Tree Physiology 30, 1096–1110
doi:10.1093/treephys/tpq018

PART OF AN INVITED ISSUE ON TREE NUTRITION

Seasonal nitrogen cycling in the bark of field-grown grey poplar is correlated with meteorological factors and gene expression of bark storage proteins

HENNING WILDHAGEN,[1] JASMIN DÜRR,[1,2] BARBARA EHLTING[1,3] and HEINZ RENNENBERG[1,4]

[1] *Institute of Forest Botany and Tree Physiology, Chair of Tree Physiology, Albert-Ludwigs-University Freiburg, Georges-Koehler-Allee 053/054, 79110 Freiburg, Germany*

[2] *Present address: Institute of Botany, Biology II, Albert-Ludwigs-University Freiburg, Schaenzlestraße 1, 79104 Freiburg, Germany*

[3] *Present address: Department of Biology, University of Victoria, PO Box 3020, Stn CSC, Victoria, BC, V8W 3N5, Canada*

[4] *Corresponding author (heinz.rennenberg@ctp.uni-freiburg.de)*

Received October 29, 2009; accepted February 23, 2010; published online March 30, 2010

Summary Seasonal tree-internal nitrogen cycling is an important strategy for trees to achieve high efficiency in the use of nitrogen (N). Key processes of this N redistribution are autumnal leaf senescence and storage of released N as bark storage proteins (BSP) in perennial tissues. While the regulation of leaf senescence has been intensively analysed in trees, the coordination of the complementary storage processes is still poorly understood. Therefore, we ascertained relationships between physiological-level and molecular-level processes and environmental factors under natural conditions in the bark of *Populus* × *canescens*. We analysed amino-N concentrations, total soluble protein concentration and transcript abundances of BSP genes in the bark of field-grown *P.* × *canescens* harvested during two annual growth cycles. By correlation analysis and linear modelling, we assessed interactions between biological data and meteorological conditions. Day length correlated with *BSP* expression, and air temperature correlated strongly with total protein concentration ($r = -0.92$), γ-aminobutyric acid (GABA; $r = 0.76$) and arginine ($r = -0.70$). GABA and arginine also correlated significantly with total protein concentration and transcript abundances of *BSP* genes. We conclude that GABA and arginine potentially contribute to adjust storage processes in the bark of poplar trees to seasonal changes in environmental conditions.

Keywords: γ-aminobutyric acid, arginine, bark storage protein, day length, nitrogen storage, P. × *canescens*, *temperature*.

Introduction

Seasonal nitrogen cycling (SNC) is an essential strategy of deciduous trees to achieve high nitrogen (N) use efficiency. This process involves the degradation of proteins during autumnal leaf senescence, transport of released amino acids into parenchyma cells of bark and wood and subsequent synthesis of bark storage proteins (BSP) in autumn (Stepien et al. 1994). In spring, BSP are broken down into amino acids which are then translocated into flushing buds and leaves where they are used for de novo protein synthesis (Cooke and Weih 2005). Over the past years, numerous studies have addressed leaf senescence in trees (Bhalerao et al. 2003, Andersson et al. 2004, Keskitalo et al. 2005, Fracheboud et al. 2009), supporting the idea that a reduction of photoperiod triggers autumn leaf senescence. Still, the complementary storage processes in bark and wood have not received the same attention.

In the dormant phase, N is stored either as BSP or in the form of amino acids in parenchyma cells of perennial tissues and as part of the cycling pool in the xylem and phloem. During dormancy, arginine is the most abundant amino acid in the bark (Sagisaka 1974, Höllwarth 1976) and in xylem sap (Sagisaka 1974). In the remobilization phase in spring, glutamine is the dominant amino acid in bark and xylem sap (Sagisaka 1974, Sauter and van Cleve 1992, Schneider et al. 1994).

In trees, N is stored as vegetative storage proteins (VSP) in small vacuoles, which accumulate in parenchyma cells of bark and wood (Sauter and van Cleve 1992). The major VSP in *Populus* are BSP (Staswick 1994, Stepien et al. 1994), which accumulate in autumn and disappear in spring (Wetzel et al. 1989, Sauter and van Cleve 1990, Langheinrich and Tischner 1991). Especially 1-year-old branches accumulate high amounts of BSP (Sauter et al. 1989). Molecular analyses have identified three subfamilies within the *BSP* family, namely, *BSP*, *wound-inducible 4* (*WIN4*) and *poplar nitrogen-inducible 288* (*PNI288*). Gene expression analyses (Coleman et al. 1992, 1994, Lawrence et al. 2001) of *BSP*, *WIN4* and *PNI288* and digital expression profiling of expressed sequence tags of *Populus* (Cooke and Weih 2005) in-

dicate that genes from these subfamilies play overlapping but non-redundant roles in N storage and cycling: mRNA amounts of *PNI288* and *WIN4* decreased in response to short-day photoperiods and were associated with actively growing tissues, whereas the *BSP* subfamily was important for N storage during dormancy.

The accumulation of *BSP* mRNA at low temperature was shown by van Cleve and Sauter (1993). Coleman et al. (1992) demonstrated that both BSP accumulation and increases in *BSP* mRNA were induced by short-day photoperiods under controlled conditions in *Populus deltoides*. Black et al. (2001) identified day length as a critical factor for *BSP* mRNA accumulation in field-grown *P. deltoides*. Zhu and Coleman (2001a) found that the *Populus* × *canescens BSPA* promoter activity is phytochrome controlled and additionally regulated by exogenously supplied N compounds. This indicates the existence of a still unknown signalling pathway sensing the actual C/N partitioning of the tree and altering *BSP* expression by C and N metabolites.

Based on published sequences of genes belonging to the three subfamilies in *Populus*, Cooke and Weih (2005) inspected the Assembly 1.0 of the *Populus trichocarpa* genome database (http://genome.jgi-psf.org/poptr1/) and found that the *BSP* family in this species consists of seven loci: three members of the *BSP* and *WIN4* subfamilies and a single member of the *PNI288* subfamily. Still, gene expression analyses carried out for the *BSP* family so far did not attempt to discriminate between members of each subfamily (Cooke and Weih 2005).

In this study, we examined the SNC of bark tissue in field-grown grey poplar trees harvested during two annual growth cycles. We aimed to (1) isolate individual members of the three *BSP* subfamilies, (2) analyse seasonal expression patterns of *BSP* genes, (3) relate these transcriptional data to N metabolite profiles and (4) assess correlations of these biological data to meteorological factors.

Materials and methods

Plant material

Experiments were carried out with poplar plants (*P.* × *canescens* (Aiton) Sm. syn. *P. tremula* × *alba*), INRA clone 717 1B4. The plants were micropropagated as described in Strohm et al. (1995) and Noctor et al. (1996). In February 2005, after 4 weeks of in vitro cultivation, cuttings were transferred into a soil mixture containing commercial potting soil (Floradur; Floragard GmbH, Oldenburg, Germany), fine quartz sand and perlite (Perligran; Knauf Perlite GmbH, Dortmund, Germany) in equal volumes and grown in a greenhouse under long-day conditions. The trees were trimmed in autumn 2005 and then placed into the tree nursery of the Institute of Forest Botany and Tree Physiology in Freiburg, Germany (48°01′ N, 7°50′ E, 236 m a.s.l.). After overwintering under natural conditions, trees were planted into the ground in April 2006. At this time, the poplars were fertilized with 120 g per tree of a slow-release fertilizer (Basa cote Plus 12 M, COMPO, Münster, Germany). During dry periods in summer 2007, trees were watered regularly with tap water. Bark samples were collected from the end of August 2006 to September 2008. In the transition phases in spring and autumn, trees were harvested weekly, whereas larger sampling intervals were chosen during the rest of the year. For sampling, three to four trees were rotationally chosen out of 16 trees to maximize the intervals between two consecutive harvests of each tree. All samples were taken at 10 a.m. The bark between leaf Numbers 5 and 15 was sampled by separating it from the wood of one twig of three (August 2006 until August 2007) or four (September 2007 until September 2008) trees at each sampling date. During active growth, it was easy to separate the bark from the wood, whereas outside of the growing season, the bark was scratched off using a razor blade. Special care was taken to avoid strips of xylem being carried over to the bark samples. Dormant buds were removed before collecting bark material. The samples were immediately frozen in liquid nitrogen and stored at −80 °C until further analysis.

Meteorological data

Data sets of air temperature (*T*), relative humidity (rH), total radiation (*R*) and precipitation (*P*) in 1 h time resolution were provided by the German Weather Service (Deutscher Wetterdienst, DWD) from the weather station Freiburg Airport in the direct vicinity of the sampling site. Following Sjödin et al. (2008), the mean, standard deviation (SD), maxima (max) and minima (min) in three time periods before harvest (4, 24 and 48 h) were computed for temperature (*T*), relative humidity (rH) and total radiation (*R*). For precipitation (*P*), cumulative sums of 4, 24, 48, 96 and 168 h before harvest were calculated. These parameters are indexed by the abbreviations for the type of data and the statistical descriptor as introduced above and the corresponding time period before harvest. Temperature sums (TS) were calculated according to Richardson et al. (2006) with a threshold temperature of 1 °C. In addition, day length in minutes was gathered from the website of the United States Naval Observatory (http://aa.usno.navy.mil/data/docs/RS_OneYear.php).

Analysis of amino acids and NH_4^+

Frozen plant material was ground under liquid N_2 with mortar and pestle to a fine powder. Aliquots of ~0.1 g fresh weight (FW) of the homogenized material were extracted and analysed using an ultra-performance liquid chromatography system (Waters Corp., Milford, MA) as described in Luo et al. (2009).

Protein extraction and quantification

Extraction of total soluble protein from bark was done as previously described by Dannenmann et al. (2009). For the determination of protein concentration, 10 µl aliquots of the protein solution were pipetted in 96-well microtitre plates

Table 1. Accession numbers of cloned *P.* × *canescens* genes and their closest homologues of *P. trichocarpa.*

Name	Acc. no.	Closest *Populus trichocarpa* gene model		
		Transcript	% identity	Highest scoring alignment
PcBSP1+2	FJ943655	POPTR_0013s10370.1	89	Storage protein, bark—cottonwood (*Populus deltoides*)
PcBSP3	FJ943654	POPTR_0013s10350.1	89	Major storage protein (*Populus deltoides*)
PcPNI288	FJ943649	POPTR_0019s07690.1	84	VSP PNI288 (*Populus trichocarpa* × *P. deltoides*)
PcWIN4	FJ943648	POPTR_0013s07800.1	93	VSP win4.5—western balsam poplar × cottonwood (fragment)
PcEF1b	FJ372570	POPTR_0015s10630.1	76	Elongation factor 1-beta (*Pimpinella brachycarpa*)

The gene models of *P. trichocarpa* genome sequence version 2.0 closest to the cloned sequences of *P.* × *canescens* and their sequence identity to the corresponding *P.* × *canescens* sequences. In addition, the highest scoring alignment of the *P. trichocarpa* sequences to annotated GenBank sequences were determined by blast alignment searches (blastp, scoring matrix BLOSUM62) with the deduced amino acid sequences in the *P. trichocarpa* genome database. Acc. no., GenBank accession number; genes encoding: *PcBSP1+2*, *P.* × *canescens* bark storage protein 1 +2; *PcBSP3*, *P.* × *canescens* bark storage protein 3; *PcPNI288*, *P.* × *canescens* poplar nitrogen-inducible 288; *PcWIN4*, *P.* × *canescens* wound-inducible 4; *PcEF1b*, *P.* × *canescens* elongation factor 1-beta.

(Greiner Labortechnik, Frickenhausen, Germany) and 200 µl Protein Assay Dye Reagent Concentrate (Bio-Rad Laboratories, Inc., Hercules, CA) were added. The concentrate was diluted with four volumes distilled water beforehand. After 10 min incubation at room temperature, the absorption at 595 nm was detected using a microplate reader (Tecan Rainbow Thermo; TECAN, Crailsheim, Germany). All samples were measured in duplicate. For calibration, bovine serum albumin (Sigma-Aldrich, Munich, Germany) was used as a standard. Data for total protein concentration are only available for the second part of the first sampling period from March to August 2007 and for the whole second sampling period.

Isolation and expression analysis of poplar genes

For the isolation of total RNA from bark, the method described by Kolosova et al. (2004) was employed. The concentration and purity of RNA was determined with a nanospectrophotometer (NanoDrop® ND-1000, Peqlab, Erlangen, Germany). First-strand cDNA was synthesized from 2 µg total RNA with Superscript™ II Reverse Transcriptase (Invitrogen, Karlsruhe, Germany) and oligo-dT primers according to the manufacturers' instructions. cDNAs for genes involved in SNC from *P.* × *canescens* were cloned by reverse transcriptase PCR (RT-PCR) using Taq DNA polymerase (Eppendorf, Hamburg, Germany) following the standard protocol of the manufacturer. For cloning of *BSP* genes, the *P. trichocarpa* gene models proposed by Cooke and Weih (2005) were used as templates for the design of primers (primer sequences are given in Supplementary Table S1). Amplified DNA segments were separated on 1% agarose gels, extracted with the QIAquick gel extraction kit (Qiagen, Hilden, Germany), ligated into the pCR2.1 vector (Invitrogen, Karlsruhe, Germany) and transformed into competent *Escherichia coli* cells (INVF′α, Invitrogen, Karlsruhe, Germany). Plasmids were isolated (QIAprep Spin Miniprep, Hilden, Germany) and the inserts were sequenced (MWG Biotech AG, Martinsried, Germany). Using the obtained sequences, blast alignment searches (blastp, scoring matrix BLOSUM62) in the *P. trichocarpa* genome database (http://www.phytozome.net/poplar) were performed, revealing homology to gene models of *P. trichocarpa* (Table 1).

The quantitative analysis of seasonal gene expression was done by quantitative real-time reverse transcription PCR (RT-qPCR) using a LightCycler® 480 Real-Time PCR System (Roche Applied Science, Mannheim, Germany). Following treatment with DNase I (Fermentas, St. Leon-Roth, Germany), first-strand cDNA was synthesized from 2 µg total RNA using Superscript™ II Reverse Transcriptase (Invitrogen, Karlsruhe, Germany) and random dodecamer primers (500 nM final concentration in a 20 µl reaction). The design of primers for RT-qPCR was assisted by Primer3Plus (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi), aiming at locating forward and reverse primers in separate exons (exons inferred from the *P. trichocarpa* genomic sequence). Specificity of the selected primer pairs for the respective gene of interest was tested by RT-PCR, subsequent agarose gel electrophoresis of the amplified gene segments and sequencing of the amplified segments (MWG Biotech AG, Martinsried, Germany). The following primer sets were chosen (forward and reverse): *EF1b*: 5′-TGAGGATCTCTGGTGTCGAAG-3′ and 5′-GTCTCAGCAGATGGAGGAGTG-3′; *PNI288*: 5′-GAGCAACACGTCAAAGAGAG-3′ and 5′-TGTCTCATCCACACACTCTCG-3′; *WIN4*: 5′-CTCAACTGCCGATCAAGAAAG-3′ and 5′-CTCAAACGACGTCTCAGAAGC-3′; *BSP3*: 5′-TGAGCCTGACAGTGAAAATCC-3′ and 5′-TCCAGAACTTCCAAAGAAGACG-3′; *BSP1+2*: 5′-AGCCCTGACAGTGAAGACTCC-3′ and 5′-GCGTTCCCAAAATAGATGACTC-3′. Using these primer pairs, segments of the following lengths (in nucleotides) were amplified: *EF1b*, 100 nt; *PNI288*, 217 nt; *WIN4*, 241 nt; *BSP3*, 183 nt; *BSP1+2*, 178 nt.

For the determination of mRNA abundance, 10 µl reactions were prepared in 384-well plates (Multiwell Plate 384; Roche Applied Science, Mannheim, Germany) using the components of the LightCycler® 480 DNA SYBR Green I Master Kit (Roche Applied Science, Mannheim, Germany) as follows: 5 µl SYBR Green Master Mix, 1 µl primer pair solution (1.0 µM final concentration), 2.5–4 µl cDNA and PCR-grade water was added to 10 µl final volume. The pre-

pared multi-well plates were centrifuged (10 min, 3500 *g*, room temperature). After a hot start for 5 min at 95 °C, 42 cycles were performed with a 10-s melting step at 95 °C, a 15-s annealing step at 55 °C and a 15-s elongation step at 72 °C. Subsequently, a melting curve analysis (5 s 95 °C, 1 min 60 °C, ramping to 95 °C with a rate of 0.11 °C s^{-1}) was performed to verify that only a single PCR product was amplified. For a sample of the reactions, this analysis was confirmed by separating the reactions on 2% agarose gels. All samples were analysed in duplicate. An appropriate number of no-template, no-RT and no-primer controls was measured in each run. Using the 'Absolute Quantification' procedure of the LightCycler® 480 Software (version 1.2.0.169 or higher), transcript levels were calculated based on standard curves generated with serial dilutions of linearized plasmids containing an insert of the corresponding cDNA. Transcript levels were normalized against the quantity of RNA used for RT (Pfaffl 2004, Nolan et al. 2006) to obtain transcripts per microgram of RNA.

Statistical analysis

Statistical analyses were done with the Windows version 2.8.1 of R software (R Development Core Team 2008). For the calculation of Pearson's correlations and multiple linear regressions, data on transcript abundance and amino acids were log transformed to achieve normal distribution. The correlations between the data on transcript abundances and amino acid concentrations and between transcript abundances and meteorological parameters (TS; day length; mean, minimum and maximum of temperature; and total radiation each for the periods of 4, 24 and 48 h before harvest) were computed using the function 'cor.test'. To account for the increasing probability of false rejections of the null hypotheses raised by multiple tests of the significance of correlations, the *P*-values were Bonferroni corrected with the function 'p.adjust', method 'bonferroni'. Linear models were fitted with the function 'lm' and the Akaike Information Criterion (AIC), a criterion assisting in the selection of models balancing fit with model size, was calculated using the function 'extractAIC'. In addition to the coefficient of determination (r^2), the function 'lm' returns the adjusted r^2 (r^2_{adj}), which adjusts for the number of explanatory variables in the models. In contrast to r^2, adding explanatory variables will only increase r^2_{adj} if the new variable improves the fit of the model more than expected by chance. Thus, for models with an appropriate number of explanatory variables, r^2_{adj} will be close to r^2, indicating a good balance between model size and model fit. Normal distribution of the residuals was inspected by quantile–quantile plots (function 'qqnorm') of the residuals (see Supplementary Figure S1). Homogeneity of variance and linearity of the residuals was assessed by plotting the residuals over the fitted values (see Supplementary Figure S1). In order to allow an unbiased estimation of the model parameters, the residuals should not show a temporal autocorrelation. This was inspected by index plots of the residuals and by plots of successive residuals (see Supplementary Figure S2). In addition, Runs Tests (function 'runs.test', R package 'lawstat', Hui et al. 2008) were performed to test that the sequence of residuals was random (see Supplementary Figure S2).

Results

Identification of BSP genes

Based on an investigation of the *P. trichocarpa* genome sequence version 1.0, Cooke and Weih (2005) proposed three gene models belonging to the *BSP* subfamily. These gene models were used as templates for the design of oligonucleotide primers which were then used in attempts to clone the homologous sequences of *P.* × *canescens*. Owing to the high sequence identity between *PtBSP1* and *PtBSP2* (98.2% based on deduced amino acid sequence), it was not possible to design differentiating primer pairs for the cloning of the homologous sequences of *P.* × *canescens*. The *P.* × *canescens* sequence obtained with a primer pair complementary to both *PtBSP1* and *PtBSP2* was, therefore, named *PcBSP1+2*. A multiple sequence alignment with the algorithm ClustalW2 (http://www.ebi.ac.uk/Tools/clustalw2/index.html, BLOSUM scoring matrix, other settings default) of *PcBSP3* and the three sequences of *P. trichocarpa* exhibited the highest score between the *BSP3* genes of the two species. Blast alignment searches in the *P. trichocarpa* genome database (version 2.0) revealed 89% identity of the deduced amino acid sequences of *PcBSP1+2* and *PcBSP3* to *P. trichocarpa* gene models showing the best hits to *P. deltoides* (bark) storage proteins (Table 1). Similar to the *BSP* subfamily, Cooke and Weih (2005) identified three gene models belonging to the *WIN4* subfamily in the *P. trichocarpa* genome sequence version 1.0. For better discrimination between putative members of the *WIN4* subfamily of *P.* × *canescens*, the reverse primers used for cloning were designed to bind to the 3′UTR of the sequences. However, in multiple sequence alignments, the three cloned *P.* × *canescens WIN4* sequences showed highest scores among each other and not to any of the *P. trichocarpa* sequences, making it impossible to assign the *P.* × *canescens* sequences to individual gene models of *P. trichocarpa*. Therefore, we considered the three cloned *P.* × *canescens* sequences as one and named it *PcWIN4*. By a blast alignment search with the deduced *PcWIN4* amino acid sequence in the *P. trichocarpa* genome database (version 2.0), a sequence with 93% sequence identity was retrieved. The best hit of this *P. trichocarpa* sequence is a poplar VSP win4.5 (Table 1). Using the same approach, a sequence identity of 84% between the putative PNI288 sequences and of 76% between the putative EF1b sequences of *P.* × *canescens* and *P. trichocarpa* was found (Table 1). The phylogenetic relationship of deduced *Populus* BSP, WIN4 and PNI288 protein sequences and sequences from other organisms as constructed using the program 'Molecular Evolutionary Genetics Analysis' (MEGA4; Tamura et al. 2007) is displayed in Figure 1. Sequences of *Zea mays* (*Zm*), *Oryza sativa* (*Os*), *Vitis vinifera*

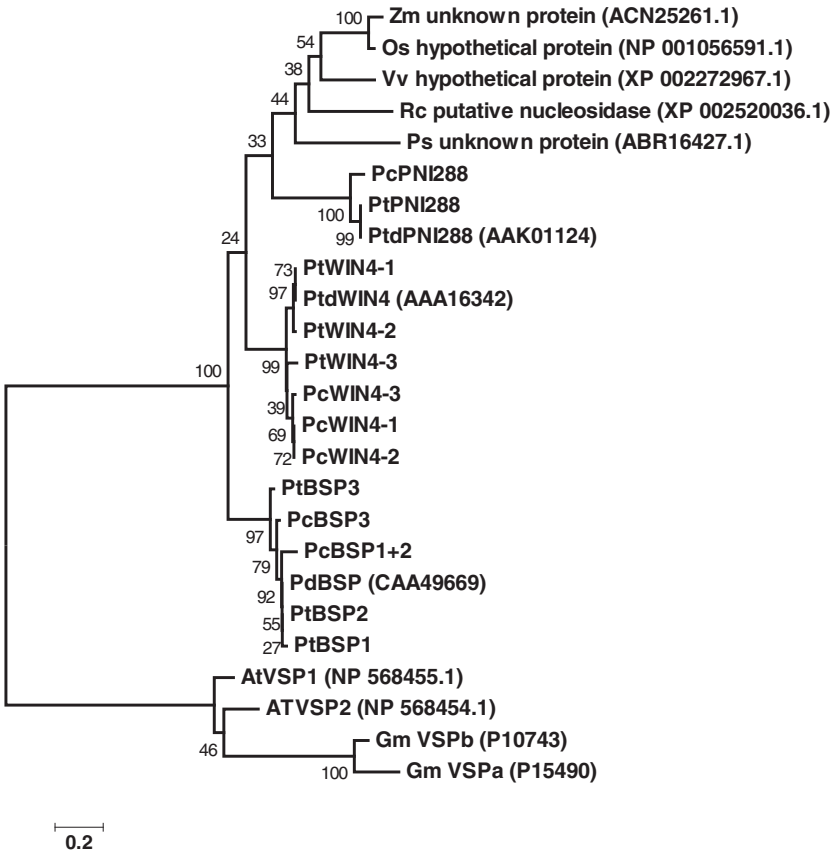

Figure 1. Phylogenetic tree of protein sequences of the *Populus BSP*, *PNI288* and *WIN4* subfamily. Sequences of *P. trichocarpa* (*Pt*) as described in Cooke and Weih (2005), *P. deltoides* (*Pd*), *P. trichocarpa* × *P. deltoides* (*Ptd*) and *P.* × *canescens* (*Pc*) and related sequences of *Z. mays* (*Zm*), *O. sativa* (*Os*), *V. vinifera* (*Vv*), *R. communis* (*Rc*), *P. sitchensis* (*Ps*), *A. thaliana* (*At*) and *G. max* (*Gm*) were included (in brackets, accession numbers of these sequence are provided). After multiple sequence alignments with the algorithm ClustalW, the trees were constructed using the program Molecular Evolutionary Genetics Analysis (MEGA4; Tamura et al. 2007), based on the neighbour-joining method (Saitou and Nei 1987). Next to the branches, results of bootstrap tests (Felsenstein 1985) are shown. As a measure of evolutionary distance, scale bars indicate the number of base substitutions per site, computed with the maximum composite likelihood method (Tamura et al. 2004). VSP, vegetative storage protein.

(*Vv*), *Ricinus communis* (*Rc*), *Picea sitchensis* (*Ps*), *Arabidopsis thaliana* (*At*) and *Glycine max* (*Gm*) were retrieved from the literature (for *G. max*, Mason et al. 1988) and protein blast analyses (http://blast.ncbi.nlm.nih.gov/Blast.cgi, protein blast against non-redundant protein sequences) using the query sequences *Pt*BSP1 and *Pt*WIN2 (Cooke and Weih 2005). This analysis confirmed that *PcBSP1+2*, *PcBSP3* and *PcPNI288* are the putative orthologues of *P. trichocarpa* genes, whereas *PcWIN4-1*, *PcWIN4-2* and *PcWIN4-3* appear to be paralogous sequences.

Meteorological conditions

From August 2006 to August 2007, the mean air temperatures ranged from 21.9 °C (6 September 2006) to −0.2 °C (21 December 2007) (Figure 2A). The climatological winter (December 2006 to February 2007) and spring 2007 (March to May 2007) were the warmest since the Year 1901. Spring temperatures in 2007 were 2.1–3 °C above the long-term reference (DWD 2008). From September 2007 to August 2008, the mean air temperatures ranged from 24.9 °C (2 July 2008) to −6.2 °C (19 December 2007) (Figure 2A). The climatological winter 2007/08 was warmer than long-term average, but to a lesser extent than the winter 2006/07. Temperatures in spring 2008 were only slightly above the long-term average (DWD 2009). Detailed plots of seasonal courses of air temperature, relative humidity, precipitation and total radiation are given in Supplementary Figure S3.

Total protein concentrations and amino compounds in the bark show substantial seasonal changes

The total protein concentration in bark increased from ~10 mg g^{-1} FW in September 2007, prior to visible leaf senescence, to ~19 mg g^{-1} FW when leaf abscission was completed (Stage II, Figure 2A) and further to levels between 20 and 25 mg g^{-1} FW during winter (Figure 2B). After a sharp decrease in April, at the time of bud break (Stages III and IV, Figure 2A), the concentration of total proteins was again in the range 5–10 mg g^{-1} FW.

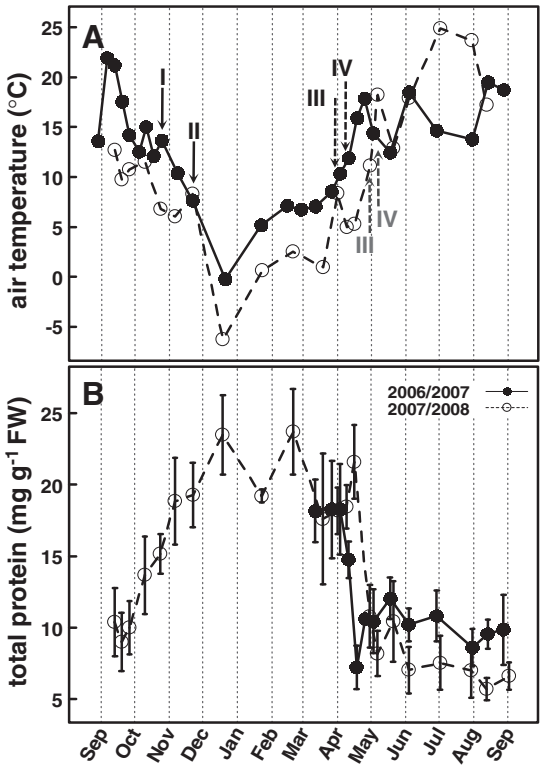

Figure 2. Seasonal courses of (A) mean air temperatures of a time period of 24 h before the time of harvest and (B) total protein concentration in the bark of field-grown *P.* × *canescens*. (A) Black solid arrows: I, first visible signs of leaf senescence; II, completion of leaf abscission in 2006 and 2007. Black dashed arrows: III, start of bud break; IV, end of bud break in 2007. Grey dashed arrows: III, start of bud break; IV, end of bud break in 2008. (B) Data shown are the means ± 1 SD of three (August 2006 to August 2007; closed circles, solid lines) or four (September 2007 to September 2008; open circles, dashed lines) replicate plants. Bark between leaf Numbers 5 and 15 of one twig per tree was harvested by carefully separating it from the wood. Time is expressed in months from September to September.

The concentrations of soluble amino acids in bark showed pronounced seasonal changes that were comparable in the two consecutive sampling periods. The four most abundant amino acids (glutamine, arginine, glutamate and asparagine), NH_4^+ and γ-aminobutyric acid (GABA) showed three general patterns of seasonality (Figure 3). Glutamine and glutamate showed two distinct peaks during the year, one in November, between the appearance of visible leaf senescence and completion of leaf abscission (Stages I and II, Figure 2A), and a second one in spring, coinciding with bud break. Asparagine had only one distinct peak in spring. A fundamentally different time course was found for NH_4^+, with increasing concentrations throughout dormancy, sharp drop-offs in mid- (2007) or end (2008) of April and low levels during summer. Similar to NH_4^+, arginine showed increasing concentrations from November, after completion of leaf fall (Stage II, Figure 2A), to mid-February and a steep decrease in April, during bud break. At their maxima, glutamine and arginine reached levels of 10–15 μmol g^{-1} FW, reflecting up to 270-fold changes when compared with their minimal values. The highest concentrations of glutamate and asparagine ranged from ~2 μmol g^{-1} FW (asparagine) to 3.5 μmol g^{-1} FW (spring peak of glutamate). The concentrations of the non-protein amino acid GABA, which is supposed to have a regulatory role in C/N metabolism (Bouché and Fromm 2004), increased in spring to ~0.5 μmol g^{-1} FW (2007) or 1 μmol g^{-1} FW (2008). After fluctuating slightly during the growing season, GABA concentrations decreased during leaf senescence to ~0.1 μmol g^{-1} FW in winter.

Seasonal changes in expression of genes involved in N storage in the bark

The transcript abundances of *PcWIN4*, *PcPNI288*, *PcBSP1+2* and *PcBSP3* were analysed throughout two annual growth cycles by RT-qPCR (Figure 4).

The mRNA abundances of *PcBSP3* and *PcBSP1+2* were highest among all analysed genes and displayed substantial seasonal changes. *PcBSP1+2* expression started to rise in September and reached a maximum in late November when leaf fall was completed, with absolute values of 2×10^9 copies per microgram of RNA. After a first decrease in December, transcript abundance remained high throughout winter. In April, at the time of bud break, a strong reduction of *PcBSP1+2* mRNA abundance occurred. In summer, *PcBSP1+2* expression was constantly low, except for an increase in July 2008. Maximum changes in *PcBSP1+2* were 73-fold in 2006/07 and 21-fold in 2007/08. With increasing transcript abundances from September to the end of November, *PcBSP3* showed a seasonal pattern very similar to that of *PcBSP1+2*. However, with highest levels of ~2×10^8 copies per microgram of RNA, the expression was about one order of magnitude smaller compared with *PcBSP1+2*. Similar to *PcBSP1+2*, maximum changes in *PcBSP3* were higher in 2006/07 (185-fold) compared with 2007/08 (17-fold).

The transcript abundances of *PcWIN4* and *PcPNI288* showed very similar seasonal changes. In spring, the mRNA of both genes increased simultaneously, just after the strong decrease of *PcBSP1+2* and *PcBSP3* mRNA. This peak was transient and the transcription started to decrease immediately after reaching its maximum. During summer, mRNA abundances of both genes were very low, in winter being below the limit of detection. Maximum changes in *PcWIN4* were 400-fold in 2006/07 and 140-fold in 2007/08. For *PcPNI288*, even higher fold changes were observed (~2600-fold, 2006/07; ~1100-fold, 2007/08).

Since we opted for an absolute quantification strategy in our RT-qPCR assays (see Materials and methods), we did not use a reference gene as the denominator for transcript abundance. However, to compare the transcript abundances of the *BSP* genes with a gene used as a reference gene for relative quantification, the mRNA expression of a gene of the elongation factor family, elongation factor 1-beta (*PcEF1b*), was chosen. Brunner et al. (2004) showed that expression levels of this

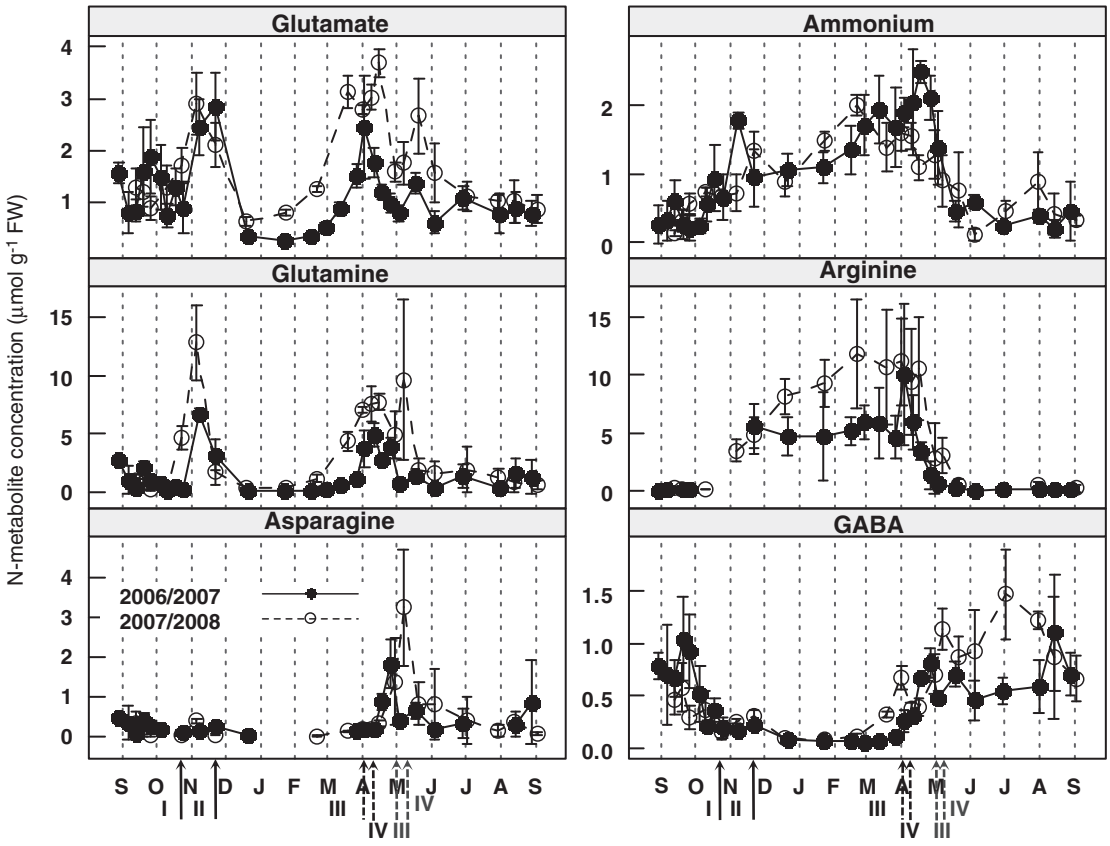

Figure 3. Concentrations of glutamate, ammonium (NH_4^+), glutamine, arginine, asparagine and GABA in the bark of field-grown *P.* × *canescens* from August 2006 to September 2008. Data shown are the means ± 1 SD of three (August 2006 to August 2007; closed circles, solid lines) or four (September 2007 to September 2008; open circles, dashed lines) replicate plants. Bark between leaf Numbers 5 and 15 of one twig per tree was harvested by carefully separating it from the wood. Black solid arrows: I, first visible signs of leaf senescence; II, completion of leaf abscission in 2006 and 2007. Black dashed arrows: III, start of bud break; IV, end of bud break in 2007. Grey dashed arrows: III, start of bud break; IV, end of bud break in 2008. Time is expressed in months from September to September.

gene in *Populus* were adequately consistent under various environmental conditions. We found that *PcEF1b* mRNA abundances were lowest from December to April and showed three peaks during the year, one in May and two in autumn. Despite this variability, *PcEF1b* was the gene with lowest *n*-fold changes (3.9, 2006/07; 3.3, 2007/08) among all studied genes. The corresponding changes in the two BSP genes analysed were at least five times as high in both sampling periods (see above).

Correlation analyses

GABA and arginine were strongly correlated to mRNA abundances of genes involved in seasonal nitrogen cycling To describe the linear relationship between transcript abundances of the genes analysed and other biological variables, bivariate Pearson's correlation coefficients were computed (for correlation coefficients, *P*-values, adjusted *P*-values (P_{adj}) and number of pairs with $P < 0.01$, see Supplementary Table S2).

Between transcript abundances of analysed genes, two correlations of statistical significance ($P_{adj} < 0.001$) were found (Supplementary Table S2): *BSP1+2* was positively correlated to *BSP3* ($r = 0.89$, $n = 48$) and *WIN4* was highly correlated with *PNI288* ($r = 0.91$, $n = 24$).

Between different transcript abundances and amino acid and total protein concentrations, some interesting correlations were found as well (for correlations with *P*-values < 0.01, see Supplementary Table S2). Arginine was the amino acid showing the strongest positive correlation with *BSP1+2* ($r = 0.73$, $n = 28$) and with *BSP3* ($r = 0.82$, $n = 28$). Among all correlations between *BSP1+2*/*BSP3* and amino acids, these were the only ones of statistical significance ($P_{adj} < 0.01$). *BSP1+2* ($r = 0.51$, $n = 29$, $P_{adj} > 0.05$) and *BSP3* ($r = 0.67$, $n = 29$, $P_{adj} = 0.013$) exhibited a strong relationship to total protein concentration. Among all amino acids analysed, GABA showed the strongest negative correlations to *BSP1+2* ($r = -0.47$, $n = 45$, $P_{adj} = 0.14$) and to *BSP3* ($r = -0.57$, $n = 45$, $P_{adj} = 0.006$). Moreover, among all amino acids, GABA was most strongly correlated to *WIN4* ($r = 0.53$, $n = 23$, $P_{adj} > 0.05$) and *PNI288* ($r = 0.82$, $n = 33$, $P_{adj} < 0.001$). No significant correlations were observed between glutamine and the two *BSP* genes.

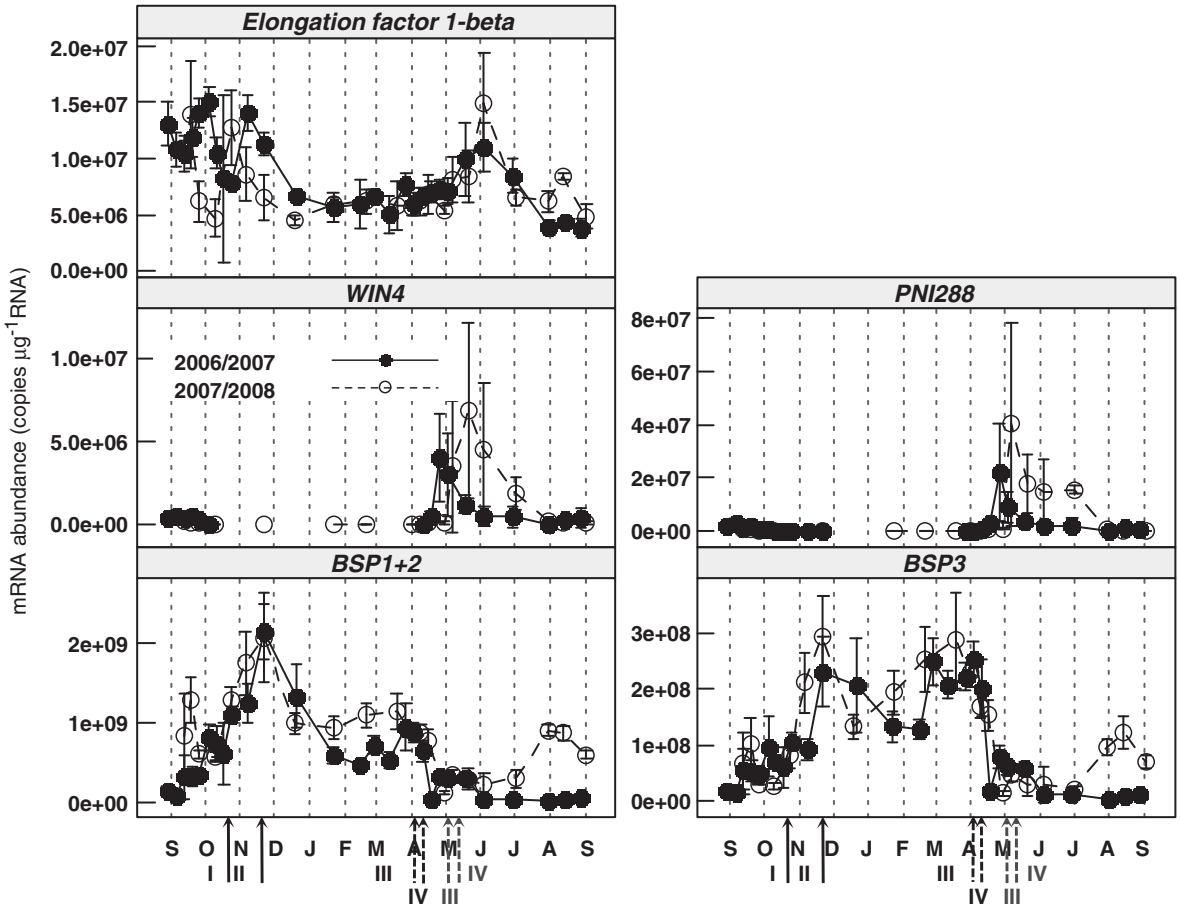

Figure 4. Seasonal changes in expression of genes involved in nitrogen cycling in the bark of field-grown *P.* × *canescens*. From August 2006 to August 2007 (closed circles, solid lines), three trees were sampled; during the following 12 months (open circles, dashed lines), four trees were harvested per sampling. Transcript abundances were analysed by RT-qPCR. Data shown are the means ± 1 SD. Black solid arrows: I, first visible signs of leaf senescence; II, completion of leaf abscission in 2006 and 2007. Black dashed arrows: III, start of bud break; IV, end of bud break in 2007. Grey dashed arrows: III, start of bud break; IV, end of bud break in 2008. *WIN4*, wound-inducible 4; *PNI288*, poplar nitrogen-inducible 288; *BSP1+2*, bark storage protein 1+2; *BSP3*, bark storage protein 3. Time is expressed in months from September to September.

BSP transcript abundances as well as GABA and arginine are strongly correlated with meteorological parameters One of the aims of this study was to identify meteorological factors interacting with metabolites and genes involved in SNC. Arginine and GABA turned out to be the amino acids most strongly correlated to meteorological parameters (see Supplementary Table S3 for all pairs with $P_{adj} < 0.05$). Both arginine and GABA were correlated to the mean air temperature during 48 h before harvest (T48-mean) ($r = -0.70$, $n = 29$, $P_{adj} = 0.012$ for arginine and $r = 0.76$, $n = 46$, $P_{adj} < 0.001$ for GABA) and day length ($r = -0.62$, $n = 29$, $P_{adj} = 0.13$ for arginine and $r = 0.75$, $n = 46$, $P_{adj} < 0.001$ for GABA).

Not only amino acid levels, but also transcript abundances and total protein concentration were found to be correlated to day length ($P_{adj} < 0.001$), e.g., *BSP1+2* ($r = -0.66$, $n = 48$), *BSP3* ($r = -0.63$, $n = 48$) and with opposite sign *PNI288* ($r = 0.78$, $n = 36$) (for all pairs with $P_{adj} < 0.01$, see Supplementary Table S4). With the same signs and comparable correlation coefficients, these genes were linked to the temperature parameter T48-mean.

Similar to *BSP1+2* and *BSP3*, the total protein concentration was negatively correlated with day length ($r = -0.71$, $n = 30$, $P = 0.0011$) and T48-mean ($r = -0.92$, $n = 30$, $P < 0.001$). All other significant correlations ($P_{adj} < 0.01$) of total protein concentration to weather parameters are included in Supplementary Table S4.

Linear models fitting seasonal changes in BSP transcript abundances and total protein concentrations The strength of the linear correlations and the consistency of their patterning prompted us to fit linear models for the expression of *WIN4*, *PNI288*, *BSP1+2*, *BSP3* and total protein concentration. We started to model the biological variables with GABA and arginine as explanatory variables (for *WIN4* and *PNI288*, arginine was excluded because no significant correlations were found at $P < 0.01$). In the case of total protein concentration, *BSP1+2* and *BSP3* expression were also integrated as

Table 2. Linear regression models relating data on mRNA abundance and total protein concentration to meteorological parameters and N metabolites as detected in bark of *P.* × *canescens* collected during two annual growth cycles.

Dependent variable	Model					
	Parameter	Estimate	SE	Sign. code	r^2/r^2_{adj}	AIC
WIN4	Intercept	5.74	0.18	***	0.25/0.21	
	GABA	1.95	0.745	*		
PNI288	Intercept	2.10	1.18	ns ($P = 0.084$)	0.77/0.76	−26.35
	Day length	0.005	0.001	***		
	GABA	2.59	0.53	***		
BSP1+2	Intercept	9.62	0.45	***	0.61/0.58	−57.5
	Day length	−0.001	0.0006	*		
	Arginine	0.31	0.11	**		
BSP3	Intercept	8.52	0.38	***	0.69/0.67	−67.27
	Day length	−0.0009	0.0004	ns ($P = 0.06$)		
	Arginine	0.41	0.09	***		
Total protein concentration	Intercept	11.15	7.02	ns ($P = 0.0125$)	0.89/0.87	37.92
	BSP3	4.82	1.6	**		
	BSP1+2	−3.26	1.42	*		
	T48-mean	−0.627	0.067	***		

SE, standard error; *PcWIN4*, *P.* × *canescens* wound-inducible 4; *PcPNI288*, *P.* × *canescens* poplar nitrogen-inducible 288; *PcBSP1+2*, *P.* × *canescens* bark storage protein 1+2; *PcBSP3*, *P.* × *canescens* bark storage protein 3; GABA, γ-aminobutyric acid; T48-mean, mean air temperature during 48 h before harvest; AIC, Akaike Information Criterion; r^2_{adj}, adjusted r^2; ***$P < 0.001$; **$P < 0.01$; *$P < 0.05$; ns, not significant.

biological predictors. As additional predictors for all variables, day length and T48-mean were used. In a backward selection, we stepwise excluded predictor variables with highest *P*-values, until all remaining explanatory variables were significant. The model for *WIN4* accounted for only 24.5% of the variability and included only GABA as predictor variable (Table 2). In contrast, the model for *PNI288* explained 77.1% of the variance, based on GABA and day length as explanatory variables. Moreover, *BSP1+2* expression was described by day length and arginine, with $r^2 = 0.607$. Using the same predictor variables, 69.4% of the variability of *BSP3* expression was explained. In this case, the *P*-value for day length was slightly above the level of statistical significance. However, its elimination led to a considerably smaller r^2, so we put it back into the model. Among all models, the one for total protein concentration reached the highest r^2 (0.886). It was built on *BSP3*, *BSP1+2* and T48-mean. For all models, differences between r^2 and the adjusted r^2 were very low, indicating that the fits were achieved with an appropriate number of explanatory variables. The data of this study represent time series, but statistical methods for time series analyses were not applicable to the data of only two complete seasons. However, the model diagnostics shown in Supplementary Figures S1 and S2 suggest that the linear models provide reliable results. The plots shown in Supplementary Figure S1 demonstrate that the model assumptions of normal distribution, homogeneity of variance and linearity of the residuals were met. The scatter plots represented in Supplementary Figure S2 do not suggest a temporal correlation of the residuals. Similarly, the *P*-values of the Runs Test were >0.05 (Supplementary Figure S2), so that uncorrelated residuals can be assumed.

Discussion

PcBSP1+2 and *PcBSP3 transcript abundances and total protein concentration increase in autumn and are high throughout winter*

In the work presented here, we analysed the processes of SNC in bark. Since *BSP* genes are central to SNC, we isolated and characterized individual members of the three *BSP* subfamilies. So far, data on mRNA abundances of the *BSP* subfamily in bark under natural conditions are only available for the transition phase from active growth to dormancy in autumn (Coleman et al. 1992, Black et al. 2001). Here, we present data on *BSP* expression in bark covering seasonal growth cycles during 2 years. The observed increase in transcript abundances of *PcBSP1+2* and *PcBSP3* (Figure 4) in autumn between the onset of leaf senescence (Stage I, 25 October 2006 and 24 October 2007; Figure 2A) and leaf abscission (Stage II, 22 November 2006 and 2007; Figure 2A) is in line with previous findings of Coleman et al. (1992) and Black et al. (2001). Analysing several ecotypes of *P. deltoides*, Black et al. (2001) reported peaks of relative *BSP* mRNA abundance in autumn with subsequent decreases. In meristematic cambial cells of aspen, Druart et al. (2007) found constantly high levels of *BSP* mRNA throughout winter, putatively outlasting the synthesis of storage proteins. In our study, *PcBSP1+2* showed a decrease after peaking in

winter, but the absolute transcript abundance was still very high. The expression of *PcBSP3* as well remained high throughout winter. These findings point to a post-transcriptional control preventing the translation of *BSP* transcripts when BSP synthesis is no longer required. Wetzel et al. (1989) reported a similar seasonal change for concentrations of putative BSP in bark of *P. deltoides* as revealed by SDS-PAGE. Further studies provide evidence for high BSP levels throughout winter (van Cleve et al. 1988, Sauter et al. 1989). As reported for putative BSP (Wetzel et al. 1989), total protein concentration (Figure 2B) and expression of *PcBSP1+2* and *PcBSP3* (Figure 4) also decline in spring. In spring 2007, this decrease coincided with bud break (occurring between 3 April and 11 April; Figure 2A), whereas in spring 2008, bud break occurred after the decrease of total protein concentration and *BSP* gene expression, between 2 May and 7 May (Figure 2A). Our own results on seasonality of total protein concentrations in bark (Figure 2B) are very similar to those found in bark of *Populus balsamifera* (Höllwarth 1976) and resemble the seasonal course of *BSP* expression. Considering our results and previous studies showing (i) that BSP comprise a large fraction of total protein concentration in bark during winter (Wetzel et al. 1989) and (ii) that strong positive correlations between BSP and total protein concentration were found in bark of *P. trichocarpa* × *P. deltoides* in midwinter (Black et al. 2001), it is very likely that the seasonal changes observed for total protein concentration are governed by the concentration of BSP, which have not been determined in the present study.

PcWIN4 and PcPNI288 mRNA abundances in the bark increase in spring, indicating a function for short-term N storage

Based on the archival assembly of the *P. trichocarpa* genome sequence (version 1.0), Cooke and Weih (2005) propose three loci in the *WIN4* subfamily. However, we did not succeed in assigning cloned sequences of *P.* × *canescens* to the corresponding *P. trichocarpa* sequences (Figure 1B). It might be possible that the cloned sequences do not represent separate paralogous genes, but allelic variants of the same gene. Interestingly, in the latest *Populus* genome release v2.0 (http://www.phytozome.net/poplar), PtWIN4-1 and PtWIN4-2 show best scores to the same gene model (POPTR_0013s_07800.1), indicating that only two *WIN4* genes exist in this species. This difference from the in silico research in the *P. trichocarpa* genome version 1.0 carried out by Cooke and Weih (2005), suggesting that there are three *WIN4* genes, is likely due to the preliminary nature of the genome version available to these authors. At that time, sequencing and mapping of the genome was still ongoing, and the current version 2.0 might, therefore, provide improved sequence information.

The expression of *WIN4* is not associated with dormant but with actively growing tissues, pointing to a function in determining the N (and therewith C) sink strength (Cooke and Weih 2005). So far, only few studies examining *WIN4* expression in trees have been published (Davis et al. 1993, Coleman et al. 1994, Lawrence et al. 1997, 2001, Cooke et al. 2003, 2005), but no study analysed seasonal changes in *WIN4* expression or WIN4 concentration. Coleman et al. (1994) detected *WIN4* transcripts only in leaves, but not in bark, concluding that the corresponding gene product modulates accumulation and mobilization of leaf nitrogen. However, we were able to detect *WIN4* transcripts in bark (Figure 4). *WIN4* transcript abundance was very low or below the limit of detection in the dormant phase. It markedly rose in spring, peaking ~2 weeks after *BSP* expression and total protein concentration decreased. In 2007, bud break (3 April to 11 April) preceded the peak of *WIN4* transcript abundance, whereas in 2008, bud break (30 April to 7 May) coincided with the transcriptional peak. Different to Coleman et al. (1994), we infer that WIN4 plays a role in short-term N storage, with N presumably being derived from degradation of BSP. This proposed function of WIN4 is in line with findings of Cooke et al. (2005), showing that *WIN4* transcript abundances in poplar stems increase with increasing N availability.

PNI288 is the most recently identified gene of the *BSP* family. Until now, only two studies on *PNI288* expression are available, describing this gene as being N-inducible (Lawrence et al. 2001, Cooke et al. 2003) and wound-inducible (Lawrence et al. 2001) in *Populus* stem sections. We provided data on seasonal expression of this gene in bark. *PNI288* has a very similar seasonal expression pattern to *WIN4*, exhibiting a distinct maximum of expression preceding or coinciding with bud break. Therefore, it is likely that also PNI288 plays a role in transient storage of N after the onset of BSP degradation, corroborating that PNI288 functions as a BSP. In summary, our data on whole-season expression patterns of genes of the three *BSP* subfamilies support previous findings showing that only the *BSP* subfamily is associated with N storage during dormancy, whereas members of the two other subfamilies are important for short-term N storage in non-dormant tissues, including bark.

N metabolites as mediators of seasonal nitrogen cycling in bark

Bark is a tissue made up of several cell types such as epidermal cells, cortex cells, peridermal cells, parenchyma cells, phloem cells and cambial cells and these cell types have differentiated functions in transport, storage and growth. Accordingly, these various cell types may vary in their content of N metabolites and in their transcriptional activity and, therefore, the investigation of relationships between N metabolites and transcript abundances might be more reliable in a cell-type-specific analysis. However, it appears that the application of techniques allowing this is very laborious and can hardly be combined with such a fine time course of sampling.

The seasonal changes in the investigated biological components and their dependency on meteorological conditions have to be considered in the context of the prevalent climatic conditions. In different climates, e.g., under more continental

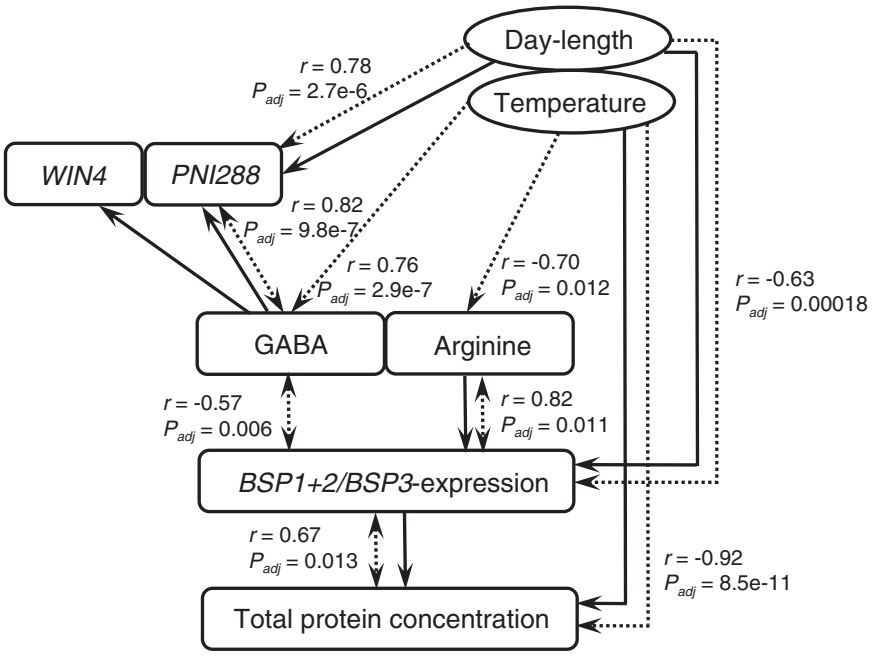

Figure 5. Hypothetical model of SNC in bark as inferred from correlation analyses and linear models. Dashed arrows indicate significant (adjusted *P*-value $P_{adj} < 0.05$) correlations. Pearson's correlation coefficients (*r*) and adjusted *P*-values of these correlations are given next to the dashed arrows. Correlations between biological variables and temperature relate to the mean air temperature of a period of 48 h before harvest. For *BSP1+2/BSP3* expression, the results of the correlation analyses were very similar. For clarity, only the data for *BSP3* are provided here. Solid arrows depict relationships supported by linear models. Components to which the solid arrows are pointing represent the dependent variables in the linear models. For details of the linear models, see Table 2. *WIN4*, wound-inducible 4; *PNI288*, poplar nitrogen-inducible 288; *BSP1+2*, bark storage protein 1+2; *BSP3*, bark storage protein 3.

conditions with lower winter and spring temperatures, different metabolic activities and, therefore, different seasonal courses of metabolites involved in SNC have to be expected.

Amino acids are capable of regulating their own metabolism and the expression of linked genes (Coruzzi and Zhou 2001, Masclaux-Daubresse et al. 2005). For instance, glutamine was found to affect the expression of glutamine synthetase (Watanabe et al. 1997, Oliveira and Coruzzi 1999) and was proposed to affect mRNA levels of *BSP* (Zhu and Coleman 2001*a*). In addition, temperature was reported to affect mRNA levels of *BSP* (van Cleve and Sauter 1993) and there is evidence for an effect of day length on *BSP* expression (Coleman et al. 1992, Black et al. 2001, Zhu and Coleman 2001*a*). To ascertain such relationships on the basis of seasonal data, we performed correlation and regression analyses (see above). Although the very conservative Bonferroni correction was applied to the correlation analyses, significant correlations involving transcript abundances, the amino acids GABA and arginine and meteorological factors were identified.

Based on the correlation analyses and the linear models, we suggest a hypothetical model of SNC in bark (Figure 5). Day length and temperature parameters showed strongest correlations with biological data. For instance, a strong positive correlation was observed between temperature parameters and GABA (Supplementary Table S3), which is considered a signal molecule balancing C/N partitioning (Bouché and Fromm 2004) and regulating the expression of cold-responsive genes in cambial meristem cells of aspen (Druart et al. 2007). This agrees with previous work reporting that GABA concentrations responded to altered temperatures under controlled conditions (Mazzucotelli et al. 2006, Kaplan et al. 2007). Among all amino acids measured, GABA appeared to show the strongest positive correlations with seasonal changes in transcript abundance of *WIN4* and *PNI288*. A reciprocal correlation was observed between GABA and *BSP* expression (Supplementary Table S2). In the linear models for *WIN4* and *PNI288*, GABA was the only significant biological predictor variable (Table 2).

The seasonal course of arginine concentrations in bark is consistent with the data of Sagisaka (1974) and Höllwarth (1976). Storing arginine during winter is advantageous because this compound contains 4 mol N/mol, thereby reducing the C demand for N storage and setting C free for maintenance respiration. Although the amount of N stored in arginine is low compared with that stored in proteins, storage of arginine might be beneficial for the plant because the amino and guanidyl groups of arginine are readily transformable to other amino compounds. The strongest negative correlation between temperature and amino acids was found for arginine. Arginine, in turn, was the amino acid exhibiting the strongest positive correlations with *BSP1+2* and *BSP3* expression (Supplementary Table S2).

The seasonal course of glutamine concentrations was characterized by a steep increase in October when leaf senescence

had already begun (Stage I, 25 October 2006 and 24 October 2007; Figure 2A) but leaf abscission was not yet completed (Stage II, 22 November 2006 and 2007; Figure 2A). This leaf senescence-associated increase in glutamine concentrations in the bark coincided with a decrease in total protein concentration in leaves (data not shown). Therefore, it is very likely that the autumnal peak of glutamine and other amino acids in bark results from a degradation of leaf proteins, interconversions of the released amino acids into the preferred transport forms, i.e., glutamine, and subsequent transport into the bark. Corroborating previous findings of Sagisaka (1974), glutamine concentrations increased in spring (Figure 3), well before bud break (happening between 3 April and 11 April 2006, 30 April and 7 May 2007; see Figure 2A). Millard et al. (2006) showed that fertilized, field-grown poplars started to take up N by the roots only when remobilization had already progressed. Malaguti et al. (2001) report a similar chronological order of remobilization and root N uptake in apple trees and point to an effect of soil temperature on the balance between root N uptake and remobilization. Although the air temperatures were above long-term average in both winters and springs under study (DWD 2008, 2009) and correspondingly high soil temperatures might have facilitated N uptake from the soil that early in the season, it is unlikely that such a process contributed to the increasing glutamine concentration because the buds had not yet burst and, subsequently, transpiration was still absent, limiting nutrient transport from the roots to the shoot. Rather, degradation of proteins might have led to increasing glutamine and glutamate concentrations. A decrease in the total protein concentration between mid-February and mid-March 2008 is in line with this interpretation (Figure 2B). In 2007, such a decrease in total protein concentrations was not observed. However, some degradation might still have occurred which is hidden in the variability of the data (the proteolysis of only 1 mg protein g^{-1} FW of a 50-kDa protein consisting of 500 amino acids will release 10 μmol amino acids g^{-1} FW). Remarkably, no statistically significant correlation was found between the seasonal courses of glutamine concentration and *BSP* expression, in contrast to the conclusions of Zhu and Coleman (2001*a*) that glutamine is of particular importance for the activation of the poplar *BSP* promoter (see below for more discussion). The strongest negative correlation between *BSP* expression and meteorological factors was found for day length (Supplementary Table S4), supporting previous work (Coleman et al. 1991, Langheinrich and Tischner 1991, Black et al. 2001, Zhu and Coleman 2001*a*). In agreement with the results of the correlation analyses, day length and arginine turned out to be good predictors in linear models of *BSP* expression (Table 2).

BSP expression was shown to correlate with and contribute significantly to the precise prediction of total protein concentration (Table 2).Wetzel et al. (1989) reported that BSP make up a large fraction of total protein concentration during dormancy and Black et al. (2001) found strong positive correlations between BSP and total protein concentration in poplar bark in midwinter. From these findings, evidence is accumulating that *BSP* expression is a major determinant of total protein concentration in the bark.

In summary, the pairs of variables forming the strongest correlations at each level of interaction can be arranged as two coherent sequences with GABA and arginine potentially acting as mediators between meteorological conditions and expression of genes involved in SNC in bark (Figure 5). At high temperatures, as found during the phase of vegetative growth, high GABA levels may act as signals promoting active growth, thus stimulating *WIN4* and *PNI288* expression. The other consistent sequence of correlations involves arginine: at low temperatures, arginine concentrations are high. High arginine levels may potentially signal a switch in the metabolic state to storage, therefore activating *BSP* expression, which in turn could lead to high total protein concentrations. In addition to this putative temperature-driven regulation of storage processes, *BSP* expression is affected by day length. This duality of the activation of *BSP* transcription was already proposed by Zhu and Coleman (2001*b*). It may allow the tree to optimally time storage processes according to the prevalent environmental conditions.

As indicated above, our data and the derived conclusions do not support the interpretation of Zhu and Coleman (2001*a*) that glutamine has an important role for the regulation of *BSP* expression. Based on the observations that, under short-day conditions, shoot growth ceases, changes in the C/N partitioning occur and *BSP* transcription is induced, Zhu and Coleman (2001*a*) proposed that N metabolites are components of signalling events from phytochrome-mediated short-day perception to induction of *BSP* transcription. From exogenously applying NH_4NO_3, glutamine, asparagine or proline, single and each in combination with sucrose, to excised shoots of *P.* × *canescens* transformed with the *BSP* promoter fused to the coding region of β-glucuronidase, the authors deduced a prominent role of glutamine in short-day induced regulation of *BSP* expression. This difference to our results might have several explanations. While our results are based on field-grown poplars, Zhu and Coleman (2001*a*) performed their study in controlled environments under long-day conditions. Since it was not verified that the supply of N compounds caused variations as a short-day treatment would do, it may be that the observed increase in *BSP* transcription was related to metabolic responses to the high N availability rather than to changes in photoperiod. It was demonstrated that glutamine supplied to roots of *P.* × *canescens* is partially metabolized, causing significant increases in asparagine, GABA, glutamate and ammonium (Dluzniewska et al. 2006, 2007). When glutamine was added to a sucrose-containing medium, at least 5-fold, significant increases in asparagine, glutamate and aspartate concentrations in *Arabidopsis* plants growing on this medium were found (Oliveira and Coruzzi 1999). This demonstrates that, in any attempts to derive functional relationships between *BSP* promoter activity and individual N compounds, the in planta levels of these compounds should be analysed. In a girdling experiment with

P. trichocarpa × *deltoides*, disruption of phloem transport to sink leaves above the girdle caused significant increases in glutamine and asparagine below the girdle (Cooke et al. 2003). Arginine, although present at lower abundance than glutamine and asparagine, showed a significant accumulation below the girdle, as well. Correlating with these increases in glutamine, asparagine and arginine, *BSP* transcript abundances increased below the girdle. This result underscores that, at present, there is a lack of experimental evidence supporting a prominent role in glutamine in transcriptional activation of *BSP* expression. Rather, additional amino compounds, like asparagine, arginine, GABA and other products of glutamine metabolism have to be considered for such a function.

Our conclusions were derived from (i) coherent sequences of correlations, each of them being the strongest at a particular level of interaction, and (ii) linear models, which further support the correlations of *BSP* transcript abundances and total protein concentrations with amino acids and meteorological factors. However, a significant correlation is only a necessary, not a sufficient, condition for causality and it is important to consider that biological variables may be spuriously correlated, with the relationship being caused by a third, not analysed factor (e.g., carbohydrates, phytohormones). In the case of correlations, the directionality of the observed interactions is only obvious in cases involving environmental variables (e.g., temperature impacts arginine concentration, not vice versa), but not for correlations between two biological variables. This was addressed by the modelling approach, which demonstrated that arginine, GABA, day length and temperature are good predictors of total protein concentration and transcript abundances of *BSP* genes. Future work will test the inferred effects of temperature and day length on N metabolites and expression of genes related to SNC in bark under controlled conditions. Moreover, a reassessment of the role of N metabolites for the regulation of *BSP* transcription and BSP deposition is needed, testing a broad range of compounds, including the provided candidate compounds GABA and arginine, under controlled conditions.

Acknowledgments

This work was financially supported by the Deutsche Forschungsgemeinschaft (DFG) (contract no. Re515/20-3) as part of the Poplar Research Group and within the DFG Graduate School 'Signal systems in plant model organisms'. H.W. and J.D. were partially financed by the Federal State Baden-Württemberg via a scholarship according to the State Law for Graduate Funding. We gratefully acknowledge the technical help of Christiane Steinki-Schwarz and Wolfgang Kornberger.

Supplementary data

Supplementary data for this article are available at *Tree Physiology* Online.

References

Andersson, A., J. Keskitalo, A. Sjödin et al. 2004. A transcriptional timetable of autumn senescence. Genome Biol. 5:R24.

Bhalerao, R., J. Keskitalo, F. Sterky et al. 2003. Gene expression in autumn leaves. Plant Physiol. 131:430–442.

Black, L.B., C.M. Parmentier-Line, L.H. Fuchigami and G.D. Coleman. 2001. Ecotypic and genetic variation in poplar bark storage protein gene expression and accumulation. Tree Physiol. 21:1289–1297.

Bouché, N. and H. Fromm. 2004. GABA in plants: just a metabolite? Trends Plant Sci. 9:110–115.

Brunner, A.M., I.A. Yakovlev and S.H. Strauss. 2004. Validating internal controls for quantitative plant gene expression studies. BMC Plant Biol. 4.

Coleman, G.D., T.H.H. Chen, S.G. Ernst and L.H Fuchigami. 1991. Photoperiod control of poplar bark storage protein accumulation. Plant Physiol. 96:686–692.

Coleman, G.D., T.H.H. Chen and L.H. Fuchigami. 1992. Complementary DNA cloning of poplar bark storage protein and control of its expression by photoperiod. Plant Physiol. 98:687–693.

Coleman, G.D., P.M. Bañados and T.H.H. Chen. 1994. Poplar bark storage protein and a related wound-induced gene are differentially induced by nitrogen. Plant Physiol. 106:211–215.

Cooke, J.E.K. and M. Weih. 2005. Nitrogen storage and seasonal nitrogen cycling in *Populus*: bridging molecular physiology and ecophysiology. New Phytol. 167:19–30.

Cooke, J.E.K., K.A. Brown, R. Wu and J.M. Davis. 2003. Gene expression associated with N-induced shifts in resource allocation in poplar. Plant Cell Environ. 26:757–770.

Cooke, J.E.K., T.A. Martin and J.M. Davis. 2005. Short-term physiological and developmental responses to nitrogen availability in hybrid poplar. New Phytol. 167:41–52.

Coruzzi, G.M. and L. Zhou. 2001. Carbon and nitrogen sensing and signaling in plants: emerging 'matrix effects'. Curr. Opin. Plant Biol. 4:247–253.

Dannenmann, M., J. Simon, R. Gasche et al. 2009. Tree girdling provides insight on the role of labile carbon in nitrogen partitioning between soil microorganisms and adult European beech. Soil Biol. Biochem. 41:1622–1631.

Davis, J.M., E.E. Egelkrout, G.D. Coleman, T.H.H. Chen, B.E. Haissig, D.E. Riemenschneider and M.P. Gordon. 1993. A family of wound-induced genes in *Populus* shares common features with genes encoding vegetative storage proteins. Plant Mol. Biol. 23:135–143.

Dluzniewska, P., A. Gessler, S. Kopriva, M. Strnad, O. Novák, H. Dietrich and H. Rennenberg. 2006. Exogenous supply of glutamine and active cytokinin to the roots reduces NO_3^- uptake rates in poplar. Plant Cell Environ. 29:1284–1297.

Dluzniewska, P., A. Gessler, H. Dietrich, J.-P. Schnitzler, M. Teuber and H. Rennenberg. 2007. Nitrogen uptake and metabolism in *Populus* × *canescens* as affected by salinity. New Phytol. 173:279–293.

Druart, N., A. Johansson, K. Baba, J. Schrader, A. Sjödin, R.R. Bhalerao, L. Resman, J. Trygg, T. Moritz and R.P. Bhalerao. 2007. Environmental and hormonal regulation of the activity-dormancy cycle in the cambial meristem involves stage-specific modulation of transcriptional and metabolic networks. Plant J. 50:557–573.

DWD. 2008. Der KLIMA-Report 2007. Deutscher Wetterdienst. http://www.dwd.de/bvbw/generator/Sites/DWDWWW/Content/Oeffentlichkeit/KU/KU2/KU22/klimastatusbericht/klimareport/

klimareport__2007.,templateId=raw.,property=publicationFile.pdf/klimareport_2007.pdf.

DWD. 2009. Der KLIMA-Report 2008. Deutscher Wetterdienst. http://www.dwd.de/bvbw/generator/Sites/DWDWWW/Content/Oeffentlichkeit/KU/KU2/KU22/klimastatusbericht/klimareport/klimareport__2008.,templateId=raw.,property=publicationFile.pdf/klimareport_2008.pdf.

Felsenstein, J. 1985. Confidence limits on phylogenies: an approach using the bootstrap. Evolution 39:783–791.

Fracheboud, Y., V. Luquez, L. Björkén, A. Sjödin, H. Tuominen and S. Jansson. 2009. The control of autumn senescence in European aspen. Plant Physiol. 149:1982–1991.

Höllwarth, M. 1976. Seasonal changes of nitrogen metabolism and its relation to temperature in the bark of poplars. Z. Pflanzenphysiol. 80:215–224.

Hui, W., Y.R. Gel and J.L. Gastwirth. 2008. lawstat: An R package for law, public policy and biostatistics. J. Stat. Softw. 28.

Kaplan, F., J. Kopka, D.J. Sung, W. Zhao, M. Popp, R. Porat and C.L. Guy. 2007. Transcript and metabolite profiling during cold acclimation of *Arabidopsis* reveals an intricate relationship of cold-regulated gene expression with modifications in metabolite content. Plant J. 50:967–981.

Keskitalo, J., G. Bergquist, P. Gardestrom and S. Jansson. 2005. A cellular timetable of autumn senescence. Plant Physiol. 139: 1635–1648.

Kolosova, T., B. Miller, S. Ralph, B. Ellis, C. Douglas, K. Ritland and J. Bohlmann. 2004. Isolation of high-quality RNA from gymnosperm and angiosperm trees. BioTechniques 36:821–824.

Langheinrich, U. and R. Tischner. 1991. Vegetative storage proteins in poplar. induction and characterization of a 32- and 36-kilodalton polypeptide. Plant Physiol. 97:1017–1025.

Lawrence, S.D., J.S. Greenwood, T.E. Korhnak and J.M. Davis. 1997. A vegetative storage protein homolog is expressed in the growing shoot apex of hybrid poplar. Planta 203:237–244.

Lawrence, S.D., J.E.K. Cooke, J.S. Greenwood, T.E. Korhnak and J.M. Davis. 2001. Vegetative storage protein expression during terminal bud formation in poplar. Can. J. For. Res. 31:1098–1103.

Luo, Z.-B., D. Janz, X. Jiang, C. Göbel, H. Wildhagen, Y. Tan, H. Rennenberg, I. Feussner and A. Polle. 2009. Upgrading root physiology for stress tolerance by ectomycorrhizas: insights from metabolite and transcriptional profiling into reprogramming for stress anticipation. Plant Physiol. 151:1902–1917. First published on October 7, 2009; 10.1104/pp.109.143735.

Malaguti, D., P. Millard, R. Wendler, A. Hepburn and M. Tagliavini. 2001. Translocation of amino acids in the xylem of apple (*Malus domestica* Borkh.) trees in spring as a consequence of both N remobilization and root uptake. J. Exp. Bot. 52:1665–1671.

Masclaux-Daubresse, C., E. Carrayol and M.H. Valadier. 2005. The two nitrogen mobilisation- and senescence-associated GS1 and GDH genes are controlled by C and N metabolites. Planta 221:580–588.

Mason, H.S., F.D. Guerrero, J.S. Boyer and J.E. Mullet. 1988. Proteins homologous to leaf glycoproteins are abundant in stems of dark-grown soybean seedlings. Analysis of proteins and cDNAs. Plant Mol. Biol. 11:845–856.

Mazzucotelli, E., A. Tartari, L. Cattivelli and G. Forlani. 2006. Metabolism of γ-aminobutyric acid during cold acclimation and freezing and its relationship to frost tolerance in barley and wheat. J. Exp. Bot. 57:3755–3766.

Millard, P., R. Wendler, G. Grassi, G.A. Grelet and M. Tagliavini. 2006. Translocation of nitrogen in the xylem of field-grown cherry and poplar trees during remobilization. Tree Physiol. 26:526–536.

Noctor, G., M. Strohm, L. Jouanin, K.J. Kunert, C. Foyer and H. Rennenberg. 1996. Synthesis of glutathione in leaves of transgenic poplar overexpressing γ-glutamylcysteine synthetase. Plant Physiol. 112:1071–1078.

Nolan, T., R.E. Hands and S.A. Bustin. 2006. Quantification of mRNA using real-time RT-PCR. Nat. Protocol. 1:1559–1582.

Oliveira, I.C. and G.M. Coruzzi. 1999. Carbon and amino acids reciprocally modulate the expression of glutamine synthetase in *Arabidopsis*. Plant Physiol. 121:301–309.

Pfaffl, M.W. 2004. Quantification strategies in real-time PCR. *In* A–Z of quantitative PCR. Ed. S.A. Bustin. International University Line (IUL), La Jolla, CA, p 87–112.

R Development Core Team. 2008. R: a language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. http://www.R-project.org.

Richardson, A.D., A.S. Bailey, E.G. Denny, C.W. Martin and J. O'Keefe. 2006. Phenology of a northern hardwood forest canopy. Glob. Chang. Biol. 12:1174–1188.

Sagisaka, S. 1974. Effect of low temperature on amino acid metabolism in wintering poplar. Plant Physiol. 53:319–322.

Saitou, N. and M. Nei. 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol. Biol. Evol. 4:406–425.

Sauter, J.J. and B. van Cleve. 1990. Biochemical, immunochemical, and ultrastructural studies of protein storage in poplar (*Populus* × *canadensis* <robusta>) wood. Planta 183:92–100.

Sauter, J.J. and B. van Cleve. 1992. Seasonal variation of amino acids in the xylem sap of "*Populus* × *canadensis*" and its relation to protein body mobilization. Trees 7:26–32.

Sauter, J.J., B. van Cleve and S. Wellenkamp. 1989. Ultrastructural and biochemical results on the localization and distribution of storage proteins in a poplar tree and in twigs of other tree species. Holzforschung 43:1–6.

Schneider, A., J. Kreuzwieser, R. Schupp, J.J. Sauter and H. Rennenberg. 1994. Thiol and amino acid composition of the xylem sap of poplar trees (*Populus* × *canadensis* 'robusta'). Can. J. Bot. 72:347–351.

Sjödin, A., K. Wissel, M. Bylesjö, J. Trygg and S. Jansson. 2008. Global expression profiling in leaves of free-growing aspen. BMC Plant Biol. 8.

Staswick, P.E. 1994. Storage proteins of vegetative plant-tissue. Annu. Rev. Plant Physiol. Plant Mol. Biol. 45:303–322.

Stepien, V., J.J. Sauter and F. Martin. 1994. Vegetative storage proteins in woody plants. Plant Physiol. Biochem. 32:185–192.

Strohm, M., L. Jouanin, K.J. Kunert, C. Pruvost, A. Polle, C. Foyer and H. Rennenberg. 1995. Regulation of GSH synthesis in leaves of transgenic poplar (*Populus tremula* × *P. alba*) overexpressing GSH synthetase. Plant J. 7:141–145.

Tamura, K., M. Nei and S. Kumar. 2004. Prospects for inferring very large phylogenies by using the neighbor-joining method. Proc. Natl. Acad. Sci. U. S. A. 101:11030–11035.

Tamura, K., J. Dudley, M. Nei and S. Kumar. 2007. MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. Mol. Biol. Evol. 24:1596–1599.

van Cleve, B. and J.J. Sauter. 1993. Induction by nitrogen and low temperature of storage-protein synthesis in poplar trees exposed to long days. Planta 189:157–160.

van Cleve, B., S. Clausen and J.J. Sauter. 1988. Immunochemical localization of a storage protein in poplar wood. J. Plant Physiol. 133:371–374.

Watanabe, A., N. Takagi, H. Hayashi, M. Chino and A. Watanabe. 1997. Internal Gln/Glu ratio as a potential regulatory parameter for the expression of a cytosolic glutamine synthetase of radish in cultured cells. Plant Cell Physiol. 38:1000–1006.

Wetzel, S., C. Demmers and J.S. Greenwood. 1989. Seasonally fluctuating bark proteins are a potential form of nitrogen storage in 3 temperate hardwoods. Planta 178:275–281.

Zhu, B. and G.D. Coleman. 2001*a*. Phytochrome-mediated photoperiod perception, shoot growth, glutamine, calcium, and protein phosphorylation influence the activity of the poplar bark storage protein gene promoter (bspA). Plant Physiol. 126: 342–351.

Zhu, B. and G.D. Coleman. 2001*b*. The poplar bark storage protein gene (*Bspa*) promoter is responsive to photoperiod and nitrogen in transgenic poplar and active in floral tissues, immature seeds and germinating seeds of transgenic tobacco. Plant Mol. Biol. 46:383–394.