

# Differential Expression of Sucrose-Phosphate Synthase Isoenzymes in Tobacco Reflects Their Functional Specialization during Dark-Governed Starch Mobilization in Source Leaves

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Sucrose (Suc)-phosphate synthase (SPS) plays a crucial role in the synthesis of Suc in photosynthetic and nonphotosynthetic tissues. Several isoforms of SPS exist in dicotyledonous plants that can be grouped into the different families A, B, and C. To explore whether functional differences between the SPS gene families might exist, we characterized a representative for each family from tobacco (*Nicotiana tabacum*). RNA-blot analysis revealed a distinct expression pattern for each of the three SPS genes. While the A-family member (NtSPSA) was found to be expressed in all tissues examined, expression of the B isoform (NtSPSB) was mainly confined to the reproductive organs and NtSPSC mRNA was exclusively detected in mature source leaves. We used RNA interference to assess the in planta function of NtSPSA and C. While silencing of NtSPSA had no detectable influence on leaf carbohydrate metabolism, reduction of NtSPSC led to an increase in leaf starch content by a factor of 3 to 8. Further analysis revealed that starch accumulation in NtSPSC-silenced plants was not due to an increased partitioning of carbon into starch, but rather showed that starch mobilization was impaired. The transgenic plants were unable to efficiently mobilize their transitory leaf starch during a prolonged period of darkness and accumulated maltose as a major intermediate of starch breakdown. NtSPSC mRNA level increased appreciably during the dark period while transcript levels of the other isoforms showed no diurnal changes. Together, these results suggest that NtSPSC is specifically involved in the synthesis of Suc during starch mobilization in the dark. The roles of the other SPS isoforms are discussed.

During the light period, starch and Suc are synthesized together as the products of photosynthetic carbon assimilation in source leaves. Suc is exported to nonphotosynthetic parts of the plants to support their growth and development, and starch is retained in the leaf. At night, starch is degraded to provide the substrates for the continued synthesis and export of Suc (Geiger and Servaites, 1994). Beyond its role in energy metabolism, Suc is often accumulated in response to environmental stresses such as cold, salinity, and drought (Yang et al., 2001; Strand et al., 2003). Suc-P synthase (SPS; EC 2.4.1.14) is assumed to be the key enzyme controlling flux of carbon into Suc (Huber and Huber, 1996). SPS catalyzes the formation of Suc-6-P from Fru-6-P and UDP-Glc. Subsequently, Suc-6-P is hydrolyzed by a specific Suc-P phosphatase (SPP; EC 3.1.3.24) to release Suc. The reaction catalyzed by SPP is essentially irreversible and displaces the reversible

SPS reaction from equilibrium into the direction of net Suc synthesis (Stitt et al., 1987). While SPP activity is abundant in source leaves and does not exert significant control of Suc synthesis under normal growth conditions (Chen et al., 2005), SPS is known to make a significant contribution to control of flux into Suc. The enzyme is regulated by a hierarchy of mechanisms involving posttranslational modification via phosphorylation, direct control by metabolic effectors, such as Glc-6-P and inorganic phosphate (Pi; Huber and Huber, 1996; Winter and Huber, 2000), and transcriptional regulation during sink-source transition (Harn et al., 1993; Klein et al., 1993; Chávez-Bárcenas et al., 2000).

The regulation of SPS from spinach (*Spinacia oleracea*) leaves has been well characterized. The enzyme contains three phosphorylation sites, Ser-158, Ser-229, and Ser-424, which are involved in light/dark regulation (Huber and Huber, 1996; Toroser et al., 1999), 14-3-3 protein binding (Toroser et al., 1998), and osmotic stress activation (Toroser and Huber, 1997), respectively. In darkness, phosphorylation of Ser-158 inactivates SPS. This effect is reversed in the light by a type 2A phosphatase resulting in an activated SPS as a consequence of an increase in the affinity for its substrates and the positive allosteric regulator Glc-6-P, and a decrease in Pi sensitivity (Huber and Huber, 1996).

There is growing evidence that higher plants contain more than one gene encoding SPS. The inspection of

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the *Arabidopsis thaliana* genome revealed the presence of four genes putatively encoding SPS enzymes, all of which are transcribed (Langenkämper et al., 2002; Lunn and MacRae, 2003). Phylogenetic analysis suggests that the four SPS sequences from *Arabidopsis* and all those known from other dicot species fall into three families: A, B, and C (Langenkämper et al., 2002; Lunn and MacRae, 2003). It was proposed that at least one representative for each family is present in the genome of a given dicot species and that a member of each family is expressed (Lunn and MacRae, 2003). Recently it was shown that monocot species contain an additional D family that probably arose after monocots and dicots diverged (Castleden et al., 2004). Comparative studies of the expression of SPS genes from different families in *Citrus unshui* and *Triticum aestivum*, respectively, revealed distinct, but overlapping, spatial and temporal expression for each of the SPS gene families (Komatsu et al., 1996; Castleden et al., 2004). A-family members have been the subject of most expression studies and most of the expressed sequence tags (ESTs) examined belong to the A family, implying that A-family genes are more abundantly expressed than those belonging to other families. Antisense repression of SPS A in *Arabidopsis* led to a reduction in SPS activity of approximately 60% to 70%. While this led to an inhibition of Suc synthesis in leaves, photosynthetic carbon partitioning was not redirected toward starch (Strand et al., 2000). Antisense or cosuppression of SPS A in potato (*Solanum tuberosum*) and the examination of the transgenic tubers revealed a crucial role for this enzyme during the adaptation to water stress and low temperature (Krause et al., 1998; Geigenberger et al., 1999); however, in these studies no data on any effects on photosynthetic carbon metabolism have been reported.

These experiments targeted only one of the SPS gene families, and the extent to which other SPS genes were targeted or may have been up-regulated to compensate has not been investigated. Thus, the challenge remains to determine if there is any functional specification between the different SPS gene families.

Toward this end, we carried out a systematic analysis of the SPS gene families in tobacco (*Nicotiana tabacum*) combining molecular, biochemical, and reversed genetics approaches. Based on the results, a specific functional role for individual families is discussed.

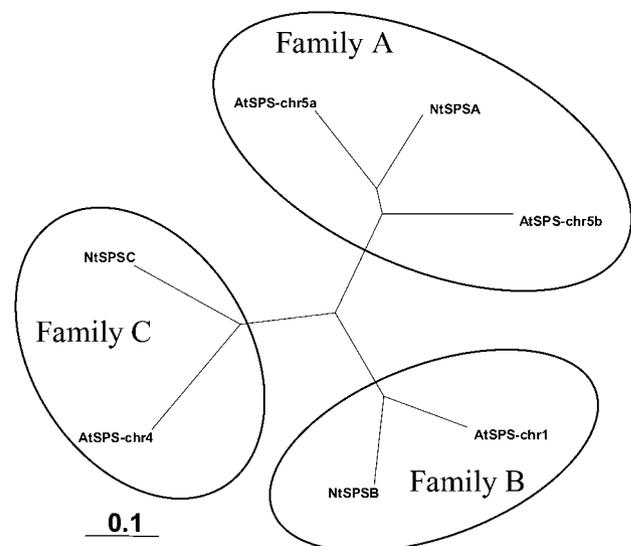
## RESULTS

### Tobacco Expresses a Representative for Each SPS Gene Family

The isolation of a cDNA encoding SPS from a tobacco leaf cDNA library using the SPS A isoform from spinach as a probe has previously been reported (Börnke, 2005; GenBank accession no. AF 194022). Based on phylogenetic analysis, this clone was subsequently classified as a member of the SPS A family

and thus designated NtSPSA. A search of the GenBank EST database using the B- and C-family members (At1g04920 and At4g10120, respectively) from *Arabidopsis* as a probe identified two additional tobacco ESTs, CB329258 and BP527800, with similarity to *Arabidopsis* SPS B and C, respectively. To obtain the full-length sequence of the respective cDNA clones, 3'- and 5'-RACE PCR were performed using cDNA prepared from tobacco leaf material as a template. Based upon the sequence data of the RACE products, the entire predicted coding region of the two tobacco cDNAs was amplified by PCR, subcloned, and sequenced. Phylogenetic analysis of the now three tobacco SPS genes revealed that each represents a member of the three SPS gene families present in dicots (Fig. 1). On amino acid level, NtSPSA showed 56.1% and 52.7% identity to NtSPSB and C, respectively, while NtSPSB showed 56.2% identity to NtSPSC. The proteins all had very similar predicted molecular mass with 119 kD for NtSPSA, 120 kD for NtSPSB, and 118 kD for NtSPSC. The theoretical pI ranged from 6.2 for NtSPSB to more than 6.3 for the A isoform, to 6.5 for SPS C.

As mentioned above, SPS A from spinach has three known regulatory phosphorylation sites involving Ser-158, Ser-229, and Ser-424. A comparison between the spinach protein and those from tobacco revealed that the light-regulated motif surrounding Ser-158 is well conserved in all sequences (Table I). However, NtSPSC lacked a basic residue at position -6 relative to the phosphoserine. The presence of a basic residue at this position has been shown to be an important



**Figure 1.** Phylogenetic analysis of full-length SPS protein sequences from tobacco and *Arabidopsis*. An unrooted neighborhood-joining tree was constructed from Dayhoff distances using the Phylogeny Inference Package. Scale bar defines the branch length derived from neighbor. The AGI codes for the *Arabidopsis* sequences are as follows: AtSPS-chr5a, At5g20280; AtSPS-chr5b, At5g11110; AtSPS-chr1, At1g04920; and AtSPS-chr4, At4g10120.

**Table 1.** Comparison of NtSPSA to C protein sequences in the regions of the regulatory phosphorylation sites

Sequences were aligned using ClustalW (<http://ebi.ac.uk/clustalw/>). Residues matching the consensus sequences (Huber and Huber, 1996) are shown in bold (B, basic; H, hydrophobic). Spinach SPS (SoSPS) was included as a reference sequence.

Protein	Ser-158	Ser-229	Ser-424
Consensus	<b>XXBHXBXXSXXH</b>	<b>HXRXXXP</b>	<b>BHXBXS</b>
SoSPS	150-KGRMRRISSVEMM	224-LTRQVSAP	418-RMRRGVVS
NtSPSA	142-KGRLPRISSVETM	216-LTRQVSSP	410-RIKRNVVS
NtSPSB	149-RKRFRQNFNSLEV	221-FTRQIAST	415-RARRGVVN
NtSPSC	171-HHVISRINSVTQM	243-LTRQITSP	431-RRRRGVVS

positive recognition element for the phosphorylation of the motif by different protein kinases (Huang and Huber, 2001). The NtSPSB protein lacks both Ser-229 and Ser-424 involved in 14-3-3 binding and osmotic regulation, respectively. The phosphoserine contained in the 14-3-3 binding site has been altered to Thr in NtSPSC, while the protein kinase recognition element remains conserved (Table I).

**Expression of SPS Genes Varies Greatly in Different Tissues**

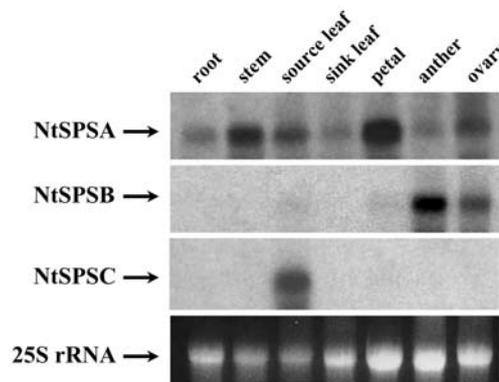
Northern-blot analysis was carried out on total RNA from different tobacco tissues using each NtSPS cDNA as a probe. All tissues examined expressed the NtSPSA mRNA, albeit to different levels (Fig. 2). Highest expression was found in petals and stems, while source leaves and ovaries showed intermediate expression. Significant levels of NtSPSA transcript were also found in heterotrophic tissues such as roots, sink leaves, and anthers. NtSPSB expression was largely confined to reproductive organs such as anthers and ovaries, although a very weak signal was also present in source leaves (Fig. 2). Interestingly, NtSPSC transcript seemed to be exclusively expressed in mature source leaves as there was no detectable expression for this isoform in any of the other tissues investigated (Fig. 2).

From these data it can be concluded that SPS families in tobacco are differentially expressed in different tissues. Furthermore, NtSPSA and C seem to represent the major leaf isoforms in tobacco.

**RNAi-Mediated Repression of NtSPSA and C in Transgenic Tobacco Plants**

To investigate specific roles of NtSPSA and C in leaf carbohydrate metabolism, their expression was decreased, either separately or in combination, using an RNA interference (RNAi) approach. To differentially decrease selected SPS isoforms, RNAi was targeted against a region within the 3' end of the respective cDNAs sharing only 27% identity at the nucleotide level between the two isoforms. The RNAi cassettes, containing the SPS fragment in antisense orientation in front and in sense orientation behind a spliceable intron sequence, were transferred into a binary vector

behind the cauliflower mosaic virus 35S promoter yielding the constructs NtSPSAi and NtSPSCi. To down-regulate NtSPSA and C simultaneously, the two fragments used to create the isoforms-specific RNAi cassettes were fused by PCR and the resulting chimeric DNA fragment was assembled into a 35S-driven RNAi construct as described above, giving rise to the construct chiSPSi. All three constructs were used to generate transgenic tobacco plants via Agrobacterium-mediated gene transfer. Approximately 80 regenerated plants for each construct were transferred into the greenhouse. To screen the transformants for reduced levels of SPS expression, western-blot analysis was carried out using polyclonal antiserum raised against an SPS A-family member from potato (Reimholz et al., 1997). In wild-type tobacco plants, two bands in the 120- to 130-kD range were immunodecorated by the antibody, which is in close agreement with the expected size of the SPS protein. A strong reduction of the lower band was observed in 29 of the transgenic plants transformed with the NtSPSAi construct (data not shown). Despite the reduction of the lower band, the expression of the upper band was largely unchanged. Using the same antibody, transgenic tobacco plants harboring the NtSPSCi construct were screened. However, in this case no difference in the band pattern



**Figure 2.** Northern-hybridization analysis of SPS gene expression in tobacco. Total RNA was isolated from different tissues of tobacco plants harvested 5 h into the light period. Thirty micrograms was subject to gel-blot hybridization using a <sup>32</sup>P-labeled probe of the respective cDNA. Ethidium bromide staining of 25S rRNA serves as control for equal loading.

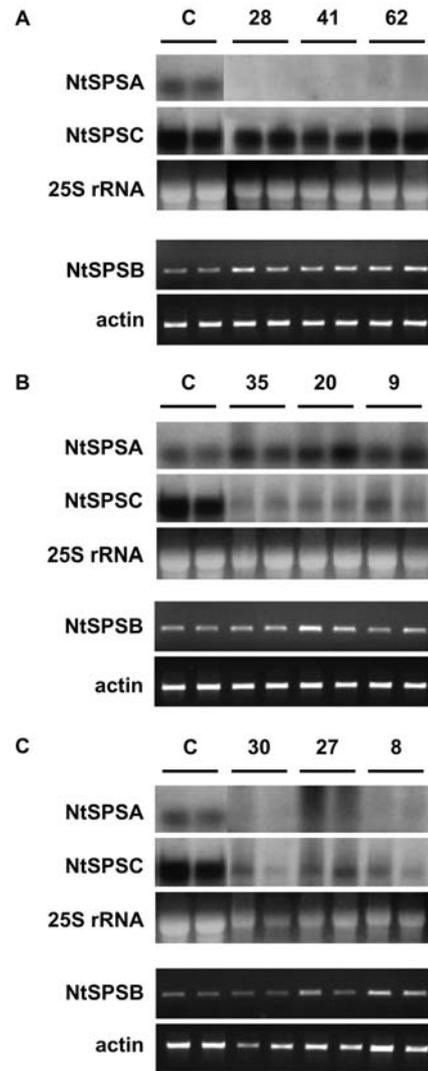
between wild-type and transgenic plants was observed, indicating that the antiserum might not recognize the NtSPSA protein. Therefore, northern-blot analysis was carried out on NtSPSCi plants to identify individuals with decreased NtSPSC mRNA level. As a result, 23 transgenic plants with strongly reduced NtSPSC expression could be identified (data not shown). To identify plants with simultaneous reduction of both SPS isoforms, transformants harboring the chiSPSi construct were initially screened by western blotting using the antiserum mentioned above. Out of 26 transgenic plants showing reduced NtSPSA protein level, eight were randomly chosen and further subjected to northern-blot analysis to demonstrate a concurrent reduction in NtSPSC expression. Semiquantitative reverse transcription (RT)-PCR carried out on cDNA prepared from these eight transgenic plants revealed that the expression of both isoforms was strongly but similarly reduced (data not shown).

Three lines from each transgenic genotype were chosen and the T1 generation was subjected to a detailed analysis. The selected transgenic lines along with a transgenic control line harboring a  $\beta$ -glucuronidase transgene under control of the cytosolic Fru-1,6-bisphosphatase (FBPase) promoter (Ebner, 1996) were germinated on kanamycin-containing medium and resistant seedlings were transferred to the greenhouse. All subsequent analyses were performed on plants 6 weeks after their transfer to the greenhouse.

### SPS Transcripts, Protein Level, and Enzymatic Activity in Selected Transgenic Tobacco Lines

The mRNA steady-state level of the three SPS isoforms was determined in all three genotypes of transgenic plants using either northern-blot analysis or semiquantitative RT-PCR. As expected, the steady-state level of NtSPSA was drastically reduced in NtSPSAi plants (Fig. 3A). Interestingly, the expression of NtSPSB was modestly induced in NtSPSAi transgenics while NtSPSC transcript levels remained unchanged as compared to the control specimen (Fig. 3A). NtSPSCi plants displayed a strong reduction of NtSPSC mRNA, however, in this case expression of NtSPSA was markedly up-regulated while NtSPSB transcript levels appeared to be unaffected (Fig. 3B). In chiSPSi plants, RNAi caused a strong reduction of both SPS isoforms targeted, while the expression of NtSPSB was induced in lines 8 and 27 (Fig. 3C).

In agreement with the strong reduction of NtSPSA transcript levels in NtSPSAi transgenic plants, NtSPSA protein amount was strongly reduced in the selected lines as compared to the control plants (Fig. 4). To investigate the influence of reduced SPS expression on enzyme activity, SPS  $V_{max}$  and  $V_{sel}$  (activity under limiting substrate conditions and in the presence of Pi) activity was determined in all three transgenic genotypes. As shown in Table II, only minor differences were found between NtSPSAi and NtSPSCi and the control plants, respectively, for both SPS activities. In

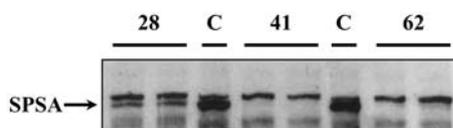


**Figure 3.** Northern-hybridization analysis of SPS gene expression in mature leaves of the three transgenic genotypes compared to control plants. To monitor NtSPSA and C expression, 30  $\mu$ g of total RNA was subject to gel-blot hybridization using a  $^{32}$ P-labeled probe of the respective cDNA. Ethidium bromide staining of 25S rRNA serves as control for equal loading. Semiquantitative RT-PCR was used to monitor NtSPSB expression. To this end, total RNA was isolated from leaves and used for RT-PCR as described in "Materials and Methods" applying 30 amplification cycles. Equal amounts of cDNA were controlled by amplification of a constitutively expressed *actin* gene (30 cycles). A, NtSPSAi transgenic lines 28, 41, and 62. B, NtSPSCi transgenic lines 35, 20, and 9. C, chiSPSi transgenic lines 30, 27, and 8.

contrast, a marked reduction of both SPS  $V_{max}$  activity (37%–41%) and  $V_{sel}$  activity (35%–38%) was observed in chiSPSi plants.

### Carbohydrate Levels in SPS-Silenced Transgenic Tobacco Plants

To determine how silencing of NtSPSA and C, separately or in combination, affects carbon metabolism, the levels of nonstructural carbohydrates were measured in mature leaves from all three transgenic



**Figure 4.** Western-blot analysis of NtSPSA protein level in selected NtSPSAi transgenic lines. Total protein was isolated from mature leaves 5 h after illumination. Thirty micrograms of total protein was separated by SDS-PAGE, transferred onto a nitrocellulose membrane, and immunodecorated using a polyclonal antiserum directed against SPS A from potato. The band representing NtSPSA is indicated by an arrow.

genotypes 5 h after illumination. In NtSPSAi transgenic plants, neither soluble sugars nor starch levels were altered when compared to the control plants (Table III). While the levels of soluble sugars in NtSPSCi plants also remained unchanged, starch content was increased by a factor of 3 to 8 in these plants (Table III).

Based on northern-blot analysis it can be assumed that from the SPS genes investigated in this study, NtSPSA and C constitute the major leaf isoforms of SPS. Nevertheless, in accordance with the rather slight effect on SPS enzyme activity, Suc content was largely unaffected in both NtSPSAi and NtSPSCi plants. One possible explanation for this phenomenon could be that loss of one isoform is compensated by one or more other isoforms. If this was to be true, we expected an effect on Suc synthesis in chiSPSi plants. As shown in Table III, Suc content in chiSPSi plants was indeed reduced by 12%, 36%, and 45% in lines chiSPSi27, chiSPSi30, and chiSPSi8, respectively, indicating that simultaneous silencing of the two major leaf isoforms of SPS in transgenic tobacco limits photosynthetic Suc biosynthesis. In addition to the reduction in Suc contents, chiSPSi plants also showed a drastic increase in starch contents (4- to 8-fold) as already observed in NtSPSCi plants (Table III).

Despite the changes in carbohydrate metabolism, silencing of SPS had no effect on the photosynthetic rate or on phenotype in any of the transgenic genotypes as compared to control plants (data not shown).

### The Accumulation of Starch in NtSPSCi Plants Was Due to a Reduced Rate of Starch Mobilization during the Night

Steady-state levels of leaf carbohydrates reflect the balance between synthesis and degradation. Therefore, the starch accumulation in NtSPSCi plants could either be brought about by an increased partitioning of newly fixed carbon into starch synthesis, or by a reduced rate of starch mobilization during the night, or both. To address the question of whether the alterations in carbon partition during photosynthesis cause the accumulation of starch, we investigated the fate of recently assimilated CO<sub>2</sub> in NtSPSCi plants as compared to NtSPSAi plants and control plants, respectively. To this end, leaf discs from dark-adapted plants were incubated in a leaf-disk electrode under saturating light and <sup>14</sup>CO<sub>2</sub> for 20 min. Following ethanol

extraction, radiolabel was determined in the insoluble and soluble fraction. As shown in Table IV, incorporation of <sup>14</sup>C in the insoluble fraction (mainly representing starch) was unchanged in both transgenic genotypes as compared to the control, indicating that silencing of neither of the two SPS isoforms had an influence on photosynthetic carbon partitioning. The soluble fraction was furthermore divided into neutrals (mainly Suc), anions (phosphorylated intermediates and organic acids), and cations (amino acids). As before, no differences were observed concerning the incorporation of radiolabel into these fractions between transgenic plants and the wild type (Table IV).

To determine whether altered starch degradation rates might be responsible for the elevated starch accumulation, NtSPSCi plants along with control plants were subjected to a prolonged dark period and, subsequently, leaves were stained for remaining starch with iodine. In leaves of control plants, starch was totally degraded after 24 h of dark treatment while leaves from NtSPSCi plants still contained sufficient starch to stain blue in the presence of iodine (Fig. 5), indicating they were unable to efficiently mobilize their transitory leaf starch during the dark.

The diurnal changes in starch content were investigated in NtSPSCi as well as in NtSPSAi plants (Fig. 6). NtSPSAi tobacco plants exhibited a similar diurnal pattern of starch content as the wild type (Fig. 6A). The diurnal changes in starch content still operated in NtSPSCi transgenic plants, indicating that the starch-excess phenotype is not accompanied by a total absence of starch turnover. However, the starch level was substantially elevated throughout the entire diurnal period (Fig. 6B). This most likely is the result of a cumulative effect over a longer growth period,

**Table II.** SPS activities in leaves of the three genotypes of transgenic tobacco plants

SPS activities were determined in leaf samples of 6-week-old plants. Samples were taken 5 h into the light period. SPS activity was measured under both optimal ( $V_{max}$ ) and limiting ( $V_{sel}$ ) assay conditions. The control (\*) plants were grown alongside with NtSPSAi transgenic tobacco plants, and the control (\*\*) plants were grown alongside with NtSPSCi and chiSPSi transgenic tobacco plants. Data represent the mean ( $\pm$ SD) of determinations on six individual plants per line.

Plant Line	SPS Activity ( $\mu\text{mol min}^{-1} \text{m}^{-2}$ )		
	$V_{max}$	$V_{sel}$	$V_{sel}/V_{max}$
			%
Control*	24.8 $\pm$ 4.2	5.4 $\pm$ 1.5	21.4 $\pm$ 2.4
NtSPSAi28	19.7 $\pm$ 2.6	5.2 $\pm$ 1.2	26.0 $\pm$ 3.5
NtSPSAi41	23.7 $\pm$ 5.3	4.4 $\pm$ 1.1	18.1 $\pm$ 3.1
NtSPSAi62	19.2 $\pm$ 4.4	5.8 $\pm$ 1.5	25.5 $\pm$ 3.7
Control**	33.3 $\pm$ 2.5	9.2 $\pm$ 0.4	27.7 $\pm$ 1.5
NtSPSCi9	29.4 $\pm$ 1.8	7.5 $\pm$ 0.7	25.4 $\pm$ 2.7
NtSPSCi20	32.6 $\pm$ 2.1	8.1 $\pm$ 0.8	24.9 $\pm$ 2.6
NtSPSCi35	27.3 $\pm$ 0.7	7.3 $\pm$ 0.5	26.7 $\pm$ 1.4
chiSPSi8	21.0 $\pm$ 1.5	6.0 $\pm$ 0.5	28.7 $\pm$ 1.7
chiSPSi27	20.6 $\pm$ 1.7	5.9 $\pm$ 0.7	28.5 $\pm$ 1.8
chiSPSi30	19.7 $\pm$ 1.0	5.7 $\pm$ 0.4	28.9 $\pm$ 1.4

**Table III.** Carbohydrate levels in leaves of three genotypes of transgenic plants compared to control plants

Leaf samples were harvested from 6-week-old plants after 5 h exposure to light. Concentrations are given in  $\text{mmol m}^{-2}$  hexose equivalents. The control (\*) plants were alongside with NtSPSAi transgenic tobacco plants, and the control (\*\*) plants were alongside with NtSPSCi and chiSPSi transgenic tobacco plants. Values represent the mean ( $\pm$ SD) from at least five different plants per line.

Plant Line	Glc	Fru	Suc	Starch
Control*	1.76 $\pm$ 0.68	2.25 $\pm$ 0.33	1.88 $\pm$ 0.35	5.99 $\pm$ 1.48
NtSPSAi28	2.02 $\pm$ 0.91	2.22 $\pm$ 0.85	2.15 $\pm$ 0.38	5.44 $\pm$ 2.10
NtSPSAi41	1.05 $\pm$ 0.39	1.31 $\pm$ 0.41	1.53 $\pm$ 0.41	4.59 $\pm$ 1.31
NtSPSAi62	2.34 $\pm$ 0.68	1.96 $\pm$ 0.43	1.82 $\pm$ 0.36	6.92 $\pm$ 2.17
Control**	1.98 $\pm$ 0.93	2.13 $\pm$ 0.86	2.18 $\pm$ 0.50	3.97 $\pm$ 1.68
NtSPSCi9	2.29 $\pm$ 0.92	2.36 $\pm$ 0.67	1.83 $\pm$ 0.53	18.05 $\pm$ 14.64
NtSPSCi20	2.48 $\pm$ 0.76	2.55 $\pm$ 0.45	2.30 $\pm$ 0.49	36.51 $\pm$ 14.31
NtSPSCi35	1.90 $\pm$ 0.76	2.37 $\pm$ 0.49	1.93 $\pm$ 0.51	16.35 $\pm$ 8.23
chiSPSi8	1.14 $\pm$ 0.56	2.47 $\pm$ 1.06	1.19 $\pm$ 0.34	32.15 $\pm$ 10.26
chiSPSi27	2.17 $\pm$ 0.88	2.43 $\pm$ 0.53	1.90 $\pm$ 0.30	18.89 $\pm$ 8.19
chiSPSi30	2.07 $\pm$ 1.01	2.49 $\pm$ 0.66	1.40 $\pm$ 0.32	36.90 $\pm$ 14.79

because the transgenics accumulate more starch in their leaves in comparison to the control specimen.

Recent evidence shows that maltose serves as a major metabolic intermediate in the conversion of starch to Suc during the night (Chia et al., 2004; Lu and Sharkey, 2004; Niittylä et al., 2004; Weise et al., 2004, 2005). To determine whether the impaired ability of NtSPSCi plants to mobilize leaf starch during the night would lead to an accumulation of primary products of starch breakdown, maltose levels were determined in NtSPSCi plants as well as in NtSPSAi plants toward the end of the dark period. It was found that maltose levels in NtSPSCi plants were elevated by a factor of 2 to 3 as compared to the control (Fig. 7B), further indicating the starch breakdown per se is operating in these plants but further metabolism of the primary products of this process is restricted. In contrast, maltose levels in NtSPSAi plants remained unchanged as compared to the control plants (Fig. 7A). This is in keeping with the unaltered starch content in these plants.

#### NtSPSC Transcript Levels Undergo Diurnal Changes

To learn more about the reasons of the different metabolic phenotypes observed in NtSPSAi and NtSPSCi transgenic tobacco plants, the expression of the three NtSPS genes was investigated in wild-type

tobacco plants throughout the diurnal cycle (Fig. 8). Northern-blot analysis revealed that NtSPSA and B transcript levels were largely independent of light/dark conditions. In contrast, the steady-state level of NtSPSC mRNA displayed a pronounced diurnal oscillation, exhibiting a doubling in signal intensity between the end-of-light period and the end-of-dark period. This result is consistent with the biochemical data from NtSPSCi plants and strongly supports the notion that NtSPSC is the major SPS isoform operating in the night path of Suc synthesis within a source leaf.

## DISCUSSION

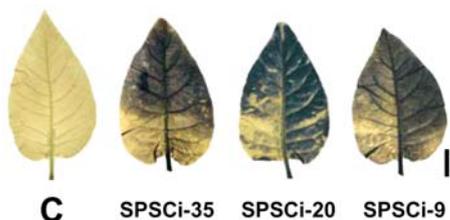
### SPS Gene Families Are Differentially Expressed in Tobacco

We show here that tobacco expresses at least three different SPS genes, denoted NtSPSA, NtSPSB, and NtSPSC, each representing one member of the three SPS gene families present in dicot plants (Langenkämper et al., 2002). RNA-blot analysis revealed that each SPS isoform had a distinct but overlapping expression pattern. NtSPSA was expressed in all tissues examined, although to a different extent. Constitutive expression has also been reported for A-family members from sugarcane (*Saccharum officinarum*; Sugiharto et al.,

**Table IV.**  $^{14}\text{CO}_2$  partitioning

Leaf discs from dark-treated tobacco plants were enclosed in oxygen electrodes under saturated  $^{14}\text{CO}_2$  and light for 20 min. The incorporation of  $^{14}\text{C}$  into soluble and insoluble fractions was determined after hot ethanol extraction. The soluble extract is further fractionated using ion-exchange chromatography into neutral, anionic, and cationic fractions. The total incorporation is given in disintegrations per min and the other values are given in percentage of total incorporation. Values represent the mean ( $\pm$ SD) from five different plants.

Fraction	Control	NtSPSAi41	NtSPSAi62	NtSPSCi20	NtSPSCi35
Total (dpm)	6,518 $\pm$ 1,855	5,339 $\pm$ 1,445	5,181 $\pm$ 1,573	8,374 $\pm$ 2,087	8,913 $\pm$ 3,079
Insoluble	39.2 $\pm$ 3.5	42.1 $\pm$ 4.1	42.7 $\pm$ 3.8	41.3 $\pm$ 3.5	42.5 $\pm$ 5.2
Soluble	60.8 $\pm$ 3.5	57.9 $\pm$ 4.1	57.3 $\pm$ 3.8	58.7 $\pm$ 3.5	57.5 $\pm$ 5.2
Neutral	46.7 $\pm$ 4.6	48.7 $\pm$ 9.5	41.6 $\pm$ 8.0	41.9 $\pm$ 4.3	40.6 $\pm$ 14.4
Anionic	8.7 $\pm$ 3.8	8.2 $\pm$ 2.0	9.9 $\pm$ 3.8	7.4 $\pm$ 0.5	7.8 $\pm$ 2.9
Cationic	6.9 $\pm$ 0.7	8.8 $\pm$ 1.4	9.1 $\pm$ 0.6	8.1 $\pm$ 0.8	7.9 $\pm$ 0.7



**Figure 5.** Visualization of starch accumulation in mature source leaves of NtSPSCi plants as compared to the control. After 12 h of illumination plants were kept in complete darkness for 24 h. Source-leaf blades were decolorized with hot 80% ethanol and subsequently stained with Lugol's solution. The eighth leaf (as counted from the top) is shown in each case.

1997), citrus (Komatsu et al., 1996), *Oncidium goldiana* (Li et al., 2003), and *Craterostigma plantagineum* (Ingram et al., 1997). All regulatory phosphorylation sites known for SPS are well conserved among A-family members from different species (Langenkämper et al., 2002). Together, these findings suggest that the A family might play a housekeeping role in plants. The multiple options for posttranslational modification of the A-family enzyme allow to rapidly adapting enzymatic activity according to environmental conditions without the need of de novo protein synthesis. This is of particular importance in actively photosynthesizing leaves where Suc synthesis has to be adjusted to the momentary rate of photosynthesis (Stitt et al., 1987), or during adaptation to osmotic stress (Toroser and Huber, 1997).

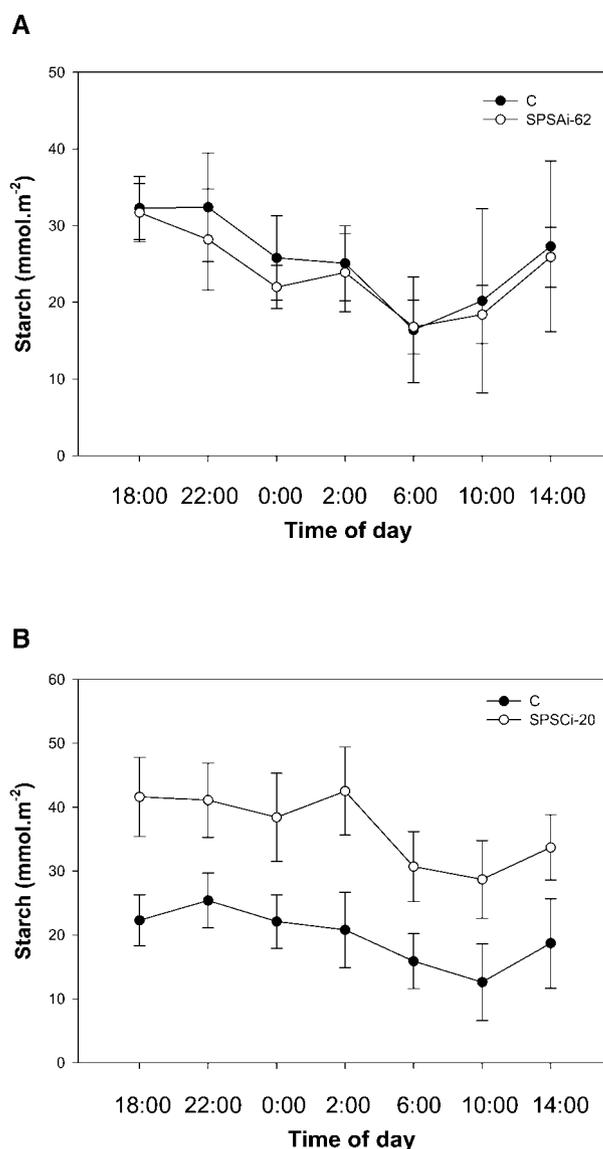
NtSPSB was only very weakly expressed in mature leaves, but high expression was found in anthers and ovaries. A similar expression pattern has been reported for a member of the rice (*Oryza sativa*) SPS B family that was highly expressed in immature inflorescences, and it has been proposed that in rice the SPS B isoform might be involved in the supply of carbon to developing pollen grains (Chávez-Bárceñas et al., 2000). Expression of the B isoform seems to be induced by stress-related environmental factors in kiwi (*Actinidia deliciosa*; Fung et al., 2003) and in *C. plantagineum* (Ingram et al., 1997), however, it is currently not known whether this holds also true for NtSPSB.

NtSPSC expression was found to be confined to mature source leaves, suggesting it has a source-specific function. This is broadly consistent with the expression of the C isoform from wheat (*Triticum aestivum*) being most abundant in the flag leaf blade and sheath, but also in germinating seeds as another type of source tissue (Castleden et al., 2004). A survey of the expression profiles of the SPS gene families from Arabidopsis using Genevestigator (<https://www.genevestigator.ethz.ch>; Zimmermann et al., 2004) revealed strong similarities between Arabidopsis and tobacco. The two Arabidopsis A-family members were found to be expressed in all organs, while the B-family member seems to be preferentially expressed in the reproductive organs, and the SPS C gene was mainly expressed in leaves (S. Chen, unpublished data). From the available data, there is a clear tendency that A- and C-family

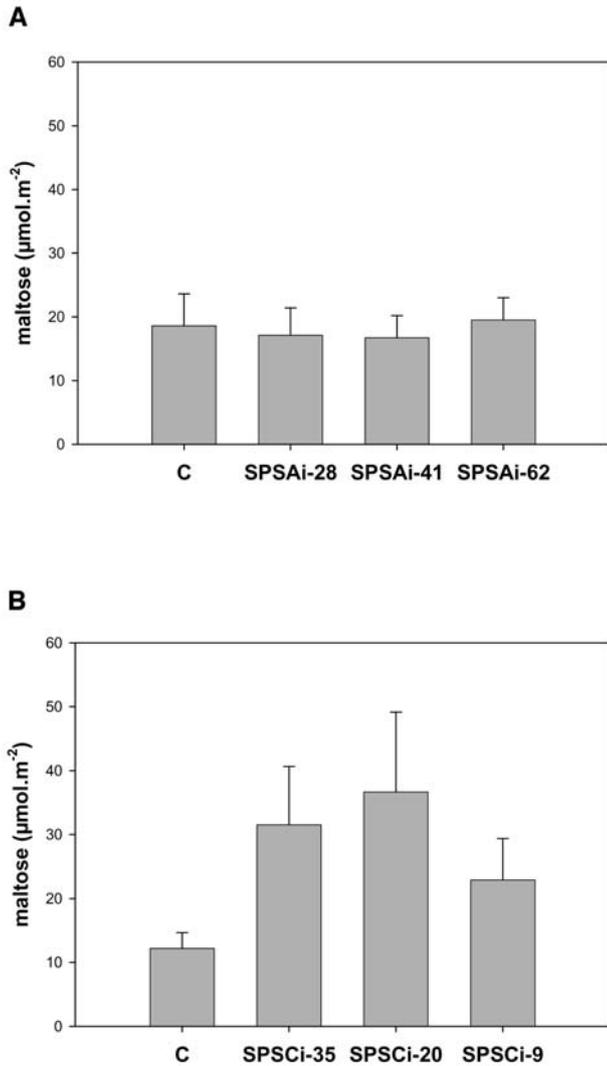
members constitute the major SPS isoforms expressed in leaves.

### NtSPSA and C Have Overlapping Functions in Source Leaf Carbohydrate Metabolism during the Day

Specific knock down of NtSPSA or NtSPSC had only slight effects on overall SPS activity and left Suc steady-state levels largely unaltered. This indicates that during the day A and C function can be mutually substituted by each other or alternatively by NtSPSB. This is further corroborated by the substantial reduction in overall SPS activity as well as Suc levels observed upon simultaneous repression of both isoforms in chiSPSi plants. Furthermore, induction of the B



**Figure 6.** Diurnal starch content in NtSPSAi (A) and NtSPSCi (B) transgenic tobacco plants. Starch content was measured in samples taken from mature leaves at various times over a diurnal cycle. The values represent the mean ( $\pm$ SD) from five individual plants.



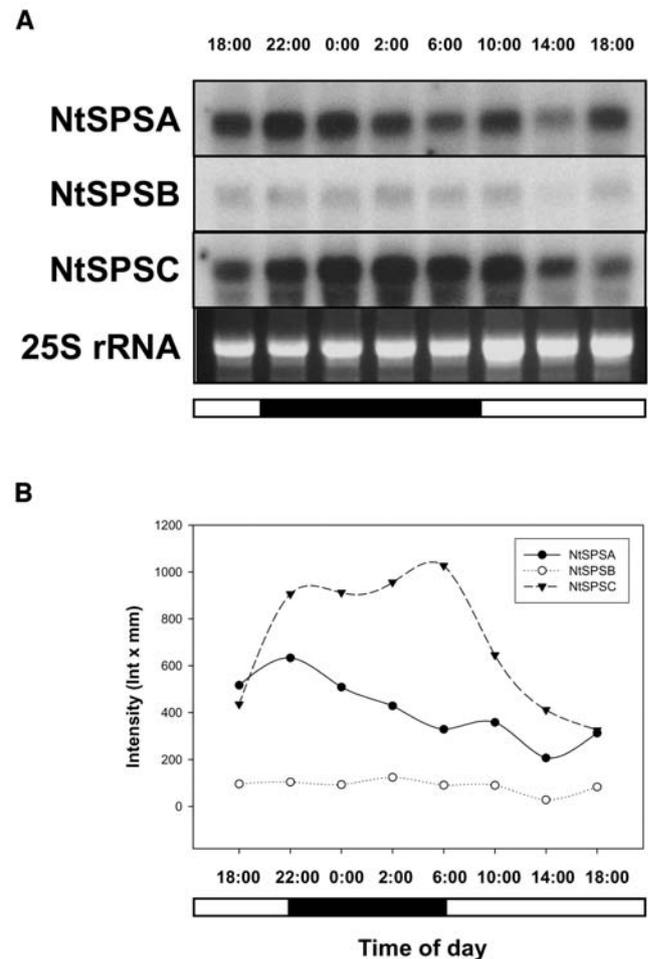
**Figure 7.** Maltose levels in NtSPSAi (A) and NtSPSCi (B) transgenic tobacco plants. Maltose levels were determined in samples taken from mature leaves at the end of the dark period. The values represent the mean ( $\pm$ SD) of five individual plants per line.

isoform as seen in NtSPSA-silenced plants and of the A isoform as observed in NtSPSC-silenced plants might compensate for loss of SPS A and C, respectively. Despite its low expression level in source leaves, it seems possible that the B family accounts at least in part for the residual SPS activity in chiSPSi plants. Given the presence of more than one A-family member in some plant species such as Arabidopsis (see Fig. 1), kiwi (Fung et al., 2003), or sugar beet (*Beta vulgaris*; GenBank accession nos. S55253 and CAC81823), the possibility remains that tobacco also contains an additional A isoform that is not targeted by the RNAi construct directed against NtSPSA and thus accounts for the residual SPS activity in chiSPSi plants. However, all attempts to identify such an additional isoform in tobacco by RT-PCR, cDNA library screening, or EST database search were so far unsuccessful.

Recently, transgenic Arabidopsis plants with reduced expression of the A-family SPS have been produced (Strand et al., 2000). In contrast to NtSPSAi plants, these plants exhibited strongly reduced SPS activity that also entailed decreased Suc levels. The reason for this difference between the two plant species is currently unknown, as the extent to which other SPS genes may have been affected by the Arabidopsis-antisense construct or, alternatively, may have been up-regulated to compensate was not investigated (Strand et al., 2000).

**NtSPSC Is the Key Isoform for Suc Synthesis during Starch Mobilization during the Night**

RNAi-mediated suppression of NtSPSC in transgenic tobacco plants lead to an increase in starch levels by a factor of 3 to 8, something that was not observed upon suppression of the tobacco A-family member. Several lines of evidence strongly indicate that starch accumulation in NtSPSCi plants was not due to



**Figure 8.** Diurnal changes of SPS expression in mature source leaves from tobacco. A, RNA gel-blot analysis of SPS mRNA level over a diurnal cycle. B, Quantification of the hybridization signals using a phosphor imager. The signals were normalized to 25S rRNA.

changes in carbon allocation during photosynthesis but rather to an impaired mobilization of starch during the night. No changes in photosynthetic carbon partitioning under saturating light and CO<sub>2</sub> conditions were observed in NtSPSAi and NtSPSCi plants, respectively, as compared to the wild type. This is consistent with the assumption that during the light period each isoform can compensate for the loss of the other and thus no restriction of Suc synthesis should occur. Interestingly, antisense inhibition of the SPS A family in *Arabidopsis* had also no effect on the ratio of Suc synthesis to starch synthesis in leaf-disc experiments (Strand et al., 2000). However, subjection of NtSPSCi plants to a prolonged dark period and subsequent iodine staining of the leaves clearly revealed that starch mobilization was impaired in these plants. The accumulation of maltose toward the end of the dark period further indicated that starch breakdown per se was operating in NtSPSCi plants but the subsequent metabolization of its breakdown products was impaired. Compared to mutant *Arabidopsis* plants lacking the maltose transporter MEX1 that displayed maltose levels at least 40 times as high as those of wild-type leaves (Nittylä et al., 2004), the 2-fold accumulation of maltose observed in NtSPSCi plants appeared relatively modest. Moreover, the diurnal changes in leaf starch content were similar in NtSPSCi plants and the wild type, although starch content in the transgenics was elevated at all time points investigated. Together, this indicates that suppression of NtSPSC does not lead to a total block of starch to Suc conversion during the night but rather limits this process to an extent that leads to the accumulation of starch over a longer growth period.

The changes in starch and maltose content observed upon silencing of NtSPSC were completely absent from NtSPSAi plants, showing that the A-family function is dispensable for maintaining Suc synthesis during the dark period.

Taken together, our data strongly suggest a crucial function for SPS C in Suc synthesis during starch mobilization at night. In contrast to the light period, C function cannot be completely substituted by other SPS families during the dark.

#### Differential Regulation of NtSPSA and C Accounts for Their Functional Specialization

During the day, Suc synthesis proceeds from triose-P exported from the chloroplast into the cytosol. Control of the pathway is achieved by coordinate regulation of cytosolic FBPase and SPS. According to the current understanding of SPS regulation, the protein's phosphorylation state is the largest determinant of catalytic activity (Huber and Huber, 1996; Winter and Huber, 2000). In the dark, SPS is phosphorylated and inactivated. Upon illumination, phospho-SPS is rapidly dephosphorylated and enzyme activity increases in parallel with a rising rate of photosynthesis. During the day, when photosynthetic end

products (i.e. Suc) accumulate within the leaf, SPS is gradually phosphorylated again and thus activity declines (Stitt et al., 1988; Huber and Huber, 1992, 1996). Furthermore, SPS is exquisitely sensitive to allosteric regulation, being activated by Glc-6-P and inhibited by Pi (Doehlert and Huber, 1983). Regulation of SPS by multiple mechanisms allows the rate of Suc synthesis to be increased in response to a rising supply of photosynthate and to be decreased in response to a falling demand of Suc. While photosynthetic Suc synthesis requires tight regulation to ensure effective operation of the reactions in the chloroplast stroma and thylakoids (Stitt et al., 1987), the situation appears somewhat different during the night when Suc synthesis is supplied from starch degradation. It is now generally accepted that the primary products of starch mobilization appearing in the cytosol are maltose and, to a lesser extent, Glc, which are further metabolized to hexose-P (Smith et al., 2005). Thus, the night path of Suc synthesis does neither involve triose-P as intermediates nor the enzymatic step catalyzed by cytosolic FBPase and it is not directly linked to the plastidic Pi pool. Depending on the plants species, carbon export during the night can be considerable, reaching 75% to 100% of the rates observed during the day (Heineke et al., 1994; Sweetlove and Hill, 2000). Maintaining a constant rate of Suc synthesis for export during the night could be achieved by an SPS isoenzyme that is regulated independently from the SPS isoform mainly operating during the day. In particular, it should be less prone to inactivation during the dark. As stated above, the phosphorylation site for light/dark regulation is conserved in NtSPSA, which is in agreement with its proposed role during the day. It has previously been shown that tobacco SPS during the day is regulated in analogy to the enzyme from spinach (Toroser et al., 1999). However, it is not clear whether NtSPSC is phosphorylated and thus regulated in an analogous manner. At least two kinases have been implicated in phosphorylation of SPS: a Suc nonfermenting 1-related protein kinase (SnRK1), which is related to the SnRK1 from yeast, and a calmodulin-like domain protein kinase (CDPK; Winter and Huber, 2000). A detailed analysis of possible variations in the phosphorylation consensus motif revealed that a Pro residue at position -4 relative to the phosphorylated Ser selectively inhibits phosphorylation by CDPK (Huang and Huber, 2001). While this Pro at position -4 is present in the phosphorylation motif contained in NtSPSA, it is absent from the NtSPSC polypeptide (Table I), which is in accordance with the assumption that in most dicot species, SnRKs, rather than CDPKs, may carry out light/dark modulation of SPS (Huang and Huber, 2001). Furthermore, it was shown that a basic residue at position -6 is a strong positive recognition element for phosphorylation and its substitution by a neutral residue significantly lowered phosphorylation of the motif. This suggests that phosphorylation of the motif contained in NtSPSC, which has a Val at position -6, is lowered relative to NtSPSA. If the two proteins were

phosphorylated as predicted they would presumably be responsive to different regulatory pathways and potentially regulated independently even within the same cell (Lunn and MacRae, 2003).

Another level of regulation where the SPS families clearly differ is at the point of transcription. While there is currently little evidence for transcriptional regulation of NtSPSA and B, mRNA steady-state level of NtSPSC showed pronounced diurnal changes. NtSPSC transcript levels peaked during the night, coinciding with the phase of starch mobilization (compare Figs. 6 and 8). Interestingly, a similar diurnal expression pattern has been observed for the Arabidopsis SPS C-family member (Harmer et al., 2000; Gibon et al., 2004).

This provides further evidence for the notion that SPS C is the key SPS isoenzyme involved in Suc synthesis during the dark period. However, this expression pattern is apparently different to that of other enzymes involved in the degradation of transitory leaf starch. In Arabidopsis the transcripts of several genes putatively involved in starch breakdown showed a coordinated decline in the dark followed by a rapid accumulation in the light (Smith et al., 2004). Despite the marked changes in their transcript levels, the amounts of some enzymes of starch metabolism do not change appreciably during the diurnal cycle and this lack of correspondence between RNA and protein has been taken as an indication for a control of the enzymes of starch metabolism at the posttranscriptional level (Smith et al., 2004). The reason for SPS C mRNA to follow a different phase than other enzymes of starch degradation is currently unclear, but it might reflect true transcriptional regulation of SPS C activity.

## CONCLUSION

Here we provide evidence that the three SPS gene families A, B, and C present in dicots are functionally distinct. In tobacco, at least one representative for each SPS family is expressed and the three genes have distinct expression patterns. Transgenic tobacco plants with selective down-regulation of the two major SPS isoforms expressed in leaves revealed a specific role for NtSPSC during starch mobilization at night. Future studies will have to elucidate differences in kinetic properties, posttranslational modifications, and protein-protein interactions of the different SPS isoforms to understand the biochemical basis for their functional specification.

## MATERIALS AND METHODS

### Transgenic Plants, Growth, and Maintenance

Tobacco plants (*Nicotiana tabacum* cv Samsun NN) were obtained from Vereinigte Saatuchten and grown in tissue culture under a 16-h-light/8-h-dark regime (irradiance 150  $\mu\text{mol quanta m}^{-2} \text{s}^{-1}$ ) at 50% humidity on Murashige and Skoog medium (Sigma) containing 2% (w/v) Suc. Plants in the greenhouse were kept in soil under a light/dark regime of 16 h light (25°C) and 8 h (20°C) dark.

### Cloning of SPS B and C from Tobacco

Standard cloning procedures were carried out as described (Sambrook et al., 1989). Two EST sequences from tobacco, CB329258 and BP527800, which encode potential SPS B- and C-family members, respectively, were identified in a GenBank query using the protein sequences of the Arabidopsis SPS B- (At1g04920) and C-family (At4g10120) members. To obtain the full-length sequence of the respective clones, the SMART RACE cDNA amplification kit (Clontech) was used according to the manufacturer's instructions.

Primers CS118 5'-GAAGATGGTGTGACTGATCTCGGAG-3' and CS119 5'-CTCCGAGATCAGTGTACACCATCTTC-3', respectively, were used in the 5'-RACE and 3'-RACE reactions to clone the missing sequence portion of NtSPSB. Primers CS25 5'-TTGGCACTTTGGCTCCTGGCGTGA-TGC-3' and CS26 5'-GCATCACGCCAGGAGCCAAAGTGCCAA-3' were designed to amplify 5' and 3' ends of SPS C, respectively. The entire predicted coding regions of SPS B and C were amplified by PCR using two pairs of primers (CS135 5'-AAGGATCCAAATGGCTGTAATGAATGGATAAA-3' / CS136 5'-ATGTCGACTCATCTAGACACTTGCTCAATGC-3' and CS38 5'-ATGGCGGAGAACGAATGGTTAAACG-3' / CS39 5'-TCACATGACCTC-TAACTTCTCCAAGGCTG-3', respectively), subcloned, and sequenced.

### Plasmid Construction and Plant Transformation

To generate the NtSPSA RNAi construct, a fragment of NtSPPA comprising nucleotides 2,565 to 3,165 was amplified by PCR using primers FB196 (5'-GGATCCTTTTGTAGTTGACTTGACTACTA-3'; a *Bam*HI site is underlined) and FB69 (5'-CGCAATTCGTCGACTTATCCTTTGAGTAC-3'; a *Sal*I site is underlined). The resulting DNA fragment was digested with *Bam*HI and *Sal*I before it was ligated in sense orientation into pUC-RNAi, a vector containing the first intron of the GA 20-oxidase gene from potato (*Solanum tuberosum*) flanked by a short polylinker sequence (Chen et al., 2003). The same fragment was inserted in antisense orientation into the *Bgl*III/*Xho*I sites of pUC-RNAi already carrying the sense fragment. The resulting RNAi fragment was excised by *Pst*I and cloned between the 35S cauliflower mosaic virus promoter and the octopine synthase terminator of the binary vector pBinAR (Höfgen and Willmitzer, 1990) using a compatible *Sbf*I site, producing the NtSPSAi construct. To generate the NtSPSCi construct, a fragment of NtSPSC comprising nucleotides 2,727 to 3,240 was amplified by PCR using primers CS21 (5'-AGGGATCCGATGCGTTGATTGTAGCAGTGAAG-3'; a *Bam*HI site is underlined) and CS22 (5'-ACGTCGACCATTTGTGAAGAAGCATCTCCG-TGG-3'; a *Sal*I site is underlined). The NtSPSCi construct was assembled as described above. To generate the chiSPSi construct, a chimeric fragment composed of NtSPSA (nucleotides 2,565–3,165) and NtSPSC (nucleotides 2,727–3,240) was amplified by PCR using primers FB196, CS28 (5'-CAAAG-GATAAGCGTTGATTGTAGCAGT-3'), CS29 (5'-CAAATCAACGCTTATC-CITTTGAGTACCC-3'), and CS22 according to Horton (1997). The resulting PCR fragment was used to assemble the chiSPSi construct as described above.

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

### Immunodetection of NtSPSA

Protein extracts were prepared by homogenization of leaf material in a buffer containing 25 mM HEPES, pH 7.0, 12 mM MgCl<sub>2</sub>, 0.5 mM EDTA, 8 mM dithiothreitol, 10  $\mu\text{M}$  phenylmethylsulfonyl fluoride, 0.1% Triton, and 10% glycerol. Protein content was determined according to Bradford (1976) with bovine  $\gamma$ -globulin as the standard. After heat denaturation, 50  $\mu\text{g}$  total protein was subjected to electrophoresis on a 10% (w/v) SDS-polyacrylamide gel and subsequently transferred onto nitrocellulose membrane (Porablot, Macherey and Nagel). Immunodetection was carried out using the ECL kit (Amersham Pharmacia Biotech) according to the manufacturer, using the rabbit antiserum raised against SPS from potato (Reimholz et al., 1994) and peroxidase-conjugated secondary antibody (Pierce).

### RNA Isolation and Northern Blot

Total RNA was extracted from tobacco leaf material as described by Logemann et al. (1987), and 30  $\mu\text{g}$  per sample were separated on a 1.5% (w/v) formaldehyde-agarose gel using conditions described by Sambrook et al. (1989). After electrophoresis, RNA was transferred to a nitrocellulose

membrane (GeneScreen, NEN Life Science Products) and fixed by UV cross-linking. Radioactive labeling of cDNA fragments was performed using the High Prime kit (Roche) and [ $\alpha$ - $^{32}$ P]-dCTP. Hybridization was carried out as described previously (Herbers et al., 1994) and signals were detected by exposure to Kodak x-ray films (Sigma).

### Semiquantitative RT-PCR

For RT-PCR experiments, 2.5  $\mu$ g of deoxyribonuclease-treated total RNA was reverse transcribed into cDNA with oligo(dT) (30-mer) using M-MLV[ $H^{-}$ ] RT (Promega). A fraction (about one-twentieth) of the first-strand cDNAs was used as a template for PCR with gene-specific primers in a volume of 100  $\mu$ L with 1 unit of Taq-polymerase (Takara), 20  $\mu$ M each dNTP, and 0.25  $\mu$ M of each primer. An initial denaturation step for 5 min at 95°C was followed by 25 to 35 cycles of 5 s at 95°C, 45 s at 55°C, and 1 min at 72°C. PCR products were separated on 1% (w/v) agarose gels containing ethidium bromide and visualized by UV light. Amplification of actin using primers 5'-ATGGCA-GACGGTGAGGATATTCA-3' and 5'-GCCTTTGCAATCCACATCTGTTG-3' served as an internal control.

### Measurement of SPS Activity

SPS activity was assayed by quantifying the fructosyl moiety of Suc using the anthrone test exactly as described by Baxter et al. (2003).

### Carbon Partitioning

The incorporation of  $^{14}C$  into tobacco leaf discs was performed as described by Quick et al. (1989). Leaf discs were incubated in an oxygen electrode (LD-2, Hansatech) under  $CO_2$ -saturated atmosphere, which was generated through the addition of 400  $\mu$ L of 2 M  $K_2CO_3/KHCO_3$ , pH 9.3, solution enriched with  $NaH^{14}CO_3$  (specific activity 0.14 MBq  $mmol^{-1}$ ). Samples were illuminated for 20 min using the beam of a slide projector and immediately frozen in liquid nitrogen.

### Determination of Soluble Sugars and Starch

Soluble sugars and starch levels were determined in leaf samples extracted with 80% (v/v) ethanol/20 mM HEPES, pH 7.5, as described (Stitt et al., 1989). Maltose was measured from the same extracts with HPLC using the conditions exactly as described by Börnke et al. (2001).

### Visualization of Starch in Tobacco Leaves

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

### Nucleotide Sequences

The nucleotide sequences reported in this paper have been lodged with GenBank/DBJ/EMBL under accession numbers DQ213015 (*NtSPSB*) and DQ213014 (*NtSPSC*), respectively.

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