Vegetative storage protein expression during terminal bud formation in poplar[1]

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Abstract: Trees recycle nitrogen (N) to conserve this valuable nutrient. The processes that regulate N recycling within trees are poorly understood at the molecular level. Because vegetative storage proteins (VSPs) are thought to play important roles in within-plant N cycling, we are investigating the expression of VSP genes to gain insights into how seasonally controlled N cycling is regulated in trees. We compared steady-state mRNA levels of three different VSP homologs during short day induced terminal bud formation in hybrid poplar (*Populus trichocarpa* Torr. & Gray × *Populus deltoides* Bartr. ex Marsh.) – WIN4 (wound-inducible protein 4), BSP (bark storage protein), and *pni288* (poplar nitrogen-regulated cDNA 288, a newly identified sequence). We determined that *win4* and *pni288* transcripts decrease, while *bsp* transcripts increase, as the terminal bud is formed. Immunolocalization analysis indicated that, during apical bud formation, BSP accumulates in the ground meristem and in parenchyma cells adjacent to xylem and proximal to the apical dome. Based on messenger RNA and protein expression analysis, we conclude that different VSPs play distinct roles in the poplar shoot apex, with BSP accumulating as a reserve near the shoot apex during terminal bud formation.

Résumé : Les arbres recyclent l'azote afin de conserver ce précieux nutriment. Les processus qui contrôlent le recyclage de l'azote dans les arbres à l'échelle moléculaire sont peu connus. Puisque les protéines végétatives d'entreposage (VSP) jouent vraisemblablement un rôle majeur dans le cycle de l'azote à l'intérieur des plantes, les auteurs ont étudié l'expression des gènes VSP afin de mieux comprendre comment le cycle saisonnier de l'azote est contrôlé chez les arbres. Les niveaux d'ARNm stable de trois homologues différents de VSP ont été comparés pendant la formation du bourgeon terminal induite sous courte photopériode chez le peuplier hybride (*Populus trichocarpa* Torr. & Gray × *Populus deltoides* Bartr. ex Marsh.), à savoir : WIN4 (la protéine 4 induite par une blessure), BSP (la protéine d'entreposage dans l'écorce) et *pni288* (l'ADNc 288 contrôlé par l'azote chez le peuplier, une nouvelle séquence venant d'être identifiée). Au fur et à mesure de la formation du bourgeon terminal, les niveaux de transcrits de *win4* et *pni288* diminuent alors que les transcrits de *bsp* augmentent. L'analyse d'immunolocalisation a indiqué que pendant la formation du bourgeon apical, BSP s'accumule dans le méristème périphérique et les cellules du parenchyme adjacentes au xylème et à proximité du dôme apical. À partir de l'analyse de l'expression des protéines et de l'ARNm, les auteurs concluent que les différentes VSP jouent des rôles distincts au sein de l'apex de la pousse terminale chez le peuplier, dont BSP qui s'accumule sous forme de réserve proche de l'apex de la pousse durant la formation du bourgeon terminal.

[Traduit par la Rédaction]

Introduction

Temperate zone hardwoods cycle nitrogen (N) and carbon (C) seasonally in association with the establishment of dormancy in the autumn and the breaking of dormancy in the spring (Millard 1993). As daylengths shorten, terminal buds are formed, and there is a net shift in N and C allocation from leaves to perennating tissues such as phloem parenchyma in bark and xylem parenchyma in wood. This shift in resource allocation is marked by the accumulation of protein, starch, and lipid reserves in terminal buds, stems, and roots. Synthesis of starch and vegetative storage proteins (VSPs) allows for the sequestration of N and C resources into an osmotically inactive form (Staswick 1994; Martin and Smith 1995). Breakdown of these reserves provides substrates for respiration and other maintenance metabolic processes that occur during the winter, as well as to support bud burst and the early stages of shoot growth during the following spring until the transpiration stream is re-established (Hansen 1971; Dickson 1991; Rowland and Arora 1997).

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Seasonal nutrient cycling is considered an important overwintering strategy for perennials (Dickson 1991; Rowland and Arora 1997), and its importance in productivity is recognized (Dickmann and Keathley 1996); however, the molecular coordination of these processes has remained elusive. VSPs are indicators of cellular N status and should serve as useful markers for within-tree N cycling (Lawrence et al. 1997).

A better understanding of VSP specialization (the cell types and times at which specific VSPs are expressed during various stages of tree development) should illuminate their potential roles in within-tree N cycling. We now have probes for three different VSP homologs to explore this question. We recently discovered one homolog, *pni288*, based on its induction by N. Two related homologs, BSP and WIN4, were previously identified in *Populus* (van Cleve et al. 1988; Wetzel et al. 1989; Lawrence et al. 1997). Some evidence already exists for VSP specialization; BSP accumulates mainly in stems and in response to short-day photoperiod (SD), whereas WIN4 accumulates mainly in shoot tips and the transcript is not SD induced (Coleman et al. 1994; Lawrence et al. 1997). In this work, we examine the regulation of *bsp*, *win4*, and *pni288* in response to environmental cues that are known to regulate VSPs. We discover that the three genes are differentially regulated both spatially and temporally in poplar shoots, with BSP being expressed in specific cell types in response to SD. This analysis suggests a gene- and cell-level dynamic that accompanies shifts between N storage and utilization pathways in trees.

Materials and methods

Plant material

Two F_1 hybrid poplar genotypes (*Populus trichocarpa* Torr. & Gray × *Populus deltoides* Bartr. ex Marsh. hybrids 53-246 and H11-11) were used in the following studies. Results for each genotype were the same. Plants were vegetatively propagated and grown to a height of 60 cm as described previously (Lawrence et al. 1997). To induce terminal bud formation, plants were transferred to a growth chamber under 8 h light, 21°C : 16 h dark, 15°C cycles. In the N-fertilization experiment, plants were provided with a complete nutrient solution containing either 0 or 50 mM NH_4NO_3 for 10 days as previously described (Lawrence et al. 1997). Leaves were identified by leaf plastochron index (LPI; Larson and Isebrands 1971). The index leaf (LPI 0) is near the shoot apex, and has a blade that is half unfolded and ~4 cm in length. Leaves are numbered consecutively and positively down the stem. In the wounding experiment, lower leaves (LPI 9 and older) were mechanically wounded with pliers as described in Parsons et al. (1989).

Differential display

Differential display of messenger RNA (mRNA; Liang and Pardee 1992) was carried out according to the manufacturer's instructions (GenHunter, Nashville, Tenn.). DNase-treated RNA (Chang et al. 1993) was used for first-strand complementary DNA (cDNA) synthesis by MMLV-RT (GibcoBRL, Gaithersburg, Md.). The cDNA designated *pni288* was amplified via PCR using the primer pair 5′-$AAGCT_{11}C$-3′ and 5′-AAGCTTTCCCAGC-3′, with 7.4 kBq/μL α-[^{33}P]dATP included in the reaction mixture. The PCR products were separated by 6% denaturing polyacrylamide gels (HR1000, Genomyx, Foster City, Calif.). The bands of interest were excised from the gel, reamplified by PCR, and ligated to pGEM-T (Promega, Madison, Wis.) for transformation into *Escherichia coli* DH5α (GibcoBRL, Gaithersburg, Md.). Sequencing was carried out at the University of Florida DNA Sequencing Core Laboratory (Gainesville, Fla.). Sequences were compared with the public data base using basic local alignment search tool (BLAST; Altschul et al. 1997), alignments were carried out using CLUSTAL W, version 1.8 (Higgins et al. 1996), and comparisons among VSP homologs were performed using the PROTPARS routine within PHYLIP (Felsenstein 1993).

RNA analysis

Tissues were collected and immediately frozen in liquid nitrogen. To test the effect of SD on VSP gene expression, primary stem tissues that contained nodes for LPI 1 – LPI 3, and LPI 4 – LPI 7 were collected under long-day photoperiod (LD) (0 week SD), and after 2- and 3-week SD. The SD-induced gene expression patterns were verified in longer term experiments in which shoot tips were collected for RNA blot and immunolocalization studies. To examine the effect of N on gene expression, secondarily vascularized stem tissues (nodes for LPI 7 and older) were analyzed. For examination of the systemic response to wounding, leaves LPI 3 – LPI 8 from wounded and unwounded plants were examined. Total RNA was isolated, blotted, and hybridized to gene-specific cDNA probes as described in Lawrence et al. (1997). DNA slot blots (5 pg/slot) with bound *bsp*, *pni288*, and *win4* cDNAs were prepared and hybridized to the three cDNAs labelled as probes, respectively, to confirm a lack of cross hybridization under the standard hybridization and washing conditions employed in this study (Church and Gilbert 1984). A cDNA encoding the small subunit of ribulose-1,5-bisphosphate carboxylase was included as a negative control on the slot blots.

Immunolocalization

Shoot tips were collected, embedded in methacrylate, sectioned and processed essentially as described in Lawrence et al. (1997). To visualize bud structure, samples were stained in 0.05% (w/v) toluidine blue O in 1% sodium borate (Sigma, St. Louis, Mo.), and viewed using brightfield microscopy. VSPs were immunolocalized using a 1:50 dilution of antibody that was raised against a vegetative storage protein enriched in overwintering bark of *Salix microstachya* Turz. (Wetzel and Greenwood 1991). The secondary antibody was a 1:60 dilution of rabbit anti-chicken IgG conjugated to 10-nm colloidal gold particles (Wetzel and Greenwood 1991). Immunolocalized samples were viewed using differential interference contrast microscopy (Nomarski optics). Images were viewed and photographed using a Zeiss-Jena Jenalumar contrast microscope (Jena, Germany).

Results and discussion

Annual monocots and dicots have hardwood VSP-like genes

As part of a larger study in which we screened for nitrogen-responsive cDNAs via differential display (J.E.K. Cooke and J.M. Davis, unpublished data), we discovered a new member of the poplar VSP gene family, *pni288*. The *pni288* cDNA is a partial clone (934 base pairs long) that encodes a protein distinct from BSP and WIN4 (Fig. 1A). The three poplar VSP homologs are related to the *Arabidopsis* hypothetical protein TT22A6.170 and an *Oryza sativa* sequence (Figs. 1B and 1C).

The similarity of the *Populus* VSP homologs to proteins in annual species is an intriguing result, because such sequence relationships may give clues to the progenitor that evolved a VSP function in trees. Soybean leaf VSPα and VSPβ have weak acid phosphatase activity (DeWald et al. 1992), while a third soybean VSP exhibits lipoxygenase ac-

Fig. 1. There are at least three different VSP homologs in *Populus*, and they are related to gene products from annual plants. (A) Translated WIN4 (Accession No. AAA16342), BSP (Accession No. CAA49669), and PNI288 (Accession No. AF330050) were aligned using CLUSTAL W, version 1.8 (Higgins et al. 1996). Consensus is shown for amino acid residues that are conserved in all three sequences (uppercase) or twice (lowercase). Sequence alignments of translated *bsp*, *win4*, and *pni288* begin at the 5′ end of the partial *pni288* cDNA clone. This occurs at amino acids 66 (for BSP) and 62 (for WIN4). (B) Percent identities between the predicted amino acid sequences of VSP-like sequences from *Populus*, *Arabidopsis thaliana* (L.) Heynh. (At), and *Oryza sativa* L. (Os). Values in parentheses indicate percent similarities, calculated using the BLOSUM 62 substitution matrix (Henikoff and Henikoff 1992) and designating as similar all nonidentical residues with scores greater than or equal to 1. (C) Unrooted tree illustrating the relationships among VSP-like sequences compared in Fig. 1B.

A

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BSP          (65aa)...SPDSEDSSVDIAGRRFHSGTLNGSSIVYVKTGSHSVNMATTLQILLARFSIHGVI
PNI288            FPSTHVPYIDLVGRRFNIGKIKDVHVVVVNVGGEIPNVVLGTQVLFDLLSIRGII
WIN4         (61aa)...TPDAETPYVDIAGRRFHIGTLNARYIVYVKIGGNSVNAAIAVQILLNRFRIQGII
Consensus         Pd e pyvDiaGRRFhiGtln   iVYVk Gg svN a  QiLl rfsI GiI

BSP          YFGNAGSLDKKTMVPGDVSVPEAVAFTGVWNWKKFRSEKGKLVFGDFNYPENGENLLGTV
PNI288       HFGSAGSVS-DSLRLGDVAVPESVAFTGNWEWKSNASTRGELKFGDFNLPQKGVNSLGSA
WIN4         HFGSAGSLDKESIVPGDVSVPLAVAFTGAWNWKKFGSDKGTLNFGEFNYPVNGENLLASV
Consensus    hFGsAGSldk s vpGDVsVPeaVAFTG WnWKkf S kG L FGdFNyP nGeNlLgsv

BSP          EYEKIKMFSPSEAPKEVFWLPITKSWYNAATEALKDMKLRKCYSDE----CLPGEPKVVF
PNI288       DFQKVKLYTSGNPSQNLLWLPVDSNWLAVASE-LQGLKLQECVNEITETNCLENTPEIVF
WIN4         DYDKVKLFSKGHSPQDVFWFPSTTSWYSAATQVLQDLELRQCYDRA----CLSSKPKIVF
Consensus    dẏ KvKlfs g  pq vfWlP t sWy aAte LqdlkLr Cy       CL   PkiVF

BSP          GSKSSTSDFYVRNKAYGDFLNDNFDAKTADTTSASVALTSLSNEKLFVVFQGVSN--VAG
PNI288       GGRGSSADIYLKNAAYGEFLANRFNATFVDTSSAAVALASLTNEVPYILFRAISNSVIQG
WIN4         GTNGSSSDSYIKNKAYGDFLHKVFNVSTADQESAAVAWTSLSNEKPFIVIRGASN--VAG
Consensus    G  gSssD Y kNkAYGdFL   Fna taDt SAaVAltSLsNEkpfivfrg SN  vaG

BSP          ETSSN-SRVSYLASYNAFLAATKFINSIPTPRLACE
PNI288       TSGPN-SH--YLATANSVKVAVKFIELIGKPNWVFKY
WIN4         EANPGFSPASYLASYNAFLAAAKFIESIPTPRLACE
Consensus    e  pn S  sYLAsyNaflaA KFIesIptPrlace
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B

Sequence	Accession No.	WIN4	BSPA	PNI288	At	Os
WIN4	AAA16342	–	64 (79)	40 (56)	33 (50)	31 (44)
BSPA	CAA49669		–	35 (55)	32 (54)	35 (51)
PNI288	AF330050			–	31 (52)	33 (49)
At TT22A6.170	T09897				–	43 (56)
Os EST	BAA93031					–

C

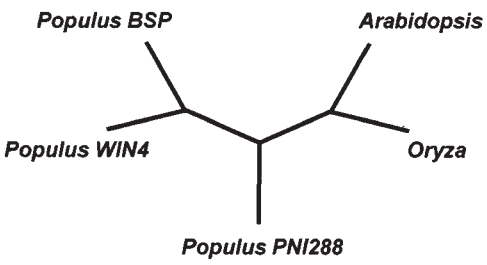
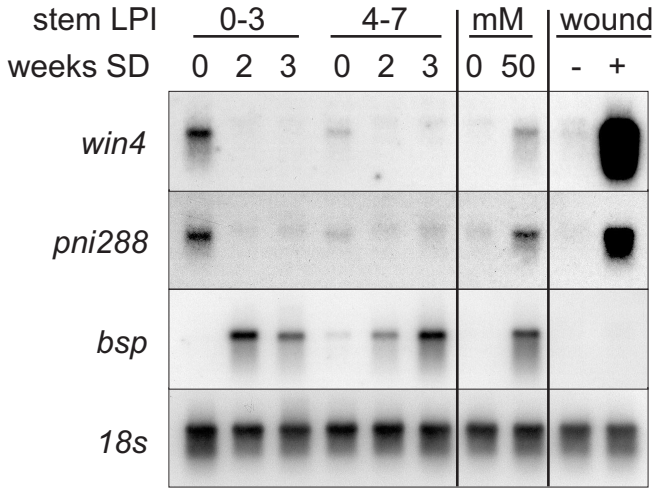


Fig. 2. *Populus* VSP homologs are all responsive to nitrogen availability but differ in their response to short-day photoperiod (SD) and wounding. Lanes 1–6: young and mature stem sections of trees under SD treatment. Lanes 7 and 8: mature stem sections of trees fertilized with limiting or luxuriant levels of NH_4NO_3 for 10 days. Lanes 9 and 10: unwounded and wounded leaves. Probes for *win4*, *pni288*, and *bsp* did not cross-hybridize under the washing conditions used in these experiments (data not shown). Ten micrograms of total RNA were loaded per lane. The bottom panel shows hybridization to 18S ribosomal RNA as a positive control for loading. LPI, leaf plastochron index (Larson and Isebrands 1971).

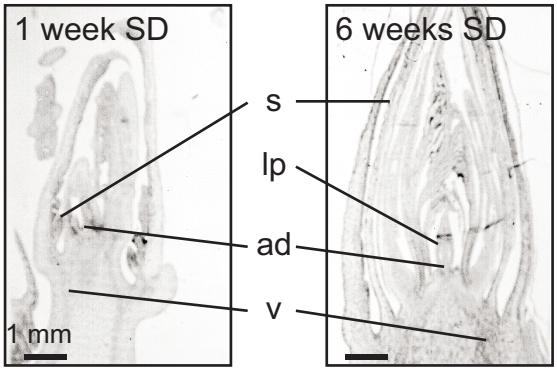


tivity (Tranbarger et al. 1991). DeWald et al. (1992) proposed a model to describe how acid phosphatase was recruited to function as a leaf VSP in soybean. Poplar VSP homologs lack sequence similarity to either acid phosphatases or lipoxygenases, suggesting that tree VSPs were derived from a different protein during evolution. This evolutionary progression should be better understood if a function is revealed for the proteins from *Arabidopsis* and rice.

Inducers of transcript accumulation

Poplar VSP homologs appear specialized for different functions, based on the results of RNA blot analysis (Fig. 2). Only one of the three transcripts, *bsp*, accumulates in stems of plants exposed to SD (Fig. 2; Coleman et al. 1991). The *pni288* and *win4* transcripts are detected at low levels in young stems of plants that are actively growing (i.e., under LD); however, they are rapidly lost from young stems under SD. Both *pni288* and *win4* are systemically wound responsive in leaves, whereas *bsp* is not. Wound responsiveness is a common feature of VSPs, although the specific role that VSPs play during a wound response is poorly understood (Davis et al. 1993; Staswick 1994). It appears that *pni288* expression is distinct from *bsp* and *win4* because the degree of wound responsiveness of *pni288* is less than that of *win4*. Furthermore, *win4* and *bsp* are induced in roots by luxuriant N but *pni288* is not (J.E. Cooke and J.M. Davis, unpublished data). A feature that is common to all three VSP homologs is their induction in mature stems of well-fertilized plants (Fig. 2, 0 vs. 50 mM). This feature is consistent with their presumed function in N storage and suggests that all three proteins would accumulate in mature stem tissues under luxuriant N conditions.

Fig. 3. Shoot architecture changes dramatically under short-day photoperiod (SD). The shoot apex is shown after 1 week (slowed growth) and 6 weeks (terminal bud fully formed) exposure to short days. s, stipules; lp, preformed leaf primordia; ad, apical dome; v, vasculature.



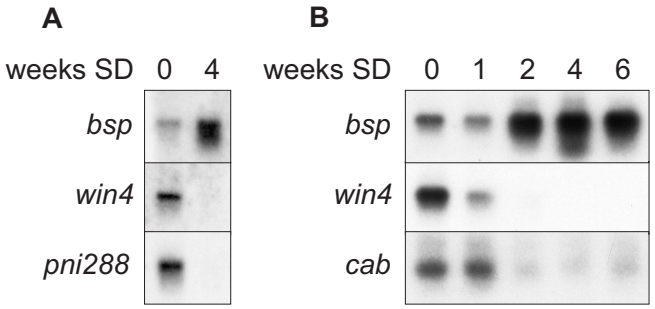
Terminal bud morphology

We focused on the shoot apex during the transition to dormancy for comparison with our previous study of the shoot apex under LD (Lawrence et al. 1997). *Populus deltoides* shoot development changes radically under the influence of SD (Goffinet and Larson 1981). Similar changes occur in hybrid poplar (Fig. 3). The left panel shows a shoot apex that had just begun the transition to terminal bud formation. After 1 week of exposure to SD, internode elongation had slowed in comparison to a similar time period under LD (Lawrence et al. 1997). However, the subtending stem was not secondarily vascularized, and no obvious manifestations of a dormant bud were apparent. After 6 weeks of SD, the terminal bud was fully formed, shoot elongation had completely ceased, and the shoot apex was secondarily vascularized. Secondary vascularization proceeds acropetally during bud formation and is associated with increased rigidity of the distal region of the stem. At this latter stage, the bud was encased by stipules that had enlarged into bud scale leaves. Leaf primordia found in the center of the bud give rise to preformed leaves that expand quickly after budbreak (Goffinet and Larson 1981).

Gene expression in the terminal bud

The SD treatment dramatically and differentially alters VSP gene expression in the shoot apex (Fig. 4). The *win4* and *pni288* transcripts are abundant in the apex prior to SD treatment but decrease under SD. In contrast, *bsp* transcripts accumulate under SD (Fig. 4A). A time course study was performed to determine when the shifts in transcript levels occurred. The *win4* transcripts (as well as *pni288* transcripts; data not shown) are abundant in the apex prior to SD treatment but decrease rapidly (within 1 week) under SD. Similarly, transcripts for chlorophyll *a*/*b* binding protein (CAB), which represents a major constituent of the photosynthetic apparatus, decrease under SD. Bud scale leaves that develop under SD have low levels of *cab* transcripts, because they

Fig. 4. The *Populus* shoot apex shows opposing regulation of *bsp* compared with *win4*, *pni288*, and *cab*. Changes in gene expression were monitored in shoot tips from trees grown under short-day photoperiods (SD) for the indicated times. Samples in Fig. 4A were derived from a different experiment than Fig. 4B, which are from the time-course experiment shown in Fig. 3. Probes were for transcripts encoding VSPs (*bsp*, *win4*, *pni288*) and chlorophyll *a*/*b* binding protein (*cab*). Three or 10 μg of total RNA were loaded per lane in Figs. 4A and 4B, respectively.



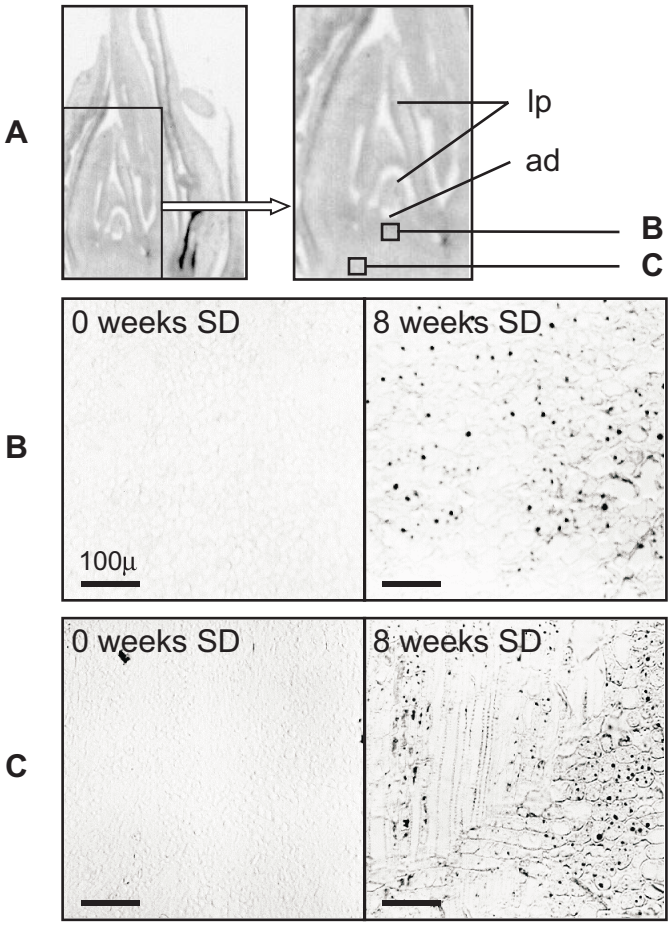
are not specialized for photosynthesis. In contrast, *bsp* transcripts in the shoot apex increase dramatically (Fig. 4B).

The time-course data show that *win4* and *bsp* genes are regulated in an opposing manner, and the temporal separation of the responses (~1 week) implies that *win4* and *bsp* genes are regulated by different signaling pathways during the SD response. The *win4* transcript levels decrease before *bsp* transcripts increase. It appears that *win4* downregulation in the shoot tip is an early response to SD, occurring before the terminal bud is fully formed. In contrast, upregulation of *bsp* occurs after the SD stimulus changes the anatomy of developing leaves (cf. Goffinet and Larson 1981; Coleman et al. 1991). The cellular factors responsible for downregulating *win4* (and *pni288*) or upregulating *bsp* in response to SD have not been identified, but presumably these factors orchestrate the shift in compartmentation of N that occurs under SD.

BSP accumulation is dramatic and cell specific

Location of VSP within a terminal bud has not been reported to our knowledge. To elucidate the cell types that contribute to N storage in autumn, we used immunolocalization to examine VSP in developing terminal buds. In fact, the cell types that accumulate storage protein during terminal bud development (Fig. 5) are fundamentally different than the cell types that express WIN4 during growth (Lawrence et al. 1997). As bud formation progressed, storage protein accumulated in the ground meristem beneath the apical dome and in cells adjacent to xylem (Fig. 5). No immunoreactive protein was detected in these cell types prior to SD treatment (Fig. 5). In addition, no label was observed when preimmune serum was used as a control (data not shown). The localization of storage protein suggests that it is available for immediate remobilization to promote shoot growth when bud dormancy is broken. In contrast, WIN4 is located in stipules and in developing leaves in actively growing shoot apices (Lawrence et al. 1997). No immunoreactive protein was observed in stipules or preformed leaves after 8 weeks of SD (data not shown). Although we cannot conclude with certainty that the *Salix* antibody is specific to *Populus* BSP, the transcript data (Figs. 2 and 4) and previously published work (Coleman et al. 1991) suggest that BSP is the major SD-induced storage protein in *Populus*. Consequently the label (Fig. 5) most likely reflects BSP accumulation. These results indicate that stem tissues, but not stipules or leaf primordia, are important sources of overwintering N in poplar buds.

Fig. 5. BSP accumulates in specific cell types within the *Populus* shoot apex under short-day photoperiod (SD). (A) Stained section of a shoot apex (0 week SD) indicating the regions shown in the immunolocalized samples. Storage protein was immunolocalized in shoot apices exposed to 0 or 8 weeks of SD: (B) within the ground meristem of the apical dome and (C) from the region below the apical dome (including a portion of the vasculature). At 0 weeks of SD, cells are meristematic and undifferentiated. However, after 8 weeks of SD, the apical dome remains undifferentiated, while the cells immediately below the apical dome show secondary vascularization. ad, apical dome; lp, leaf primordium.



Roles of VSPs in seasonal N cycling

The *Populus* VSP homologs appear to have specialized functions based on their differential regulation and tissue-specific expression. Interestingly, the most important question about VSPs remains unanswered: what is their actual function in trees? Two models may be proposed to explain

our current thoughts on the role of VSPs. In the first model, VSPs act as N sink strength determinants: they are active drivers of N import into cells where they are being synthesized. Such a scenario would demand fine control of VSP gene expression to direct appropriate N sink strength in specific cell types and at specific times of the year when N allocation is shifted among tree organs. Multiple VSP genes could afford that fine level of control. In the second model, other factors determine the relative N sink strength of a cell, and VSPs are passive N reserves that are synthesized as a consequence of excess N present in the cells. The presence of multiple VSP genes, with distinct cell specificities and induction characteristics, may be adaptive for riparian species such as *Populus* that are exposed to episodic incursions of nutrients at different times of the year. Multiple VSPs may allow more efficient nutrient capture.

Ultimately, determining the function of VSPs will require a transgenic approach. The first model predicts that transcriptional activation of VSPs in a tissue precedes increased import of N into those cells. In contrast, the second model predicts that increased N import into a tissue would result in transcriptional activation of VSPs. To our knowledge, no evidence has been published determining that either WIN4 or BSP is a cause (first model) or consequence (second model) of increased N allocation to a tissue. A series of transgenic approaches in which specific VSP homologs are over- or under-expressed, or altered in their regulation, should allow these contrasting models to be tested.

Acknowledgments

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