ALESSANDRO JAQUIEL WACLAWOVSKY

CHARACTERIZATION OF SOYBEAN *SBP2* (*SUCROSE BINDING PROTEIN*) GENE IN TRANSGENIC TOBACCO PLANTS: PROTEIN FUNCTIONAL ANALYSES AND PROMOTER ACTIVITY

Tese apresentada à Universidade Federal de Viçosa, como parte das exigências do Programa de Pós-Graduação em Fisiologia Vegetal, para obtenção do título de *Doctor Scientiae.*

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(Orientadora)

Ao meu filho **Gabriel**

À minha esposa **Karla**

Aos meus pais, **Sebaldo** e **Inês**

Ao meu irmão, **Aguinel** e ao seu filho, **João**

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RESUMO

WACLAWOVSKY, Alessandro Jaquiel, D.S., Universidade Federal de Viçosa, maio de 2005. **Caracterização do gene de SBP2 (Sucrose Binding Protein) de soja em plantas transgênicas de tabaco: análise funcional da proteína e atividade do promotor *SBP2*.** Orientadora: Elizabeth Pacheco Batista Fontes. Conselheiros: Fábio Murilo da Matta, Marcelo Ehlers Loureiro e Wagner Campos Otoni.

Em plantas, a energia luminosa é transformada em energia química na forma de carboidratos, os quais são alocados entre os diferentes tecidos vegetais através do floema. Durante este processo, a sacarose, principal carboidrato transportado em plantas superiores, é "carregada" e "descarregada" no floema com o auxílio de transportadores localizados na membrana plasmática. A proteína SBP ("sucrose binding protein") foi inicialmente identificada pela sua capacidade em ligar a sacarose e, pelo menos, dois homólogos da proteína, SBP1 e SBP2, têm sido descritos em soja. Neste trabalho, nós analisamos a função de SBP2 em plantas transgênicas de tabaco expressando o cDNA na orientação antisenso e caracterizamos os *cis*-elementos presentes no promotor que controlam a expressão espacial do gene *SBP*. Plantas anti-senso na geração T2 apresentaram crescimento e desenvolvimento reduzidos. Este fenótipo, característico de plantas com inibição do transporte de sacarose a longa distância, foi associado a alterações fisiológicas e metabólicas ocorridas, principalmente, na fase vegetativa do desenvolvimento. As plantas apresentaram reduzida fotossíntese e alteração no particionamento de carboidratos, com preferência para o acúmulo de amido nas folhas em detrimento à síntese de sacarose. Nossos dados suportam a hipótese que SBP provavelmente atua no transporte de sacarose a longa distancia no floema, alterando a expressão ou a atividade de proteínas envolvidas em sistemas alternativos de transporte de carboidratos, já que a manipulação dos níveis de SBP também alterou a atividade da invertase e da sintase da sacarose. Consistente com o papel da proteína no transporte de sacarose, um fragmento de 2 kb do promotor de *SBP2* dirigiu a expressão do gene repórter de *b-glicuronidase* (*GUS*) para as células do floema de folhas, caules e raízes. A caracterização dos *cis*-elementos presentes no promotor de *SBP2* foi realizada por

deleções sucessivas na região 5’ e por deleções internas. A deleção da seqüência de -2000 a -703 causou o acúmulo de GUS em todos os tecidos de folhas, caule e raízes analisados, indicando a presença de *cis*-elementos que reprimem a atividade do promotor em tecidos, que não o floema, a jusante da posição -703 pb. Deleções sucessivas até a posição -92 indicaram que a atividade tecido-específica do promotor é coordenada pela interação complexa entre elementos negativos e positivos. De fato, elementos negativos fortes foram identificados na seqüência delimitada pelas posições -495 e -370, os quais foram confirmados por experimentos de ganho de função, e também, entre -243 e -193. Foi identificada também uma região silenciadora do meristema radicular na região entre -136 e -92. Uma seqüência curta de 92pb a partir do códon de iniciação de tradução foi capaz de manter altos níveis de expressão basal em todos os tecidos analisados e, portanto, provavelmente representa um promotor eucariótico mínimo em plantas.

ABSTRACT

WACLAWOVSKY, Alessandro Jaquiel, D.S., Universidade Federal de Viçosa, May, 2005. **Characterization of soybean *SBP2* (*sucrose binding protein*) gene in transgenic tobacco plants: protein functional analyses and promoter activity.** Adviser: Elizabeth Pacheco Batista Fontes. Committee members: Fábio Murilo da Matta, Marcelo Ehlers Loureiro e Wagner Campos Otoni.

The plants are autotrophic organisms with specialized organs that transform the sun light energy in chemical energy as organic compounds, the carbohydrates, which are transported in part to the other organs by phloem. In plants, sucrose is the major transported form of photoassimilated carbon and its translocation involves loading and unloading of the phloem by membrane specific transporters. A sucrose binding protein, SBP, was initially identified by its capacity to bind sucrose and, at least, two SBP homologues, SBP1 and SBP2, have been described in soybean. Here, we analyzed the SBP2 homologue function in tobacco transgenic plants expressing the cDNA in the antisense orientation and characterized the *cis*-acting elements involved in spatial regulation of the *SBP2* promoter. Typical phenotypes of an inhibition of long distance sucrose translocation were observed in the antisense T2 transgenic lines. In general, the growth and development of the transgenic lines was retarded when compared with control plants. This growth-related phenotype was associated with physiological and metabolic alterations during the early plant development. The photosynthetic rate was decreased and the leaf starch content was higher in antisense lines in comparison with control plants. Our data support the hypothesis that SBP may have a regulatory role in phloem sucrose transport by regulating the expression or activity of alternative carbohydrate uptake systems, since the manipulation of the level of the SBP homologue also altered invertase activity and sucrose synthase activity. Consistent with the involvement of SBP in long-distance sucrose transport, the *SBP* promoter directed the expression of the β-*glucuronidase* (*GUS*) reporter gene with high specificity to phloem of leaves, stems and roots of the transgenic tobacco plants. In order to identify potential *cis*-regulatory elements controlling the spatial expression of

SBP promoter, we performed 5' and internal deletion promoter analyses in transgenic tobacco. The repression of the *SBP2* promoter activity in all other tissues of root, stem and leaf is alleviated by deletion of sequences upstream of -703. This indicates that the phloem-specific expression of *SBP2* promoter is contributed by tissue-specific silencers in distal sequences as well as phloem-specific elements within proximal sequences of *SBP2* promoter. Further deletions of 5' flanking sequences indicate that *SBP2* promoter activity and tissue-specificity is coordinated by negative and positive combinatorial modules that interact to each other in a complex way. Strong negative elements were found in sequences delimited by positions -495 to -370, which were further confirmed by gain-of-function experiments and also between positions -243 and -193. In addition, a root meristem-specific silencer was identified within the region between -136 and -92. A short sequence, spanning from -92 to the start codon, was able to maintain high level of basal expression and may represent a potential eukaryotic minimal promoter.

GENERAL INTRODUCTION

Plants are autotrophic organisms with specialized organs that transform the sun light energy in chemical energy as organic compounds, the carbohydrates, which are transported in part to the other organs. The development and growth of sink tissues, such as seeds, fruits, flowers, stem and root, depend on carbon import from photosynthetically active tissues. About 80% of the carbon assimilated during photosynthesis is exported from leaves to satisfy the metabolic needs of the non-photosynthetic cells (Geiger *et al.*, 1974). In plants, sucrose is the major transported form of photoassimilated carbon (Giaquinta, 1983) and it is transported at long-distance through the vascular system that interconnects the source tissues, mature leaves, with sink tissues. In the vascular system, the phloem sap flow drives sucrose in a specialized network of cells, called sieve elements. Sieve elements lose their nucleus and many organelles during differentiation, but stay connected to companion cells, which has a high metabolic activity. Sieve elements are connected to form sieve tubes that oppose very little resistance to the flow of sap. The driving force for this flow occurs through phloem loading and unloading processes, which correspond to the entry of sucrose and subsequently water in the sieve tubes of the source organ and, at the other end of the conduit in the sink organs, the continuous unloading of solutes and water (van Bel, 1996).

So, the translocation of sucrose from its site of synthesis, the mesophyll cells, to sink tissues involves phloem loading in the source tissues, long-distance transport by mass flux and sucrose unloading from the phloem cells to the sink tissues (revised by Frömmer and Sonnevald, 1995). These processes of phloem loading and unloading with sucrose occur via cell-to-cell transport either directly through plasmodesmata interconnecting adjacent cells (symplastic transport) (Lucas *et al.*, 1993; Russin, *et al.*, 1996) or across plasma membranes mediated by protein carriers (apoplastic transport)

(Lemoine, 2000; Williams *et al.*, 2000). Both routes can contribute for phloem loading and unloading processes, although in some plants one route of sucrose loading may predominate over the other (Frömmer and Sonnerwald, 1995).

Some plants have a high number of plasmodesmatal connections between mesophyll cells and the sieve element/companion cell complex (SE-CCC). In these species, sugars of the rafinose family and not sucrose are often the preferentially exported carbohydrates (Turgeon and Beebe, 1991). In most species, at least in crop species, the SE-CCC is simplastically isolated from the surrounding cells. The importance of an apoplastic step for phloem loading is demonstrated by strong plant growth-related phenotypes caused by the expression of a yeast invertase in the apoplast of transgenic solanaceous species, such as tobacco, tomato and potato (von Schaewen *et al.*, 1990; Sonnewald *et al.*, 1991; Dickinson *et al.*, 1991; Heineke *et al.*, 1992). Such phenotypic effects include leaf curling and local bleaching, reduced root growth and tuber yield. Furthermore, expression of a heterologous invertase in the apoplast dramatically affected assimilates 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 some plants.

In support of these conclusions, biochemical studies with isolated cells and plasma membrane vesicles have identified sucrose transport activities across plasma membrane in several plant species (Lemoine and Delrot, 1989; Williams *et al.*, 1991, Lemoine *et al.*, 1992). These studies have demonstrated that sucrose uptake kinetics in leaves is complex and consists of different components. For example, in *Vicia faba*, two saturable (high- and low-affinity) components and one linear, low-affinity component have been described (Delrot and Bonnemain, 1981).

The understanding of plant sucrose translocation has advanced considerably over the last years with the molecular and biochemical characterization of the sucrose transporter (SUT) family of low- and high-affinity sucrose transporters (Lalonde *et al.*, 1999). The SUT1 protein has been described as the proton-motive-force-driven sucrose symporter that mediates phloem loading and long-distance transport, the key transport step in assimilate partitioning for many plants (Riesmeir *et al.*, 1992; Riesmeier *et al.*, 1994; Bürkle *et al.*, 1998). SUT1 serves as a high-affinity transporter, whereas SUT4, a second member of this sucrose transporter family, corresponds to the low-affinity/high capacity saturable component of sucrose uptake found in leaves

(Weise *et al.*, 2000). A third structurally related-member of the family has been identified and designated SUT2 (Barker *et al.*, 2000). The SUT2 protein has been proposed to act as a sugar sensor that controls sucrose fluxes across the plasma membrane of sieve elements by regulating expression, activity and turnover of SUT1 and SUT4 (Barker *et al.*, 2000). This hypothesis was raised based on the lack of transport activity of SUT2 and its colocalization with the high and low-affinity sucrose transporter in sieve elements. Nevertheless, direct evidence for a SUT2 sucrose sensor and regulatory function has not been provided.

At present, dozens of the cDNAs encoding SUT homologous have been identified in different plant species, both in dicots and monocots. *SUT* cDNAs have been isolated from various species such as spinach (Riesmeier *et al.*, 1992), potato (Riesmeier *et al.*, 1993), common plantain (Gahrtz *et al.*, 1994), Arabidopsis (Sauer and Stolz, 1994; Weise *et al.*, 2000), ricinus (Weig *et al.*, 1996), rice (Hirose *et al.*, 1997), *Faba bean* (Weber *et al.*, 1997), tomato (Kühn *et al.*, 1997; Barker *et al.*, 2000; Weise *et al.*, 2000), tobacco (Bürkle *et al.*, 1998), carrot (Shakya and Sturm, 1998), grape (Davies *et al.*, 1999; Ageorges *et al.*, 2000), pea (Tegeder *et al.*, 1999), corn (Aoki *et al.*, 1999) and others. Although the whole family of sucrose transporter genes of a given species has not been identified, the sucrose transporters make a large gene family, as at least seven distinct sequences that encode putative sucrose transporters are present in the Arabidopsis data base (Williams *et al.*, 2000).

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 (Williams *et al.*, 2000). Their activity is pH dependent and inhibited by protonophores, thiol-group modifying agents and diethylpyrocarbonate and they have been described as sucrose:proton cotransporters (Bush, 1990). Complementation assays using an invertase-deficient yeast mutant has been successfully used to characterize H^+-ATPase:sucrose cotransporters (Riesmeier *et al.*, 1992; 1993). The yeast-produced SUT1 recombinant protein exhibits biochemical properties similar to those described for sucrose uptake in protoplasts and in leaf plasma membrane vesicles from a number of plant species (Riesmeier *et al.*, 1993). Functional expression in *Xenopus* sp. oocytes also has demonstrated further that SUT1 functions as a proton symporter (Boorer *et al.*, 1996).

Furthermore, these acid transporters are localized in the SE-CCC and probably mediate sucrose uptake in the phloem from the apoplast (Riesmeier *et al.*, 1992; 1993;

1994; Gahrtz *et al.*, 1994; Kühn *et al.*, 1996). Some members are found to be preferentially expressed in import zones of sink organs where they may catalyze either influx and/or retrieval of sucrose. Sink-specific sucrose transporters have been characterized in seeds of *Faba bean* (Weber *et al.*, 1997; Harrington *et al.*, 1997). A proton ATPase that supplies the driving force for sucrose transport against a gradient of concentration has also been found in the plasma membrane of the companion cells (Frommer, 1995). In potato, the pattern of *SUT* mRNA expression follows the sink-to-source transition and is coordinated with accumulation of sucrose transport activity (Riesmeier *et al.*, 1993). Furthermore, physiological analyses of plants demonstrate that sucrose transporters are essential components of the sucrose translocation pathway. *In vivo* antisense repression of SUT1 in transgenic tobacco and potato plants inhibits sucrose export from leaves, which alters leaf morphology, plant development and carbohydrate accumulation in leaves (Riesmeier *et al.*, 1994; Kühn *et al.*, 1996; Lemoine *et al.*, 1996; Bürkle *et al.*, 1998).

Another class of sucrose transporters has been identified because of its strong affinity 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). This protein was designated sucrose binding protein (SBP). Subsequent progresses in characterizing SBP led to the isolation of two cDNAs from expression libraries prepared from soybean cotyledon mRNA, here denominated *SBP1* (Grimes *et al.*, 1992) and *SBP2* (Pirovani *et al.*, 2002). Molecular characterization of the cDNA-encoded products revealed that the SBP homologs were quite dissimilar from the H^+/sucrose symporter SUT. Analysis of the SBP deduced amino acid sequence indicates that the protein contains a single hydrophobic domain at its N terminus but otherwise is a hydrophilic protein lacking the expected membrane-spanning hydrophobic segment typically present in transport proteins (Grimes *et al.*, 1992; Pirovani *et al.*, 2002). SBP shares similar structural characteristics with members of the vicilin family that belongs to the cupin superfamily of proteins. Very likely SBPs and vicilins were originated from the same precursor of the vicilin family (Heim *et al.*, 2001; Contim *et al.*, 2003). Despite the lack of similarity between SBP and other known membrane transport proteins, several lines of evidence have implicated the SBP protein as the linear, low affinity component of sucrose uptake system in plants.

In fact, the SBP has many features that suggest its involvement in sucrose transport. First, SBP has been localized in the plasma membrane of soybean 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 (Grimes *et al.*, 1992). In spinach, a SBP homologue was immunolocalized in the plasma membrane of sieve elements in fully expanded leaves, shoots and roots (Warmbrodt *et al.*, 1989, 1991) and in V. faba developing seeds, SBP was colocalized with the H^+/sucrose symporter in the plasma membrane of transfer cells (Harrington *et al.*, 1997). A SBP homologue was also detected in the microsomal fraction of young leaves from *Nicotiana tabacum* (Pedra *et al.*, 2000). The observation that affinity-purified antiserum against the SBP inhibits sucrose uptake into *Vicia faba* transfer cells further exalts the involvement of SPB in sucrose transport (Fieuw *et al.*, 1992).

Overexpression and antisense repression studies have been conducted in transgenic tobacco (*Nicotiana tabacum* L. Cv Havana) to analyze the function of SBP in the long-distance sucrose transport (Pedra *et al.*, 2000). The antisense transgenic plants developed symptoms consistent with inhibition of sucrose translocation and displayed a reduction in plant growth and development. Furthermore, both antisense repression and overexpression of a SBP homologue in transgenic lines altered carbohydrate partitioning in mature leaves. These results indicated that SBP might represent an important component of the sucrose translocation pathway in plants. The role of SBP in plant cell sucrose transport has also been analyzed performing radiolabeled sucrose uptake experiments with transgenic tobacco cell lines expressing the SBP sense or antisense gene (Delú-Filho, 2000). In this condition, the level of a SBP homologue correlated with the efficiency of radiolabeled uptake by the transgenic tobacco cells. Furthermore, manipulation of SBP levels altered sucrose-cleaving activities in a metabolic compensatory manner. Enhanced accumulation of SBP caused an increase in intracellular sucrose synthase activity with a concomitant decline in cell-wall invertase activity. This alteration in sucrose-cleaving activities is consistent with a metabolic adjustment of the sense cell lines caused by its high efficiency of direct sucrose uptake as disaccharide. Although these studies clearly demonstrated that SBP is involved in sucrose translocation-dependent physiological processes, still unresolved is whether the underlying mechanism involves SBP-mediated sucrose transport or SBP-mediated regulation of alternative carbohydrate uptake systems.

Direct evidence implicating SBP in sucrose transport has been obtained with complementation studies using a secreted-invertase-deficient mutant yeast strain, incapable of growth on medium containing sucrose as the only carbon source.

Expression of a *SBP* cDNA alone reverted the mutant phenotype (Grimes and Overvoorde, 1996; Overvoorde *et al.*, 1996; 1997; Pirovani *et al.*, 2002). Kinetics analysis of SBP mediated sucrose uptake in this yeast system indicates that the uptake is specific for sucrose but is proton-independent and relatively nonsaturable, thus defining a novel mechanism for sucrose uptake (Grimes and Overvoorde, 1996; Overvoorde *et al.*, 1996). These biochemical features closely resemble the kinetics properties of the previously characterized linear component of sucrose uptake in higher plants (Maynard and Lucas, 1982a, 1982b; Lin *et al.*, 1984). Although these results might suggest that SBP directly mediates sucrose transport, analysis of its primary structure indicates that SBP do not show typical structural motifs of any membrane transporter proteins.

Analysis of the SBP2 deduced amino acids sequence allowed us to predict a signal peptide and its processing site, a consensus sequence for nucleotide binding and a site for N-linked glycosylation, as potential sites for post-translational modifications of the protein (Pirovani *et al.*, 2002). Despite the hydrophilic nature of SBP, solubilization and partitioning studies of plasma membrane proteins have demonstrated that approximately 25 % of SBPs are associated with a hydrophobic portion of the plasma membrane (Overvoorde and Grimes, 1994). This observation has led to the suggestion that the putative leader peptide, which corresponds to the only hydrophobic region of the protein, is not quantitatively cleaved from the mature protein. In fact, it has been demonstrated that the leader peptide is partially cleaved *in vitro* (Overvoord *et al.*, 1994) and *in vivo*, since the SBP protein was detected in microsomal fraction of soybean cotyledon (Pirovani *et al.*, 2002). A *Vicia faba* SBP-like protein (VfSBPL) was found to accumulate predominantly in the protein storage vacuole, but a small fraction of the protein was also detected in the plasma membrane of cotyledonary cells (Hein *et al.*, 2001). Biochemical analysis of the topology of the SBP demonstrates that it is tightly associated with the external leaflet of the plasma membrane (Overvoorde and Grimes, 1994).

SBP2 possesses nucleotide binding activity, which exhibited a high degree of selectivity to guanine nucleotides (GTP, GDP,GTPγS) over adenine and pyrimidine nucleotide triphosphates (Pirovani *et al.*, 2002). Furthermore, this GTP-binding activity doesn't seem to affect the sucrose transport capacity of SBP. While mutations in the *SBP* nucleotide-binding site abolished GTP binding, it didn't prevent the ability of the protein to transport sucrose, since mutant protein promoted growth of the secreted

invertase-deficient yeast strain on medium containing sucrose as the only carbon source (Pirovani *et al.*, 2002). These results indicate that GTP binding and sucrose transport by SBP are separable and function independently. Consistent with this observation, the nucleotides are absent extracellularly and as such might not affect sucrose transport, since SBP has been proposed to be an extracellular integral membrane protein. In contrast, any biological significance for the presence of a functional GTP binding site would be strictly dependent on the intracellular localization of the protein where high nucleotide concentration is present. While previous result based on NHS-biotin labeling membrane proteins demonstrated that SBP was associated with the external surface of the membrane (Grimes *et al.*, 1992), they did not exclude other sites of cellular localization that would provide the appropriate compartmentalization for a functional GTP binding properties, like demonstrated with VfSBPL (Hein *et al.*, 2001). In conclusion, the function of the GTP binding activity stays obscure. These previous biochemical studies have shown also that SBP is organized in vivo as dimers and trimers whose subunits interact to each other through disulfide linkage (Overvoorde *et al.*, 1997; Pirovani *et al.*, 2002).

Based in these results, topological models were proposed. In the first model, SBP behaves as a type II membrane protein, which spans the bilayer once and has the bulk of the protein exposed to the extracellular environment, since a portion (about 25%) of the protein is tightly associated with the external leaflet of the plasma membrane (Overvoorde and Grimes, 1994). According to this topological model, the oligomerization properties of the protein would provide the means for assembling protein conduits across the membrane to mediate sucrose transport. This possibility has been previously considered with the observation that SBP is structurally related to vicilin-like storage proteins (Overvoorde *et al.*, 1996; Braun *et al.*, 1996), which assemble into trimers to form an 86–88 Å toroid complex with an internal hole of 18 Å (Ko and McPherson, 1993; Braun *et al.*, 1996). The proposed topology for SBP/S64, as an intrinsic membrane transporter, would also predict that GTP binding might not affect sucrose transport as nucleotides are absent extracellularly.

The second topological model takes into account the fact that any biological significance for the presence of a functional GTP binding site would be strictly dependent on the intracellular localization of the protein where high nucleotide concentration is present (Pirovani *et al.*, 2002). One possibility is that a proportion of the soybean SBP is indeed localized extracellularly at the cell surface as a type II

membrane transport protein and a fraction of correctly processed protein remains intracellularly as membrane-associated protein where its capacity to bind GTP and sucrose may implicate a regulatory role (Pirovani *et al.*, 2002). Further experiments will be necessary to confirm the proposed topology for SBP and its subcellular localization.

Many studies in plants have described sugar-mediated changes in gene expression and recent research has provided convincing evidence for a sucrose-dependent signaling pathway, as an important regulatory step in resource allocation (Lalonde *et al.*, 1999; Gibson, 2000; Smeekens, 2000). The demonstration that SBP2 exhibits GTP binding activity (Pirovani *et al.*, 2002) together with its capacity to bind sucrose specifically and reversibly (Ripp *et al.*, 1988) raises the possibility that SBP may also serve a regulatory role in sucrose translocation-dependent physiological processes in plants. The resulting phenotypes from alteration on SBP levels in transgenic plants may support such function (Pedra *et al.*, 2000; Delú-Filho *et al.*, 2000). SBP2 repression studies in tobacco have indeed shown some of the typical phenotypes caused by impairment of sucrose translocation (Riesmeier *et al.*, 1994; Kühn *et al.*, 1996; Lemoine *et al.*, 1996), such as accumulation of carbohydrates within source leaves, inhibition of photosynthesis and stunted growth (Pedra *et al.*, 2000). Nevertheless, the pattern of sugar accumulation in the *SBP2* antisense leaves was not identical to that caused by antisense repression of H+/SUT1 symporter (Riesmeier *et al.*, 1994). This observation suggests that SBP and SUT have distinct functions in sucrose translocation and favors the argument that SBP serves a regulatory role in the plant sucrose uptake system (Pirovani *et al.*, 2002). Consistent with this hypothesis, the increase in SBP2 levels had a stronger effect on sucrose synthase activity than on sucrose uptake (Delú-Filho *et al.*, 2000). These observations further support the idea that SBP functions in the sucrose translocation pathway by regulating the expression or activity of alternative carbohydrate uptake systems (Pirovani *et al.*, 2002). Further studies will be necessary to discern whether SBP mediates sucrose transport or functions directly as regulator of sucrose metabolizing enzymes or both.

An important physiological alteration in plants with impairment of the sucrose long-distance transport is the decrease in photosynthesis and the delay in flowering (Riesmeier *et al.*, 1994; Kühn *et al.*, 1996; Lemoine *et al.*, 1996; Bürkle *et al.*, 1998; Pedra *et al.*, 2000). After CO_2 fixation in the chloroplasts of the leaf cells, through the reactions of Calvin's cycle, the resulting triose-phosphates are addressed to starch, lipids, amino acids or sucrose biosynthesis. The pathways of sucrose and starch syntheses occur in the cytosol and in the chloroplast stroma, respectively, and are

separated by the metabolite-impermeable inner membrane of the chloroplast envelope. The direction of biosynthesis is determined by the exchange ratio of triose-phosphate from the chloroplast with inorganic phosphate from the cytoplasm (Flügge *et al.*, 1989), through the phosphate-triose translocator (phosphate translocator) (Flügge and Heldt, 1976). This phosphate translocator catalyzes the exact stoichiometric exchange of triose phosphate or 3-PGA with Pi (Fliege *et al.*, 1978) and it is the key mediator of a regulatory network for the distribution of triose phosphate among the Calvin cycle, sucrose synthesis, and starch synthesis (Stitt and Heldt, 1985). Since the levels of inorganic phosphate depend predominantly of sucrose synthesis and sucrose demand, any alteration of sucrose exportation alters the inorganic phosphate concentration. Therefore, a decrease of the sucrose synthesis rate reduces the levels of inorganic phosphate, and, in turn, the transport of triose phosphate to the cytosol that leads to the deposition of secondary starch in the chloroplast and, consequently, the photosynthesis is repressed. This regulatory cycle works as an adaptive mechanism of carbon metabolism, for momentary applications of transient stock or long-distance transport (Flügge *et al.*, 1989).

However, it has been proposed that this mechanism can just work short term, while for long term the accumulation of sugars in leaves represses the photosynthetic apparatus through the transcriptional regulation of their genes (Krapp *et al.*, 1991). In fact, sugars can act as regulatory key that affect the expression of genes and consequently the development of the plant (Dijkwel *et al.*, 1997). In general, sugars favor the expression of enzymes in connection with biosynthesis, and storage of reserves (including starch, lipid, and proteins), while repressing the expression of enzymes involved in photosynthesis and reserve mobilization (Koch, 1996). In tobacco, an accumulation of sugars in the leaves drives a decrease in the transcripts and protein levels of Calvin´s cycle enzymes (Stitt and Sonnewald, 1995). The mechanisms of activation or repression of sugar-responsive genes include regulation at transcriptional, post-transcriptional and translational level (Tung *et al.*, 1992; Krapp *et al.*, 1993; Sheu *et al.*, 1994; Shen and Tye, 1995; Trevelein and Hohmann, 1995; Cereghino and Scheffler, 1996; Rook *et al.*, 1998; Ho *et al.*, 2001)

Understanding the global mechanisms involved in sugar sensing, sugar transduction and sugar regulation of gene expression in plants is still at early stages. Regarding sugar sensing, one well-characterized example in plants involves a hexokinase-mediated sugar sensing system in the repression of photosynthetic genes by hexose (Jang and Sheen, 1994; Jang *et al.*, 1997; Moore and Sheen, 1999). Likewise, the

importance of hexose phosphorylation by hexokinase was proposed for sugar suppression of non-photosynthetic genes (Graham *et al.*, 1994; Prata *et al.*, 1997; Umemura *et al.*, 1998). However, multiple sugar sensing pathways have also been proposed to exist in plants (Halford *et al.*, 1999; Sheen *et al.*, 1999; Smeekens, 2000). Another key component in sugar signaling, a homolog of SNF1, has been isolated from a variety of plants (Halford and Hardie, 1998). Several plant homologs have been shown to complement snf1 mutations in yeast, suggesting that there might be an SNF1-dependent sugar-signaling pathway in plants (Halford and Hardie, 1998; Halford *et al.*, 1999). Besides the sugar-sensing system mediated by hexokinase, the existence of a sucrose-specific sensor and a hexose transporter-associated sensor has been suggested (Smeekens and Rook, 1997; Lalonde *et al.*, 1999).

Despite considerable progress in recent years, many crucial elements in these pathways are still unknown (Koch *et al.*, 2000; Pego *et al.*, 2000). Biochemical studies have provided evidence for the involvement of a variety of protein kinases, protein phosphatases, 14-3-3 proteins, and Ca^{2+} as a second messenger. Although several transcription factors and regulatory cis-elements have been found to mediate sugar control of gene expression, their precise roles in sugar signal transduction pathways require further investigation (Rolland *et al.*, 2002). Furthermore, plant sugar-response pathways form part of a complex regulatory web that also includes phytohormone and environmental-response pathways, such as pathways mediating ABA, auxin, cytokinin, ethylene and nitrogen regulation (Gibson, 2000; Rolland *et al.*, 2002).

The effects of sugars on floral transition have been studied in more detail recently and appear to be very complex. In *Arabidopsis,* increased leaf carbohydrate export and starch mobilization are required for flowering, suggesting that phloem carbohydrates have a critical function in floral transition (Corbesier *et al.*, 1998). Interestingly, the addition of sucrose can rescue the late-flowering phenotype of several mutants (Araki and Komeda, 1993; Roldan *et al.*, 1999) and even promotes leaf morphogenesis and flowering in the dark (Roldan *et al.*, 1999). However, high exogenous concentrations of sugars have been shown to delay flowering significantly (Zhou *et al.*, 1998). A recent report confirmed the pleiotropic effects of sugars on floral transition, which are dependent on sugar concentration, vegetative growth phase, and genetic background. Sugars may control floral transition by positively and negatively regulating the expression of floral identity genes (Ohto *et al.*, 2001).

Finally, in order to understand better the participation of SBP2 on the sucrose translocation-dependent physiological processes in plants, two *SBP* genomic clones, *gsS641.1* and *gsS641.2*, which correspond to allelic forms of the *GmSBP2* gene, have been isolated and characterized (Contim *et al.*, 2003). Fluorescence in situ hybridization (FISH) suggested that the soybean *SBP* gene family is represented by at least two non-allelic genes corresponding to the previously isolated *GmSBP1* and *GmSBP2* cDNAs (Grimes *et al.*, 1992; Pirovani *et al.*, 2002). These two cDNAs share extensive sequence similarity but are located at different loci in the soybean genome. The nucleotide sequence of a 2.0 kb 5'-flanking sequence from *gsS641.1* was cloned, sequenced and analyzed for the presence of *cis*-elements. The analysis of these promoter regions revealed a number of conserved motifs of most eukaryotic promoters, in addition to several potential regulatory elements of plant promoters (Contim *et al.*, 2003). The authors also investigated transcriptional activation of these genomic clones. The SBP2 promoter directed expression of both *GUS* and *GFP* reporter genes with high specificity to the phloem of leaves, stems and roots. Thus, the overall pattern of *SBP-GUS* or *SBP-GFP* expression is consistent with the involvement of SBP in sucrose translocation-dependent physiological processes (Contim *et al.*, 2003).

The objectives of this work were to select and to characterize T2 generation of SBP2 antisense tobacco plants and also to analyze *SBP2* promoter regulation in transgenic plants. So, the second chapter describes the effects of SBP2 inactivation on carbon partitioning and photosynthesis regulation as related to the proposed role of SBP2 in sucrose translocation-dependent physiological processes. In the third chapter, *SBP2* promoter analyses are conducted to identify *cis*-acting DNA sequences that confer spatially-regulated activation of *SBP* gene expression.

CHAPTER 1

EVIDENCE FOR A SUCROSE BINDING PROTEIN ROLE IN SUCROSE-DEPENDENT PHYSIOLOGICAL PROCESSES DURING EARLY PLANT DEVELOPMENT

Introduction

The phloem plays an essential role in the delivery of resources (photoassimilates, amino acids, and signaling molecules) to heterotrophic plant tissues. In angiosperms, the phloem is composed of sieve elements and their associated companion cells. As the molecules enter the sieve elements, they move in the osmotically driven translocation stream from source (sites of production and export) to sink (sites of import) tissues along the vascular pathway. The osmotic pressure gradient is created and maintained by loading and unloading of photoassimilates at the source and sink tissues, respectively. Sucrose is the dominant form of carbohydrates that is transported through the phloem. The current understanding of sucrose transport biochemistry has increased significantly over the past 10 years due to the successful cloning of several sucrose transporter genes isolated from various plant species (Sauer and Stolz, 1994; Shakya and Sturm, 1998; Lemoine *et al.*, 1999; Weschke *et al.*, 2000; Eksittikul *et al.*, 2001). Two families of sucrose/H^+ symporters (SUT) are currently recognized in plants and are defined as high-affinity–low-capacity (HALC) and low-affinity–high-capacity (LAHC) transport systems (Delrot and Bonnemain, 1981). SUTs are members of the Major Facilitator Superfamily (MFS) and are characterized by the presence of 12 membrane-spanning domains arranged in a 6-α-helical + 6-α-helical configuration separated by a cytoplasmic loop (Marger and Saier, 1998) with both the N-terminus and the C-terminus on the cytoplasmic side of the plasma membrane (Stolz *et al.*, 1999). Previous reports demonstrate that the *SUT* gene products represent an important component of the carrier-mediated apoplastic transport process. Furthermore, antisense repression of SUT1 in transgenic plants inhibits sucrose export from leaves, which alters leaf morphology and plant development (Riesmeier *et al.*, 1994; Kühn *et al.*, 1996; Lemoine *et al.*, 1996).

A third sucrose transport mechanism have been suggested, which is independent of H+ movement and is nonsaturable (Lin *et al.*, 1984). Maynard and Lucas (1982a, 1982b) have estimated that ~43% of sucrose uptake into leaves is mediated by this linear component in the presence of 25mM sucrose. A possible carrier candidate for this sucrose transport mechanism is the sucrose binding protein, or SBP, which was previously isolated by photoaffinity labeling from membranes isolated of the soybean cotyledon with photolyzable sucrose analog (Ripp *et al.*, 1988). An evidence implicating SBP in proton-independent sucrose transport has been obtained with complementation

studies using a secreted-invertase-deficient mutant yeast strain, incapable of growth on medium containing sucrose as the only carbon source (Overvoorde *et al.*, 1996; 1997; Pirovani et al., 2002). Ectopic expression of the SBP cDNA alone reverses the mutant yeast phenotype and SBP mediated specific sucrose uptake in yeast displays linear, nonsaturable kinetics up to 30mM external sucrose, being relatively insensitive to pH gradient across the membrane (Overvoorde *et al.*, 1996; Grimes *et al.*, 1996; Pirovani *et al.*, 2002). These biochemical features closely resemble the kinetics properties of the previously characterized linear component of sucrose uptake in higher plants (Maynard and Lucas, 1982a; 1982b; Lin *et al.*, 1984).

However, characterization of the two cDNA (named *SBP1* and *SBP2*) encoding sucrose binding proteins and analyses of their deduced amino acid sequence indicate that SBP is not similar to other membrane transport proteins (Grimes *et al.*, 1992; Pirovani *et al.*, 2002). The hydrophilic SBP, which contains a single putative transmembrane domain at the N-terminus, has been demonstrated to be tightly bound to the external surface of the plasma membrane (Overvoorde and Grimes, 1994), suggesting that it may behave as either a peripheral or type II membrane protein (i.e., N-terminus$_{in}$, one membrane-spanning domain, the bulk of the protein exposed to the extracellular environment, and the C-terminus$_{out}$).

Despite the lack of similarity between the SBP and other known membrane transport proteins, several characteristics of this protein link it to the process of sucrose uptake. 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 (Grimes *et al.*, 1992; Overvoorde and Grimes, 1994). In addition, expression of the *SBP* gene and accumulation of the encoded protein are temporally coordinated with the rate of sucrose uptake in cotyledons (Grimes *et al.*, 1992). The overexpression and antisense repression studies in transgenic tobacco plants and suspension cultured tobacco cells demonstrated that SBP homologue protein represents an important component of the sucrose translocation pathway in plants (Pedra *et al.*, 2000; Delú-Filho *et al.*, 2000). The antisense transgenic plants developed symptoms consistent with inhibition of sucrose translocation and displayed an altered carbohydrate partitioning in mature leaves and altered sucrose-cleaving activities in a metabolic compensatory manner. Furthermore, the antisense repression was associated with a drastic reduction in plant growth and development that led to a significant delay in the induction of flowering (Pedra *et al.*, 2000). Although these studies clearly demonstrated that SBP is involved

in sucrose translocation-dependent physiological processes, still unresolved is whether the underlying mechanism involves SBP-mediated sucrose transport or SBP-mediated regulation of alternative carbohydrate uptake systems.

The flowering induction involves shoot transition from vegetative to reproductive. Shoot morphogenesis is typically characterized by three developmental phases: juvenile, adult, and reproductive (for review, see Poethig, 1990). These phases are primarily distinguished from one another on the basis of the capacity of the shoot apical meristem to generate reproductive structures, but other traits are also diagnostic of one phase versus another. These traits vary from species to species, but include leaf morphology, rate of leaf initiation, and phyllotaxy (Poethig, 1990). It has been proposed that shoot meristem transition in plants with reduced photosynthetic capacity due to an antisense inhibition of RUBISCO is delayed because source strength regulates the duration of an early phase of tobacco shoot development and the transition to a later phase (Tsai *et al.*, 1997). Furthermore, plants with impaired sucrose transport exhibit similar changes in photosynthetic rate and sink or source strength. In a previous study, we showed that reduced photosynthesis correlated with a delay in flowering time of the SBP antisense tobacco; however, photosynthesis and growth parameters were not evaluated along the development. Here, we further characterized plants in T2 generation expressing a soybean *SBP* cDNA in antisense orientation by monitoring their physiologic and biochemistry behavior along the growth and development. The resulting phenotypes of the antisense plants were similar to those described by Pedra *et al.* (2000). The inhibition in the CO_2 assimilation was restricted to vegetative phase when an impaired transport of the sucrose was more harmful due to smaller relationship between source and sink tissues. The physiological and biochemistry effects of reduced accumulation of the SBP homologue in transgenic plants are also reported. Possible associations of the SBP with the sucrose translocation-dependent physiological processes in plants are discussed.

Materials and Methods

Recombinant DNA

DNA manipulations were performed essentially as described by Sambrook *et al.* (1989).The isolation of soybean *s-64* cDNA (GeneBank™ accession number AF191299), also denominated *SBP2*, has been previously described (Pirovani *et al.*, 2002). The SBP2 deduced protein shares 91% sequence identity with the sucrose binding protein and is also referred to as SBP homologue. The plasmid pUFVs64S, containing the *SBP2* coding region in the right orientation, and the plasmid pUFVs64AS, harboring the *SBP2* coding region in the reverse orientation into the binary plant transformation vector pBI121 and under the control of *35S cauliflower mosaic virus* (*CaMV-35S*) promoter and the polyadenylation signal of the *T-DNA* nopaline synthase (nos) gene, have been previously described (Pedra *et al.*, 2000). The sense and antisense constructs are designated here as 35S-sbp2S and 35S-sbp2AS, respectively. A schematic diagram of these constructions is shown in Figure 1.

Plant transformation and selection

Transgenic tobacco (*Nicotiana tabacum* L. cv. Havana) plants expressing the *SBP2* gene under the control of the *CaMV-35S* promoter, either in the sense or antisense orientation, were derived from several independent transformants as already previously described (Pedra *et al.*, 2000). Lines were also selected for the SBP2 protein accumulation as described by Pedra *et al.* (2000).

Homozygous T2 lines generation and selection

For these studies, we used *in vitro* propagated T2 generation of transgenic plants expressing the sbp2 sense or antisense gene selected from previously obtained lines (Pedra *et al.*, 2000). Firstly, T1 seeds were germinated on MS 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 T2 seeds after self-pollination. Secondarily, a PCR diagnostic was accomplished for those plants with specific primers linked to *NPTII* gene, as described previously (Pedra *et al.*, 2000).

Finally, accumulation of SBP2 was monitored in each generation by immunoblotting analysis. The selected plants were then maintained under *in vitro* conditions.

Plant growth conditions

Untransformed and homozygous T2 lines from transformed control (pBI121 vector alone), transformed sense (35S-sbp2-S lines), and antisense (35S-sbp2-AS lines) tobacco plants were propagated in MS medium supplemented with 3 % (w/v) sucrose without phytohormones. *In vitro* conditions were 14h photoperiod and 25 °C of temperature. After acclimatization, plants were grown in a mixture of soil, sand and dung (3:1:1) for 2 weeks in greenhouse conditions under natural conditions of light, relative humidity and temperature or in growth chamber at 25 °C of temperature and 200 µmol photons m^{-2} s^{-1} of photosynthetic photon flux density (PPFD). All the experiments were conducted with 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, USA) in an open system. Measurements of leaf gas-exchange included net CO_2 assimilation rate (A, µmol CO_2 m^{-2} s^{-1}), stomatal conductance (gs, mol H_2O m^{-2} s^{-1}), transpiration rate (E, mmol H_2O m^{-2} s^{-1}) and intercellular CO_2 concentration (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 pre-adjusted for flow rate (500 µmol s^{-1}), leaf temperature (25 °C) and PPFD (1000 µmol photons m^{-2} s^{-1}). Reference CO_2 (~360 µmol mol^{-1}) and H_2O (~65 at 85 % relative humidity) concentration were uncontrolled (ambient conditions). For A versus 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 versus PPFD curves (0, 50, 100, 200, 400, 600, 800, 1000, 1500 and 2000 µmol photons m^{-2} s^{-1}) and the others 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, USA) controlled with a quantum sensor located inside the leaf cuvette. All measurements were carried out between 07:30 and 11:30h except for day course experiments (7:00 to 18:00h).

Carbohydrate determinations

Carbohydrates were extracted and determined according to Trethewey *et al.* (1998). Soluble sugars and starch were extracted from 50 mg of leaf discs. Each fraction was ground to a fine powder in liquid nitrogen and then transferred to a microcentrifuge tube. After evaporation of the liquid nitrogen, carbohydrates were extracted three times by boiling with 80 % (v/v) ethanol by 20 min. After each centrifugation (14000 *g* by 5 min), supernatants were mixed and stored at -20 °C until soluble sugars determination. The starch was extracted from the pellet by addition of 500 µL of 0.2 M KOH and incubation at 95 °C for 60 min when the medium was neutralized with 140 µL of 1 M acetic acid. After 10 min of centrifugation at 14000 *g*, the supernatant was collected and stored at -20 °C until starch determination.

Soluble sugars were determined in buffer containing 100 mM imidazole, pH 7.0, 5 mM $MgCl_2$, 2 mM NAD^+, 1 mM ATP, 2 units of Glc-6-P dehydrogenaseb mL^{-1} and 15 µL of extract in the assay volume of 300 µL. Glucose, fructose and sucrose were spectrophotometrically determined (340 nm) by the successive addition of 0.5 units of hexokinase mL^{-1}, 0.6 units of phosphoglucoisomerase mL^{-1} and 50 units of invertase mL^{-1}.

Starch was determined by measuring the amount of glucose (100 mM imidazole, pH 7.0, 5 mM $MgCl_2$, 2 mM NAD^+, 1 mM ATP, 2 units of Glc-6-P dehydrogenase mL^{-1}, 0.5 units of hexokinase mL^{-1}, assay volume of 300 µL) hydrolyzed from starch in the specific step (300 mM citrate buffer, pH 4.6, 6 units amiloglicosidase mL^{-1}, assay volume of 100 µL, 55 °C for 45 min).

Enzyme assays

Enzymes were extracted according to Geigenberger and Stitt (1993) from 200 mg of leaf discs. Frozen samples were ground to a fine powder in liquid N_2, and enzymes were extracted in ice-cold medium containing 50 mM HEPES buffer (pH 7.4), 5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 5 mM DTT, 2 mM benzamidine, 2 mM aminocaproic acid, 0.5 mM PMSF, 10 % (v/v) glycerol, 0.1 % (v/v) Triton X-100 and 10 % (w/v) PVPP. The extract was centrifuged at 14,000 *g* for 10 min. The supernatant (1 mL) was desalted in Sephadex G-25 columns (Amersham Pharmacia Biotech) and stored at -20 °C until enzyme activity assay.

Invertase activity was assayed by measuring the amount of glucose (100 mM imidazole, pH 7.0, 5 mM $MgCl_2$, 2 mM NAD^+, 1 mM ATP, 2 units of Glc-6-P dehydrogenase mL^{-1}, 0.5 units of hexokinase mL^{-1}, assay volume of 300 µL) hydrolyzed from sucrose in the specific step (20 mM sodium acetate buffer, pH 4.2, 100 mM sucrose, assay volume of 100 µL, 37 °C for 60 min).

Sucrose synthase activity was assayed by measuring the amount of UDP-Glc (200 mM Glycine, pH 8.9, 2 mM NAD^+, 5 mM $MgCl_2$, 0.02 units of UDP-Glic dehydrogenase mL^{-1}, assay volume 300 µL) hydrolyzed from sucrose in the specific step (20mM HEPES, 400mM sucrose, 4mM UDP, pH 7.0, total volume of 100µL, 30°C for 60min).

ADP-Glucose pyrophosphorylase (AGPase) activity was assayed by measuring the amount of glucose-1-phosphate formed from ADP-glucose. Assay buffer contained 80 mM HEPES, pH 7.4, 10 mM $MgCl_2$, 10 mM NaF, 10 µM glucose-1,6-biphosphate, 10 mM PGA, 0.6 mM NAD^+, 1 unit phosphoglucomutase mL^{-1}, 2.5 units Glc-6-P dehydrogenase mL^{-1}, 1 mM ADP-glucose, 3 mM DTT and 2 mM Na-PPi.

Protein and chlorophyll determination

Protein was determined using a protein assay reagent and BSA as the standard (Bradford, 1976). Total chlorophyll content (chlorophyll a and b) was determined spectrophotometrically after quantitative extraction with 80 % (v/v) acetone in the presence of approximately 1 mg of $NaCO_3$ according to the method of Lichtenthaler (1987).

Data Analyses

The least statistic difference (*LSD)* test based in the Student's t test was performed at $P<0.05$ using the Software Scientific (EMBRAPA, Brazil).

Results

T2 generation of SBP antisense transgenic tobacco plants displays growth-related phenotypes consistent with impairment of sucrose translocation

A *SBP* homologue cDNA has been isolated in our laboratory from a soybean expression library (Pirovani *et al.*, 2002), which shares 91% sequence identity with the previously reported *SBP* gene from soybean (Grimes *et al.*, 1992). The predicted gene product is referred here as either the SBP homologue or SBP2 protein. To elucidate the physiological role of SBP, transgenic tobacco (*N. tabacum* cv. L. Havana) plants expressing the *SBP2* gene in either sense or antisense orientation were generated previously (Pedra *et al.*, 2000). Figure 1 shows the pBI121-derived plasmids harboring the soybean *SBP2* gene under the control of *35S cauliflower mosaic virus* (CaMV) promoter and the *3' nos* polyadenylation signal that were used to transform tobacco via *Agrobacterium tumefaciens*. The accumulation of an endogenous tobacco SBP homologue in untransformed plants as well as the extend of antisense repression and overexpression in T0 primary antisense and sense transformants have been already characterized in detail in previous reports (Pedra *et al.*, 2000; Delú-Filho *et al.*, 2000). Several independent transgenic lines were established, transferred into soil, and grown in greenhouse to generate seeds (T1 seeds). T1 transformed plants were selected by PCR analysis with *nptII*- and *sbp2*-specific primers. This procedure was repeated until the production of the T3 seeds, which were used for the segregation analyses of the T2 generation of the plants on the basis of their kanamycin resistance. Because the T2 generation sense plants displayed similar phenotypes to the untransformed plants in preliminary assays, they were not considered further.

The integration of the constructs in the T2 antisense plants was further confirmed by PCR analysis of the *SBP2* transgene (Figure 2). The *SBP2* gene-specific primers amplified the predicted sized fragment from DNA prepared from antisense lines, but not from DNA prepared from untransformed, control plants (lane C-) and from 35S-sbp2-AS4-8 and 35S-sbp2-AS8-1 plants (lanes AS4-8 and AS8-1). More likely the T-DNA locus segregated out in this negative antisense plants and, therefore, 35S-sbp2-AS4-8 and 35S-sbp2-AS8-1 were not considered further. 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, since 75 % of

their T3 segregating seedlings were resistant to kanamycin (Table 1). 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 didn't show mendelian segregation were not considered further.

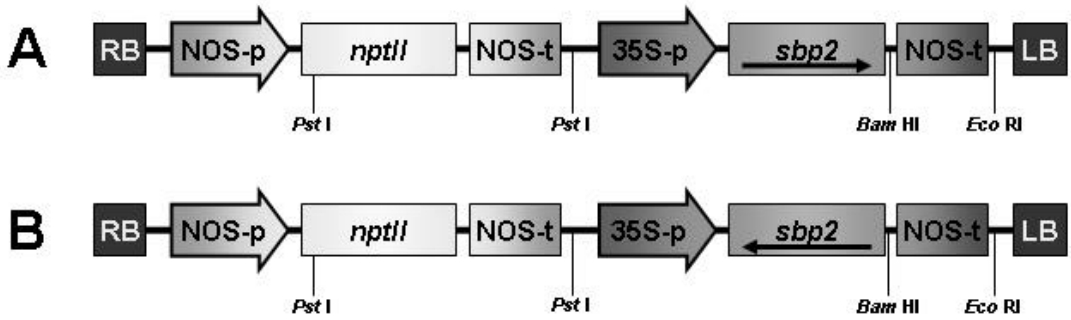

Figure 1. Schematic diagram of the chimeric *sbp2* constructs transformed into tobacco (*Nicotiana tabacum* L. cv. Havana) via pBI121-derived binary vector. The *sbp2* gene in sense (A and denominated 35S-sbp2-S) and antisense (B and denominated 35S-sbp2-AS) orientation was placed under the control of the constitutive CaMV 35S promoter (35S-p) and the 3' *nos* polyadenylation signal (NOS-t). The *npt*II gene expression is driven by *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.

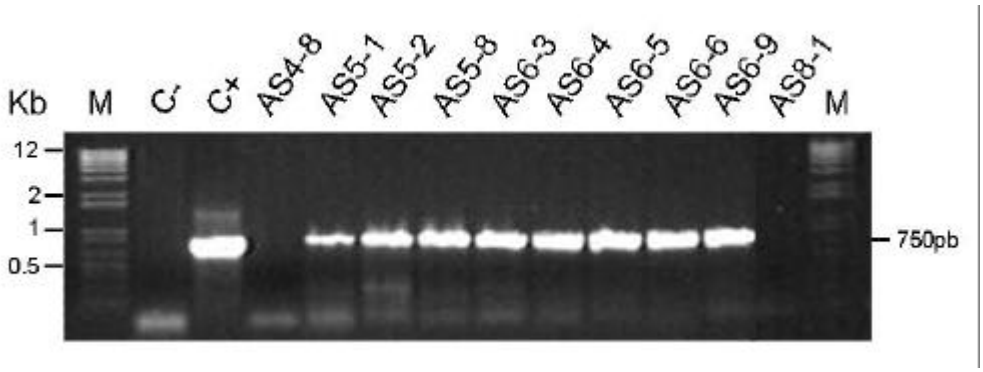

Figure 2. PCR analysis of transgenic plants in T2 generation. Total DNA was isolated from *in vitro* grown transgenic tobacco plants and provided the template in PCR reactions using *sbp2* gene-specific primers. AS refers to the plants transformed with the antisense construct (35S-*sbp2*-AS). Different numbers following AS symbols indicate that the transgenic plants were originated from independent events of transformation and segregation. C- corresponds to the results of a PCR reaction performed with DNA from control plants (WT). In C+, the *SBP2* cDNA was used as template. M corresponds to DNA standard markers whose sizes are shown on the left in kb.

Table 1. Segregation of the antisense transgene (T-locus, 35S-sbp2-AS) based on kanamycin resistance.

Plant Lines Tested	Kanamycin-Resistant Seedlings	Observed ratio	Tested ratio	c^2
Wild type	$0^+/352^-$	-	-	-
35S-sbp2-AS5-1	$1301^+/417^-$	3,11	3:1	1,09
35S-sbp2-AS5-2	$2282^+/824^-$	2,77	3:1	0,78
35S-sbp2-AS5-8	$2299^+/0$-	2299	≥63:1	0,05
35S-sbp2-AS6-3	$2845^+/462^-$	6,15*	-	-
35S-sbp2-AS6-4	$3752^+/0^-$	3752	≥63:1	0,02
35S-sbp2-AS6-5	$2053^+/701^-$	2,93	3:1	0,95
35S-sbp2-AS6-6	$1490^+/405^-$	3,67	3:1	1,70
35S-sbp2-AS6-9	$1140^+/322^-$	3,54	3:1	1,34

Obs. The plants were analyzed for 3:1 (Kan^r:Kan^s), 15:1 or 63:1 ration by the X^2 (6.63) test.
* It did not follow mendelian segregation.
≥ the copy number may be higher than 3, but it requires a larger sampling to be precise.

In vitro cultivated transgenic and control plants did not display differences on growth (data not shown). However, typical phenotypes of an inhibition of sucrose translocation were observed in the antisense T2 transgenic lines when they were transferred to the greenhouse conditions. In general, the growth of the transgenic lines was retarded when compared with control plants (Figure 3A). The growth-related phenotypes of the antisense transgenic lines were reflected in the induction of flowering that was delayed in comparison to the control plants (WT and pBI) and sense plants (Figure 3A and 3B). 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). 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 intensity of the growth-related phenotypes exhibited by the antisense transgenic lines was very similar, regardless their different copy number and gene dosage (Table 1). Therefore, the data of the lines 35S-sbp2-AS5-1, 35S-sbp2-AS5-2 and 35S-sbp2-AS5-8 were integrated as 35S-sbp2-AS5 and the data of the lines 35S-sbp2-AS6-4, 35S-sbp2-AS6-5, 35S-sbp2-AS6-6 and 35S-sbp2-AS6-9 were integrated as 35S-sbp2-AS6 in the further analyses.

The developmental performance of the antisense lines was visibly distinguishable from the normal development of the wild type plants (Figure 3A) but the difference did not persist throughout development as indicated by growth analyses (Figures 3C and 3D).

Because the younger internodes elongated more quickly after flowering, the height increase rate was delayed in transgenic lines until the flowering induction when they reached a similar height as control plants, exhibiting the same final size as wild type (Figure 3C). Likewise, leaf emergence was delayed in transgenic lines until the onset of flowering when their total number of leaves equaled to that of control plants (Figure 3D). No significant morphological differences in young and mature leaves of the transgenic lines were observed when compared with the wild type.

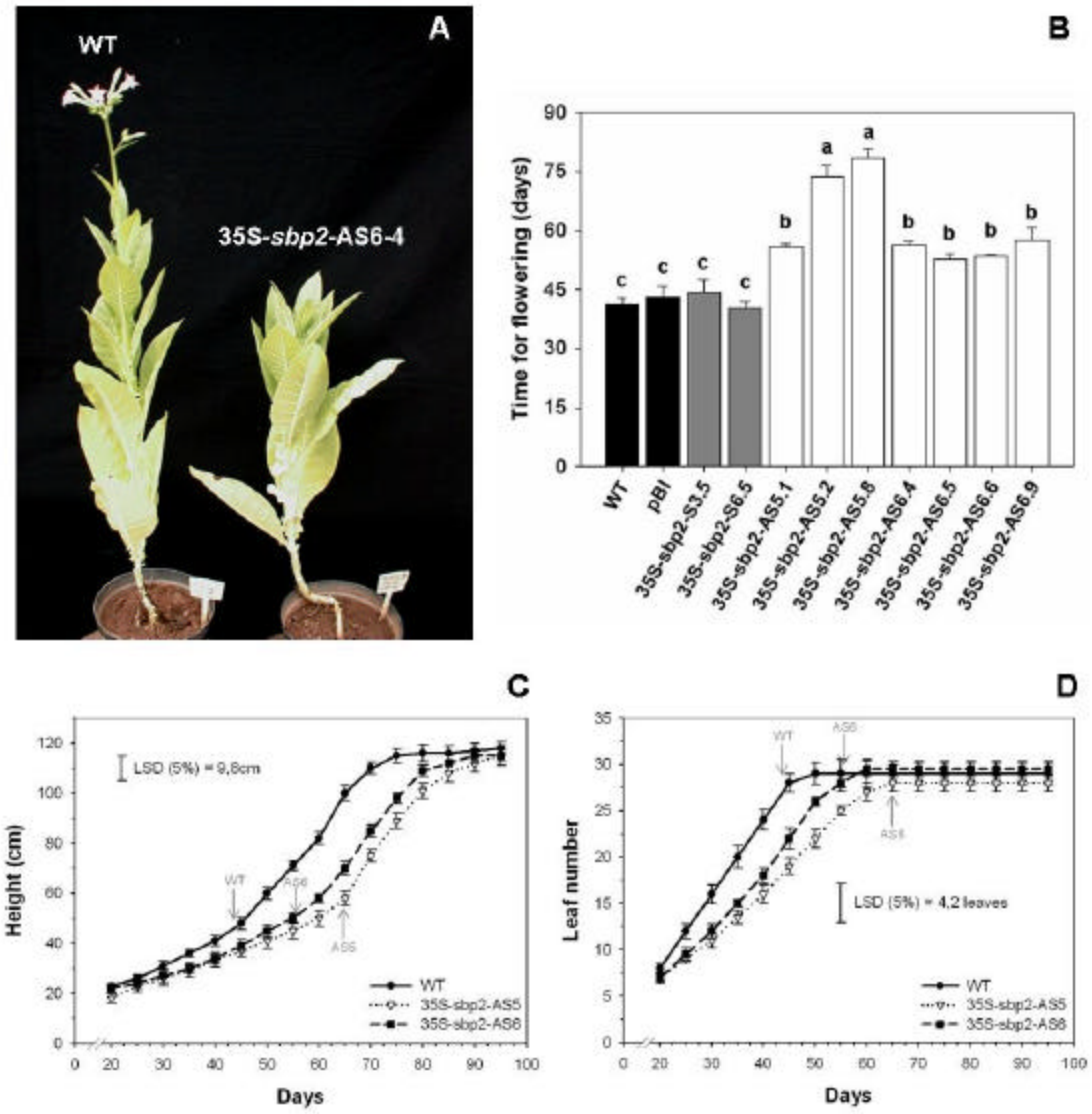

Figure 3. Comparison of developmental performance between control (WT and pBI), sense (35S-sbp2-S) and antisense (35S-sbp2-AS) T2 transgenic plants. (A) Transgenic tobacco plants after 8 weeks in the greenhouse conditions. (B) The time of flowering induction in sense and antisense plants (±SE; n=10). (C) Plant height (±SE; n=6). (D) Number of leaves per plant (±SE; n=6). Days in greenhouse. In (B), bars with different letters are significantly different with $P < 0.05$ (*t* test). In (C) and (D) LSD: least statistic difference (LSD test, $P<0.05$). Arrows indicate the flowering time.

Reduced SBP homologue accumulation by antisense repression delays leaf senescence

After flowering and fructification, tobacco plants exhibit accelerated leaf senescence. Due to the developmental delay of the antisense plants, we evaluated the leaf senescence based on chlorophyll and protein content. Plants that flowered later, e.g. antisense lines, had less leaf chlorophyll and protein content in the end of the life cycle (Figure 4). These results indicate a clear difference in senescence of the plants, which is developmentally coordinated by several factors, such as light, phytohormones and sugars (Wingler *et al.*, 1998). Interestingly, the leaf chlorophyll content after 20 days in greenhouse was much higher in transgenic plants than in the control plants (Figure 4A).

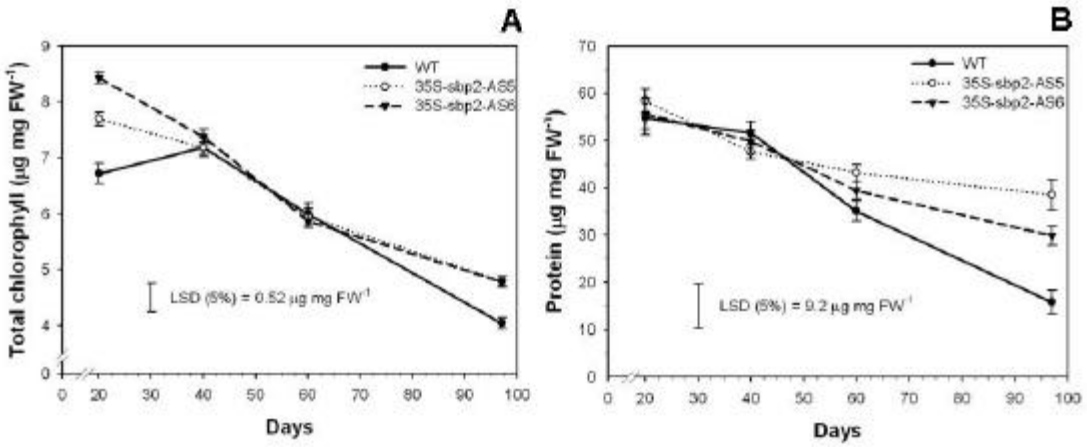

Figure 4. Contents of total chlorophyll (A) and protein (B) in the leaves from wild-type tobacco (WT) and antisense (35S-sbp2-AS) T2 transgenic plants (±SE; n=6). Days in greenhouse. LSD: least statistic difference (LSD test, $P<0.05$).

During the vegetative phase, the photosynthetic rate is biochemically limited in antisense plants and correlates with their delay in flowering induction

The retarded development of the antisense primary transformants containing reduced amounts of SBP homologue was correlated with a reduction in the rate of net photosynthesis, indicating that SBP inactivation promoted a decrease either in sucrose export activity or sink strength (Pedra *et al.*, 2000). The reduction of photosynthetic rate paralleled a decline on stomatal conductance. Thus, it was of our interest to extend these observations to understand whether the inhibition of photosynthesis follows a biochemical mechanistic by feedback regulation or was due to regulation of

stomatal closure. We first characterized the photosynthesis rate of our T2 generation transgenic lines in comparison to control plants. Consistent with the transgenic parental phenotype, the rate of net photosynthesis during the vegetative phase was reduced in both antisense lines relative to controls (Figure 5A). For both control and antisense plants, the photosynthetic rate continuously decreased as the overall development progressed with a slight increment after the induction of flowering (Figure 5A). Although the measurements were always accomplished in the leaf with greater photosynthesis capacity within the profile of the plant, e.g. fifth leaf from of the top, its reduction in the CO_2 assimilation during the development may be explained by the increase in the number of leaves (source organs) (Figure 3D) in relation to the sink organs. Sink tissues in tobacco are represented by the root system, young and immature leaves and shoot apical meristem. In greenhouse conditions, plants are cultivated in recipients that quickly limit root growth. The number of the young and immature leaves stays constant until flowering when the plant stops emitting new leaves. Besides, the vegetative shoot apical meristem is a strong sink tissue in the beginning of the development, when there are few leaves, and as it turns into a floral meristem. In fact, after flowering the leaf photosynthesis increased, probably to supply the demand created by the flowers and fruits (Figure 5A). In the reproductive phase, however, the CO_2 assimilation was sensibly higher in antisense lines, probably because of the delay in the flowering time and senescence of the transgenic plants. 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 *SBP2* cDNA probe detected hybridizing bands at low stringency and an anti-SBP2 serum detected a protein homologue in tobacco leaves (Pedra *et al.*, 2000).

Photosynthetic rate can be regulated by several factors, including light and metabolic regulation (Paul and Foyer, 2001; Paul and Pellny, 2003) in addition to stomatal movement (Schroeder *et al.*, 2001). The reduction of the photosynthetic rate during the vegetative phase in antisense transgenic lines in comparison with control plants was accompanied by a parallel decline on stomatal conductance (*gs*) (Figure 5B). In contrast, during reproductive phase, stomatal conductance of transgenic lines was higher than control leaves. This change of behavior was associated with the delay in the development and senescence of the transgenic plants. These data may indicate that a stomatal aperture may be blocking the CO_2 exchange through the stomatal pore as the cause of photosynthesis reduction in the vegetative phase of antisense plants. However, no differences in intercellular CO_2 partial pressure (*Ci*) and *Ci*/*Ca* rate (*Ca*:

ambient CO_2 partial pressure) among plants were observed, which varied in the range of 250 to 350 µmol mol^{-1} and 0.6 to 0.8, during the experiment, respectively (data not shown). Furthermore, diurnal photosynthesis analyzed in the vegetative and reproductive phases confirmed the previous results (Figure 6A and 6B) and the photosynthesis decline in the vegetative phase of the transgenic plants was reflected by a lower *A*/*Ci* ratio as compared to control plants demonstrating that there was a biochemistry limitation in the CO_2 fixation (Figure 6C and 6D), which was also confirmed by the ratio *A*/*gs* (data not shown).

Two of the most commonly reported responses of CO_2 uptake are the responses of photosynthesis to photon flux (*Q*) and to intercellular CO_2 mole fraction (*Ci*). These parameters were analyzed during the vegetative phase of control and antisense plants (Figure 7). No differences in the initial slope of the A/C_i curves among plants were observed (Figure 7A). Nevertheless, as CO_2 concentration increased, a larger inflection in the curves of the transgenic plants could be detected (Figure 7A). This result indicates that ribulose-1,5-bisphosphate (RuBP)-regeneration and triose-phosphate utilization may be limiting (Long and Bernacchi, 2003). As typical of a C_3 plant, the light response photosynthetic curve (*A*/*Q* curve) of tobacco produced a saturation of photosynthesis at 600 to 800 mmol m^{-2} s^{-1} in all plants. However, in antisense lines, the photosynthesis under saturating light conditions was lower than in control leaves (Figure 7B). Therefore, antisense transgenic plants exhibited a reduced efficiency of light-energy utilization.

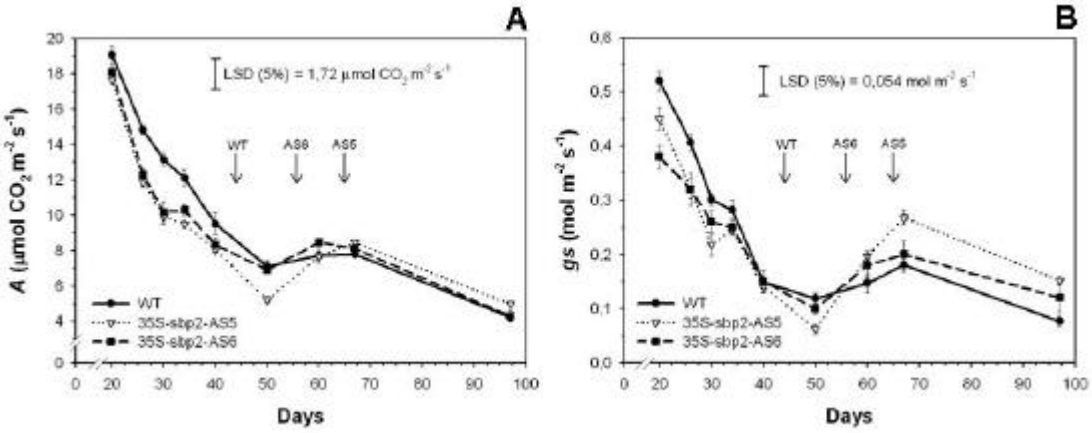

Figure 5. Photosynthetic rate (*A)* (A) and stomatal conductance (*gs*) (B) of fifth leaf from the top of control (WT) and antisense (35S-sbp2-AS) T2 transgenic plants (±SE; n=6). Days in greenhouse. LSD: least statistic difference (LSD test, $P<0.05$). Arrows indicate the flowering time.

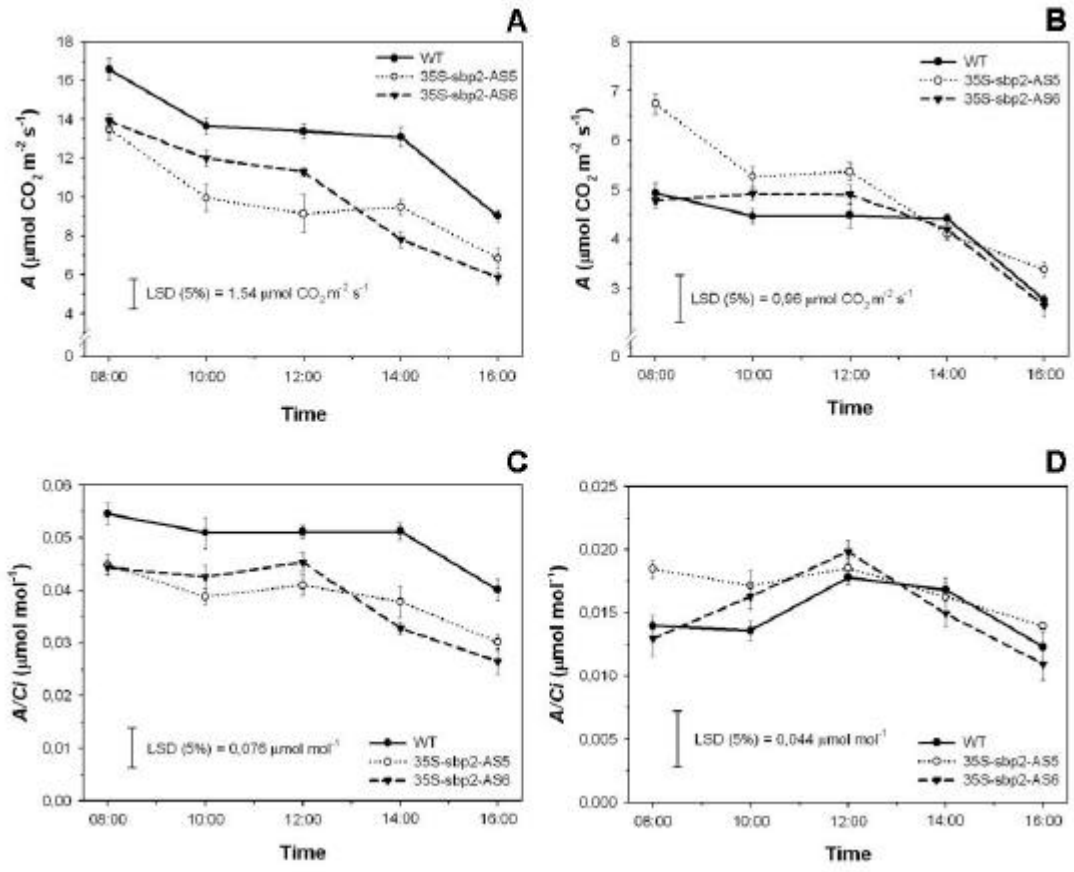

Figure 6. Diurnal photosynthetic rate (A and B) and A/*Ci* ratio (C and D) analyzed in the vegetative (20 days in greenhouse) (A and C) and reproductive phase (97 days in greenhouse) (B and D) on the fifth leaf from the top of control (WT) and antisense (35S-sbp2-AS) T2 transgenic plants (±SE; n=6). LSD: least statistic difference (LSD test, $P<0.05$).

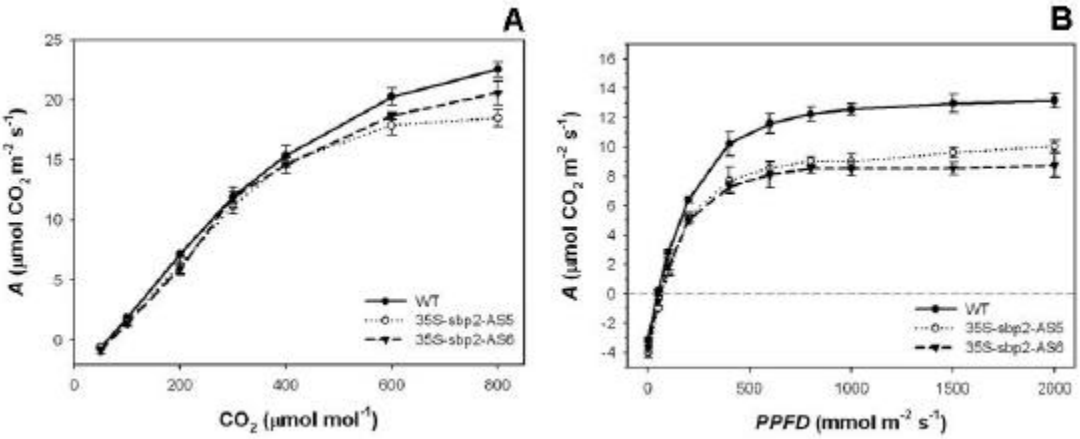

Figure 7. *A/Ci* (A) and *A/Q* (B) response curves of the fifth leaf from the top of control (WT) and antisense (35S-sbp2-AS) T2 transgenic plants in the vegetative phase (40 days in greenhouse) (±SE; n=8).

Antisense inhibition of tobacco SBP homologue gene alters carbon allocation in mature leaves

In order to evaluate the effects of antisense repression of the SBP homologue gene on carbohydrate metabolism, the content of soluble sugars and starch was determined in fully expanded leaves (Figure 8). Hexoses and sucrose content were reduced in the antisense leaves during the course of the experiment (Figures 8A, C and E). During a diurnal cycle, both the hexoses and sucrose content as well as the rate of sugars accumulation were significantly reduced in the antisense lines when compared with wild type plants (Figure 8B, D and F). In contrast, the content of starch in the leaves of antisense transgenic lines was significantly higher than in control plants, mainly in the vegetative phase of the plants (Figure 9A). This tendency persisted during a diurnal cycle, in which a significant increase both in the starch content and the rate of starch accumulation was observed in the antisense lines as compared to wild type plants (Figure 9B). This difference was so significant that even after the dark period the starch content of the transgenic lines was higher than that of control plants. Partitioning of recently fixed photosynthate between the insoluble starch pool and the soluble sucrose pool was affected in the transgenic lines, because the sucrose to starch ratio in the leaves of antisense lines was clearly 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 (Grusak *et al.*, 1990). Nevertheless, the alteration of carbohydrate content in SBP 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 (Riesmeier *et al.*, 1994; Kühn *et al.*, 1996; Lemoine *et al.*, 1996). 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 (Overvoorde *et al.*, 1996; Grimes and Overvoorde, 1996).

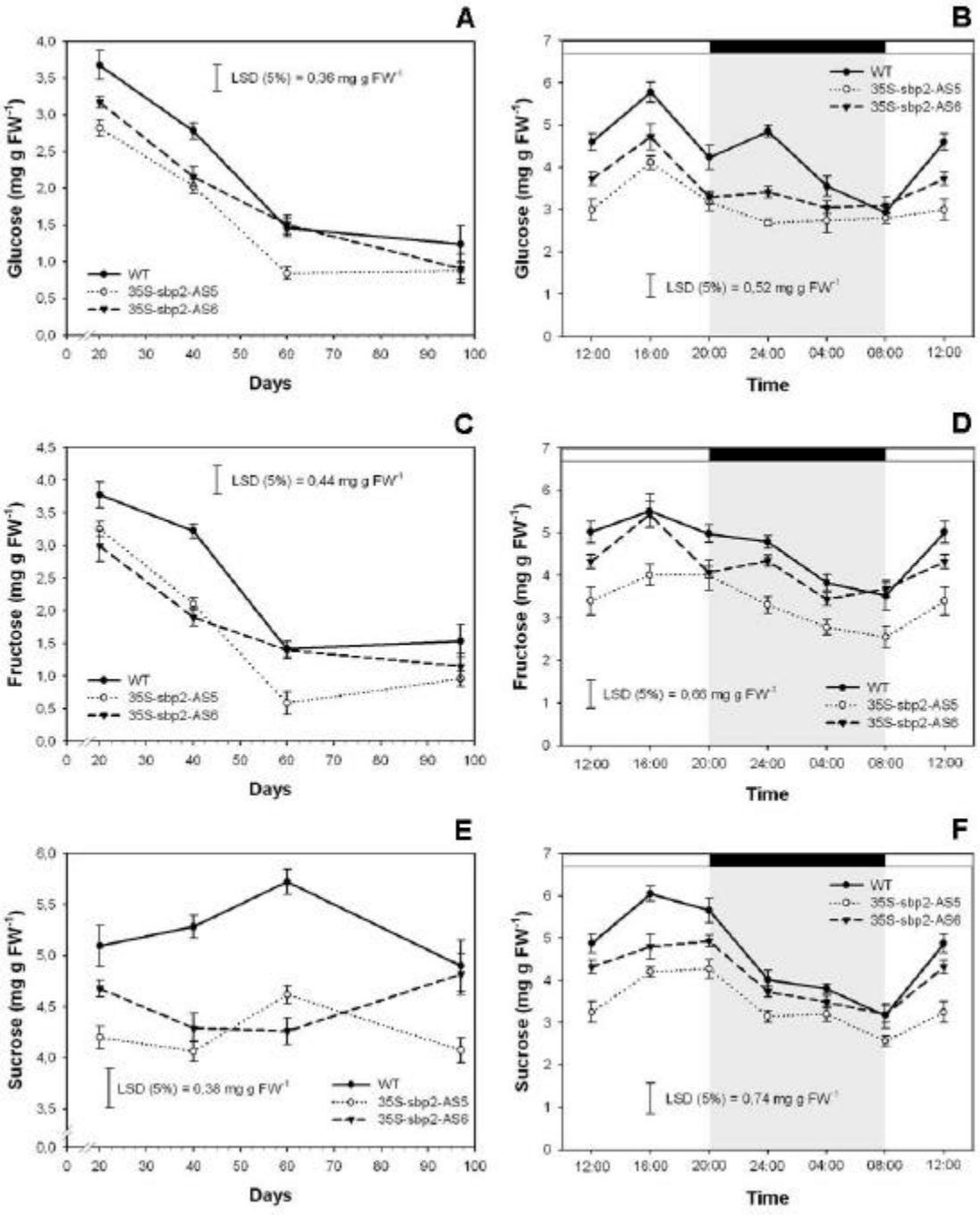

Figure 8. Concentration of glucose (A and B), fructose (C and D) and sucrose (E and F) of fifth leaf from the top of control (WT) and antisense (35S-sbp2-AS) T2 transgenic plants (±SE; n=5). A, C and E: developmental changes in leaf sugars (Days in greenhouse). B, D and F: diurnal changes in leaf sugars during the vegetative phase of the plants. LSD: least statistic difference (LSD test, $P<0.05$).

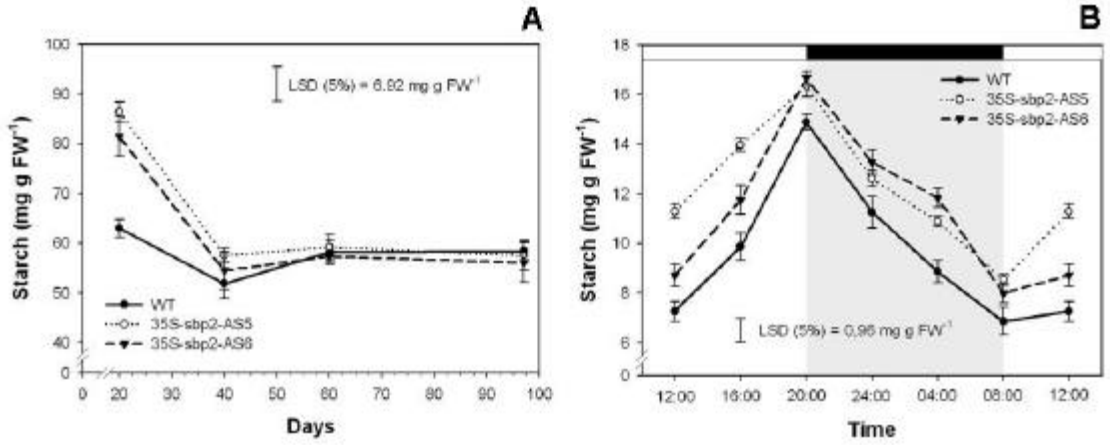

Figure 9. Developmental (A) and diurnal changes (B) on the concentration of starch of fifth leaf from the top of control (WT) and antisense (35S-sbp2-AS) T2 transgenic plants (±SE; n=5). Days in greenhouse. LSD: least statistic difference (LSD test, *P*<0.05).

The activity of endogenous sucrose and starch metabolism enzymes is altered in transgenic leaves

To determine whether the alteration in partitioning of carbohydrates was mainly due to manipulation of SBP homologue levels and not due to activation of compensatory carbohydrate uptake systems, the activities of acid invertase of mature leaves were determined (Figure 10A). The activity of acid invertase of the antisense plants was sensibly higher than that of control leaves, mainly in the beginning of the development. While invertase activity was inversely correlated with the sucrose content in antisense leaves, it did not correlate with its hexose content, which was lower than that of control leaves (Figure 8). The glucose and fructose products of sucrose hydrolysis by acid invertase may not accumulate to high levels due to high efficiency of C allocation into starch. In fact, the starch content was very high in transgenic plants during the vegetative phase (Figure 9A). Thus, the reduced content of sucrose observed in antisense lines may be a consequence of increased invertase activity rather than inhibition of sucrose transport. In this case, SBP would exert an indirect role in sucrose translocation by activation of sucrose hydrolyzing activities. The total activity of acid invertase is contributed by cell wall invertase and vacuolar invertase. A direct role of cell wall acid invertase (CWI) in sucrose partitioning between source and sink regions

has been demonstrated in transgenic tobacco overexpressing CWI in a constitutive manner (Heineke *et al.*, 1992). In general, elevated levels of the enzyme in the leaves reduce the sucrose transport between sources 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. It has been previously demonstrated that CWI activity in SBP2 antisense plants is increased, and therefore, CWI would control the exit of assimilated carbon by cleaving sucrose to support leaf growth.

The other sucrose hydrolytic enzyme of the cells and localized in the cytosol is the sucrose synthase. If decreased accumulation of SBP homologue in antisense plants promoted a lower rate of sucrose transport, one would expect a high activity of intracellular sucrose cleaving activities in mesophyll cells due to a transient accumulation of sucrose in mature leaves. In fact, increase of sucrose uptake and accumulation in cultured tobacco cells has been demonstrated to be coordinated with enhanced intracellular sucrose synthase activity (Delú-Filho *et al.*, 2000). In view of this observation, we analyzed the sucrose synthase activity in antisense and control cells (Figure 10B). The sucrose synthase activity in mature leaves of these plants was remarkably lower than in control plants, which was inconsistent with a low efficiency on the sucrose transport in transgenic lines.

Sucrose and starch are the prominent products of CO_2 assimilation in many plants. However, phloem loading, transport, or unloading may restrict the capacity for sucrose synthesis. A portion of the fixed carbon is also allocated for formation of starch in many plants. AGPase is an important regulatory enzyme controlling starch biosynthesis and it catalyzes the first committed step in the pathway of starch synthesis (Preiss, 1988; Martin and Smith, 1995). In fact, the capacity of starch synthesis was reduced by a leaf-specific antisense inhibition of the AGPase plants (Leidreiter *et al.*, 1995). Leaf starch in SBP antisense lines was very greater than in control plants (Figure 9). This result was correlated with a higher AGPase activity (Figure 10C) in the leaves of antisense plants in the initial phase of the development, exactly when it was observed the largest differences in starch accumulation among the plants.

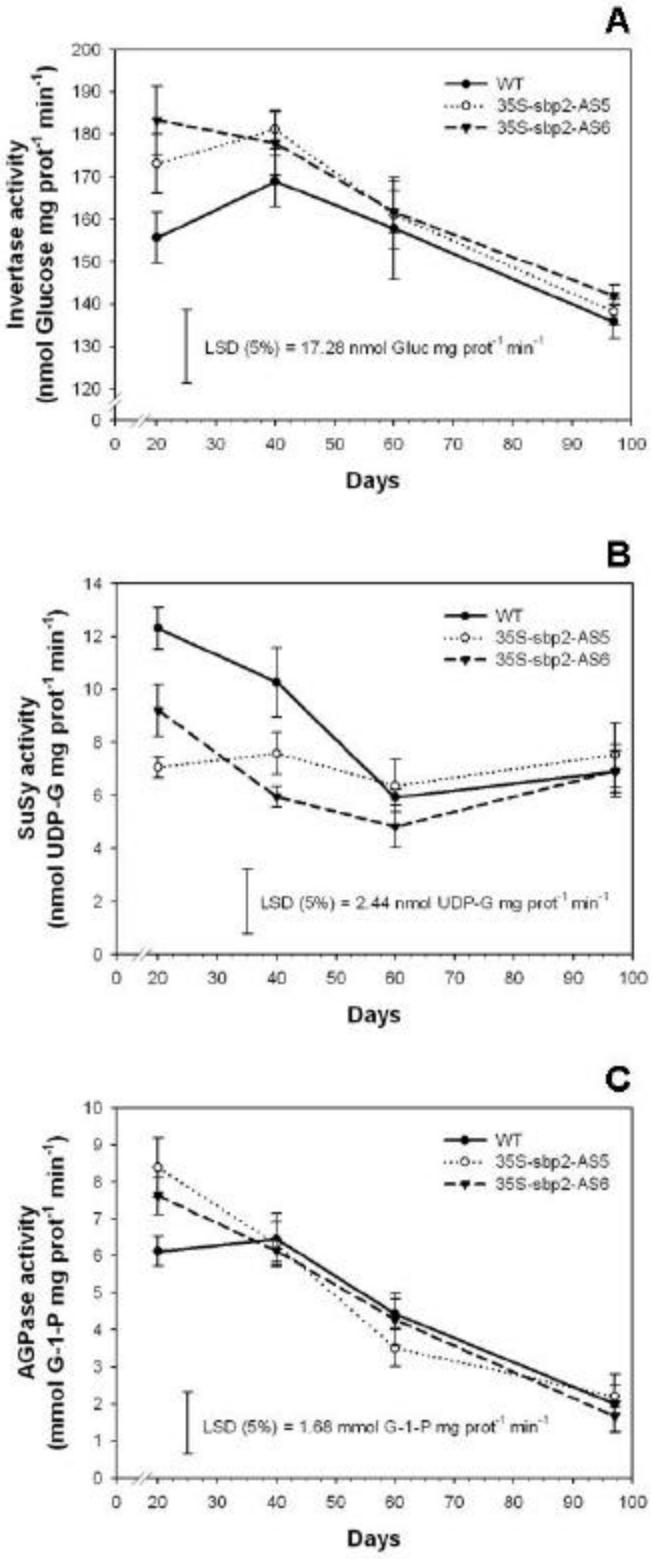

Figure 10. Acid invertase (A), sucrose synthase (SuSy) (B) and ADP-glucophosphorylase (AGPase) activities of fifth leaf from the top of control (WT) and antisense (35S-sbp2-AS) T2 transgenic plants (±SE; n=5). Days in greenhouse. LSD: least statistic difference (LSD test, $P<0.05$).

Discussion

SPB antisense tobacco lines show growth-related phenotypes similar to plants with a decrease in sucrose exportation and/or in sink strength

SBP from soybean is an intriguing and enigmatic protein due to the apparent biochemical inconsistency between its assigned biological function and its predicted topological structure. Although biochemical analyses indicate that SBP was capable of transporting sucrose in a heterologous system (yeast), the protein is not structurally related to any other membrane transport protein (Overvoorde *et al.*, 1996; Pirovani *et al.*, 2002). Previous functional analyses have demonstrated that *SBP* repression in tobacco promoted growth-related phenotypes consistent with impairment of sucrose translocation (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. Here we further characterized these *SBP* antisense plants in T2 generation by measuring growth rate parameters, carbohydrate partitioning and carbohydrate-metabolizing activities throughout development. As expected, the overall altered plant development in the antisense lines supports the notion that sucrose translocation was indeed impaired. The phenotypes were similar to those described by antisense repression of the H^+:symporter (sucrose exportation) (Riesmeier *et al.*, 1994; Kühn *et al.*, 1996; Lemoine *et al.*, 1996) and overexpression of apoplastic invertase (sink strength) (Heineke *et al.*, 1992; Sonnewald *et al.*, 1997). The *SBP* transgenic lines showed a reduction in plant growth and development with a delay in the onset of flowering induction (Figure 3) and senescence (Figure 4).

The reduced photosynthesis in antisense lines may be associated with sink regulation and/or altered carbon partitioning

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. 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 *SBP* antisense leaves, the level of starch was higher, but the content of hexoses and sucrose was remarkably lower than in control leaves. Therefore, antisense repression of *SBP* homologue expression resulted in an increased allocation of fixed carbon in the direction of starch synthesis. The bulk of the photosynthetically fixed carbon in mature leaves is partitioned between sucrose and starch. This process is controlled by triose-phosphate (triose-P) and inorganic orthophosphate (Pi) since they are important allosteric effectors of enzymes in the pathways of sucrose and starch synthesis (Preiss, 1982; Stitt *et al.*, 1984). In a situation in that sucrose synthesis or demand is affected, as in *SBP* antisense lines, starch synthesis may be stimulated (see Herold, 1980, Stitt, 1987). In fact, AGPase activity in *SBP* antisense plants was higher exactly when the starch accumulation was greater than control plants (Figure 9 and 10).

The growth-related phenotypes were correlated with reduced photosynthesis in the beginning of the development, during the vegetative phase of the antisense plants (Figure 5). After flowering, however, the CO_2 assimilation was similar in both, wild type and transgenic lines. Similar results were found with *Rubisco* mutant plants in which the consequent reduction in the photosynthetic capacity of tobacco led to a slower growth and developmental delay; however, the plants eventually flowered and reproduced normally (Jiang and Rodermel, 1995; Whitney *et al.*, 1999). The decease in photosynthesis during the development occurs especially when photosynthesis becomes limited by the inability of the plant to form additional sink tissues (Stitt, 1991). In this study, 3L pots used for the experiments limited the growth of the root system decreasing sink tissues (data not show). In addition to the reduction of the sink tissues, new source leaves emerged, thereby altering the source/sink relationship. Likewise, upon appearance of the first flower bud, which functions as a strong sink for photoassimilates, an increase in the photosynthetic rate was observed (Figure 5).

Photosynthetic rate may be regulated by several factors, including light and metabolic regulation (Paul and Foyer, 2001; Paul and Pellny, 2003) besides of stomatal movement (Schroeder *et al.*, 2001). Because the alterations in stomatal conductance are not accompanied by the corresponding changes in *Ci*, it is unlikely that stomatal aperture plays a central role in controlling photosynthetic activity of the *SBP* antisense leaf, at least under theconditions of the experiments.

As an alternative explanation for the decreased photosynthesis rate in antisense leaves, a failure in its end-product destination would downregulate photosyntheis by a feedback mechanism. Feedback regulation of photosynthesis include: (1) a short-term

metabolic feedback control through Pi recycling by end-product synthesis; (2) carbohydrate accumulation in leaves when there is an unbalance between source and sink at the whole plant level that can lead to decreased expression of photosynthetic genes (reviewed by Paul and Foyer, 2001). However, the soluble sugars content in antisense leaves doesn't support this hypothesis, since sucrose, glucose and fructose contents were lower than in control leaves (Figure 8). Nevertheless, this hypothesis cannot be excluded as yet, since we didn't evaluate the transcription level of photosynthetic genes. Independently of the mechanism that regulates the starch synthesis in *SBP* antisense plants one may also suppose that the accumulation of starch grains can disrupt chloroplast structure (Cave *et al.*, 1981) and increase diffusive resistance to CO_2 (Nafzigerand Koller, 1976; Grub and Mächler, 1990). There is often a more pronounced down-regulation of photosynthesis in starch-accumulating species when sink capacity is limiting (Goldschmidt and Huber, 1992). Besides, an immediate effect of the accumulation of starch is a decrease in the activation state of Rubisco, without any change in the total and maximal activities and hence amounts of the protein (Paul and Foyer, 2001). However, others have found no relationship between starch accumulation and a decrease in photosynthesis (Van Oosten *et al.*, 1994; Moore *et al.*, 1998). In fact, a reduction in starch content by antisense inhibition of leaf AGPase in potato promoted a parallel decline in CO_2 assimilation under elevated CO_2; therefore, in this case, acclimatation of the photosynthesis is not caused by an accumulation of starch (Ludewig *et al.*, 1998). So far, there is little understanding about the role of starch in the photosynthesis regulation.

Leaf senescence

Senescence of the fifth leaf from the top of the plants also was affected in antisense plants (Figure 4). In the antisense line 35S-sbp2-AS5, a strong delay of leaf senescence was observed as function of the delay in flowering (Figure 3). Dicot leaf ontogeny is characterized by an initial phase that coincides with leaf expansion in which the photosynthesis capacity progressively increases until it reaches a plateau of maximal photosynthetic capacity, followed by a senescence phase with a decline in the photosynthesis capacity (reviewed by Gepstein, 1988) Because the delay in senescence, the photosynthesis in the antisense leaf was kept slightly higher after flowering as compared to control plants, very likely due to alteration in the life-period of antisense leaves. Likewise, the leaves of the *rubisco* mutant tobacco, with reduced photosynthesis capacity and similar phenotypes of *SBP* antisense tobacco, have life-period longer than wild type leaves (Tsai *et al.*, 1995; Muller *et al.*, 2000).

The time of flowering induction in antisense lines may reflect a decrease in sucrose transport rate

During plant development, the factors involved in the regulation of the shoot developmental phase transitions are poorly understood. However, some mechanisms have been described. Shoot morphogenesis is typically characterized by three developmental phases: juvenile, adult, and reproductive phase (for review, see Poethig, 1990). These phases are primarily distinguished from one another on the basis of the capacity of the shoot apical meristem to generate reproductive structures, but other traits are also diagnostic of one phase versus another. Tsai *et al.* (1997) have proposed that shoot meristem transition in plants, which display a reduced photosynthetic capacity due to an antisense inhibition of rubisco, is delayed because source strength regulates the duration of an early phase of tobacco shoot development and the transition to a later phase. In our case, we suggest that this phase change may occur in response to the attainment of a threshold source strength, which is delayed in the *SBP* antisense plants.

Compelling evidence in the literature suggests that sucrose promotes flowering in most species (for review, see Bernier *et al.*, 1993). Arabidopsis mutant studies indicate that sugar may affect floral transition by activating or inhibiting genes that act to control floral transition, depending on the concentration of sugars, the genetic background of the plants, and when the sugar is introduced in the medium (Ohto *et al.*, 2001). Generally, after induction of flowering, the concentration of sucrose in the phloem reaching the shoot apex increases rapidly and transiently. This pulse of sucrose translocation precedes the increase in cell division that normally is observed in the shoot apical meristem during floral evocation (Bernier *et al.*, 1993; Corbesier *et al.*, 1998). Therefore, the delay in the flowering time of the *SBP* antisense plants may be a direct result of sucrose transport deficiency rather than a global developmental effect.

Alteration of invertase and sucrose synthase activities in antisense source leaves may lead to a reduced sucrose transport in phloem

Recently, the physiological effects of invertase have been investigated by expression of a yeast derived invertase in various organs and different subcellular compartmens of transgenic plants (Sonnevald, *et al.*, 1991). Expression of this yeast derived invertase in cell wall compartment of potato plants caused a typical phenotype of long-distance

sucrose transport inhibition. Because phloem loading in these leaves involves an apoplastic step, sucrose is cleaved before long-distance transport. As a consequence, export is blocked and high levels of soluble sugars and starch accumulate, leading to the repression of photosynthetic genes. Simultaneously, the sink organs are starved of carbon, and the plants produce fewer tubers (Heineke *et al.*, 1992). Because cell wall invertase activity of *SBP* antisense source leaves was higher than control leaves, an analogous mechanism may be operating in these plants. Therefore, a reduction in sucrose phloem loading or unloading in *SBP* transgenic plants, which is consistent with the *SBP* antisense phenotypes, may be caused by an indirect effect via apoplastic hydrolysis of sucrose into hexoses, which are not transported efficiently at long-distance, rather than by a direct reduction in SBP-mediated sucrose transport.

Sucrose synthase activity in mature leaves of antisense plants was remarkably lower than in control plants, which was consistent with the reduced sucrose content in these leaves. Sucrose synthase, the direct counterpart of the neutral cytosolic invertase, has been demostrated to be directly involved in sucrose cycling in sink tissues (Geigenberger and Stitt, 1993) and in sucrose cleavage for glycolysis within the phloem complex of *Ricinus* seedlings (Geigenberger *et al.*, 1993). In addition, the promoter sequences of maize and Arabidopsis sucrose synthase genes have been shown to drive phloem-specific expression of reporter genes in transgenic tobacco plants (Yang and Russell, 1990; Martin *et al.*, 1993). Therefore, sucrose synthase is the main sucrolytic enzyme responsible for supplying substrates for glycolysis and, consequently, ATP for the maintenance of sucrose phloem loading via H^+:symporter. A similar scenario may be envisaged in *SBP* transgenic plants, as sucrose synthase activity in the source leaves of these plants was lower than in control leaves. In this situation, low levels of sucrose-cleaving activities in the phloem cells counteract the glycolysis route, thereby preventing ATP production for the maintenance of the sucrose phloem loading by a proton gradient–dependent carrier.

Carbon partitioning, sucrolytic enzyme activities and structural analysis of the protein lead to an alternative model for the role of SBP in the long-distance sucrose translocation

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 (SUT) and overexpression of apoplastic invertase. However, the pattern of sugar accumulation in antisense plants

was not identical to that observed in plants expressing a H+:sucrose symporter antisense transgene. Furthermore, significant structural and biochemical discrepancies between SBPs and SUTs are observed. SUTs are members of the Major Facilitator Superfamily (MFS) and are characterized by the presence of 12 transmembrane-spanning domains arranged in a 6-α-helical + 6-α-helical configuration that are separated by a cytoplasmic loop (Marger and Saier, 1993) with the N-terminus and the C-terminus on the cytoplasmic side of the plasma membrane (Stolz *et al.*, 1999). In contrast, analysis of its deduced primary structure indicated that SBP is not similar to any other membrane transport proteins (Grimes *et al.*, 1992; Pirovani *et al.*, 2002). These differences may indicate that SBP and the H^{+}-symporter have distinct functions in sucrose translocation. It has been difficult to assign a function to the sucrose binding protein due to its unusual topology for a membrane carrier and its lack of similarity with other membrane transport proteins.

The first proposed model for SBP function postulated that the protein might have a regulatory role in phloem sucrose transport through interaction with SUT1 (Grimes *et al.*, 1992). Consistent with this, SBP was co-localized with sucrose/H+ cotransporters in the plasma membrane of *Vicia faba* transfer cells in developing seeds, and in cotyledons of *Vicia faba* (Harrington *et al.*, 1997a; 1997b). In soybean, *GmSUT1* and *SBP* expression are temporally coordinated during cotyledon development in a pattern that closely parallels sucrose availability within the developing soybean seed (Aldape *et al.*, 2003; Grimes *et al.*, 1992). Recently a sucrose/H^{+}:symporter cDNA, denominated *NtSUT1*, was isolated from tobacco and shown to be highly expressed in leaves (Bürkle *et al.*, 1998), from where a tobacco SBP homologue was also observed (Pedra *et al.*, 2000). Furthermore, antibodies against the sucrose binding protein have been demonstrated to specifically inhibit sucrose-proton symporter from transfer cell protoplast of developing *Vicia faba* seeds (Fieuw *et al.*, 1992). Protein:protein interaction assays and coexpression of *SUT1* and *SBP* in oocytes might be a suitable approach to test the hypothesis that SBP also has a regulatory function. The observation that affinity-purified antiserum against the SBP inhibits sucrose uptake into *Vicia faba* transfer cells further exalts the involvement of SPB in sucrose transport (Fieuw *et al.*, 1992).

A second model for SBP function was derived from heterologous expression of the *SBP* cDNA in mutant yeast, deficient in sucrose uptake. These studies clearly demonstrated that SBP was capable of mediating sucrose uptake across the plasma membrane in the absence of any other plant protein (Overvoorde *et al.*, 1996; Pirovani

et al., 2002). Consistent with its function as a transport protein, topological characterization indicated that the leader peptide of SBP is not quantitatively cleaved and may function as a transmembrane domain (Overvoorde and Grimes, 1994; Pirovani *et al.*, 2002). Thus, a fraction of SBP may behave as a type II membrane protein, which the uncleavable putative N-terminal leader peptide (the only hydrophobic region of the protein) works as a membrane-spanning domain that spans the bilayer once and has the bulk of the protein exposed to the extracellular environment (Overvoorde and Grimes, 1994). According to this topological model, the oligomerization properties of the protein would provide a toroid complex with an internal hole, forming conduits across the membrane of 18Å to mediate sucrose transport (Pirovani *et al.*, 2002). In fact, SBP is able to form trimers (Pirovani *et al.*, 2002) and is structurally related to vicilin-like storage proteins (Braun *et al.*, 1996; Overvoorde *et al.*, 1997), which assemble into trimers to form this toroid complex (Ko *et al.*, 1993; Lowrence *et al.*, 1994). As SBP and vicilins share an extraordinary conservation of primary structure that extends to include identical tertiary motifs, it is very likely that the SBP protein might also fit into the canonical model proposed for the structure of the vicilin-like protein family (Grimes *et al.*, 1992; Pirovani *et al.*, 2002). Consistent with this model, the initial rate and the final extent of the pH-independent sucrose uptake were positively correlated with the level of SBP in tobacco cultured cells (Delú-Filho, 2000). However, one may assume that if SBP transports sucrose directly across the membrane, *SBP* antisense plants should exhibit a high sucrose accumulation in leaves, similar to the SUT antisense phenotype. Therefore, this hypothesis seems unlikely as the sucrose content was lower in the antisense leaves than in control plants.

The phenotypes of the *SBP* antisense plants support an alternative functional mechanism in which SBP may have a regulatory role in phloem sucrose transport by regulating the expression or activity of alternative carbohydrate uptake systems. Consistent with this model, the manipulation of SBP levels in transgenic plants and cultured cells correlated inversely with cell-wall invertase activity (Figure 10A and Pedra *et al.*, 2000) and directly with sucrose synthase activity (Figure 10B and Delú-Filho *et al.*, 2000). Elevated levels of CWI activity in the source leaves of antisense plants could account for their phenotypes 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, lead to stunted growth. Furthermore, low levels of sucrose synthase activity in the source leaves would oppose the glycolysis route and ATP building up, which is needed for the maintenance of the sucrose phloem loading by a proton gradient-dependent carrier.

The demonstration that SBP2 exhibits GTP binding activity (Pirovani *et al.*, 2002), together with its capacity to bind sucrose specifically and reversibly (Ripp *et al.*, 1988), further support the possibility that SBP may serve a regulatory role in sucrose translocation-dependent physiological processes in plants.

Conclusions

In this work, the reduced accumulation of the SBP homologue in antisense tobacco plants was associated with the decrease in the developmental and growth performance, low photosynthetic rate, high starch content and altered sucrolytic activities. These phenotypes and physiological effects caused by antisense expression of *SBP2* gene are consistent with the involvement of the SBP homologue in the long distance sucrose translocation pathway. Our data support the hypothesis that SBP may have a regulatory role in phloem sucrose transport by regulating the expression or activity of alternative carbohydrate uptake systems, since the manipulation of the level of the SBP homologue also altered invertase activity and sucrose synthase activity. Our data also indicated that SBP functions predominantly at early plant development when the sink:source relationship is high due to the growth of shoot and root meristems and to the reduced number of source leaves. Therefore, during this phase the sucrose transport systems should operate at high efficiency to support normal growth and development.

CHAPTER 2

MULTIPLE POSITIVE AND NEGATIVE *CIS*-ACTING REGULATORY ELEMENTS INTERACT AS COMPOSITE MODULES TO CONFER A SPATIALLY-REGULATED *SBP2* PROMOTER ACTIVITY

Introduction

In higher plants, CO_2 fixation occurs predominantly in autotrophic mesophyll cells of mature leaves, which are net exporters of sugars and, therefore, known as 'carbon sources'. Heterotrophic cells in roots, reproductive structures, storage and developing organs are known as 'carbon sinks' or net importers since they depend on a supply of sugars for their nutrition. Long-distance transport of carbohydrates between sources and sinks occurs in specific cells of the vascular system, the phloem sieve elements. Sucrose represents the major form of carbohydrate that is transported in sieve elements (Zimmermann and Ziegler, 1975), although some plants can also transport raffinose, stachyose and polyols. After its synthesis, sucrose can move cell-to-cell from the mesophyll to the sieve element–companion cell complex (SE-CCC) via plasmodesmata (symplastic route). Alternatively, sucrose may be released from the mesophyll cells and actively loaded from the apoplast into the SE–CCC (apoplastic route). Unloading of sucrose at the sinks might occur either symplastically or apoplastically and mechanisms must exist to ensure that all sink tissues receive an adequate supply of sugars for growth and development. In the apoplastic route, sugar transporters play a pivotal role in the transport of sugars through membranes.

Plants appear to have several sucrose transporters to coordinate sugar transport in diverse tissues, at different developmental stages and under varying environmental conditions. Plasma membrane sucrose:H^+ symporters (SUT) are fundamental to this process. The members of the SUT family encode highly hydrophobic disaccharide transporters with two sets of six membrane-spanning domain structures, separated by a large cytoplasmic loop (Williams *et al.*, 2000). SUT1 serves as a high-affinity transporter, whereas SUT4, a second member of this sucrose transporter family, corresponds to the low-affinity/high capacity saturable component of sucrose uptake found in leaves (Weise *et al.*, 2000). A third structurally related-member of the family has been identified and designated SUT2 (Barker *et al.*, 2000). Although the whole family of sucrose transporter genes of a given species has not been identified, the sucrose transporters make a large gene family, as at least seven distinct sequences that encode putative sucrose transporters are present in the Arabidopsis database (Williams *et al.*, 2000). Other protein that has been implicated in the transport of sugars is the sucrose-binding protein (SBP/SBP2) (Grimes *et al.*, 1992; Pirovani *et al.*, 2002). SBP is structurally unrelated to the members of the SUT family and the SBP-mediated

specific sucrose uptake in yeast displays linear, non-saturable kinetics up to 30 mM external sucrose, being relatively insensitive to the pH gradient across the membrane (Grimes and Overvoorde, 1996; Overvoorde *et al.*, 1996). These biochemical features closely resemble the kinetic properties of the previously characterized linear-component of sucrose uptake in higher plants (Maynard and Lucas, 1982; Lin *et al.*, 1984). In spite of the absence of typical membrane transporter structural motifs on SBP (Grimes *et al.*, 1992) several evidences implicating SBP in sucrose transport has been obtained (Grimes and Overvoorde, 1996, Pedra *et al.*, 2000; Delú-Filho *et al.*, 2000, Pirovani *et al.*, 2002).

As sugar transporters play a pivotal role in the membrane transport of sugars, their distribution throughout the plant may be tightly controlled to guarantee an adequate supply of sugars for growth and development of all sink tissues. This regulation may occur at several levels (Bush, 1999; Delrot *et al.*, 2000). The transcriptional regulation of sucrose transporters have been attributed to factors involved into sink-source transition (Riesmeier *et al.*, 1993; Truernit and Sauer, 1995), seed development (Weber *et al.,* 1997) and pollen maturation and germination (Truernit *et al.,* 1999; Ylstra *et al.,* 1998). Abiotic and biotic factors, such as mechanical and pathogens injuries, light, water and osmotic stress and phytohormones, have also been shown to control sugar transporter expression (Truernit *et al.*, 1996; Delrot *et al.*, 2000; Noiraud *et al.*, 2000). For instance, phytohormone application in potato detached leaves increased the *StSUT1* mRNA levels (Harms *et al.*, 1994). Sugar transporters have also been demonstrated to be regulated by post-transcriptional control mechanisms, such as mRNA stability (turnover), mRNA translation and post-translational control. Examples include the diurnal regulation of sucrose transporters (Kühn *et al.*, 1997; Hirose *et al.*, 1997), phosphorylation–dephosphorylation (Roblin *et al.*, 1998), and possible modification of activity by the lipid environment (Delrot *et al.*, 2000).

There is evidence that changing the sugar levels might have an effect on sugar transporter expression and activity. In fact, the existence of a putative sucrose-sensing pathway modulating the expression levels and activity of a proton–sucrose transporter has been demonstrated as a result of changing sucrose concentrations in the leaf (hexoses do not elicit the response) (Chiou and Bush, 1998). This is expected to have a direct impact on assimilate partitioning at the level of phloem translocation. However, the data dealing with sugar control of gene expression of the sucrose transporters are scarce and sometimes contradictory. The expression of the *Vicia faba* sucrose carrier gene, *VfSUT1*, but not the glucose transporter, *VfSTP1*, decreased in cotyledons

following exposure to high concentrations of sucrose or glucose (150 mM), whereas lower levels (10mM) had no effect on transcript levels (Weber *et al.*, 1997). In contrast, in detached maize leaves the increase in sucrose availability led to an increase in *ZmSUT1* transcript levels (Aoki *et al.,* 1999). Likewise, the sucrose transporter from sugar beet (Chiou and Bush, 1998) is repressed by sucrose, whereas a companion cell-specific sucrose transporter of rice (Matsukura *et al.*, 2000) is up-regulated by its own substrate. Thus, for sugar transporters, the emerging scenario is that multiple levels of control allow plant cells to regulate the fluxes of sugars across the plasma membrane during normal growth and development, and also in response to perturbations in their natural environment.

In spite of these observations, little attention has been given to the complete characterization of the regulatory sequences of genes involved in sucrose transport. Recently, we have isolated and characterized two genomic clones of soybean SBP2 (Contim *et al.*, 2003). Fluorescence in situ hybridization (FISH) suggested that the soybean SBP gene family is represented by at least two non-allelic genes corresponding to the previously isolated *GmSBP1* and *GmSBP2* cDNAs. These two cDNAs share extensive sequence similarity but are located at different loci in the soybean genome. To investigate transcriptional activation of the *GmSBP2* gene, 2 kb 5'-flanking sequences of *gsS641.1* and *gsS641.2* were fused to the *b-glucuronidase* (*GUS*) reporter gene and to the *green fluorescent protein* (*GFP*) reporter gene. The SBP2 promoter directed expression of both *GUS* and *GFP* reporter genes with high specificity to the phloem of leaves, stems and roots. Thus, the overall pattern of *SBP-GUS* or *SBP-GFP* expression was consistent with the involvement of SBP in sucrose translocation-dependent physiological processes (Contim *et al.*, 2003). In this investigation, we further characterized the SBP promoter through serial and internal deletions linked to the gene reporter (*GUS*) to identify *cis*-acting DNA sequences that confer spatially-regulated activation and repression of *SBP* gene expression.

Materials and Methods

Generation of SBP2 promoter-reporter gene constructs

The isolation of *SBP2* genomic clone, *pgsS641.1*, has been described previously (Contim *et al.*, 2003). This pBluescript II SK-derived clone contains a 6.4 kb-full length sbp2 genomic clone with 2.0-kb promoter sequence (Figure 1). All DNA manipulations

were performed essentially as described by Sambrook *et al*. (1989). A *sbp2 promoter-GUS* fusion gene was constructed by cloning a 2.0 kb *Eco*RI/*Nco*I fragment from *pgsS641.1* into the *Eco*RI/*Nco*I sites of pCAMBIA1381Z (Roberts *et al.*, 1996) to yield -*2000pSPB2-GUS*, which contains a *GUS* cDNA under the control of 2.0-kb 5'-flanking sequence of *pgsS641.1* (Contim *et al.*, 2003). Deletions of *sbp2* 5'-flanking sequences were obtained by PCR-based mutagenesis using *Pfu* DNA polymerase (Stratagene, La Jolla, CA, USA) and *pgS641.1* as DNA template. The promoter deleted fragments were obtained by combining the reverse primer pS64ATGR (5'- gcttgggtctggtcgccatgg-3', coordinates -2 to +19 relative to the translational initiation codon, with a *Nco*I site underlined) with either the forward primer pS64Ec750F (5'-gacttaaatttgagaattctgattgag-3', coordinates -714 to -688, that creates an *Eco*RI site at position -701), pS64Pt505F (5'-ctcacgctggcctgcagctg-3', coordinates -502 to -483, that creates a *Pst*I site at position -492), pS64Ec373F (5'-ataaagaattcgaaactcacaatg-3', coordinates -370 to -347, that creates an *Eco*RI site at position -365), pS64Hd240F (5'-atttttttataagcttctctatctaggtg-3', coordinates -250 to -225, that creates a *Hind*III site at position -243), pS64Bl207F (5'-acgcacgcacacccagatctac-3', coordinates -205 to -184, that creates a *Bgl*II site at position -191), pS64Hd138F (5'-ttaaagcttgtccttgtgcatg-3', coordinates -137 to -116, that creates a *Hind*III site at position -134) or pS64Hd90F (5'-cattcctcactccaagcttttc-3', coordinates -103 to -82, that creates a *Hind*III site at position -90). After purification (Wizard® SV Gel and PCR Clean-Up System, Promega, Madison, WI, USA) and treatment with appropriated restriction endonucleases, the amplified fragments were cloned into the same endonucleases sites of the binary vector pCAMBIA1381Z (Roberts *et al.*, 1996), except for *Bgl*II that was substituted by *Bam*HI. The resulting clones were denominated, respectively, -*703pSBP2-GUS* (pUFV578), -*489pSBP2-GUS (*pUFV428), -*367pSBP2-GUS (*pUFV433), -*243pSBP2-GUS (*pUFV577), -*193pSBP2-GUS (*pUFV574), -*136pSBP2-GUS (*pUFV429) and -*92pSBP2-GUS (*pUFV579) and contain *SBP2* 5'-flanking sequences that extend until the position indicated by their numbers.

To create internal promoter deletions, two additional upstream fragments were generated by PCR and linked to *sbp2* sequences at positions -136 or -92 relative to the translational initiation codon. The first fragment (207pb, named fragment I) was amplified with the forward primer pS64Ec750F and the reverse primer pS64Hd489R (5'-ccatcagcggaagcttagcgtgag-3', coordinates -502 to -479, that creates a *Hind*III site at position -489). The second fragment (125pb, named fragment II) was amplified with the forward primer pS64Ec489F (5'-ctcacgctggaattccgctgatgg-3', coordinates -502 to -479, that creates a *Eco*RI site at position -493) with the reverse primer pS64Hd363R (5'-

gtgagtttcgtaagctttattgta-3', coordinates -374 to -351, that creates a *Hind*III site at position -362). After purification (Wizard® SV Gel and PCR Clean-Up System, Promega, Madison, WI, USA) and treatment with appropriated restriction endonucleases, both fragments were cloned into the same endonucleases sites of either *-136pSBP2-GUS* or *-92pSBP2-GUS* constructs. The clones generated were denominated *fragI/-134pSBP2-GUS* (pUFV575), *fragII/-134pSBP2-GUS* (pUFV576), *fragI/-90pSBP2-GUS* (pUFV580) and *fragII/-90pSBP2-GUS* (pUFV581). Figure 2 and 5 illustrates the *SBP2* promoter deletions.

Plant transformation and selection

The pCAMBIA-derived recombinant plasmids and the pCAMBIA1381Z binary vector alone were used to transform tobacco (*Nicotiana tabacum* L. cv. Havana) plants by *Agrobacterium tumefaciens*-mediated leaf disc transformation (Alvim *et al.*, 2001). Transformed plants were selected and regenerated on MS medium (Murashige and Skoog, 1962) containing hygromycin (50mg.L^{-1}) (Buzeli *et al.*, 2002). For the *pSBP2-GUS* constructs, plantlets were assayed for GUS activity and primary transformants were either maintained *in vitro* or transferred into soil and grown in standardized greenhouse conditions (T0 plants) to generate seeds. Most of the rooted plants were tested for the incorporation of the hygromycin (*hptII*) and *sbp2* promoter-GUS fusion genes by PCR analysis. The *hpt*II-specific primers used were HPTF (5'-cgcttctgcgggcgatttgtgtacg-3', coordinates 110 to 134) and HPTR (5'-tcagcttcgatgtaggagggcgtgg-3', coordinates 916 to 940). The intensity of GUS staining among the selected lines was similar and the expression pattern was consistent between independent transgenic lines. Detailed sectional analyses for tissue-specific expression were carried out on five independent *pSBP2-GUS* transgenic lines of each constructs. One hygromycin-resistant plant for the pCAMBIA1381Z incorporated binary vector and one wild type plant were used as negative controls, whereas three hygromycin-resistant plants for the pCAMBIA1301 incorporated binary vector were used as positive control.

Determination of GUS activity and histochemical in situ localization of GUS in tobacco organs

Protein extraction and fluorometric assay for GUS activity were performed essentially as described by Jefferson *et al.* (1987) with methylumbelliferone (MU) as a standard. Extracts were prepared from the leaf that had been frozen in liquid nitrogen and kept at

-80 °C until processing. For the standard assay, plant tissues were ground in 0.5mL of GUS assay buffer (100 mM $NaH_2PO_4.H_2O$, pH 7.0, 10 mM EDTA, 0.1 % (w/v) sarcosyl, 0.1 % (v/v) Triton X-100) and 50 μL of the supernatant were mixed with 50 μL of GUS assay buffer containing 2 mM of 4-methylumbelliferyl-β-D glucuronide (MUG) as a substrate. The mixture was incubated at 37 °C for 15 min and GUS activity was measured using a DYNA Quant 200 Fluorometer (Amersham Pharmacia Biotech, UK). For the transgenic lines carrying *sbp2* promoter-GUS fusion, histochemical analysis of β-glucuronidase activity was performed as previously described (McCabe *et al.*, 1988). The tissues (shoot apex, leaves, petiole, stems and roots) were sampled and sectioned using a hand microtome. Tissue sections were embedded in the GUS assay buffer (100 mM $NaH_2PO_4.H_2O$, pH 7.0, 0.5 mM $K_4Fe(CN)_6.3H_2O$, 10 mM $Na_2EDTA.2H_2O$, 0.1 % (v/v) Triton X-100) containing 1.5 mM 5-bromo-4-chloro-3-indolyl-β-D glucuronide (X-Gluc) (McCabe *et al.*, 1988) and incubated at 37°C in the dark for 4 h. Pigments were extracted from stained tissues with methanol:acetone (3:1, v:v). After extensive washing, the clarified tissues were stored in 50% (v/v) glycerol until photodocumentation. The micrographies were taken under an Olympus AX-70 microscope.

Results and Discussion

Potential regulatory elements are found in the soybean SBP2 promoter

Two genomic clones, gsS641.1 and gsS641.2, carrying an SBP gene from a soybean genomic library constructed in λZAPII have been previously isolated (Contim *et al.*, 2003). Characterization of these clones indicated that they are allelic forms of the *SBP2* gene. The analysis of a 1kb 5'-flanking sequence from *gsS641.1* revealed that this region corresponds to the *SBP* promoter. Sequence analysis of this promoter region indicated a number of conserved motifs of most eukaryotic promoters, in addition to several potential regulatory elements of plant promoters (Figure 1, from Contim *et al.*, 2003). In summary, typical TATA box and CCAAT box were identified on gsS641.1 at position -73 and -337 upstream of the ATG translation start codon, respectively. The potential regulatory elements found in the soybean *SBP2* promoters fall into three categories: (1) tissue-specific controlling elements, such as the GLUB1 sequence AACAAAC (Wu *et al.*, 2000) in sense (coordinates -68 to -74) and reverse

orientation (-33 to -39), the legumin box CATGCAY (-116 to -127; Fujiwara and Beachy, 1994), and a reverse SEF1 motif ATATTTAWW (-519 to -527; Allen *et al.*, 1989); (2) light-responsive elements, such as the GT1 core sequence GGTTAA in reverse orientation (coordinates -767 to -756; Zhou, 1999) and a reverse IBOX core sequence GATAA (-275 to -281; Terzaghi and Cashmore 1995); (3) stress-responsive elements, such as a MYB2AT sequence TAACTG in reverse orientation (-418 to -428; Urao *et al.*, 1993), a WBOXATNPR1 sequence TTGAC (-215 to -221; Yu *et al.*, 2001) and two repeated ASF1 sequences, TGACG, positions -541 and -575 (Katagiri *et al.*, 1989). The conserved stress-responsive elements are found in a number of genes, involved in sugar metabolism or transport.

In this previous study, a region of 2kb of *SBP2* promoter was used to driven the expression of the *GUS* and *GFP* cDNAs in tobacco transgenic plants. Quantitative analyses of promoter activity demonstrated that the *SBP2* promoter was more active in transgenic seeds and in developing fruits. These results were consistent with the high levels of SBP mRNA accumulation in immature seeds (Grimes *et al.*, 1992; Overvoorde *et al.*, 1997). Besides, *SBP2* promoter directed *GUS* and *GFP* expression in a tissue-specific manner. Detailed analysis of leaves, shoot apex, stems and roots revealed that the gene reporter expression was restricted to the phloem, with clear staining of the sieve elements (Contim *et al.*, 2003). Here, we also verified the ability of *SBP2* promoter to drive GUS staining in phloem tissues (Figure 4). Therefore, *GmSBP2* promoter directed expression of *GUS* and *GFP* reporter genes to the vascular tissues of roots, stems and leaves, corroborating with the involvement of SBP in the long-distance sucrose translocation pathway (Contim *et al.*, 2003 and our studies). In fact, the transport of sucrose between source and sink tissues occurs in the vascular tissue and is mediated by the sieve-elements and the closely associated companion cells of the phloem tissue (Truernit, 2001). Nevertheless, these results, based on expression of SBP2-reporter gene transcriptional fusions in transgenic plants, did not allow the possibility to be ruled out that the accumulation of SBP2 protein in soybean tissues might be different from the reporter gene activity pattern due to the lack of transcriptional regulatory elements and post-transcriptional control. However, the spatial regulation of the *SBP2-GUS* and *SBP2-GFP* reporter genes accurately reproduced the accumulation of SBP that has been observed in the phloem cells of soybean leaves (Grimes *et al.*, 1992). Likewise, in spinach, an SBP homologue was immunolocalized in the plasma membrane of sieve elements in fully expanded leaves, shoots and roots (Warmbrodt *et al.*, 1989, 1991). These observations support the

argument that the *SBP2* derived sequence may control tissue-specific regulation of *SBP2* expression.

Figure 1. Putative *cis*-regulatory elements on *gsS641.1* promoter regions. The sequences presented extend until the ATG translational initiation codon of gsS641.1. Numbers indicate the position relative to the translation start codon. The putative transcriptional start site is indicated, followed by the putative TATA box. Several putative regulatory *cis*-acting elements are underlined and indicated by their appropriate names. The arrows indicate the borders of the deletions.

SBP2 promoter contains negative and positive regulatory cis-elements

To characterize functional regulatory cis-acting elements on *SBP2* promoter, we generated progressive 5' deletions of the *SBP2* promoter from -2000 to -92. This deletion series yielded eight fragments which were transcriptionally fused to *GUS* in pCAMBIA1381Z binary vector (Figure 2). Transgenic tobacco plants were then generated via *Agrobacterium tumefaciens*-mediated transformation and GUS activity was determined in at least four independent transformants for each construct. All the fragments tested were able to induce GUS enzyme (Figure 3). However, the strength

of the promoter fragment, as measured by absolute GUS activity, was not correlated with the size of the fragments, suggesting the presence of positive and negative elements. In fact, the magnitude of GUS activity seemed to be influenced by the specific group of regulatory elements present in each construct. Deletion of sequences between -2000 and -703 resulted in a strong increment in promoter activity, while additional deletion until position -489 led to a decrease in GUS activity. Nevertheless, this result was reverted with the further deletion of -489 to -367 sequences. These observations suggest that negative *cis*-regulatory elements are contained in this region (-489 to -367) and/or the low activity of the -489 construct may be due to deletion of positive *cis*-regulatory elements in the region -703 to -489.

An analogous hypothesis may be applied from the analyses of the -243 construct, as deletion of -367 to -243 sequences reduced promoter activity approximately seven fold, whereas an additional deletion from -243 to -191 practically reestablished full promoter activity (Figure 3). Therefore, the region delimited by positions -243 and -191 may also contain negative *cis*-regulatory elements. Alternatively or additionally positive *cis*-regulatory elements are present between positions -367 and -243. The high promoter activity of the -367 fragment supports this hypothesis. A CCAAT box, responsible by high transcriptional levels in plant promoters (Kusnetsov *et al.*, 1999), was identified on *gsS641*.1 at position -337 upstream of the ATG translation start codon. In fact, plants cultivated under autotrophic conditions showed high expression of transactivator associated with the CCAAT box (Edwards *et al.*, 1998). However, the promoter activity of the -489 construct was lower than that of the -367 construct, indicating that the putative positive *cis*-regulatory elements present in the -367/-243 region are not able to overcome the inhibitory effect of the -489 to -367 sequences.

We also observed that sequences up to -92 are enough to produce a high level of basal expression (Figure 3). In fact, the 92-bp fragment of the SBP2 promoter contains a TATA box in a favorable context for transcription, at 21 nucleotides downstream of the putative transcriptional start site (Figure 1). Therefore, the sequence spanning from -92 to the start codon is able to maintain high level of basal expression and may represent a potential eukaryotic minimal promoter.

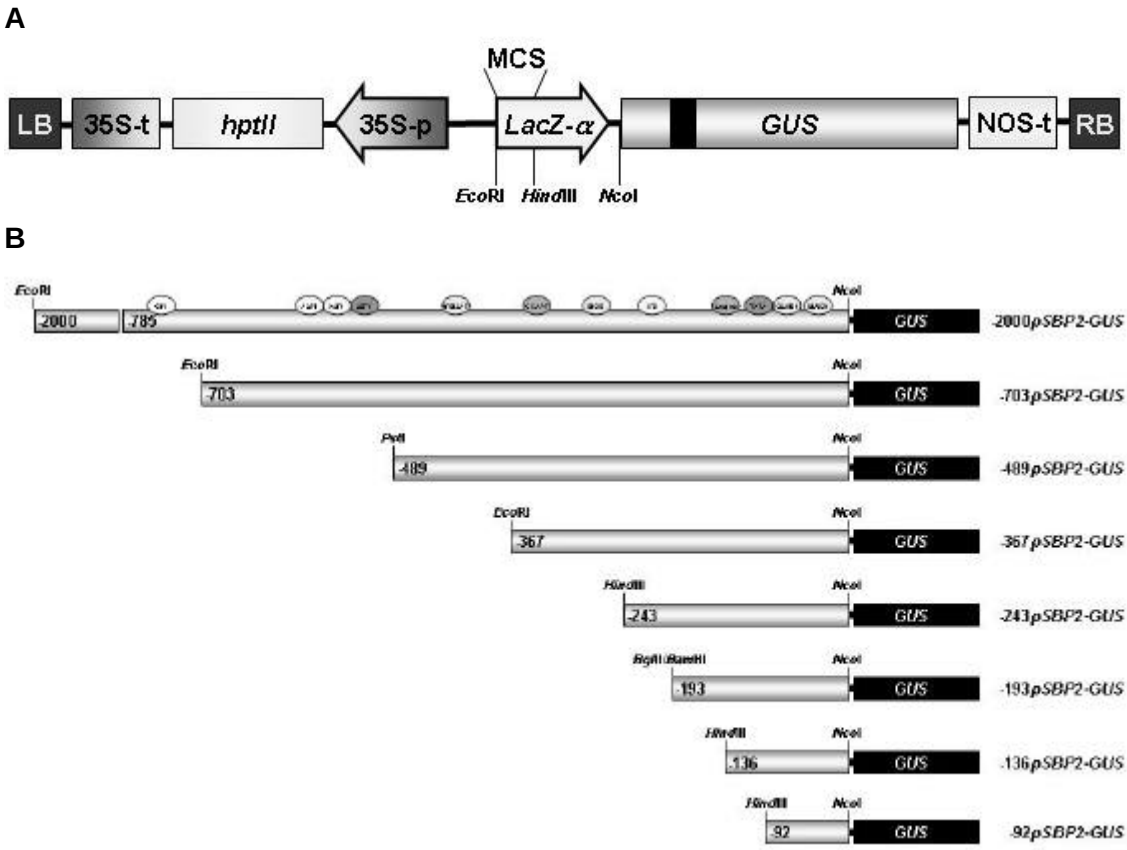

Figure 2. A. Map of the binary vector pCAMBIA1381Z. In the binary vector, the promoterless -glucuronidase coding region (*GUS*) is interrupted by a catalase intron (black bars) and is followed by the 3' nopaline synthase polyadenylation signal (NOS-t). The hygromycin phosphotransferase gene (*hptII*) expression is driven by the constitutive *CaMV 35S* promoter (35S-p) and 3' *CaMV35S* polyadenylation signal (35S-t). MCS correspond to the multiple cloning sites. LB and RB correspond to the T-DNA left and right borders, respectively. The positions of some restriction enzyme sites are indicated.
B. Schematic representation of 5' flanking sequences of *gsS641.1* fused to *GUS*. The successive deletions and the respective endonucleases sites were generated by PCR-based mutagenesis and cloned into the appropriate sites of pCAMBIA1381Z.

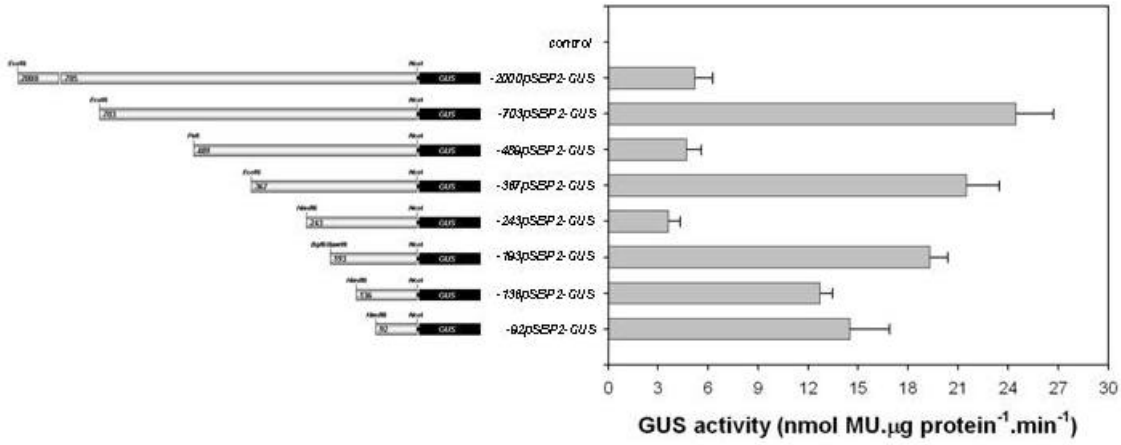

Figure 3. GUS activity in mature leaves of transgenic tobacco plants expressing SBP2-GUS fusion genes. Specific GUS activity was determined by fluorometric assays with total extracts from leaves and expressed as nmol of 4-methylumbelliferone μg protein^{-1} min^{-1}. The bars represent average (±SE) of four independent measurements using extracts from independent transgenic lines. Control represents the promotorless binary vector (pCAMBIA1381Z) transformed plants.

Sequences between -2000 to -700 are essential for phloem-specific expression of the SBP2 promoter

The *SBP2* promoter was able to drive the GUS and GFP expression specifically to the vascular tissue of tobacco (Contim *et al.*, 2003). Therefore, independently of the external factors, the tissue-specific expression is controlled by regulatory elements presents in *SBP2* promoter. In fact, several tissue-specific controlling elements are found in the *gsS641.1* (Figure 1). To further examine these elements, we analyzed the transgenic tobacco plants expressing the *SBP2-GUS* fusion genes for tissue-specific GUS activity. Except for -489pSBP2-GUS and -243pSBP2-GUS constructs, which contain negative control elements (Figure 3 and 4), all the other deletions provided an ubiquitous pattern of cell expression with intense GUS staining in all tissues and organs analyzed (Figure 4). These results indicate that the -703 upstream region contains silencer elements that confine the promoter activity to the phloem, by preventing gene expression in the other tissues. Deletion of this region de-represses *SBP2* promoter in all tissues analyzed and, therefore, all the *cis*-acting elements necessary for expression in these tissues must be located downstream of -703. In fact, several potential tissue-specific controlling elements were identified within this region (Figure 1). Further deletions demonstrated that sequences located downstream of -92 are sufficient to promote a constitutive expression of the *SBP2* promoter (Figure 4), exhibiting 60 % activity relative to the -703 fragment (Figure 3).

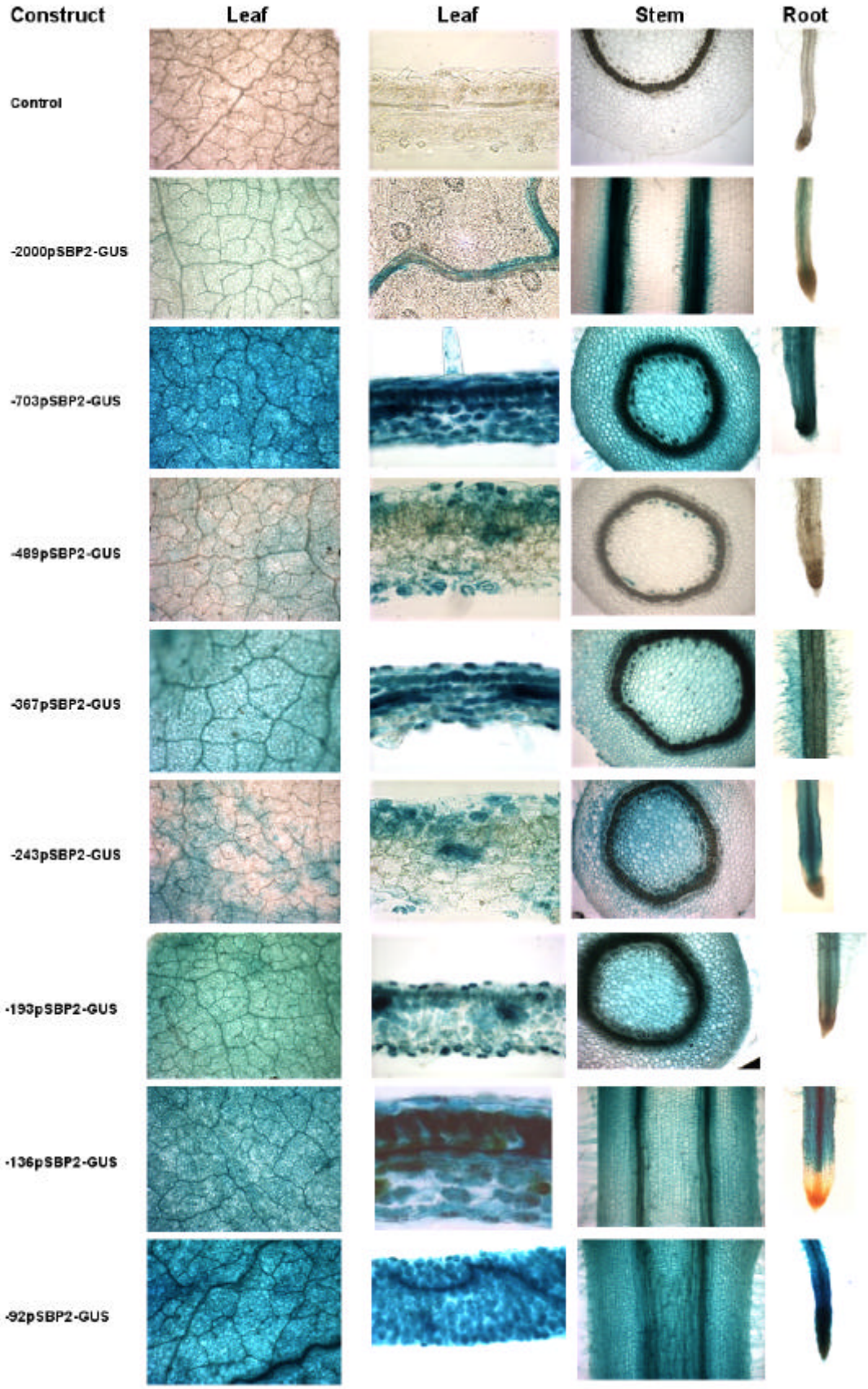

Figure 4. Histochemical analysis of tissue-specific regulation of pSBP2-GUS fusion gene expression in transgenic tobacco plants. Photographs of leaf surface and leaf transverse sections, stem transverse or longitudinal sections and root. Control corresponds to promotorless pCAMBIA-transformed plants.

The -489pSPB2-GUS construct possesses strong silencers that suppress the positive regulation of SBP2 promoter

Histochemical analyses of plants harboring the -489pSBP2-GUS construct demonstrated that deletion of *SBP2* promoter up to the -489 position abolished detectable expression in the root and, in the stem, GUS staining was confined to the inner phloem (Figure 4). In contrast, a week expression was observed in leaves. This pattern indicates either a presence of root-specific and stem-specific elements within the region between -703 and -489 or the presence of strong silencers downstream of -489 or both. Consistent with the second hypothesis, further deletion up to -370 caused a seven-fold induction of gene expression (Figure 3) and promoted a constitutive expression in leaves, stem and roots (Figure 4).

To further distinguish between there two possibilities we performed gain-of-function experiments for the sequences between -703 and -489 and the sequences between -489 and -370. The 270-bp fragment (named fragment I), coordinates -703 to -496, and the 125-bp fragment (named fragment II), coordinates -495 to -370, were directly fused to the -136 and -92pSBP2-GUS constructs (Figure 5). Although the fragment I caused a 24 % increase in the -136 fragment-mediated GUS expression, the fusion of fragment I to -92pSBP2-GUS construct resulted in almost 18 % reduction in gene expression (Figure 6). These results may indicate that fragment I contains enhancer-like elements that may act in an interactive combinatorial manner with other *cis*-elements and the promoter activity may depend either on correct spacing among them or on sequences between -136 and -92. In contrast, the fragment II caused a 2-fold reduction in both -136 and -92-mediated GUS expression (Figure 6) and recapitulated the tissue-specific expression pattern of -489pSBP2-GUS in leaves (Figure 7). Therefore, the histochemical examination (Figure 4 and 7) together with the quantitative measurements of GUS activity (Figure 3 and 6) supply evidences that the -489pb fragment possesses regions capable of silencing the gen expression.

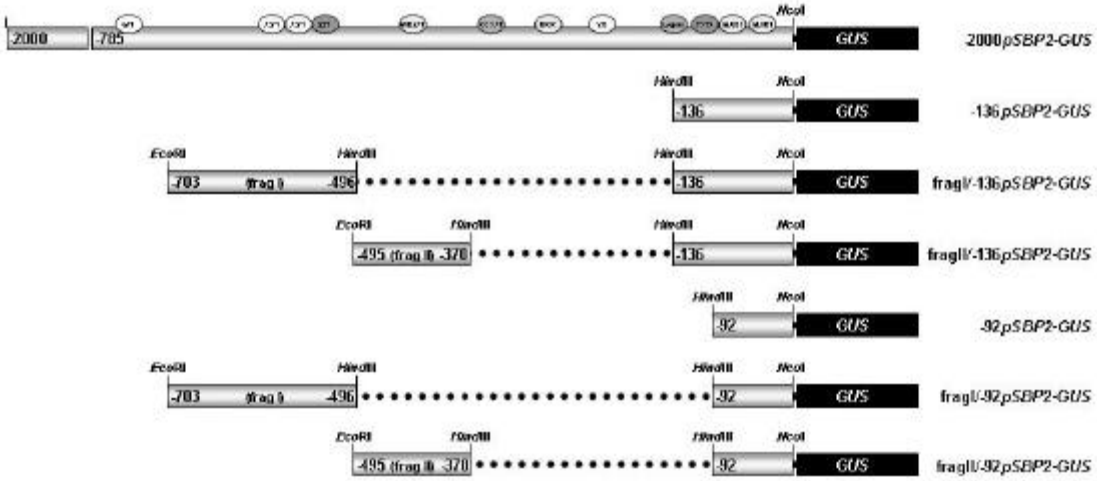

Figure 5. Schematic representation of 5' flanking sequences of gsS641.1 fused to *GUS*. The fragments (fragI and fragII) and the respective endonucleases sites were generated by PCR-based mutagenesis and cloned into the appropriate sites of -136pSBP2-GUS and -92pSBP2-GUS constructs.

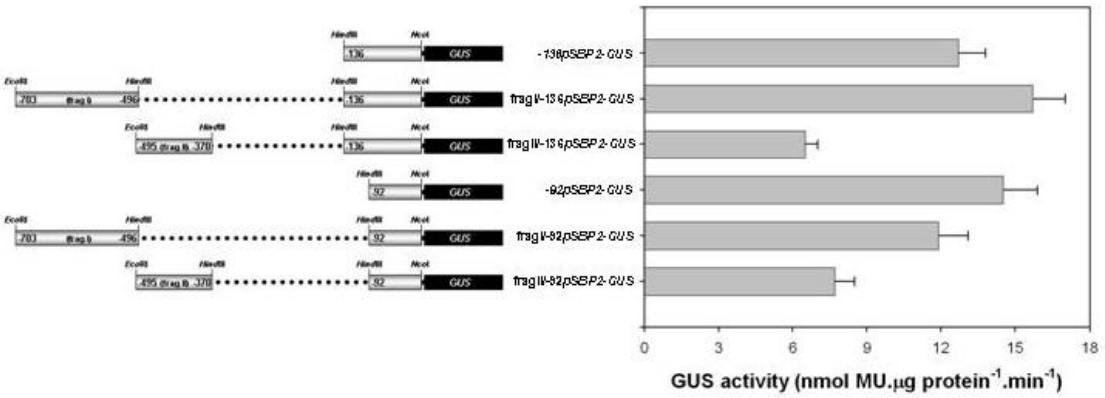

Figure 6. GUS activity in mature leaves of transgenic tobacco plants expressing SBP2-GUS fusion genes. Specific GUS activity was determined by fluorometric assays with total extracts from leaves and is expressed as nmol of 4-methylumbelliferone.µg protein^{-1}.min^{-1}. The bars represent average (±SE) of four independent measurements using extracts from independent transgenic lines.

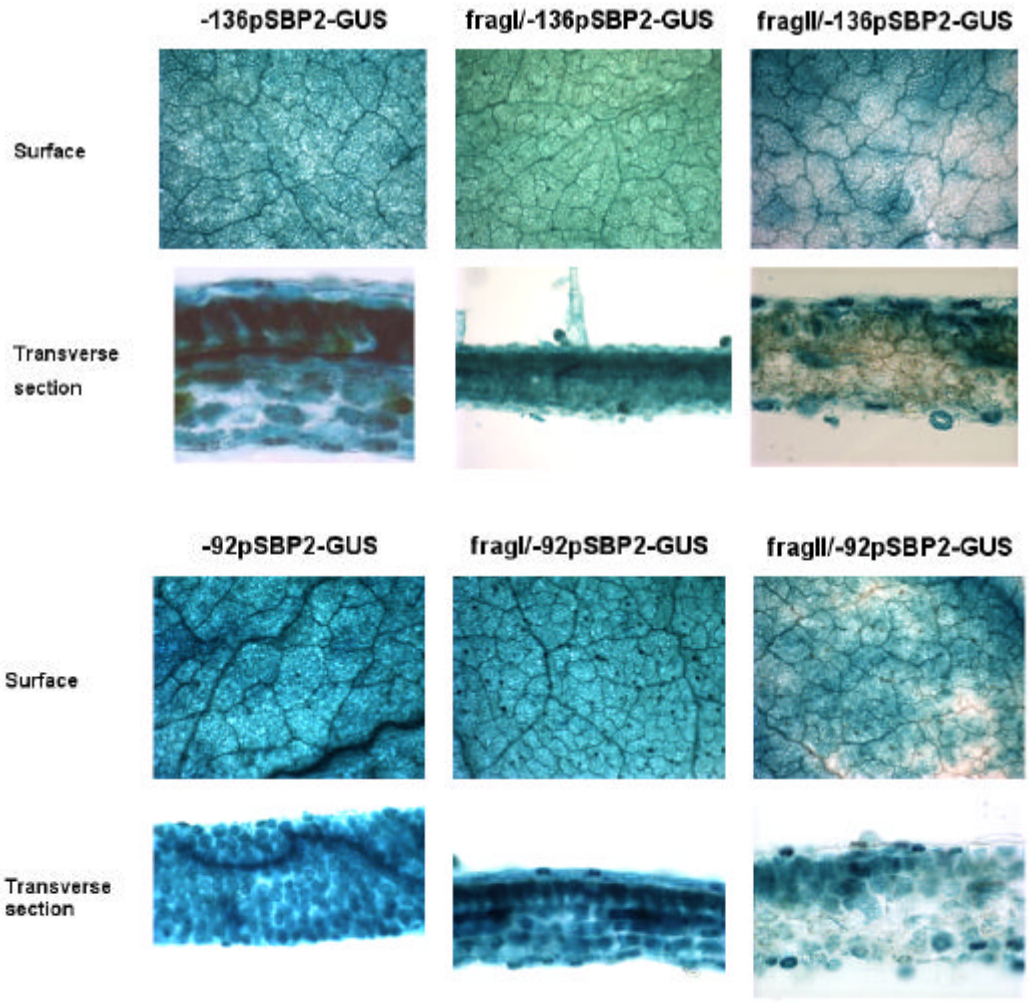

Figure 7. Histochemical analysis of tissue-specific regulation of pSBP2-GUS fusion gene expression in transgenic tobacco leaves. Photographs of leaf surface and leaf transverse sections.

The -243, -193 and -136pSBP2-GUS constructs fail to drive GUS expression in the root meristem

Constitutive expression of *SBP2* promoter was recovered with the deletion up to -367, as the -367pSBP2-GUS generated the same ubiquitous pattern of cell staining in leaves, stem and root as the -703pSBP2-GUS, although to a less extent . However, deletions upstream to the position -243 abolished GUS staining in the root meristem, so did deletions up to -193 and -136 (Figure 4). Very likely, this was due to the loss of *cis*-acting elements that may be targets of specific transcriptional factor of the root meristem. In fact, a transcriptional factor that is necessary for the root meristem expression has been described in tobacco (Baumann *et al.*, 1999). In contrast, a robust GUS staining in the root meristem was observed in -92pSBP2-GUS plants, indicating

that a root meristem-specific silencer may be contained in the region between -136 and -92. Furthermore, the sequences upstream of -243 may contribute to the expression in root meristems by overcoming the repression of this silencer. Consistent with this observation, the fusion of the fragment I and fragment II regions to the -136 end of *SBP2* sequences restored GUS expression in root meristem (Figure 8).

Figure 8. Histochemical analysis of tissue-specific regulation of pSBP2-GUS fusion gene expression in transgenic tobacco. Photographs of roots.

Conclusions

The SBP2 promoter shows a functional structure characteristic of eukaryotic promoters, containing general *cis*-acting elements for promoter activity and additional elements for spatially regulated plant gene expression. The full-length *SBP2* promoter sequence drives the expression of a linked *GUS* gene specifically to the phloem tissues of tobacco transgenic lines. The repression of the *SBP2* promoter activity in all other tissues of root, stem and leaf is alleviated by deletion of sequences upstream of -703. This indicates that the phloem-specific expression of *SBP2* promoter is contributed by tissue-specific silencers in distal sequences as well as phloem-specific elements within proximal sequences of *SBP2* promoter. Further deletions of 5' flanking sequences indicate that *SBP2* promoter activity and tissue-specificity is coordinated by negative and positive combinatorial modules that interact to each other in a complex manner. Strong negative elements were found in sequences delimited by positions -495 to -370, which were confirmed by gain-of-function experiments, as well as in the sequence spanning from position -243 to -193. Also, a root meristem-specific silencer was identified within the region between -136 and -92. A short sequence, spanning from -92 to the start codon, was able to maintain high level of basal expression and may represent a potential eukaryotic minimal promoter.

GENERAL CONCLUSIONS

Sucrose Binding Protein has been involved in higher plant sucrose transport mechanism. To further examine the protein role, SBP antisense tobacco plants in T2 generation were characterized phenotypical, physiological and biochemically. Transgenic plants showed a decrease in growth rate and delay in development. This growth-related phenotype was associated with reduced photosynthesis in antisense leaves during the vegetative phase. Carbohydrate partitioning was sharply altered, as transgenic plants accumulated higher starch content in leaves in comparison with wild type control. Accordingly, AGPase activity was increased in antisense transgenic plants. These phenotypes and physiological effects caused by antisense expression of *SBP2* gene are consistent with the involvement of the SBP homologue into the long distance sucrose translocation pathway.

The growth-related phenotypes and pattern of sugar accumulation exhibited by antisense lines were clearly distinguishable from control during early developmental stages, but did not persist as development progresses. These results strongly suggest that SBP functions predominantly in early plant development and are consistent with the developmental accumulation of SBP. Furthermore, at early vegetative phase the sucrose transport systems must operate at high efficiency to supply the carbon demand of sink tissues, due to a high sink:source relationship.

In the antisense lines, the cell wall invertase activity was increased, whereas sucrose synthase activity was decreased as compared to control plants. Therefore, a reduction in sucrose phloem loading or unloading in *SBP* transgenic plants, which is consistent with the *SBP* antisense phenotypes, may be caused by an indirect effect via apoplastic hydrolysis of sucrose into hexoses, which are not transported efficiently at long-distance, rather than by a direct reduction in SBP-mediated sucrose transport. Therefore, our data support the hypothesis that SBP may have a regulatory role in phloem sucrose transport by regulating the expression or activity of alternative carbohydrate uptake systems.

Consistent with the involvement of SBP in long distance sucrose transport, the *SBP2* promoter directed the expression of both *b-glucuronidase* (*GUS*) and *green fluorescent protein* (*GFP*) reporter genes with high specificity to phloem of leaves, stems and roots of the transgenic tobacco plants. In order to identify potential *cis*-regulatory elements controlling the spatial expression of *SBP2* promoter, we performed 5' and internal deletion promoter analyses in transgenic tobacco.

The *SBP2* promoter shows a functional structure characteristic of eukaryotic promoters, containing general *cis*-acting elements and additional elements for the plant tissue-specific controlling expression. The full-length *SBP2* promoter sequence drives the expression of a linked *GUS* gene specifically to the phloem tissues of tobacco transgenic lines. The repression of the *SBP2* promoter activity in all other tissues of root, stem and leaf is alleviated by deletion of sequences upstream of -703. This indicates that the phloem-specific expression of *SBP2* promoter is contributed by tissue-specific silencers in distal sequences as well as phloem-specific elements within proximal sequences of *SBP2* promoter. Further deletions of 5' flanking sequences indicate that *SBP2* promoter activity and tissue-specificity is coordinated by negative and positive combinatorial modules that interact to each other in a complex way. Strong negative elements were found in sequences delimited by positions -495 to -370 and a root meristem-specific silencer was identified within the region between -136 and -92. A short sequence, spanning from -92 to the start codon, was able to maintain high level of basal expression and may represent a potential eukaryotic minimal promoter.

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