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Sucrose phosphate synthase expression influences poplar phenology

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Summary The objective of this study was to manipulate the intracellular pools of sucrose, and investigate its role in regulating plant growth, phenology (leaf senescence and bud break) and fibre development. This objective was achieved by differentially expressing an *Arabidopsis* (*Arabidopsis thaliana* L. Heynh.) sucrose phosphate synthase (SPS) gene in hybrid poplar (*Populus alba* × *Populus grandidentata* Michx.), a model system for tree biology with substantial industrial relevance in the context of short rotation forestry and a target bioenergy crop. Phenotypic differences were evident in the transgenic trees, as both the timing of bud flush and leaf senescence were altered compared to wild-type (WT) trees. Tree height and stem diameter were similar in WT and in the AtSPS transgenic trees, however, there were differences in the length of xylem fibres. Elevated concentrations of intracellular sucrose in both leaf and stem tissue of the transgenic trees are associated with a prolonged onset of senescence and an advancement in bud flush in the following spring. The association among sucrose content, tree phenology and elevated SPS gene expression implicates both enzyme and product in regulating poplar developmental processes.

Keywords: carbohydrate metabolism, dormancy, fibre development, senescence, soluble sugar, sucrose.

Introduction

Sucrose is central to plant metabolism and the most dominant metabolite involved in the growth and development of plant cell walls. It is the primary product of photosynthesis and in vascular plants the most commonly translocated carbohydrate, serving an integral role as both a source of carbon and energy for non-photosynthetic tissues. When imported into sink tissues, the influx of sucrose is critical for the maintenance of cellular metabolism, cell wall biosynthesis and respiration, and this can be converted to starch for storage and use at a later time (Sturm 1999, Kutschera and Heiderich 2002, Canam et al. 2006). Furthermore, intracellular sucrose moieties also serve as the precursor for the synthesis of the raffinose oligosaccharide families (ROFs), which are alpha-galactosyl-substituted sucrose molecules that participate in carbon translocation and allocation in plants with symplastic phloem loading, as has been proposed of *Populus* (Turgeon and Medville 2004). The ROFs fulfill two important physiological roles in poplar, establishing sink strength in phloem loading for translocation and carbohydrate storage, and more importantly in stress physiology (biotic or abiotic). For example, ROFs have been shown to respond to cold (and drought) stress as 'antifreeze' agents and as osmotica (Haritatos et al. 2000).

Equally important, sucrose also serves as a signal molecule regulating gene expression (Smeekens 2000, Wiese et al. 2004), and consequently influences associated metabolic pathways and thus morphological development (Lunn and MacRae 2003). There is substantial information regarding the flux of sucrose in leaves in relation to photosynthesis, including kinetics and feedback inhibition data on most of the genes and proteins involved in the synthesis of sucrose. Transcriptionally, the genes [e.g., sucrose phosphate synthase (SPS) and invertase] encoding these enzymes often coincide with developmental state, especially in developing leaves transitioning from sink to source, as well as during the mobilization of carbohydrates during bud break, seed germination and fruit development. Sucrose synthesis is also strongly influenced by post-transcriptional events such as phosphorylation, which is known to influence the rate of synthesis, as well as protein–protein interactions (Lunn and MacRae 2003).

The reaction catalysed by SPS (EC 2.4.1.14), the synthesis of sucrose-6-phosphate from fructose-6-phosphate and UDP-glucose, is an important regulatory step in controlling sucrose synthesis in plants (Stitt et al. 1988). SPS plays a crucial role in carbohydrate metabolism by regulating the partitioning of carbon between starch production and carbohydrate accumulation in many physiological and developmental processes, including responses to water stress (Geigenberger et al. 1997), diurnal carbohydrate allocation within plants (Huber and Huber 1996, Chen et al. 2005),

and fruit (Laporte et al. 2001) and flower development (Baxter et al. 2003). Substantial research investigating the role of SPS in photosynthetic tissue has been conducted, and as such, many aspects of its function and regulation in these tissues are known (Huber and Huber 1996, Lunn and MacRae 2003). In contrast, the role of SPS in non-photosynthetic tissue, where sucrose import and degradation occurs, has received less attention and has only recently been more actively investigated (Park et al. 2007). SPS may serve two roles in non-photosynthetic tissues: the resynthesis of sucrose after import via apoplastic cleavage, or direct involvement in carbohydrate regulatory cycles in which sucrose or starch or both are degraded and sucrose is resynthesized via SPS from one or more products (Geigenberger et al. 1997) or both apoplastic cleavage and direct investment. Examples of non-photosynthetic tissues expressing SPS include developing endosperm in maize (*Zea mays* L.; Im 2004), potato tubers (*Solanum tuberosum* L.; Geigenberger et al. 1999), sugarcane stems (*Saccharum* spp. hybrids; Zhu et al. 1997) and kiwi roots (*Actinidia chinensis* Planch.; Fung et al. 2003).

Babb and Haigler (2000) showed a correlation between SPS activity and secondary cell wall formation in cotton (*Gossypium hirsutum* L.); observing that sucrose was synthesized in developing cotton fibres when glucose was the sole carbon source, while the activity of both SPS and cellulose synthase increased. Haigler et al. (2001) reported that increased SPS activity in cotton fibres was associated with the onset of secondary cell wall formation. Fibre length could be altered with elevated sucrose concentrations resulting from the over-expression of SPS in tobacco (*Nicotiana tabacum* L.; Park et al. 2007). However, despite the importance of sucrose to plant development and as a key regulatory signal, little information exists with respect to the effects of sucrose manipulation on growth and development of woody plants.

Research involving endogenous SPS in woody plant species has been underrepresented, in comparison to agricultural crops (cotton, maize and potato). However, there have been studies involving endogenous SPS expression levels in both leaf and xylem tissue. Egger and Hampp (1993) have demonstrated that mature needles (net sucrose exporters) displayed significantly higher SPS activities than developing needles (net sucrose importers), implying that SPS plays a role in generating sucrose for export in source leaves. In contrast to herbaceous plants, SPS activity in spruce needles decreased in the presence of ATP (Loewe et al. 1996), whereas ATP had a less dramatic effect on spinach leaf SPS (Huber et al. 1989). It was also apparent that the seasonal variation of spruce SPS activity was largely unaffected by protein phosphorylation, and is instead largely a function of enzyme abundance (Loewe et al. 1996). However, spruce needle SPS activity appears to differ from other tree species, as *Prosopis juliflora* (Sw.) DC. leaf SPS exhibits light activation (Sinha et al. 1997), which implies broadly that the kinetic properties of spruce SPS may be specific to Norway spruce or gymnosperms in general.

SPS activity has also been examined in the woody stem of both a gymnosperm, Scots pine (Uggla et al. 2001), and angiosperms, including *Poplar*, *Robinia*, *Salix*, *Acer* and *Fagus* (Hauch and Magel 1998, Magel et al. 2001, Schrader and Sauter 2002). *Robinia* SPS has been shown to display a strong correlation between activity and abundance, similar to the observations in spruce needles (Loewe et al. 1996). Additionally, SPS activity was shown to be greater in the middle and inner sapwood than in the bark and outer sapwood, indicating spatial variation in *Robinia*. Temporally, a relationship has been established demonstrating the highest activity in the winter months (Hauch and Magel 1998), which can be correlated with an accumulation of sucrose during the winter months (Magel et al. 1994) and illustrating the significant sucrose synthesis in parenchymous cells (Schrader and Sauter 2002). It has also been shown that the activation state of SPS in *Poplar, Acer, Salix* and *Fagus* is increased in response to chilling, providing further evidence of seasonal SPS control (Schrader and Sauter 2002). SPS activity has recently been linked with cambial development in early spring, suggesting a role in supplying sucrose to developing cells (Hauch and Magel 1998, Magel et al. 2001). These studies clearly highlight the critical role of SPS in the regulation of sucrose levels and carbon metabolism in general – at a spatial and temporal level in both stem and leaf tissue of tree species.

Herein, the effects of over-expressing an *Arabidopsis* (*Arabidopsis thaliana* L. Heynh.) family A SPS (AtSPS) gene in hybrid poplar (*Populus alba* L. × *Populus grandidentata* Michx.) were investigated to study the role(s) of SPS in tree phenology, growth and carbohydrate allocation over a complete growing season. Furthermore, the influence of SPS over-expression on stem tissue was evaluated in an attempt to better clarify the relationship between carbon allocation and xylem development in woody plants.

Materials and methods

Vector construct and plant transformation

Cloning of the *Arabidopsis* SPS gene and vector construct for plant transformation was carried out as previously described (Park et al. 2007). In short, the AtSPS gene (*At5g20280*) was targeted from the four known SPS genes in *Arabidopsis* due to its high levels of expression in all tissues (Langenkämper et al. 2002), and cloned using polymerase chain reaction (PCR) from total RNA using SPS gene-specific forward (5′-CGATTCTCGATCTTT GATCGTCCCACC-3′) and reverse (5′-CCGGCAGCT TATGTACGACGAAGTA-3′) primers. This PCR fragment was cloned into a pCR-Blunt II-TOPO vector (Invitrogen, Carlsbad, CA), and the nucleotide sequence

confirmed to be identical to the *At5g20280* GenBank sequence accession. The gene was then cloned into pSM2Ca and transformed into *Agrobacterium tumefaciens* (Smith & Townsend, 1907) strain EHA105 (Hood et al. 1993).

Hybrid poplar was transformed with *A. tumefaciens* EHA105, employing standard leaf disc inoculation. The binary plasmid (pSM2CaSPS and pSM4CLSPS) was inserted into EHA105 and the bacteria were incubated overnight in a liquid woody plant medium (WPM; McCown and Lloyd 1981) with 2% sucrose and 100 μM acetosyringone. Leaf discs were cut and cocultured with EHA105 for 1 h, blotted dry, plated onto WPM in 3% (w/v) agar and 1.1% (w/v) phytagel and supplemented with 0.1 μM each of α-naphthalene acetic acid (NAA), 6-benzylaminopurine (BA) and thiodiazurone (TDZ) (WPM + NAA/BA/TDZ). The explants were incubated for 3 days and transferred to WPM + NAA/BA/TDZ containing carbenicillin disodium (500 mg l^{-1}) and cefotaxime sodium salt (250 mg l^{-1}). After three additional days on selective media, the discs were transferred to WPM + NAA/BA/TDZ containing carbenicillin, cefotaxime and hygromycin (20 mg l^{-1}). Following two consecutive 5-week periods on this medium, viable shoot tips were transferred to WPM without antibiotics. Plants were confirmed as AtSPS transgenics by PCR screening of genomic DNA using gene-specific oligonucleotides (forward 5′-GGCTATCGTTCAAGATGCCTCTG-3′ and reverse 5′-AGGCCTCGCAAGGGCAAGTA-3′). All shoot cultures, including both transgenic and non-transformed wild-type (WT) trees, were maintained on solid WPM with 3% sucrose in GA-7 vessels at 22 °C under a 16-h photoperiod with an average photon flux of 40 μmol m^{-2} s^{-1}. Plants were maintained by transferring apical regions at 4-week intervals, until sufficient replicates were generated, which were then transferred to soil in the greenhouse for growth trials.

Plant growth analysis

Tissue culture plantlets were transferred to 7.5-l pots containing a 50% peat, 25% fine bark and 25% pumice soil mixture in the glasshouse and covered with ∼ 500 ml clear plastic vessels for 1 week to aid in acclimation. Each line, transgenic and WT, was represented by 10–16 individual trees. Trees were placed under a 16 h photoperiod, with supplemental overhead lighting when ambient intensity was < 300 μmol m^{-2} s^{-1}. Plant height (from plant apex to base) and stem diameter (measured 10 cm above the root collar using a calliper) were measured throughout the growing season (May–September), over two consecutive years (2005 and 2006). Developmental stages of tissues were standardized by employing a plastichron index, where leaf plastichron index PI = 0 was defined as the first leaf > 7 cm in length from the apex, and where PI = 1 is the leaf immediately below PI = 0. To establish the timing of senescence, the trees were divided into four equal sections from top to base, and the extent of senescence was calculated as the percentage of discoloration of leaves per section, and then averaged across trees. All trees were trimmed at the end of autumn, leaving a stem 1.5 m in height from the base. Bud flush frequency following spring was monitored, and the number of buds displaying leaves > 1 cm was recorded.

Quantitative RT-PCR

Total RNA was isolated in triplicate from PI = 5 and PI = 6 leaves, as well as the stem tissue 15 cm above the root collar. Tissue was ground in liquid nitrogen using a mortar and pestle, and extracted with TRIzol® reagent (Invitrogen) according to the manufacturer's instructions. An equal volume of phenol:chloroform:isoamyl alcohol (25:24:1), pH 6.7, was added to DNase-treated RNA. The samples were then vortexed and centrifuged for 5 min at 12,000*g*. Ten microlitres of 3 M sodium acetate (pH 4.5) and 200 μl of 100% ethanol were added to the supernatant, incubated at −80 °C for 1 h and centrifuged at 12,000*g* for 15 min at 4 °C. The RNA pellet was re-precipitated in 75% ethanol, centrifuged at 12,000*g* for 10 min at 4 °C and resuspended in RNase-free distilled water after air drying.

cDNA was synthesized using Superscript™ II First Strand Synthesis (Invitrogen) in a 20 μl reaction containing 1 μg of DNase-treated RNA with 0.5 μg dT_{16} oligonucleotides. An identical reaction without the reverse transcriptase was performed to confirm the absence of genomic DNA. AtSPS transcript abundance was quantified with Brilliant® SYBR® Green QPCR Master Mix (Stratagene, La Jolla, CA) on an Mx3000P™ PCR system (Stratagene) employing the forward and reverse primers: ASPS-F3 (5′-CCACAGTGGCAAAGTGATGATGGC-3′) and ASPS-R4 (5′-TCTGACCTCTCCAGTGATCCC-3′), respectively. Poplar β-actin (GenBank Accession No. AB025795), which served as an internal control, was amplified using forward and reverse primers: PtactF (5′-TGCTGAGCGATTCCGTTGC-3′) and PtactR (5′-GGGCTAGTGCTGAGATTTCC-3′), respectively. Thermocycler conditions for all real-time analyses were 95 °C for 10 min, followed by 40 cycles of 95 °C for 30 s, 55 °C for 1 min and 72 °C for 30 s. Data were analysed using Mx3000P™ Real-Time PCR system software (Stratagene). Transcript abundance of AtSPS was normalized to β-actin by subtracting the Ct value of β-actin from the Ct value of AtSPS gene, where $\Delta Ct = Ct_{AtSPS} - Ct_{\beta\text{-actin}}$. Transcript abundance of the AtSPS gene in WT and transgenic plants was obtained from the equation $(1 + E)^{-\Delta Ct}$, where E is the PCR efficiency, as described by Ramakers et al. (2003) which is derived from the log slope of the fluorescence versus cycle number in the exponential phase of each individual amplification plot, using the equation $(1 + E) = 10^{slope}$.

SPS assay

Leaf and cambial tissue from each of three different plants per transgenic line and the corresponding WT trees were

used for quantification of SPS activity. Frozen tissue samples were ground in liquid nitrogen with 1.8 ml of extraction buffer containing 50 mM HEPES–KOH (pH 7.4), 5 mM $MgCl_2$, 1 mM EGTA, 5 mM DTT, 2 mM aminocaproic acid, 0.5 mM PMSF, 0.1% Triton X-100 and 10% glycerol. Samples were then transferred to pre-chilled microcentrifuge tubes and centrifuged at 4 °C and 12,000*g* for 15 min; 1.5 ml of the supernatant was then desalted on a Econo-Pac® 10DG column (BioRad, Hercules, CA), of which 100 µl was immediately used for the SPS assay. SPS activity was assayed as described by Iraqi and Tremblay (2001) and Baxter et al. (2003). Samples were incubated for 20 min at 25 °C in 50 µl of reaction buffer (50 mM HEPES–KOH, pH 7.5, 20 mM KCl and 4 mM $MgCl_2$) containing 12 mM UDP-glucose and 10 mM fructose-6-phosphate (in a 1:4 ratio with glucose-6-phosphate). The reaction was terminated at 95 °C for 10 min with 70 µl of 30% KOH to destroy any unreacted hexose phosphates. The samples were then centrifuged at 4 °C and 12,000*g* for 5 min, and the reaction absorbance was measured at 620 nm following the addition of four volumes of 0.14% (w/v) anthrone reagent (in 14.6 M H_2SO_4). A standard curve (0–200 nmol sucrose) was used to calculate the absolute amount of sucrose-6-phosphate generated. Controls containing boiled protein extract were included to provide appropriate blanks. Total protein content was measured according to Bradford (1976).

Sucrose and starch analysis

Stems and leaf tissues were harvested and immediately frozen in liquid nitrogen and freeze-dried for 48 h. Tissue (20–50 mg) was ground with a mortar and pestle in liquid nitrogen, and incubated for 24 h at −20 °C with 4 ml of methanol:chloroform:water (12:5:3). The samples were centrifuged for 10 min at 5000*g* and 4 °C, and the supernatants were collected. The pellets were washed with 8 ml of methanol:chloroform:water (12:5:3), centrifuged for 10 min at 5000*g* and 4 °C, and the supernatants were pooled. An aliquot of 5 ml of distilled water was then added to the pooled supernatants and phases were partitioned, at which point 1 ml of the aqueous phase containing the soluble sugars was dried in a speedvac at 40 °C. The pellet was resuspended in 1 ml of nanopure water and filtered through a 4-mm nylon filter (0.45 µm). The soluble sucrose concentrations were quantified using anion-exchange HPLC (Dionex, Sunnyvale, CA) on a DX-600 equipped with a CarboPac™ PA1 column and an electrochemical detector. Sugars were eluted with water at room temperature at a flow rate of 1 ml min^{-1}. Each concentration was determined using regression equations from calibration curves that were derived from external standards.

Fibre quality analysis

To determine the fibre length, a portion of the lower stem of poplar trees (spanning PI = 15 and the root collar) was cut into representative samples of approximate dimensions of 2 mm × 2 mm × 30 mm, and incubated in Franklin solution (1:1, 30% peroxide:glacial acetic acid) for 48 h at 70 °C. The solution was decanted and the fibrous tissue was washed under vacuum with deionized water until a neutral pH was achieved. The samples were then resuspended in 10 ml of deionized water and diluted to obtain a count of 25–40 fibres per second on a Fibre Quality Analyzer (FQA; OpTest Inc., Hawkesbury, Ont.). All samples were run in triplicate.

Statistical analyses

All analyses were carried out using unpaired two-tailed *t* tests at 95% confidence.

Results

SPS transcript abundance

AtSPS transcript abundance was quantified and normalized to β-actin levels for both leaf and stem tissue in all transformed lines (Figure 1). No amplification product was observed in the WT tissues with the primer pair employed for RT-PCR as expected, and therefore all transcripts observed were due to the expression of the exogenous family A AtSPS transgene. AtSPS transcript abundance was similar among the lines when comparing specific tissues. When comparing the two promoters used to drive the expression of the AtSPS gene, the most significant differences were in leaf tissue, where 2×35S::SPS lines had greater

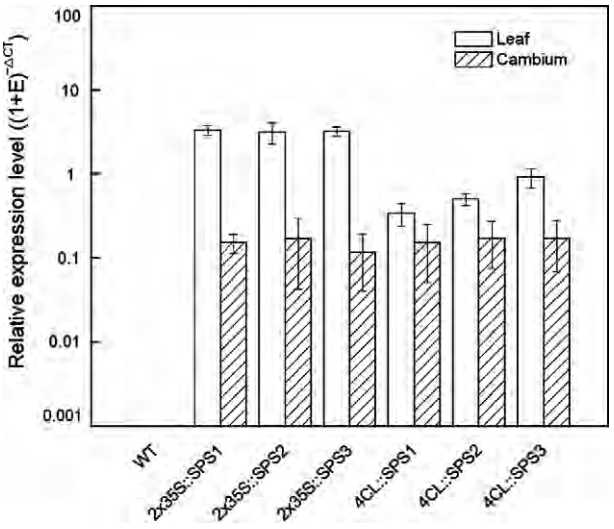

Figure 1. AtSPS transcript abundance in transgenic and WT hybrid poplar trees. RNA isolated from PI = 4 and PI = 5 leaves and cambial tissue was analysed using quantitative RT-PCR. AtSPS expression was calculated relative to β-actin expression. Data are mean values of three replicates from individual leaves or cambium (±SEM) taken from five individual plants from representative transgenic and WT trees (July 2006).

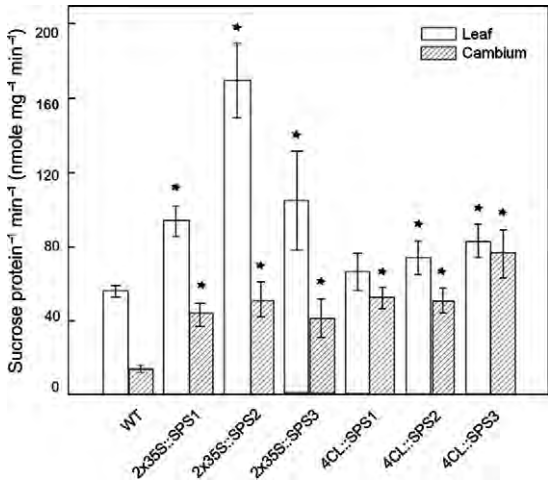

Figure 2. Total SPS activity in leaves (PI = 4 to PI = 5) and cambial tissue of transgenic and WT hybrid poplar trees. Data are mean values of three replicates from individual leaves or cambium (±SEM) taken from five individual plants from representative transgenic and WT trees (July 2006). Asterisk denotes significance at $P = 0.1$.

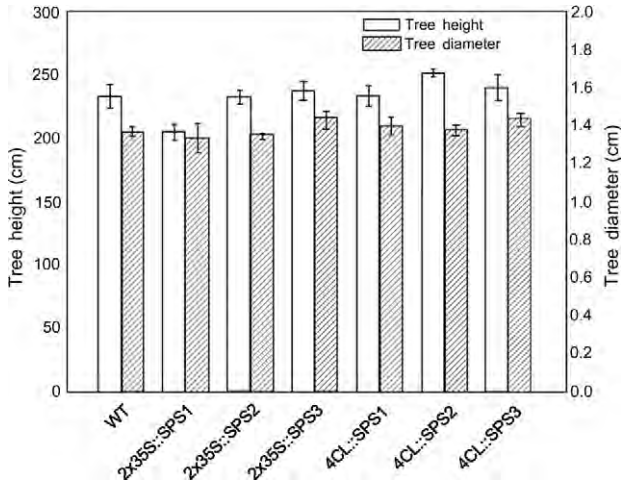

Figure 3. Average tree height and stem diameter (measured 10 cm above the root collar using a calliper) of transgenic and WT hybrid poplar trees, as determined in July 2006. Data are mean values of 10 clonally replicated individuals (±SEM) of each line, grown in a greenhouse for a complete year.

transcript abundance than the 4CL::SPS lines. Both promoter constructs showed equal levels of expression in cambial scrapings. The transcript abundance in the cambium was consistently lower than the corresponding leaf tissue from the same trees, regardless of the promoter.

SPS activity

All transgenic lines showed a clear statistical increase in SPS activity relative to the non-transformed WT trees (Figure 2), in both leaf and cambial tissue (exception being 4CL::SPS1 leaf tissue). The results of SPS enzyme activity quantification were in good agreement with the overall observed gene expression patterns. The 2×35S::SPS transgenic lines showed the most significant increases in SPS activity compared to the WT trees, in both leaf and cambial tissue. However, the increase in activity was greater in the leaf tissue, which is consistent with the transcript levels observed. Although there was an increase in SPS activity in both tissue types when SPS was driven by the 4CL promoter, a larger increase was apparent in the cambial tissue. However, there does not appear to be a correlation with increased SPS activity and growth (Figure 3).

Sucrose and starch content

Sucrose and starch contents were quantified throughout the active growing period (bud flush through to senescence). Leaf sucrose content in all WT trees appeared to be similar, with a gradual increase as the growing season proceeded. In contrast, despite the increase in exogenous SPS activity, all transgenic lines did not show substantial increases in soluble sucrose content in leaf tissue during active growth (May–August; Table 1). However, the cellular pools of sucrose were substantially increased in August through to senescence. The highest sucrose content was observed in September when chlorophyll content had already started to decrease. The leaf sucrose concentration remained con-

Table 1. Sucrose concentrations of transgenic and WT hybrid poplar leaf tissue. Data are mean values (±SEM) of five independent plants per line (July 2006). Bold denotes significance at $P = 0.1$.

	June	July	August	September	October
	Sucrose (μg mg^{-1} tissue)				
WT	4.02 (0.09)	4.39 (0.88)	4.55 (0.05)	5.14 (0.08)	5.23 (0.73)
2×35S::SPS1	4.29 (0.35)	3.35 (0.18)	4.00 (0.40)	6.79 (0.47)	**7.56 (0.49)**
2×35S::SPS2	4.84 (0.44)	4.03 (0.53)	4.01 (0.05)	**7.47 (0.42)**	7.14 (0.97)
2×35S::SPS3	4.06 (0.46)	3.98 (0.54)	4.29 (0.16)	**7.61 (0.53)**	6.07 (0.39)
4CL::SPS1	5.47 (0.66)	4.40 (0.12)	4.40 (0.56)	7.59 (0.38)	5.06 (0.36)
4CL::SPS2	4.70 (0.40)	4.63 (0.58)	4.74 (0.15)	**7.70 (0.54)**	**7.87 (0.77)**
4CL::SPS3	5.22 (0.74)	4.10 (0.51)	5.20 (0.42)	**8.93 (0.32)**	**9.25 (0.94)**

Table 2. Starch concentrations of transgenic and WT hybrid poplar leaf tissue. Data are mean values (±SEM) of five independent plants per line (July 2006). Bold denotes significance at $P = 0.1$.

	June	July	August	September	October
	Starch (μg mg^{-1} tissue)				
WT	0.86 (0.15)	1.52 (0.32)	2.68 (0.07)	2.37 (0.73)	3.44 (0.05)
2×35S::SPS1	1.10 (0.06)	**3.88 (0.57)**	1.02 (0.09)	2.25 (0.46)	2.85 (0.18)
2×35S::SPS2	1.14 (0.06)	2.65 (1.73)	3.07 (0.72)	2.39 (0.41)	2.51 (0.27)
2×35S::SPS3	0.98 (0.03)	2.80 (0.76)	2.87 (0.80)	4.15 (0.94)	4.04 (0.30)
4CL::SPS1	1.13 (0.17)	2.44 (1.05)	3.41 (1.12)	2.31 (0.46)	2.68 (0.50)
4CL::SPS2	0.93 (0.13)	2.02 (1.13)	2.69 (0.19)	4.44 (0.52)	**4.50 (0.16)**
4CL::SPS3	1.17 (0.12)	2.37 (0.35)	2.97 (0.64)	2.43 (0.25)	2.47 (0.33)

Table 3. Concentration of sucrose and starch in the cambium of AtSPS transgenic and WT hybrid poplar, which was harvested during the growing season (June 2006) and senescence (October 2006). Data are mean values (±SEM) of five independent plants per line. Bold denotes significance at $P = 0.1$.

	June		October	
	Sucrose (μg mg^{-1})	Starch (μg mg^{-1})	Sucrose (μg mg^{-1})	Starch (μg mg^{-1})
WT	12.42 (0.08)	1.16 (0.03)	2.03 (0.08)	2.19 (0.33)
2×35S::SPS1	12.19 (0.05)	0.88 (0.01)	**3.66 (0.14)**	2.56 (0.14)
2×35S::SPS2	**13.56 (0.06)**	1.15 (0.01)	**10.07 (1.61)**	**8.45 (1.45)**
2×35S::SPS3	**15.02 (0.12)**	0.98 (0.05)	3.36 (0.49)	3.04 (0.50)
4CL::SPS1	**14.20 (0.09)**	1.16 (0.06)	**4.13 (0.32)**	**3.45 (0.26)**
4CL::SPS2	12.36 (0.11)	**1.42 (0.01)**	**3.89 (0.47)**	3.09 (0.10)
4CL::SPS3	**17.89 (0.08)**	**1.42 (0.04)**	**4.63 (0.55)**	2.59 (0.16)

sistently higher in all transgenic lines compared to the WT trees from September onwards. Starch accumulation increased over the growing season, in all trees. The transgenic trees had slightly more starch than the WT throughout the growth trial (Table 2).

Stem sucrose and starch concentrations were also monitored (Table 3). Similar to leaf tissue, early in the growing seasons, there did not appear to be a substantial difference in sucrose content when comparing the transgenic and WT trees (transgenic levels were only slightly higher). However, later in the season, as with leaf tissue, all transgenic lines had significantly elevated pools of stem sucrose (in some cases double). A similar scenario was apparent when quantifying starch (Table 3).

Fibre development and senescence

Both WT and transgenic lines were examined twice a month from May to September to evaluate plant growth development. All transgenic lines grew similarly to the WT trees, with the exception of line 2×35S::SPS2, which had substantially higher enzyme activity in leaf tissue as well as elevated sucrose concentrations in cambium tissue. Transgenic plants had slightly longer fibres than the WTs (Table 4). Even though there was not a distinct difference in the phenotypes of the transgenic trees in whole plant growth, the tree phenology varied between the transgenic lines and the corresponding WT lines (Figures 4–6). In March (2007), when the poplar trees initiated bud break, four of the six transgenic lines had a significantly greater proportion of their buds flushed when compared to the WT trees. For example, on 27 March, the WT trees had on average 24% of their buds flushed, while some transgenic lines (4CL::SPS2) had greater than 80% of the buds flushed. In contrast, in the fall, when 80% of WT tree leaves had yellowed or dropped, as much as 50–90% of the AtSPS transgenic trees still retained green leaves (Table 5; Figure 5). Furthermore, the onset of spring flush was also

Table 4. Fibre length (length weighted fibre length) and coarseness of AtSPS transgenic and WT hybrid poplar stem tissue. Stem samples (PI = 14) were taken on December 6, 2006. Data are mean values (±SEM) of five independent plants per line. Bold denotes significance at $P = 0.1$.

Line	Fibre length (mm)	Coarseness (mg m^{-1})
WT	0.426 (0.009)	0.039 (0.001)
2×35S::SPS1	0.442 (0.012)	0.039 (0.002)
2×35S::SPS2	**0.464 (0.006)**	0.041 (0.001)
2×35S::SPS3	**0.462 (0.007)**	0.040 (0.002)
4CL::SPS1	0.450 (0.006)	0.041 (0.001)
4CL::SPS2	**0.473 (0.003)**	0.037 (0.001)
4CL::SPS3	**0.472 (0.009)**	0.040 (0.001)

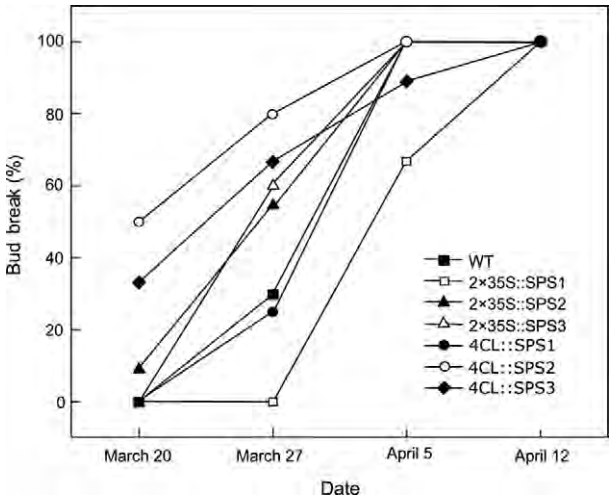

Figure 4. Timing of bud flush in AtSPS transgenic and WT hybrid poplar lines between 20 March and 12 April 2007.

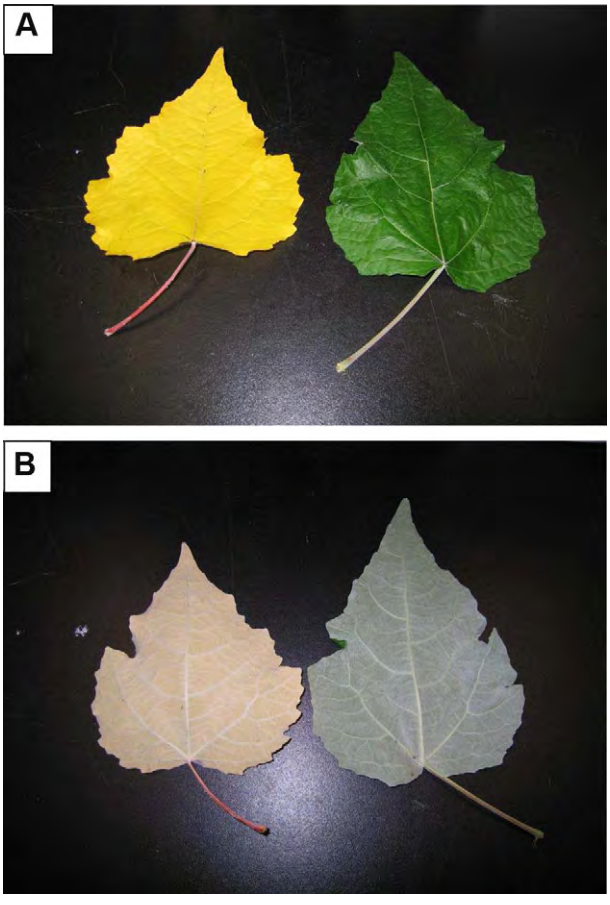

Figure 5. Photo demonstrating the delayed timing of senescence in transgenic AtSPS hybrid poplar. WT (left) and 2×35S::SPS2 (right): (A) represents an adaxial view, whereas (B) is the corresponding abaxial view on 6 December 2006.

affected by the action of the AtSPS gene insertion, where most of the transgenic trees flushed ~ 2 weeks earlier than the corresponding WT trees (Figure 6).

Figure 6. Photo demonstrating differences in timing of bud flush (March 2008). WT (left), 2×35S::SPS2 (centre) and 4CL::SPS2 (right).

Table 5. The extent of senescence (percentage of leaves displaying leaf discoloration) of AtSPS transgenic and WT hybrid poplar. Data are mean values (±SEM) of five independent plants per line (October 2007). Bold denotes significance at $P = 0.1$.

Line	Rate (%)
WT	79.2 (2.4)
2×35S::SPS1	**0.0 (0.0)**
2×35S::SPS2	**16.7 (2.6)**
2×35S::SPS3	**20.8 (4.3)**
4CL::SPS1	**42.5 (3.1)**
4CL::SPS2	**11.1 (2.7)**
4CL::SPS3	**42.9 (5.1)**

Discussion

Differential expression of a family A *Arabidopsis* SPS gene (SPS; EC 2.3.1.14) was achieved in both hybrid poplar leaf and stem tissue by over-expression of the AtSPS gene under the regulation of either the constitutive 2×35S or the vascular-specific 4CL promoter. Generally, higher transcript abundance was observed in leaves relative to the stem in all the transgenic lines evaluated. The elevated transcript abundance and the associated elevated enzyme concentrations that were manifested delayed senescence and earlier bud flush in the transformed trees, regardless of promoter. However, the over-expression of exogenous SPS did not appear to substantially affect the tree height and diameter, which were only slightly increased. Xylem fibre length of the transgenic trees was increased compared to the corresponding WT plant, which is likely a consequence of a longer overall growing period. During the active growing phase, cellular soluble sugar and starch concentrations were only marginally changed when comparing the transgenic SPS over-expressers to non-transgenic WT trees, however, a substantial increase in both compounds was clearly evident in the late fall, before senescence. Consequently, it appears that increased SPS

activity results in increased carbohydrate accumulation (sucrose and starch) in leaf and stem tissue later in the growing season, which ultimately affects tree phenology (bud flush and senescence).

There are many reports that demonstrate a relationship between SPS and plant growth, even though the exact role of SPS in plant growth and yield is not clearly understood. Haigler et al. (2000) observed altered fibre characteristics in cotton fibre cells over-expressing a spinach (*Spinacia oleracea* L.) SPS gene. Similarly, tomato (*Lycopersicon esculentum* L.) transformed with a maize SPS gene led to increased shoot and decreased root biomass (Galtier et al. 1993), while antisense *Arabidopsis* SPS showed a 50% reduction in plant growth (Strand et al. 2003). Additionally, SPS has been linked to a quantitative trait locus controlling growth and yield in rice (*Oryza sativa* L.), and SPS-transgenic rice has been reported to grow taller than non-transformed plants (Castleden et al. 2004). However, consistent changes among all transgenics are not always observed, as Laporte et al. (1997) reported increased biomass in only one of four SPS transgenic tomato lines. A similar lack of growth enhancement was reported in independent studies of tobacco (Baxter et al. 2003) and tomato (Worrell et al. 1991). In this study, hybrid poplar trees transformed with an *Arabidopsis* SPS gene did not generally have altered plant growth attributes (height and diameter), but did influence the fibre length, which is consistent with observations in cotton (Haigler et al. 2000) and tobacco (Park et al. 2007).

There are two transitional phases in the annual growth of deciduous trees: one is between bud break/flush and the termination of shoot extension and the other is between the termination of shoot extension and leaf abscission. Senescence is often regarded as an energy-requiring process in which ATP is needed for degradation, recycling and transportation of nutrients out of the cell. When photosynthetic capacity decreases, the mitochondria become the primary energy source, as the chloroplasts and other cell components are degraded (Yamashita 1990). By monitoring changes in the levels of key metabolites (ATP/ADP ratios and pools of starch and various soluble sugars), Keskitalo et al. 2005 observed the highest total carbohydrate content of aspen (*Populus tremula* L.) leaves in the middle of September, when chlorophyll content had already started to decrease. After this time, sucrose and starch rapidly decreased to shift the main energy sources of the cell from chloroplasts to mitochondria. Smart (1994) showed that respiratory energy is required during senescence for chlorophyll and protein degradation, protein synthesis and the translocation of carbohydrates, nitrogen and mineral nutrients. The results of this study are consistent with these studies, demonstrating that the highest total carbohydrate concentrations coincide with the onset of poplar senescence. The data of this study clearly demonstrate a difference in stem carbohydrate levels in the transgenic trees during senescence, regardless of promoter. It appears that these trees employ these soluble carbohydrate sources, via phloem transport from leaves, as a storage reserve for next spring and ultimately bud flush.

The initiation and duration of natural senescence is regulated by environmental as well as by internal factors. The most critical environmental factors are photoperiod, temperature and fertility of the soil. Autumn senescence in most trees is triggered by a reduction in photoperiod. Although the leaves do not continue to perform photosynthesis, phloem transport from source leaves is needed to export nutrients. Therefore, senescing leaves are still source leaves. Eventually phloem transport stops, and a protective layer is formed on the inner side of the petiole, a separation or abscission layer is formed proximal to it and finally the cell walls in the separation layer are gradually loosened and the leaf eventually falls (Roberts et al. 2002).

In temperate woody species, bud break is marked by the beginning of vegetative growth, which is characterized by intense physiological activity that creates a high demand for soluble carbohydrates (Maurel et al. 2004). Bud break and spring flush consume energy and reserve material stored in the stems of hardwood trees (Yamashita 1990). In this study, all transgenic lines flushed earlier than WT plants in the spring, consistently over two growing seasons (Figure 6). It appears that the entire growth development phase may have shifted as a consequence of the induced change in concentrations of stored and soluble carbohydrate available. Given that poplar is known to be an inherently strong and rapid mobilizer of starch reserves via the activation of amylases (Witt and Sauter 1994), the rapid bud flush observed in this study may be attributed to the increased availability of starch in the transgenic plants, which may have been hydrolysed more rapidly during spring flush. The flushing vegetative bud is a photosynthetically inactive sink, so it must import soluble sugars to meet its carbon and energy demands (Kelner et al. 1993, Maurel et al. 2004). Decourteix et al. (2008) suggest that before the formation of an efficient photosynthetic apparatus in spring, the vegetative bud grows mainly at the expense of carbohydrate reserves. Loescher et al. (1990) proposed that xylem sap is the main route by which the reserve-derived soluble sugars are transported to different sink organs during the leafless period, and more specifically Alves et al. (2004) reported that sucrose and hexoses comprise 95% of the total soluble sugars available in xylem sap during bud break in walnut. Moreover, Decourteix et al. (2008) indicated that xylem parenchyma sucrose transporters make a substantial contribution to the imported carbon supply of bursting vegetative buds. In this study, it may be that the over-expression of an exogenous SPS, not under the regulation of the native promoter, may be synthesizing sucrose in non-photosynthetic tissues, such as the xylem.

The over-expression of exogenous SPS in trees has the potential to delay leaf senescence and the timing of bud flush. It is less clear how trees entering senescence remobilize their nutrients such as nitrogen at the expense of photosynthetic yield. In short, the timing of autumn senescence

can be regarded as a delicate trade-off between the conflicting requirements for optimizing the carbon status of the plant (Keskitalo et al. 2005).

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