants were similar to those caused by antisense inhibition of the H^+/sucrose transporter gene in tobacco [19,20]. The phenotypic and physiological effects of enhanced accumulation of the SBP homologue in transgenic plants are also reported.

2. Materials and methods

2.1. Isolation of a SBP homologue cDNA and construction of plasmids

DNA manipulations were performed essentially as described by Sambrook et al. [28]. The *s*-64 cDNA (GeneBank accession number AF191299) was unintentionally isolated from a soybean seed expression library using an antibody raised against a partially purified microsomal membrane fraction

from immature soybean seeds. The identity of this clone was obtained by sequence comparison analysis using the BLAST program [29]. The computer program CLUSTAL-W was used for sequence alignment. The S-64 deduced protein shares 91% sequence identity with the SBP and is also referred as SBP homologue.

The SBP homologue insert was released from the λ recombinant DNA with *Eco*RI digestion and subcloned into the *Eco*RI site of pUC 118 to obtain the clone pS64. An *Eco*RI site immediately adjacent to the stop codon was created by PCR using the Pfu DNA polymerase, the forward primer 5′-cctcactgacctcaccatatggcgaccaga-3′ (initiation codon underlined) and the reverse primer 5′-gaattctcacgcaacagcgcgacgacc-3′ (*Eco*RI site underlined). The amplified sequence, spanning the entire protein-coding region and lacking the 3′ untranslated sequences, was subcloned into the *Sma*I site of pUC118 in the right orientation, obtaining the clone pUFV32.

The insertion of the antisense and sense *s*-64 coding sequences into a plant expression cassette was accomplished through the replacement of the *gus* coding region within the binary plant transformation vector pBI121 by the *s*-64 cDNA in the reverse and sense orientations. The pBI121-derived binary plant transformation vector was obtained by releasing the gene *gus* with *Sac*I/*Sma*I double digestion, repairing *Sac*I site with Klenow and then circularizing it in vitro. To place the *s*-64 gene in the sense orientation into the pBI121 derivative, pUFV32 was digested with *Eco*RI, repaired with Klenow and then the *s*-64 coding region was liberated with *Xba*I digestion. The released 1.6-kb fragment was subcloned into the pBI121-derived binary vector that had been digested with *Bam*HI, repaired with Klenow and then digested with *Xba*I. The resulting plasmid, pUFVs64S, contains the *s*-64 coding region in the sense orientation placed between the CaMV 35S promoter and the polyadenylation signal of the T-DNA nopaline synthase (*nos*) gene. The chimeric *s*-64 antisense gene was constructed by inserting the 1.6-kb *Bam*HI/Klenow-repaired *Eco* RI fragment from pUFV32 into *Bam*HI/Klenow-repaired *Xba*I sites of the pBI121 derivative. The resulting plasmid, pUFVs64AS, harbors the *s*-64 coding region in the reverse orientation placed between the CaMV 35S promoter and 3′ *nos* sequences.

2.2. Plant transformation

Leaf discs from in vitro grown *Nicotiana tabacum* plants were co-cultivated for 15 min with *Agrobacterium tumefaciens* strain LBA4404 containing the binary plasmid pUFVs64AS or pUFVs64S. Transformed shoots were selected on MS medium [30] supplemented with 6-benzylaminopurine (500 mg l^{-1}), cefotaxime (400 mg l^{-1}) and kanamycin sulfate (150 mg l^{-1}). Regenerated shoots were rooted on phytohormone-free medium, containing kanamycin sulfate (150 mg l^{-1}), transferred into soil and grown in standardized greenhouse conditions. Tobacco plants were also transformed with the pBI121 binary vector and are referred as control plants.

2.3. PCR analysis of transgenic plants

PCR was carried out on 20 ng of genomic DNA isolated from 4-week old greenhouse grown transgenic plants, using 1.5 μM each of *s*-64 or *npt*II gene-specific primers and 1 U of Taq polymerase in a final volume of 25 μl. The s-64 primers were 5′-accaacatcatctagagatctatgag-3′ (coordinates 1000–1026, upstream) and 5′-atacattcccc-gaattcagccacctcc-3′ (positions 1498–1524, downstream). The primers specific for the *npt*II gene were 5′-tcgacgttgtcactgaagcgcg-3′ (position 627–648, upstream) and 5′-gcggtcagcccattcgccgcc-3′ (coordinates 1082–1102, downstream). The PCR reactions were conducted for 30 cycles (50 s at 94°C, 75 s at 47°C and 120 s at 72°C) with a final extension at 72°C, for 10 min.

2.4. Antibody production

The S-64 protein was expressed as a fusion protein using the pET-16b vector (Novagen) which provides an *N*-terminal His tag. For this purpose, the *s*-64 cDNA was amplified form pS64 with Pfu polymerase (Stratagene) with the sense primer 5′-aagaaactcgaggtcgaaga-3′ (*Xho*I site underlined) and the antisense primer 5′-atacattcccc-gaattcagccacctcc-3′ (*Eco*RI site underlined). The amplification fragment was cloned into the *Eco*RI/*Sma*I-restricted pGEM7Zf(−) vector (Promega) from which was released with *Xho*I digestion and subsequently inserted into the *Xho*I site of pET-16b vector in the sense orientation. The synthesis of the recombinant protein was induced by iso-

propylthio-β-D-galactoside (IPTG). The induced protein was affinity-purified using Ni-chelating sepharose resin (Pharmacia) and used as antigen for antibody production in rabbits, which were immunized through subcutaneous injections during 2-week intervals.

2.5. SDS-PAGE and immunoblotting analysis

A membrane-enriched fraction from leaves was prepared as described by Ripp et al. [22]. Equivalent amounts of membrane proteins were resolved by SDS-PAGE [31], transferred to nitrocellulose membrane using a blot apparatus (BIORAD) according to the manufacturer's instructions. The membrane was blocked with 3% BSA in TBST (100 mM Tris–HCl pH 8, 150 mM NaCL, 0.05% (v/v) Tween-20). S-64 was detected using a polyclonal anti-S64 antibody at a 1:1000 dilution, followed by a goat anti-rabbit IgG conjugated to alkaline phosphatase (Sigma, St. Louis, MO) at a 1:5000 dilution. Alkaline phosphatase activity was assayed using 5-bromo-4-chloro-3-indolyl phosphate (Gibco BRL) and *p*-nitroblue tetrazolium (Gibco BRL).

2.6. Expression of S-64 in yeast

Heterologous expression of the *s*-64 gene in yeast was achieved using the *Pichia pastoris* expression system (Invitrogene). The full-length *s*-64 cDNA was cloned into the *Eco*RI site of the yeast/*Escherichia coli* shuttle vector pPIC3.5 (Invitrogen) under the control of the alcohol oxidase 1 (*AOX*1) promoter. The resulting clone, pUFV159, harboring the cDNA insert in the sense orientation was used to transform *P. pastoris*. The expression of the recombinant gene was induced by 0.5% (v/v) methanol for 48 h according to the manufacturer's instructions. Accumulation of the methanol-induced recombinant protein was monitored by immunoblotting.

2.7. Physiological measurements

Transgenic seeds were germinated in kanamycin-containing medium for 1 week before transplantation. Plants were grown in a mixture of soil, sand and dung (3:1:1) for 8 weeks in standardized greenhouse conditions, under natural conditions of light, relative humidity 70% and controlled temperature, 18 and 30°C (night and day). Photosynthetic rate, transpiration rate and stomatal conductance of fully expanded leaves were measured by IR gas analysis using a portable analyzer (model LCA-2, Analytical Development, Hoddeston, UK), at growth irradiance.

2.8. Determination of starch and soluble sugars in leaves

Freshly harvested leaves were immediately frozen in liquid nitrogen and extracted three times for 30 min each in 3 ml 80% (v/v) ethanol at 80°C. The extracts were cleared by centrifugation, evaporated and dissolved in sterile water. Total soluble sugars were measured according to Hodge and Hodfreiter [32] and reducing sugars were measured as described by Somogy [33]. The remaining leaf insoluble material was resuspended with 52% perchloric acid and used for starch determination [34].

2.9. Invertase assay

Soluble and insoluble forms of acid invertase were assayed as described by Lowell et al. [35]. Both soluble and insoluble invertase activities were assayed for 15–30 min at 37°C. Reaction medium contained 100 mM sucrose and 0.67 M acetate buffer, pH 4.7. The liberated reducing sugars were measured according to Somogy [33].

3. Results

3.1. Transformation of N. tabacum with a SBP homologue gene

We have isolated a cDNA from a soybean expression library, which shares 91% sequence identity with the previously reported SBP gene from soybean. The predicted gene product is referred here as either the S-64 protein or SBP homologue. To elucidate the physiological role of the SBP homologue, transgenic tobacco plants expressing the *sbp* homologue gene in either sense or antisense orientation were generated. Fig. 1 shows the pBI121-derived plasmids harboring the soybean *s*-64 gene that were used to transform *N. tabacum* via *A. tumefaciens*. T0 primary transformants were selected in tissue culture on the basis

of their kanamycin resistance and most of the rooted plants were tested further for the incorporation of the *npt*II gene (data not shown) and *s*-64 gene by PCR analysis (Fig. 2). In few kanamycin-resistant transformants, the incorporation of the *s*-64 gene was not detected (lane AS2) and, therefore, these plants were not considered further. For each construct, several independent transgenic lines were established, transferred into soil, and grown in greenhouse to generate seeds (T1 seeds). The integration of the constructs in the transformed plants were further confirmed by Southern blot analysis of genomic DNA (data not shown) and segregation analyses of the *npt*II gene in the T0 progenies (T1 plants). Four antisense and three sense independently transformed lines were arbitrarily selected for further analyses.

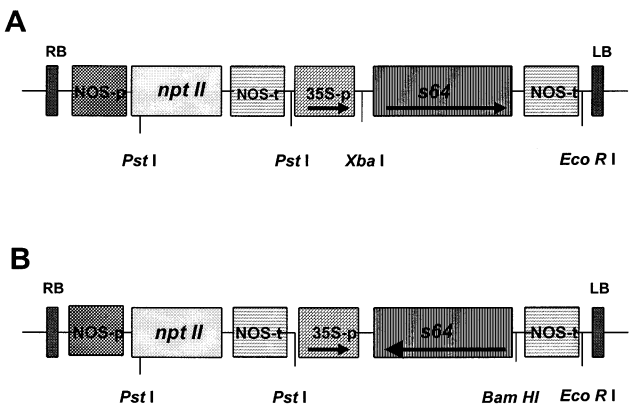

Fig. 1. Schematic diagram of the chimeric SBP homologue constructs transformed into *N. tabacum* via pBI121-derived binary vector. The *s*-64 gene either in the sense (A) or antisense (B) orientation was placed under the control of the constitutive CaMV 35S promoter (35S-p) and the 3′ *nos* polyadenylation signal (NOS-t). The *npt*II gene expression is driven by the *nos* promoter (NOS-p). LB and RB correspond to the T-DNA left and right borders, respectively. The positions of some restriction enzyme sites are indicated.

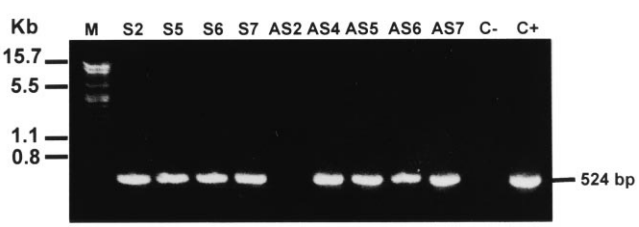

Fig. 2. PCR analysis of transgenic plants. Total DNA was isolated from greenhouse grown transgenic plants and provided the template in PCR reactions using *s*-64 gene-specific primers. S refers to the plants transformed with the sense construct (pUFVs64S) and AS are plants transformed with the antisense construct (pUFVs64AS). Different numbers following S and AS symbols indicate that the transgenic plants were originated from independent events of transformation. C− corresponds to the result of a PCR reaction performed with DNA from control plants, transformed with the pBI121 vector. In C+, the *s*-64 cDNA was used as template. M corresponds to DNA standard markers whose sizes are shown on the left in kb.

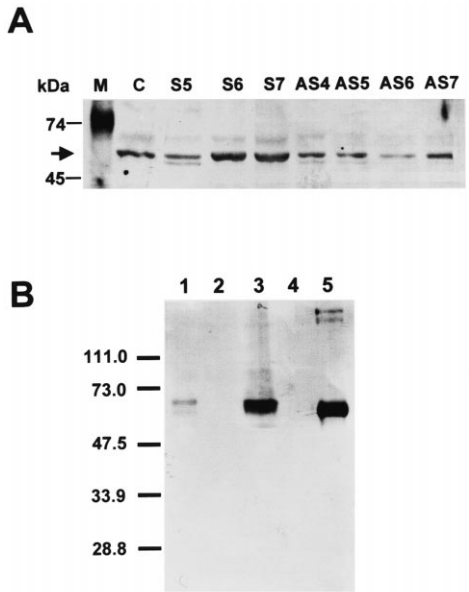

Fig. 3. (A) SBP homologue accumulation in transgenic and wild type leaves. Membrane-enriched protein fraction was isolated from control plant (C), sense 35S-s64S transgenic plants (S5, S6 and S7), antisense 35S-s64AS transgenic plants (AS4, AS5, AS6 and AS7), fractionated by SDS-PAGE, transferred to nitrocellulose membrane and probed with an anti-S64 antibody. M corresponds to pre-stained molecular markers indicated on the left in kDa. The arrow shows the position of SBP homologue polypeptide. (B) Specificity of the anti-S64 serum in yeast. Whole cell protein extracts (1 and 2) and total microsome membranes (3 and 4) were isolated from *P. pastoris* transformed with the *s*-64 cDNA under the control of the methanol-induced *AOX*1 promoter. In lanes 1 and 3, the expression of the heterologous protein was induced by 0.5% methanol and in lanes 2 and 4, yeast transformants were grown in non-induced conditions. Lane 5 corresponds to the *E. coli*-produced recombinant protein that was used to prepare the anti-S64 antibody. The positions of pre-stained molecular markers are indicated on the left in kDa.

The level of S-64 protein in transgenic plants was examined by immunoblotting of microsomal membrane-enriched fractions from leaves probed with an anti-S64 serum (Fig. 3A). A 64 kDa immunoreactive species was detected in membrane-enriched fraction from young leaves of control plants (lane C) and, therefore, confirmed the presence of a membrane-associated SBP homologue in tobacco leaves, which was not recognized by the preimmune serum (data not shown). The

accumulation of the native tobacco SBP homologue was reduced to different extents in 35S-s64AS$_4$ (lane AS4), 35S-s64AS$_5$ (lane AS5), 35S-s64AS$_6$ (lane AS6) and 35S-s64AS$_7$ (lane AS7) independent antisense transformants. The S-64 protein levels in the leaves of 35S-s64S$_6$ and 35S-s64S$_7$ sense lines (lanes S6 and S7) were significantly higher than in control tobacco leaves (lane C), whereas the level of the protein in 35S-s64S$_5$ sense line (lane S5) appeared to be unaltered.

Based on the subcellular localization and electrophoretic mobility of the anti-S64 cross-reactive polypeptide, we inferred that the soybean S-64 antibody recognized a S-64/SBP homologue in tobacco leaves. This represents the first demonstration that a SBP homologue is present in Solanaceae. To strengthen our results, the specificity of the anti-S64 serum was further evaluated in a yeast expression system. The S-64 protein was expressed in *P. pastoris* under the control of the methanol-induced *AOX*1 promoter (Fig. 3B). The anti-S64 antibody recognized only a methanol-induced 64-kDa protein in whole cell protein extracts (lane 1) and in membrane fractions (lane 3) from yeast transformants and did not cross-react with any endogenous yeast protein (lanes 2 and 4). This result confirms the specificity of the anti-S64 serum and provides further support for the detection of a tobacco SBP homologue.

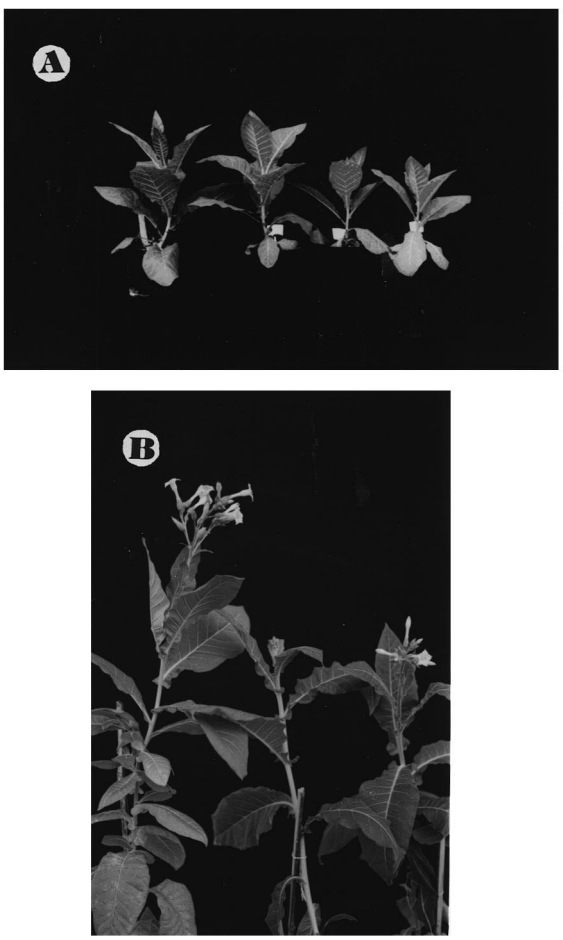

Fig. 4. Comparison of developmental performance of sense and antisense transgenic plants. (A) Transgenic tobacco plants after 4 weeks in the greenhouse. Two 35S-s64S sense independently transformed tobacco plants are shown on the left and two 35S-s64AS antisense plants on the right. (B) Transgenic tobacco plants after 8 weeks in the greenhouse (from left to right): 35S-s64S sense transgenic tobacco plant, 35S-s64AS antisense plant and control plant. Sense plants show a premature flowering, whereas the onset of flowering in antisense plants is retarded in comparison to control plants.

3.2. Growth-related phenotypes of the antisense and sense transgenic plants

Typical phenotypes of an inhibition of sucrose translocation were observed in the antisense transgenic lines. In the primary regenerants, a severe leaf curling phenotype was observed which did not persist in the mature leaves of the greenhouse grown transgenic plants. In the first 3 weeks of the plant regeneration process, leaf expansion in the antisense regenerants was higher than in control and sense regenerants (data not shown). The phenotype was reversed, however, in greenhouse grown transgenic lines and, in general, the growth of the vegetative parts of the antisense transgenic lines was retarded when compared with sense transgenic plants (Fig. 4A) and control plants (data not shown). The growth rate of the sense plants was higher than control plants (data not shown). The growth-related phenotypes of the transgenic lines were reflected in the induction of flowering (Fig. 4B). While the onset of flowering in sense lines was slightly accelerated in comparison to control plants, the induction of flowering in the antisense lines was retarded. The developmental phenotypes were evaluated in 20 progenies of each independent transformant and were found to be linked to the expression of the transgene. Reduced vegetative growth and retarded reproductive development exhibited by the antisense lines may reflect an impairment of phloem loading and/or decrease in sink activity. The contrasting phenotypes of sense lines support an inverse correlation of effects. However, the intensity of the growth-related phenotypes exhibited by the antisense transgenic

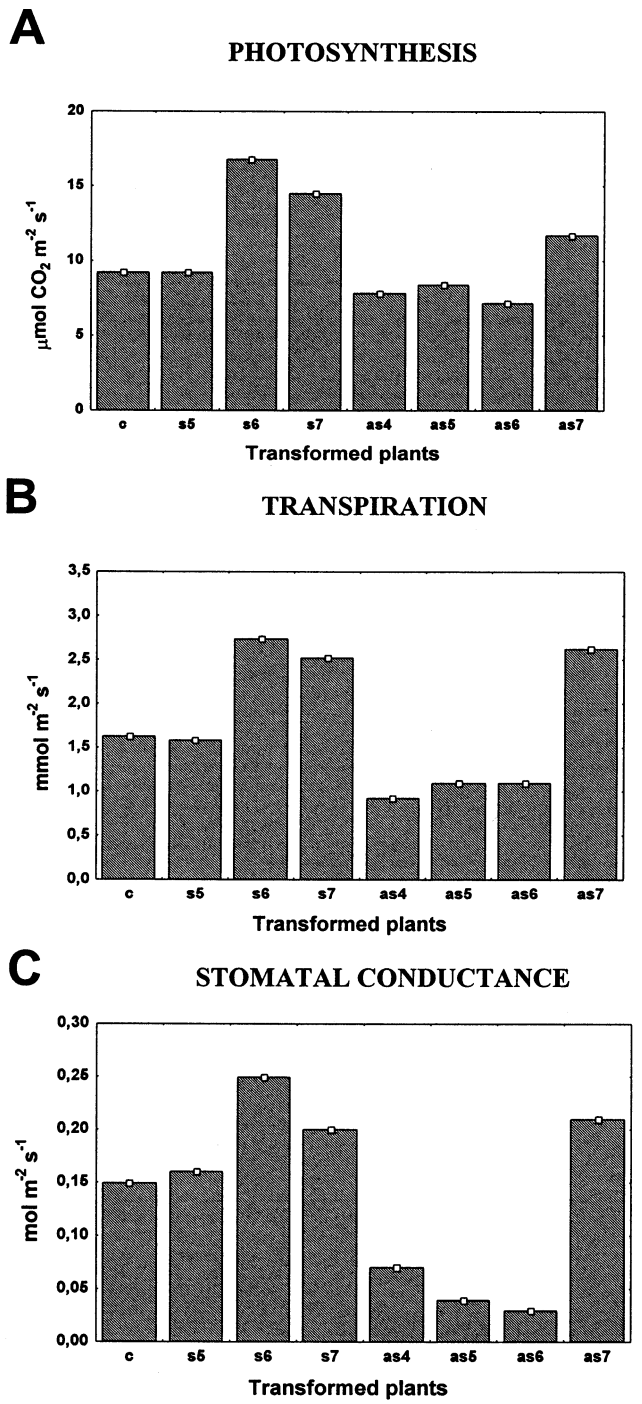

Fig. 5. Physiological measurements of sense and antisense transgenic tobacco plants. Photosynthetic rate (A), transpiration rate (B) and stomatal conductance (C) of fully expanded leaves of control (c), 35S-s64S sense (s5, s6, s7) and 35S-s64AS antisense (as4, as5, as6, as7) independently transformed plants were measured by the LCA-2 IR gas analyzer at growth irradiance.

lines was very similar, regardless the different degree of inhibition of the SBP accumulation in their leaves (Fig. 3). Thus, the decrease in the accumulation of SBP homologue led to a significant but non-proportional alteration in the overall plant development.

3.3. Sense and antisense expression of the SBP homologue gene affects the photosynthetic rate of transgenic plants

Retarded development in antisense lines as well as accelerated development in sense lines indicated that photosynthesis could have been affected in the transgenic mature leaves. If the *s*-64 gene is involved in sucrose transport for phloem loading and/or unloading, a reduction in transport activity should lead to a feedback inhibition of photosynthesis. In fact, the photosynthesis rate of mature leaves measured under ambient normal conditions by gas exchange was slightly reduced in 35S-s64AS$_4$ (as4), 35S-s64AS$_5$ (as5), 35S-s64AS$_6$ (as6) antisense lines and almost unaltered in leaves of the 35S-s64AS$_7$ (as7) transgenic line in comparison to control plants (Fig. 5A). Reduction of the photosynthetic rate in antisense transgenic lines was accompanied by a parallel decline on transpiration rate and stomatal conductance under these same conditions (Fig. 5B and Fig. 5C). In contrast, photosynthetic rates of the 35S-s64S$_6$ (s6) and 35S-s64S$_7$ (s7) sense lines were higher than control plants (c), suggesting that enhanced accumulation of SBP homologue promoted an increase either in sucrose export activity or sink strength (Fig. 5A). In 35S-s64S$_5$ (s5) leaves, in which an increase in SPB homologue accumulation is almost undetectable (Fig. 3), photosynthesis, transpiration and stomatal conductance remain unaltered. Collectively, these data are consistent with the involvement of the SBP in long-distance sucrose transport. In addition, they suggest that a functional SBP homologue is encoded by the tobacco genome. In fact, Southern blot analysis of tobacco genomic DNA with the *s*-64 cDNA probe detected hybridizing bands at low stringency (data not shown) and an anti-S64 serum detected a protein homologue in tobacco leaves (Fig. 3).

3.4. Antisense and sense expression of the SBP homologue gene alters carbon allocation in mature leaves

In order to evaluate the effects of antisense repression and overexpression of the SBP homo-

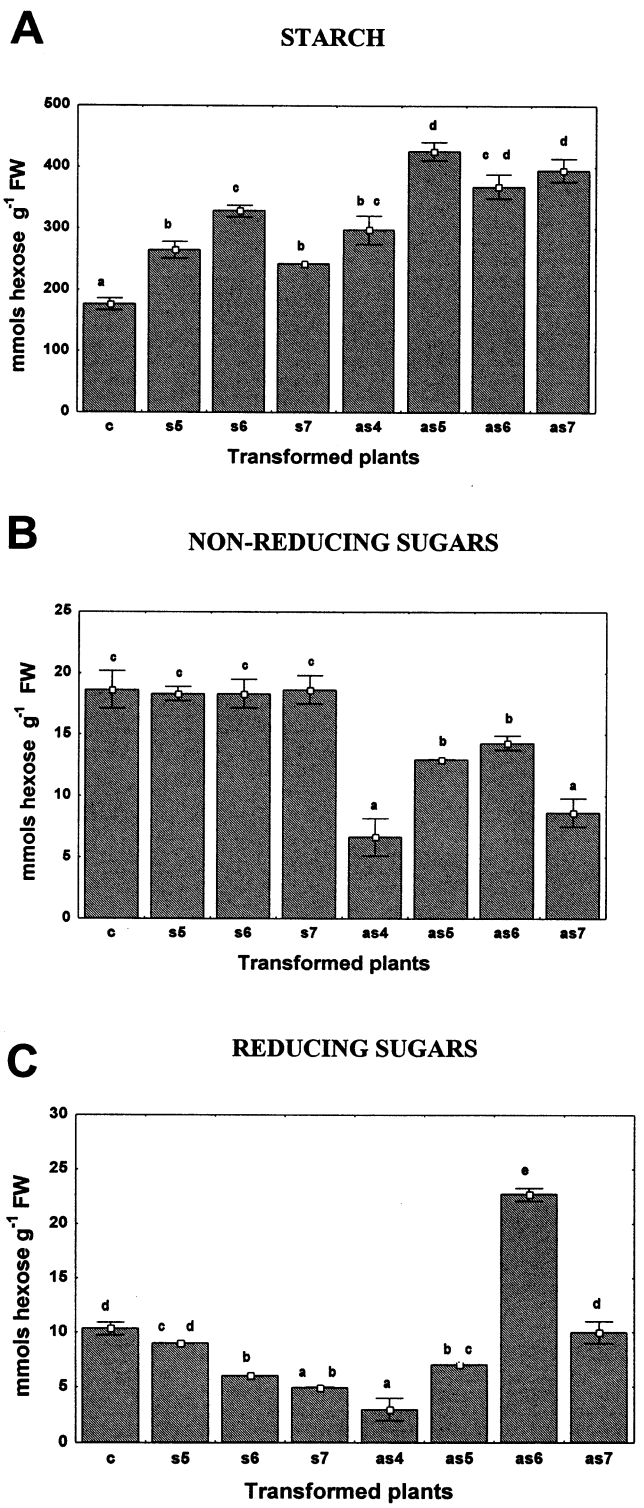

Fig. 6. Concentration of starch (A), non-reducing sugar (B) and reducing sugar (C) in leaves from control plants (c), 35S-s64S sense (s5, s6 and s7) and 35S-s64AS antisense (as4, as5, as6 and as7) transgenic tobacco plants. Values for starch, reducing and non-reducing sugars are given in mmol hexose per gram fresh weight. Values are given as mean $\pm$ S.D. of five determinations from independent leaves of equivalent fresh weight. Different letters indicate significant differences at $P \leq 0.05$.

logue gene on carbohydrate metabolism, the content of starch and soluble sugars was determined in fully expanded leaves (Fig. 6). The content of starch in the leaves of all four antisense transgenic lines (as4, as5, as6 and as7) was significantly higher than in control plants (Fig. 6A), whereas the sucrose content, determined as non-reducing sugar, was reduced in the antisense leaves (Fig. 6B). The sucrose to starch ratio in the leaves of antisense lines was correlated with an increased partitioning of photoassimilates toward starch. Enhanced carbon allocation into insoluble carbohydrates was also found in studies in which export was blocked by heat girdling [36]. Nevertheless, the alteration of carbohydrate content in SBP/S-64 antisense lines does not follow the same pattern as in antisense H^+/sucrose transporter transgenic lines, in which the levels of sucrose, hexoses as well as starch were consistently higher than in wild type plants [19–21]. We interpret these differences as a sign that the SBP and the H^+/sucrose transporter function via distinct mechanisms. In fact, unlike SUT-mediated sucrose uptake, sucrose uptake mediated by ectopic SBP expression in a yeast system is proton-independent and displays linear, non-saturable uptake kinetics [25,27].

In the sense lines (s5, s6 and s7) the content of starch in leaves was higher than in control leaves (Fig. 6A), but unlike the antisense lines, their sucrose content, determined as non-reducing sugar, was unaltered as compared to control leaves (Fig. 6B). A high content of carbohydrates in the sense leaves was expected since the photosynthetic rate was higher in these lines than in control plants (Fig. 5A). In this case, we would expect a higher sucrose content as well. Because the level of sucrose was unaltered, we assumed that the high efficiency of sucrose export in these lines prevented a detectable increase in sucrose accumulation in mature leaves. Thus, the relative content of soluble sugars and starch in sense leaves may be a result of an increase in both photosynthetic and sucrose transport rates when compared with control leaves.

3.5. The activity of endogenous invertases is altered in transgenic leaves

In the leaves of 35S-s64AS$_6$ (as6) and 35S-s64AS$_7$ (as7) antisense transgenic lines, the ratio of reducing to non-reducing sugars was at least

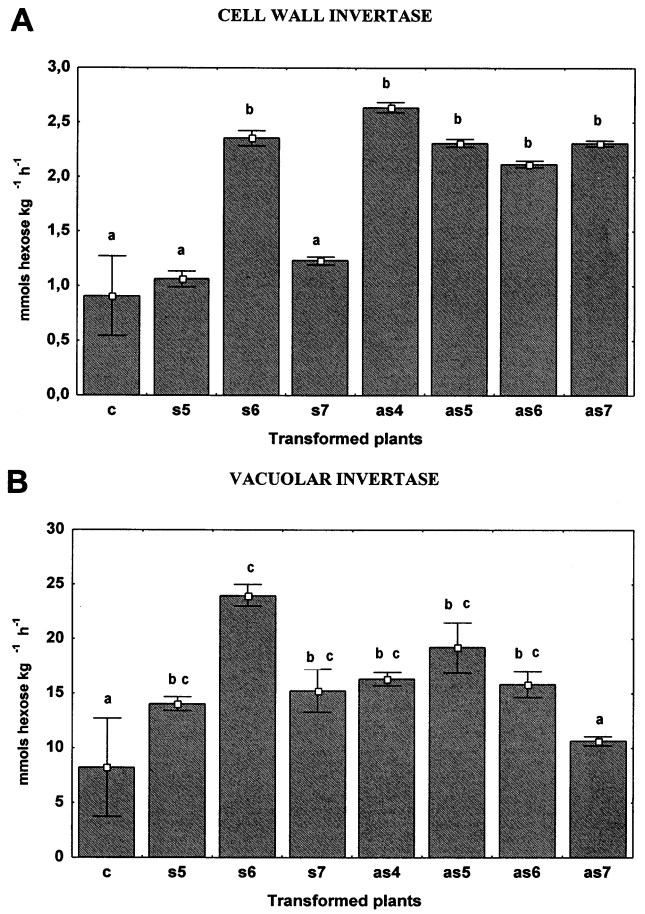

Fig. 7. CWI (A) and vacuolar invertase (B) activities of tobacco leaves from control and transgenic plants. Invertase activities was measured in extracts from leaves of control (c), 35S-s64S sense (s5, s6 and s7) and 35S-s64AS antisense (as4, as5, as6 and as7) transgenic plants. Values for invertase activities are given in mmol hexoses kg^{-1} h^{-1} and are the mean $\pm$ S.D. from five replicates. Different letters indicate significant differences at $P \leq 0.05$.

two-fold higher than in control leaves, whereas an inverse result was observed in sense leaves. The relative levels of sucrose and hexoses could reflect an unbalance between sucrose hydrolysis and synthesis in the transgenic lines (Fig. 6B and Fig. 6C). In this case, SBP would exert an indirect role in sucrose translocation by sequestering it from sucrose hydrolyzing activities. In order to test this hypothesis, the activity of cell wall invertase (CWI) was measured in mature leaves of sense, antisense and control plants. In all antisense lines (as4, as5, as6 and as7), the activity of CWI was significantly higher than in control leaves (Fig. 7A). A direct role of CWI in sucrose partitioning between source and sink regions has been demonstrated in transgenic tobacco overexpressing CWI in a constitutive manner. In general, elevated levels of the enzyme in the leaves reduce the sucrose transport between source and sink tissues and in turn leads to stunted growth and overall altered plant morphology. Thus, the level of invertase activity in antisense plants could account, at least in part, for the growth-related phenotypes of those plants. In 35S-s64S_5 (s5) and 35S-s64S_7 (s7) sense leaves, the activity of CWI remained practically unaltered (Fig. 7A), whereas the activity of vacuolar invertase in all three sense leaves was significantly higher than in control leaves (Fig. 7B, s5, s6 and s7). The variations of invertase activities in sense transgenic leaves may reflect the result of metabolic adjustment to accommodate an increase in sucrose translocation promoted by sense transgene expression without compromising leaf growth. While high activity of vacuolar invertase would favor sucrose export by preventing sucrose storage in leaves, CWI would control the exit of assimilated carbon by cleaving sucrose to support leaf growth.

4. Discussion

To elucidate the function of the *s*-64 gene of soybean, which shares 91% sequence identity with the *sbp* gene, we used an antisense repression strategy in tobacco. We chose tobacco for the repression and overexpression studies because (a) *s*-64 and/or *sbp* homologue sequences are present in the tobacco genome, (b) the apoplastic mechanism represents the primary route for sugar uptake into the long-distance translocation pathway in this plant species, and (c) tobacco can easily be transformed by *A. tumefaciens*. The overall altered plant development in the antisense lines supports the notion that sucrose translocation was indeed impaired. The phenotypes were very similar to those described by antisense repression of the H^+/ symporter and overexpression of apoplastic invertase. Nevertheless, the decrease in the accumulation of SBP homologue led to a significant but non-proportional alteration in the overall plant development. These results may reflect the complexity of the sucrose translocation pathway, which may have multiple transport mechanisms operating synergistically and in a compensatory manner. In contrast to the antisense repression phenotypic effects, enhanced expression of the *s*-

64 transgene resulted in a slight improvement of plant growth and development. The photosynthetic rates of the transgenic plants correlated very well with the developmental performance of the plants. While photosynthesis in the antisense lines was slightly decreased, in the sense lines photosynthetic rates were increased.

Consistent with inhibition of long-distance sucrose translocation, antisense repression of *sbp* homologue gene led to the accumulation of high amounts of starch in leaves, inhibition of photosynthesis and stunted growth. Nevertheless, the pattern of sugar accumulation in antisense plants was not identical to that observed in plants expressing a H^+/sucrose symporter antisense transgene. While in the leaves of SUT antisense plants both soluble sugars and starch were increased, in S-64/SBP antisense leaves, the level of starch was higher, but the content of non-reducing sugars was remarkably lower than in control leaves. In fact, antisense repression of *sbp* homologue expression resulted in an increased allocation of fixed carbon in the direction of starch synthesis. These differences may indicate that SBP/S-64 and the H^+/ symporter have distinct functions in sucrose translocation. One possibility is that SBP serves a regulatory role as an accessory protein in the sucrose uptake system in plants [37]. Alternatively, SBP may function by sequestering sucrose from hydrolyzing activities, which would favor a carrier-mediated sucrose uptake into the long distance translocation pathway. In this case, a high SBP activity would bypass the need of stoichiometric amounts of the protein. These possibilities are not mutually exclusive and favor the argument that SBP/S-64 acts in concert with the H^+/symporter to mediate sucrose uptake in plant cells. Consistent with this model, SBP colocalizes with sucrose/H^+ cotransporters and H^+-ATPase in the plasma membrane of *Vicia faba* transfer cells in developing seeds [37]. Furthermore, in the plasma membrane of abaxial epidermal cells, SBP accumulation is temporally coordinated with sucrose transport activity and the Vmax of sucrose symporter [38]. This proposed model would also predict that accumulation of SBP in sink tissues might be coordinated with the activity of an invertase-independent sugar uptake system. Several lines of evidence are consistent with this hypothesis. First, SBP protein accumulated predominantly in membranes of cells actively engaged in sucrose uptake. Second, in cotyledons of soybean and *V. faba*, accumulation of SBP transcripts parallels the period of cell expansion and storage, concomitant with a developmentally programmed decline in CWI activity [39]. Finally, we demonstrated that low levels of SBP in antisense lines are associated with high levels of CWI activity. Isolation of the tobacco SBP homologue gene and identification of its expression pattern will provide insight into the role of SBP in the sucrose transport pathway.

As an alternative explanation, the retarded growth and delayed flowering of antisense plants may not be a direct effect of the lowered accumulation of the SBP homologue because antisense leaves also exhibited a higher CWI activity. In transgenic plants expressing a yeast invertase in the apoplastic space, sucrose translocated into the cell wall is cleaved by the apoplastic invertase into its constituents, glucose and fructose [4–7]. Because hexose sugars do not appear to be transported efficiently into the phloem, they are re-imported into the cells, causing a large reduction in the sucrose export from transgenic leaves which, in turn, leads to stunted growth. Therefore, elevated levels of CWI activity in the leaves of antisense plants could account for their phenotypes. Nevertheless, a direct function of SBP in sucrose transport has been demonstrated by its ability to mediate sucrose uptake when ectopically expressed in an artificially mutant yeast, which is deficient in utilizing extracellular sucrose [25,27]. In addition, SBP antibody has been shown to selectively inhibit sucrose uptake by transfer cells of *V. faba* cotyledons [40]. Further support of a direct involvement of SBP in sucrose transport was provided by our results of *s*-64 overexpression studies in which enhanced accumulation of the soybean SBP homologue in tobacco appeared to increase the efficiency of sucrose transport and was not correlated with CWI activity (Fig. 7A).

Mature leaves of the three sense lines had higher starch levels than control leaves, whereas their level of sucrose which was measured as non-reducing sugar was not markedly changed (Fig. 6). Since photosynthesis was increased in these plants, an excess of sucrose might not be detected because it was apparently utilized for long-distance translocation, supporting the enhanced developmental performance of the sense lines. Consistent with this observation, the activity of vacuolar invertase in the sense leaves was significantly in-

creased (Fig. 7B). In leaves of plants growing under normal conditions, sucrose is transiently stored in the vacuole before being exported to the sink organs. The increase of vacuolar invertase activity could prevent sucrose storage in the vacuole, a situation that would prevail if its export rate was accelerated.

In summary, the growth-related phenotypes and physiological effects caused by sense and antisense expression of *s*-64 gene are consistent with the involvement of the SBP homologue into the long-distance sucrose translocation pathway. Because SBP is localized mainly in sink tissues actively engaged in sucrose uptake, it is more likely to be involved in phloem unloading processes. However, SPB was also found to accumulate in the companion cell plasma membrane of soybean mature leaves and in the sieve-elements plasma membrane of spinach fully expanded leaves [41], indicating that it may also have functions associated with the phloem loading mechanism. Isolation of the tobacco homologue gene and analysis of its expression pattern will allow us to directly address these possibilities. Despite the phenotypes of transgenic plants, our results do not rule out the possibility that these physiological symptoms were due to pleiotropic effects, as manipulation of the level of the SBP homologue also altered CWI activities. In addition, the ratio of soluble sugars/starch in antisense leaves may support the notion that sucrose export slows down in these lines as a result of stimulation of starch production in mature leaves. However, the observation that SBP directly mediates sucrose uptake in yeast and the physiological effects caused by enhanced accumulation of a SBP homologue in transgenic tobacco support a direct involvement of SBP in sucrose transport. Future experiments using stable sense and antisense cell suspension lines will allow us to conduct direct sucrose uptake studies in plant cells in order to answer questions concerning the precise mechanism of the role of SBP in sucrose translocation.

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