Poplar Bark Storage Proteins (BSP) are important in storing nitrogen (N) recovered from autumn-senescent leaves, and supply N for spring growth. Plants of *Populus tremula* x *P. alba* were transformed with a poplar BSP antisense cDNA fused to a double 35S promoter. Regenerated lines were screened for reduced BSP accumulation in short day photoperiod, and two lines were selected for further studies. Under long-day conditions, both BSP-antisense lines were characterized by larger leaves, longer internodes and slower growth rates, for a net shift in dry-matter partitioning from stem to leaf. Antisense lines also show reduced N concentration in leaf and stem tissue, and altered nitrate uptake. Grafting studies show that these effects on leaf and stem partitioning and N content are determined by the shoot genotype. These results indicate an important role of BSP in long day growth and partitioning. BSP-antisense plants did not show altered Nitrate Reductase activity, as determined by *in vivo* assay. Wild-type poplar plants were grown hydroponically on solutions of 0 to 30 mM nitrate, and NR activity determined on leaf, stem and root tissue. Leaf activity was >20x higher than root or stem, with the highest activity found in young expanding leaves. NR activity of both leaves and roots increased with N supply. During autumn, BSP-antisense leaves abscised earlier than the untransformed *wt*. Abscised *wt* leaves contained a
higher proportion of pre-senescent N levels. Comparisons among ecotypes of P. deltoides and among clones of P. trichocarpa x P. deltoides demonstrated genetic variation in both time of BSP induction, and amount of BSP accumulation. In six P. deltoides ecotypes grown at a common site, time of maximum BSP mRNA was inversely correlated with latitude of origin. Eight to ten clones from each of six full-sib families of P. trichocarpa x P. deltoides were screened for SD BSP accumulation. Clonal differences in BSP accumulation were significant in 5 of 6 families, and clones with high BSP levels also had higher total stem N content. These results further confirm the importance of BSP in autumn N resorption.
SEASONAL AND SHORT-TERM NITROGEN CYCLING IN *POPULUS*

by

Brent L. Black

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Approved: 

Major Professor, representing Plant Physiology

Chair of Plant Physiology Program

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Dean of Graduate School

I understand that my dissertation will become part of the permanent collection of Oregon State University libraries. My signature below authorizes the release of my dissertation to any reader upon request.

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Brent L. Black, Author
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Experiments documenting the ecotypic variation in time of BSP induction (chapter 5) were conducted by Gary Coleman prior to my involvement in the project. They are included here because these experiments and my genetic-variation study are closely related and will be submitted together for publication.
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bark storage protein(s)  BSP(s)
bicinchoninic acid  BCA
bovine serum albumin  BSA
corrected image density value  CIDV
leaf dry mass per area  LMA
leaf plastochron index  LPI
long day  LD
low temperature  LT
nitrate reductase  NR
nitrate reductase activity  NRA
nitrogen  N
photosynthetic photon flux  PPF
resorption efficiency  RE
short day  SD
Tris-buffered saline  TBS
vegetative storage protein(s)  VSP(s)
wild type  wt
SEASONAL AND SHORT-TERM NITROGEN CYCLING IN POPULUS

CHAPTER 1: Introduction

Plants optimize nitrogen (N) use by redistributing N internally to meet the changing demands of different tissues and organs during growth and development (reviewed by van den Driessche, 1984). In some cases, specific proteins are synthesized as temporary storage sites to facilitate this N redistribution (Staswick, 1994). For example, storage proteins are synthesized in the seeds during seed development and maturation. The catabolism of these seed storage proteins later provides a source of carbon and nitrogen during seed germination and seedling growth (for review see Higgins, 1984). Other types of storage proteins accumulate in specialized storage organs such as potato tubers (Paiva et al., 1993). Plants may also store reduced N, not in specialized storage organs, but by accumulating storage proteins in vegetative tissues including the leaves (Benedetti et al., 1995; Wittenbach, 1983). In herbaceous plants, these vegetative storage proteins (VSP) are later catabolized providing a ready supply of amino acids for protein synthesis in developing reproductive organs (Staswick, 1994).

Deciduous hardwood trees accumulate VSPs as part of a seasonal N cycle. This seasonal cycle involves resorption of N from senescing leaves in the autumn, and storing this N in the overwintering portions of the plant. These stored reserves are later remobilized for spring growth (reviewed by Stepień et al., 1994). During vegetative growth, about 75% of total tree N is contained in the leaves (Pregitzer et al., 1990). An estimated 50 to 90% of this leaf N is translocated from senescing leaves during autumn in the form of amino acids (Chapin and Kedrowski, 1983; Kang and Titus, 1980; Taylor and May, 1967; Titus and Kang, 1982). This reduced N is then stored in the perennial tissues primarily as protein (Alban 1985; McColl
1980; Pastor and Bockheim 1984; Killingbeck et al., 1990). During autumn leaf senescence and abscission in apple, leaf protein levels decline by >50% and stem protein levels increase by >200% (Kang and Titus, 1980; Tromp, 1970). This seasonal increase in stem protein levels during autumn leaf senescence has also been reported in other temperate hardwoods. In a survey of six genera representing four families, Wetzel and Greenwood (1991) found that total soluble protein in the inner bark was 160 to 400% higher in the winter than in the summer.

Stored protein reserves are an important source of carbon and nitrogen for spring growth, as total spring growth has been correlated with the total amount of stored N in the bark (Taylor and May, 1967). Work with isotopically labeled N has shown that 45 to 75% of the N in new shoots comes from stored reserves (Deng et al., 1989; Sanchez et al., 1991). This partitioning of stored N to new growth has been shown to be independent of available soil N (Millard, 1994; Millard and Neilsen, 1989; Millard and Proe, 1991), further signifying the importance of stored reserves in spring growth.

O'Kennedy and Titus (1979) suggested that proteins which serve a seasonal storage role would fit the following criteria: (a) seasonal storage proteins would accumulate to high levels during autumn leaf senescence, (b) represent a major component of the overwintering nitrogen reserves, and (c) would be degraded at the onset of vegetative growth in the spring. Based on these criteria, seasonal VSPs have been identified in a number of temperate hardwoods including: Acer saccharum (Wetzel et al., 1989), Malus (O'Kennedy and Titus, 1979), Ginkgo biloba (Shim and Titus, 1985) Prunus persica (Arora et al., 1992), Robinia pseudoacacia (Nsima-Lubaki and Peumans, 1986) Salix x smithiana (Wetzel et al., 1989), Sambucus nigra (Nsima-Lubaki and Peumans, 1986), Sophora japonica (Baba et al., 1991), and in a number of Populus species (Coleman et al., 1991; Langheinrich and Tischner, 1991; Stepien and Martin, 1992; Wetzel et al., 1989). These storage proteins are found primarily in the bark, but in some cases
have also been reported in the wood (Arora et al., 1992; Langheinrich and Tischner, 1991; van Cleve et al., 1988) and roots (Langheinrich and Tischner, 1991). Due to their prevalence in the bark, these seasonal VSPs from tree species have also been called bark storage proteins (BSPs).

Seasonal protein accumulation occurs in protein-filled vacuoles known as protein bodies. (Stepien et al., 1994). These protein bodies have been identified in the parenchyma cells of the phloem, cortical tissue and xylem rays (Baba et al., 1991; Clausen and Apel, 1991; Sauter et al., 1989; Stepien et al., 1991; van Cleve et al., 1988; Wetzel and Greenwood, 1991; Wetzel et al., 1989), and in cambial cells (Wetzel and Greenwood, 1991). The BSPs of poplar and willow have been immunolocalized within these protein bodies (Clausen and Apel, 1991; Sauter and van Cleve, 1989; Stepien and Martin, 1992; van Cleve et al., 1988; Wetzel and Greenwood, 1991). Protein bodies change in shape and appearance from autumn to winter to spring (Sauter and van Cleve, 1990) corresponding to changing levels of stored protein. Protein bodies were not detected in the bark cells of Populus deltoides or Acer saccharum during the summer (Wetzel et al., 1989).

The disappearance of BSPs and protein bodies from the cells of the stem tissue corresponds with spring mobilization of stored reserves (Wetzel et al., 1989). In poplar, the spring disappearance of storage proteins has been correlated with an increase in amino acid levels (primarily glutamine) in the xylem sap (Sauter and van Cleve, 1992). Further, if poplar shoot growth is inhibited or shoots are removed, the degradation of storage proteins is inhibited (Coleman et al., 1993), suggesting that new shoots communicate with bark storage sites to regulate degradation of BSPs.

A 32-kDa poplar BSP has been identified which accumulates to as much as 70% of soluble protein during autumn leaf senescence, and declines to nearly undetectable levels shortly after vegetative growth resumes in the spring (Coleman et al., 1991; Langheinrich and Tischner, 1991; Tromp and Ovaa, 1973). Besides
this 32-kDa BSP, additional 36 kDa and 38 kDa storage proteins have also been reported (Langheinrich and Tischner, 1991; Stepień and Martin, 1992). However, Langheinrich and Tischner (1991) determined that the three different molecular weight proteins were glycosylated, and that all three were glycolytic isoforms of the same polypeptide. Both a cDNA and gene for this poplar BSP have been isolated and sequenced (Coleman and Chen, 1993; Coleman et al., 1992). The deduced amino acid sequence indicates a polypeptide rich in Ser, Lys, Leu, Phe. Lys and Ser together make up 17.5% of the total protein by molecular weight (Coleman et al., 1992), and have a C:N ratio of 3:1. The C:N ratio of the complete protein and how this compares to other proteins was not presented. The primary transport form of N in poplar is glutamine (Sauter and van Cleve, 1992). Therefore the efficiency of breaking down the storage protein and converting the constituent amino acids to glutamine may be more important than C:N ratio in determining the effectiveness of BSP as a storage site for N. However, except for the initial characterization of several break-down products (Banados 1992), little is known about the process of BSP degradation.

The regulation and expression patterns of the poplar BSP gene have been documented for both short-day (SD) and long-day (LD) conditions (for review see Coleman, 1997). BSP gene expression in the stem increases dramatically under SD photoperiod and decreases under LD (Coleman et al., 1992). This SD induction is modulated by N availability, where reduced N availability delays SD-induced accumulation of BSP mRNA and protein (Coleman et al., 1994). This interaction of SD photoperiod and N availability in regulating BSP gene expression is consistent with a seasonal N storage role.

Under LD conditions, BSP mRNA and protein levels increase with increased N supply (Banados, 1992; Coleman et al., 1994). LD BSP gene expression is also induced by wounding (Davis et al., 1993; Stepień and Sauter, 1994). Both the wounding- and high N-induced expression were localized in the stem tissue with no
measurable BSP gene expression in mature leaves (Coleman et al., 1994; Davis et al., 1993). More recently, Zhu and Coleman (in review) transformed poplar with the promoter region of the BSP gene fused to a reporter gene. Transformed plants were used to localize BSP gene expression under different environmental conditions by assaying for the presence of the reporter-gene product (GUS activity). They found the highest background BSP gene expression and the highest N-inducible expression localized in the stem and youngest expanding leaves. However, low levels of N-inducible expression were also detected in mature leaves (Coleman, personal communication). This differential expression of BSP genes under LD conditions suggests an important role for BSP in LD growth. However, previous research efforts have focused on the seasonal function of poplar BSPs, and have not directly investigated their potential role in long-day growth and development.

In some cases, storage of reduced N may be a secondary function of these proteins. For example, ribulose 1,5-bisphosphate carboxylase/oxygenase (Rubisco) is the predominant protein in active photosynthetic tissue and accumulates in response to N supply in both herbaceous (Millard, 1988) and woody plants (Millard and Thomson, 1989), and is therefore considered a form of summer storage for reduced N. In addition, several proteins identified for their vegetative storage function in soybean (Glycine max; Wittenbach, 1983) also appear to have important enzymatic functions. Two of these, vspa and vspβ, have been shown to exhibit acid phosphatase activity (DeWald et al., 1992). Another (vsp94) has similar molecular mass, and similar DNA and amino acid sequences to a lipoxygenase (Tranbarger et al., 1991) which is involved in fatty acid metabolism and in wounding responses (Hildebrand et al., 1988). After reviewing the available literature on woody-plant VSPs, Stepien et al. (1994) suggest that the patterns of woody-plant BSP gene expression and protein accumulation indicate the possibility of multiple physiological functions. However, unlike VSPs from annual plants,
woody plant BSPs have no known enzymatic activity. Therefore, they conclude that more information is needed on the physiological function(s) of BSPs.

Molecular genetic techniques provide new tools for studying the function of individual genes and gene products. Antisense RNA can inhibit gene expression of a complementary mRNA (Green et al. 1986), and has been used to study the function of a number of plant genes (van der Krol et al. 1988; Smith et al., 1988; Stockhaus et al., 1990). We have transgenic poplars which express an antisense copy of the BSP cDNA. Coleman (unpublished) constructed a gene consisting of a selectable marker and the poplar BSP cDNA fused in antisense orientation to tandem 35S promoters. (See Chapter 2 materials and methods). This construct was transformed into hybrid poplar (P. tremula x P. alba) and transformed lines were regenerated. Previous work suggested that maximum rate of BSP accumulation occurred 4 weeks after SD exposure (Coleman et al., 1992; 1994). Therefore, transgenic lines were screened for reduced BSP accumulation at 4 weeks of SD photoperiod, with two independent lines selected for characterization. Part of the research presented here is directed at determining the function of BSP under both LD and SD conditions by characterizing these two BSP-antisense lines.

Imsande and Touraine (1994) suggest that the function of VSPs in soybean are as a supplemental N sink at the site of N assimilation. Nitrate reductase (NR) catalyzes the first step in the energy-intensive process of nitrate assimilation. N reduction and assimilation patterns and the localization of NR activity have been extensively studied in a wide range of plant species. Among non-legume temperate annuals, some genera reduce NO₃⁻ primarily in the leaves while others localize nitrate reduction and assimilation in the roots (Andrews, 1986). Biochemical advantages of shoot- versus root-localized NRA have been proposed (see Lexa and Cheesman, 1997) based on observed leaf: root NR partitioning among species which differ in ecological adaptation (Stewart et al., 1988). Roots have traditionally been considered the predominant site of nitrate reduction in temperate
perennial (Andrews, 1986) based on measurements of xylem sap constituents (Bollard, 1960). However, several temperate hardwoods including Quercus rubra and Fraxinus pennsylvanica have significant NR activity in the leaves (Traux et al., 1994). Little is known about the activity and localization of NR in poplar. Based on evidence suggesting a LD function for poplar BSP (see discussion above and Chapter 2), studies were conducted to determine the site of NR activity in poplar. NR activity localized in the shoot, near the site of BSP synthesis, would be consistent with the LD function proposed for herbaceous-plant VSPs.

Aside from the remaining physiological questions, little is known about the ecological costs and benefits associated with BSP accumulation. Genetic variation in BSP accumulation could be used to investigate the ecological role of BSP. Several reports have documented clonal variation in total BSP accumulation (Coleman et al., 1991; Langheinrich, 1993). However these studies compared BSP levels among different species and interspecific hybrids, or among different ecotypes grown at two different field sites. Since mid-winter BSP levels are also a function of N availability, neither of these studies adequately determine the extent to which BSP accumulation is genetically determined. Further, despite the extensive knowledge of the accumulation patterns and properties of BSP and the regulation of BSP gene expression, little direct physiological evidence exists as to the precise function of BSP (Stepien et al., 1994).

The primary objectives of this research were:

1. To use antisense technology to determine whether or not poplar BSP plays a role in long-day growth and development.
2. To determine whether NR in poplar is leaf or root localized, and whether or not NR activity is altered in BSP-antisense plants.
3. To determine the role of BSP in resorption of N during autumn leaf senescence, by comparing N resorption efficiency of BSP-antisense plants to that of the wild type.

4. To determine whether or not there is genetic variability in SD-induced BSP accumulation and identify poplar clones which accumulate different amounts of BSP during SD treatment.
CHAPTER 2: Antisense Bark Storage Protein Gene in Poplar Alters Growth and Dry Weight Partitioning Under Long-day Photoperiod

ABSTRACT

Plants of *Populus tremula x P. alba* were transformed with a poplar bark storage protein (BSP) antisense cDNA fused to a double 35S promoter. Regenerated lines were screened for reduced BSP accumulation in short day photoperiod, and a number of BSP-antisense lines were identified with 70 to 90% reduction in BSP accumulation. Two of these lines were selected and the growth and partitioning in these lines compared to wild-type plants. Both BSP-antisense lines were characterized by larger leaves, longer internodes and a slower rate of node production. Taken together, these changes result in a shift in dry-matter partitioning from stem to leaf. Antisense lines also showed reduced N content in leaf and stem tissue, and altered nitrate uptake. Grafting studies showed that effects on leaf and stem partitioning and N content are determined in the shoot. These results suggest that BSP has an important function in growth and partitioning which may be related to nitrogen acquisition and partitioning.

INTRODUCTION

To optimize use of limited available nitrogen (N), plants redistribute N internally to meet demands of new vegetative growth or reproductive development (reviewed by van den Driessche, 1984). A number of plant species store reduced N by accumulating high levels of specific proteins. Annual seed plants like soybean (*Glycine max*; Wittenbach, 1983) and *Arabidopsis thaliana* (Benedetti et al., 1995)
accumulate proteins in the leaves during vegetative growth. These vegetative
storage proteins (VSP) are then catabolized during reproductive growth, providing a
ready supply of amino acids for protein synthesis in developing reproductive organs
(Staswick, 1994).

Deciduous hardwood trees accumulate VSPs for seasonal N storage. During
vegetative growth, about 75% of total tree N is contained in the leaves (Pregitzer et
al., 1990). An estimated 50 to 90% of this leaf N is translocated from senescing
leaves during autumn (Chapin and Kedrowski, 1983; Kang and Titus, 1980; Taylor
and May, 1967) and stored in the perennial tissues (Alban 1985; McColl 1980;
Pastor and Bockheim 1984; Killingbeck et al., 1990). This seasonal storage occurs
primarily in the bark and xylem ray cells of the stem as bark storage proteins
(BSPs) (Kang and Titus 1980; Sauter et al., 1988, 1989; Wetzel et al. 1989a,
1989b). A 32-kD BSP has been identified in poplar which accumulates to as much
as 70% of soluble protein during autumn leaf senescence and declines to nearly
undetectable levels shortly after vegetative growth resumes in the spring (Coleman
et al., 1991; Langheinrich and Tischner, 1991; Tromp and Ovaa, 1973). Both a
cDNA and gene (bspa) for poplar BSP have been isolated and sequenced (Coleman
et al., 1992; Coleman and Chen, 1993). Expression of bspa is regulated by short-
day photoperiod (Coleman et al., 1992), consistent with a seasonal role.

Under long day conditions, BSP mRNA and protein are detectable primarily
in the stem (Coleman et al., 1994; Davis et al., 1993) and growing shoot tip and to a
lesser extent, in the youngest expanding leaves (Zhu and Coleman, in review).
Increased N availability can induce bspa expression and increase BSP levels in the
bark (Coleman et al., 1994). However, previous research efforts have focused on
the seasonal function of poplar BSPs, and have not directly investigated their
potential role in long-day growth and development.

Antisense RNA can inhibit gene expression of a complementary mRNA
(Green et al. 1986), and has been used to study the function of a number of plant
genes (van der Krol et al. 1988; Smith et al., 1988; Stockhaus et al., 1990). We have produced transgenic poplars which express an antisense copy of the BSP cDNA. Several of these lines selected for reduced SD BSP accumulation were used to investigate the function of poplar BSP in long-day growth and biomass partitioning.

MATERIALS AND METHODS

BSP antisense gene construction

A HindIII - XbaI fragment containing tandem copies of the CaMV 35S promoter and the untranslated leader sequence from RNA4 of the alfalfa mosaic virus (AMV) from pBI505 (Warkentin et al., 1992), was ligated into a HindIII - XbaI digested binary vector, pGPTV-BAR (Becker et al., 1992). The resulting binary vector was then digested with Smal, ligated to XhoI linkers, digested with XhoI and SacI and gel purified. This purified binary vector was then ligated with an XhoI - SacI DNA fragment that contained the BSP cDNA (Coleman et al., 1992). The resulting binary vector consisted of tandem copies of the 35S promoter followed by the AMV sequence fused to the BSP cDNA in antisense orientation. (Figure 2.1). The pGPTV-BAR vector contains the bialaphos resistance gene (bar) as a selectable marker (Figure 2.1).

Plant Material

pGPTV-BAR containing the 35S35S-AMV::BSP-antisense construct was co-cultivated with either stem or root sections of *Populus tremula* x *P. alba* (INRA clone 717 1-B4) following the procedure of Leple et al. (1992). Both stem-transformed ("BR" series) and root-transformed ("RT" series) poplar lines were regenerated, propagated *in vitro*, and moved to the greenhouse. Southern analysis
using bar as a probe was used to verify poplar transformation (data not shown). Previous work has shown that the highest levels of BSP mRNA and the highest rate of BSP accumulation occur after 4 weeks of SD exposure. Therefore, more than 30 independent transgenic lines were screened by comparing BSP levels in the bark after four weeks of SD photoperiod (Figure 2.2 shows the comparison of “RT” clones). Following SD induction, bark samples were collected from replicate plants, frozen in liquid nitrogen and stored at -80°C for later protein extraction. For protein extraction, samples were ground in liquid nitrogen with mortar and pestle and transferred to chilled 1.5 mL microfuge tubes. Warm extraction buffer (62.5 mM Tris-HCl - pH 6.8, 2% SDS, 10% glycerol and 5% 2-mercaptoethanol; Laemmli, 1970) was added and samples immediately vortexed, held on ice for 15 to 20 min, and then moved to a boiling water bath for 15 min. After boiling, samples were cooled to room temperature, centrifuged at 14,000 rpm (Eppendorf model 5417R centrifuge, Brinkmann Instruments, Westbury NY) and the supernatant collected and stored at -20°C.

Protein concentration was determined using bicinchoninic acid (BCA) colorimetric assay (Brown et al., 1989). Interfering substances were removed by diluting aliquots 100x in distilled water and then precipitating protein with deoxycholate and trichloroacetic acid followed by centrifugation. Proteins were resuspended in 5% SDS and 0.1 N NaOH and concentration determined using the BCA colorimetric assay (Pierce, Rockford IL). The BCA procedure was as per manufacturer recommendations, with bovine serum albumin as a standard and absorbance measured at 570 nm.
Figure 2.1. BSP-antisense gene construct. The BSP cDNA from poplar was fused in antisense orientation to tandem copies of the CaMV 35S promoter and the untranslated leader sequence from RNA 4 of the alfalfa mosaic virus (AMV). See materials and methods for details of gene construction.

Figure 2.2. Bark storage protein (BSP) levels of BSP-antisense plants after 4 weeks of short-day photoperiod. Comparison of lines regenerated from root transformation (RT) experiments. Each lane on protein gel blots received equal amounts of protein. BSP levels were detected as described in materials and methods.
BSP levels were compared among antisense lines by western-blot analysis. Equal amounts of protein per lane were loaded on a 12% polyacrylamide gel (Mini-Protein II, BioRad) and electrophoresed according to manufacturer recommendations (45 min at 200 volts). Subsequently, gels were blotted to nitrocellulose (NitroBind, Micron Separations Inc., Westborough MA) using standard blotting procedures. After blotting, membranes were washed for 10 min in Tris-buffered saline (TBS, 20 mM Tris-HCl - pH 7.5 and 150 mM NaCl) and blocked overnight at 4°C in TBS with 3% bovine serum albumin (BSA). After blocking, blots were warmed at 30°C for 1 h, washed in TBS at room temperature for 15 min, and then incubated in primary antibody solution for 1 h. Serum containing anti-BSP antibody (Coleman et al., 1991) was diluted (1,000x) in TBS containing 0.05% Tween (TTBS) and 1% BSA. After incubation in primary antibody solution, blots were washed three times for 15 min each in TTBS at room temperature, and then incubated in secondary antibody solution (goat-anti-rabbit linked to alkaline phosphatase, diluted 25,000x in TTBS + 1% BSA). Blots were then washed 3 times for 15 min in TTBS, once in TBS and color developed as described by Sambrook et al. (1989). Two independent lines (14BR and RT5), were selected for further study based upon reduced SD BSP levels.

Plants were propagated in vitro by taking 2-node cuttings from plantlets in sterile culture and rooting these cuttings on 0.5x Murashige and Skoog media. Rooted plantlets were transplanted to flats containing soil-less potting media (ProGro 300-S; consisting of peat moss, vermiculite, composted pine bark, shredded polystyrene) and placed in the greenhouse under intermittent mist for approximately four weeks. After plants had acclimated to the greenhouse environment, they were removed from the mist bench.
Growth and partitioning analysis

At a height of approximately 30 cm, 15 plants from each of three clones (wild type, 14BR, RT5) were selected for uniformity, repotted in approximately 4.5 L of potting media and blocked by plant size and by location in the greenhouse. Low levels of continuous supplemental incandescent light were provided to ensure long day growth. Plants were fertilized at weekly intervals with 5 mM NH4NO3, and at approximately 3-week intervals with a combination fertilizer solution consisting of 0.5x modified Hoagland’s solution without N (1.0 mM MgSO4, 0.50 mM KH2PO4, 44.8 µM FeNa-EDTA, 23.1 µM H3BO3, 4.57 µM MnCl2, 0.404 µM ZnCl2, 0.266 µM CuCl2, 51.6 nM Na2MnO4).

After 8 weeks, five plants of each clone were harvested by collecting leaves, bark, wood and roots. Fresh weight of each tissue was determined, samples were dried at 65°C for two weeks and dry-weight determined. Leaf plastichron index (LPI; Dickson, 1986) was used to designate leaves at similar stages of development and age. Leaves were numbered sequentially from the growing point basipetaly, with the youngest leaf longer than 2 cm assigned an LPI of 1.

Samples were collected from each of the remaining 10 replicates for total N determination. After leaf area and fresh weight determinations, these samples were immediately frozen in liquid nitrogen and stored at -80°C until lyophilization. Lyophilized samples were milled with a high speed mill equipped with a 1.0 mm screen (Tecator Cyclotec 1903 Sample Mill). Total N was determined on triplicate sub-samples by Dumas combustion using an automated C/N analyzer (NC 2100 - Soil, CEInstruments, Italy). The amount of N present in leaves can be expressed as a function of leaf weight (McColl, 1980; Tew, 1970) or leaf area (Alban, 1985; Killingbeck et al., 1990). For the purpose of the present discussion, N per dry weight is defined as N concentration, and N per leaf area as N content.

The experimental design was a randomized complete block (blocked by greenhouse location and sampling time), and statistical analysis was performed.
using the General Linear Model procedure in the SAS program package (SAS Institute, Cary North Carolina).

**Reciprocal grafting**

Wild type (wt) and 14BR plants were used in a reciprocal grafting study. When the plants were approximately 30 cm in height, wt scions (shoots) were grafted to 14BR (a) rootstocks (wt/a), and 14BR scions grafted to wt rootstocks (a/wt). Control plants were created by grafting wt and 14BR scions back to their own roots (wt/wt and a/a). Plants were grafted by removing all but the youngest leaf, and severing the stem 10 to 15 cm from the base. The lower portion of the plant was used as the rootstock, and split vertically from the top for approximately 2 cm. The severed shoot was used as a scion with 2 cm at the base cut into a wedge shape and inserted into the split portion of the appropriate rootstock. The resulting graft was wrapped tightly with parafilm and plants placed on a propagating bench under intermittent mist. Over the following weeks, shoots emerging from below the graft union were removed. When terminal growth of the scion resumed, plants were removed from the propagating bench and planted in 4.5-L pots. Fifteen replicate plants of each scion/rootstock combination were selected for uniformity and blocked by location in the greenhouse. Plants were grown for 8 weeks with fertilization, harvesting, and sample analysis as described above.

Results were analyzed as a randomized complete block design, where scion and rootstock genotypes were treated as a 2 x 2 factorial treatment structure. Tests for main effect (scion or rootstock) and interaction significance suggest the location (shoot or root) where a particular response is determined.

**Nitrate uptake**

*In vitro* propagated wt and antisense plants were established in perlite and grown on dilute fertilizer solution (Excel 21-5-20) to a height of approximately 15
cm. Plants were then transferred to a hydroponic system and moved to a growth chamber (200 μmol m⁻² s⁻¹ light, 23 °C). In transferring to the hydroponic system, each plant stem was secured between two halves of a split #12 rubber stopper, perlite was washed from the root system, and washed roots were placed in a 1-liter opaque plastic bottle. Each bottle contained a 0.5x modified Hoagland's solution with 7.5 mM NO₃⁻ as the only N source (2.5 mM KNO₃, 2.5 mM Ca(NO₃)₂, 1.0 mM MgSO₄, 0.50 mM KH₂PO₄, 44.8 μM FeNa-EDTA, 23.1 μM H₃BO₃, 4.57 μM MnCl₂, 0.404 μM ZnCl₂, 0.266 μM CuCl₂, 51.6 nM Na₂MnO₄). Commercially available aquarium pumps (80 gallon rated, Top Fin) supplied a continuous flow of air to the bottom of each bottle to aerate the hydroponic solution. A single pump provided air to three plants through a series of valves. These valves were adjusted to equalize flow rate to all bottles. The solution in each bottle was replenished daily with distilled water and replaced biweekly with fresh solution.

After an additional 8 to 10 leaves had formed, plants were sorted for uniformity and one plant from each clone (717, 14BR, RT5) assigned to one of two treatments and six blocks (replications), with one plant per block. Blocking was by plant size, location in the growth chamber, and aeration pump. For one treatment, normal 0.5x modified Hoagland's solution (as described above) was continued. The second treatment solution was a modified Hoagland's solution contained 3 mM nitrate (1.0 mM KNO₃ and 1.0 mM Ca(NO₃)₂, with the balance of K⁺ and Ca²⁺ ions supplied by adding 1.5 mM KCl and 1.5 mM CaCl₂). After 1 week of exposure to the treatment solutions, nitrate uptake was determined over six 24-h periods by quantifying the disappearance of nitrate from the hydroponic solution. 950 mL of solution was initially placed in each bottle. At 24-h intervals, 1-mL samples of hydroponic solution were collected from each container for determining nitrate concentrations and solution volume was measured and replenished to 950 mL with distilled water. Solution samples were stored at -20°C for later determination of nitrate concentration. Fresh solutions were added after 72 h, providing 2 3-day
cycles of measurements. Plants were then destructively harvested for measurement of total leaf area and total leaf, stem and root fresh and dry weights.

Aliquots from hydroponic solution samples were diluted in 19 volumes of distilled water and nitrate concentration was determined on replicate dilutions using an automated nitrate analyzer equipped with a reduced cadmium column (Flow Solution 3000, Alpkem, Wilsonville OR). Nitrate uptake rates were calculated from differences in nitrate concentration and solution volume over each 24-h period and corrected for plant size (leaf area). Preliminary experiments indicated that among leaf area, and leaf, root and whole-plant weights, leaf area most accurately represented variation in plant size. Rate determinations were averaged over the 6 periods, and treatment and clone differences determined by analysis of variance.

RESULTS AND DISCUSSION

To determine if poplar BSP has a function in long day growth, hybrid poplar plants transformed with BSP-antisense cDNA were selected for reduced BSP. As shown in Figure 2.2, a number of transformed poplars had reduced levels of BSP after 4 weeks of SD treatment, when BSP gene expression and accumulation rate are known to be at a maximum (Coleman et al., 1993). Based on this screening, two independent lines (RT5 from root transformation and 14BR from stem section transformation) were selected for further study.
Figure 2.3. Greenhouse-grown plants of *wt* (left) and BSP-antisense (right) poplar. BSP-antisense plants have larger leaves and longer internodes.
**Growth and partitioning analysis**

Five replicate plants of two BSP-antisense lines and the *wt* were harvested for determining growth pattern and dry weight partitioning. Leaves of BSP-antisense lines were 20 - 25% larger (Figure 2.3) and internodes 10 - 15% longer than *wt* (Table 2-I). Rate of node emergence at the shoot tip (inverse of plastochron) was significantly reduced for BSP-antisense plants resulting in a net reduction in plant height, despite longer internodes (Table 2-I).

Table 2-I. Plant size and growth characteristics of *wt* and BSP-antisense plants grown in the greenhouse under supplemental light for 8 weeks. Five replicate plants were harvested for each line. Leaf area values are means of 20 youngest fully expanded leaves (LPI 11-30).

<table>
<thead>
<tr>
<th></th>
<th><em>wt</em> (717)</th>
<th>14BR</th>
<th>RT5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leaf area (cm²/leaf)</td>
<td>167.4</td>
<td>208.1 ***</td>
<td>204.7 ***</td>
</tr>
<tr>
<td>Internode length (mm)</td>
<td>34.0</td>
<td>37.3 *</td>
<td>39.6 ***</td>
</tr>
<tr>
<td>Plastochron* (days/node)</td>
<td>1.25</td>
<td>1.57 **</td>
<td>1.71 **</td>
</tr>
<tr>
<td>Height (m)</td>
<td>2.11</td>
<td>1.78 ***</td>
<td>1.89 ***</td>
</tr>
</tbody>
</table>

*Plastochron determined on similar treatments in a separate experiment. Means significantly different from the *wt* at *P < 0.05, **P < 0.01, ***P < 0.001.*

Although the BSP-antisense plants had significantly larger leaves with higher leaf dry mass per unit area (LMA), the total leaf biomass was similar to that of the *wt* because of reduced leaf number (Table 2-II). Total stem biomass was significantly reduced in the BSP-antisense clones (Table 2-II). The net effect of these changes was an 18% to 33% increase in leaf : stem (dw : dw) ratio. Root : shoot ratio was not significantly altered in the BSP-antisense lines. Bark samples
collected from BSP-antisense lines had a higher water content (lower percent dry matter) than the *wt*, whereas leaf and wood water content was not altered (Table 2-II).

Table 2-II. Biomass partitioning of *wt* and BSP-antisense plants grown in the greenhouse under supplemental light. Five replicate plants were harvested after 8 weeks, total leaf area measured, and fresh and dry weights of leaves, stem and roots determined. Leaf weight per unit area and leaf percent dry matter are means of 20 youngest fully expanded leaves (LPI 11-30).

<table>
<thead>
<tr>
<th></th>
<th>wt (717)</th>
<th>14BR</th>
<th>RT5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Above ground biomass (g dw)</td>
<td>47.8</td>
<td>43.3</td>
<td>46.5</td>
</tr>
<tr>
<td>Leaf biomass (g dw)</td>
<td>21.0</td>
<td>22.2</td>
<td>22.4</td>
</tr>
<tr>
<td>Stem biomass (g dw)</td>
<td>26.8</td>
<td>21.1</td>
<td>**</td>
</tr>
<tr>
<td>Leaf:Stem ratio (dw:dw)</td>
<td>0.788</td>
<td>1.047</td>
<td>***</td>
</tr>
<tr>
<td>Leaf mass per area (g/m²)</td>
<td>27.9</td>
<td>30.9</td>
<td>*</td>
</tr>
<tr>
<td>Leaf water content (%)</td>
<td>76.0</td>
<td>77.4</td>
<td></td>
</tr>
<tr>
<td>Bark water content (%)</td>
<td>79.1</td>
<td>81.3</td>
<td>**</td>
</tr>
<tr>
<td>Wood water content (%)</td>
<td>74.1</td>
<td>74.8</td>
<td>74.2</td>
</tr>
<tr>
<td>Root biomass (g dw)</td>
<td>11.9</td>
<td>11.7</td>
<td>12.2</td>
</tr>
<tr>
<td>Root:Shoot ratio (dw:dw)</td>
<td>0.249</td>
<td>0.266</td>
<td>0.264</td>
</tr>
</tbody>
</table>

Means significantly different from the *wt* at *P < 0.05, **P < 0.01, ***P < 0.001.

Vegetative storage proteins are thought to be involved in directing partitioning and internal distribution of N (Imsande and Touraine, 1994). To determine whether or not N partitioning was altered by BSP-antisense expression, 10 replicate plants of each clone were sampled and various tissues were analyzed for N concentration. Leaves, bark and wood of the BSP-antisense clone RT5 had significantly reduced N concentration, while N concentration of 14BR was
intermediate to that of RT5 and the wt (Table 2-III). Root N concentration was not significantly affected in the antisense lines. Leaf N concentration (mg N per g dry weight) was reduced by 9% and 20% compared to the wt for 14BR and RT5, respectively (Table 2-III). After accounting for clonal differences in leaf mass per area (LMA), N content was reduced by only 7% and 11%. It is interesting to note that in separate experiments, 14BR and RT5 had shown reductions in rate of CO₂ assimilation per leaf area of 11% and 15%, respectively (Black and Coleman, unpublished data). Bark N concentration was 6% and 13% lower than wt for 14BR and RT5, respectively, while wood N concentration was reduced by 12% and 14% (Table 2-III).

Table 2-III. Nitrogen content of wt and BSP-antisense plants harvested after 8 weeks of growth. 10 replicate plants of each line were sampled, and N concentration was determined by Dumas combustion analysis of lyophilized samples.

<table>
<thead>
<tr>
<th></th>
<th>wt (717)</th>
<th>14BR</th>
<th>RT5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leaf N concentration (mg/g)</td>
<td>27.5</td>
<td>25.1 *</td>
<td>21.9 *</td>
</tr>
<tr>
<td>Leaf N content (g/m²)</td>
<td>0.898</td>
<td>0.836 *</td>
<td>0.799 **</td>
</tr>
<tr>
<td>Bark N concentration (mg/g)</td>
<td>12.1</td>
<td>11.4</td>
<td>10.5 **</td>
</tr>
<tr>
<td>Wood N concentration (mg/g)</td>
<td>8.99</td>
<td>7.85  *</td>
<td>7.74 *</td>
</tr>
<tr>
<td>Root N concentration (mg/g)</td>
<td>7.70</td>
<td>7.87</td>
<td>8.21</td>
</tr>
</tbody>
</table>

*aLeaf values are means of determinations on LPI 6, 12, 18, 24, 30.

*bStem samples taken between LPI 6 and LPI 30. Means significantly different from the wt. at * P < 0.10, * * P < 0.05, * * * P < 0.01, * * * * P < 0.001.

Estimates of total plant N were calculated for the 10 replicate plants sampled for N content. Estimates were calculated from tissue fresh weights, N concentration, and tissue water content. Estimates of total stem N were from bark and wood N concentrations, water content, and from total stem fresh weight, using a best approximation of bark to wood ratio. Total plant N was significantly lower
in both BSP-antisense plants than in the control, primarily due to a 10% reduction in total leaf N (Table 2-IV). Stem N was slightly lower than the wt, but differences were not statistically significant. Total root N was increased in BSP-antisense lines but represented < 8% of total plant N.

Table 2-IV. Estimated total plant N content and N partitioning among leaves, stem, and roots. N totals were estimated from measurements of total fresh weights, tissue N concentration, and percent water content.

<table>
<thead>
<tr>
<th>Clone</th>
<th>wt</th>
<th>14BR</th>
<th>RT5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fresh wt (g)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Leaf fw</td>
<td>85.1</td>
<td>95.5 **</td>
<td>101.9 **</td>
</tr>
<tr>
<td>Stem fw</td>
<td>85.2</td>
<td>93.0</td>
<td>89.1</td>
</tr>
<tr>
<td>Roots</td>
<td>73.4</td>
<td>71.9</td>
<td>67.0</td>
</tr>
<tr>
<td>N (mg)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Leaf (fw)</td>
<td>602</td>
<td>539 *</td>
<td>541 *</td>
</tr>
<tr>
<td>Stem</td>
<td>176</td>
<td>164</td>
<td>156</td>
</tr>
<tr>
<td>Root</td>
<td>44.4</td>
<td>47.7</td>
<td>56.6 *</td>
</tr>
<tr>
<td>Total N</td>
<td>822</td>
<td>751 *</td>
<td>754 *</td>
</tr>
</tbody>
</table>

Grafting study

Analysis of reciprocally grafted plants indicated that BSP influences growth and partitioning primarily in aerial portions of the plant. Those parameters characteristic of the BSP-antisense plants, namely internode length and leaf : stem ratio were determined by the scion genotype (Table 2-V). However, total shoot growth was determined by the roots. BSP-antisense rootstocks reducing scion biomass (Table 2-V), which affected other size-related parameters such as plant
height and leaf size. However, scion genotype remained the single largest factor in
determining growth pattern and biomass partitioning.

Table 2-V. Plant size, growth characteristics, and dry weight partitioning
of reciprocally grafted wt and BSP-antisense plants after 8 weeks of
growth in the greenhouse. Scions from 14BR (a) were grafted to 717 (wt)
rootstocks and the reciprocal grafts were made. For controls, both wt and
BSP-antisense plants were grafted back to their own roots.

<table>
<thead>
<tr>
<th>Scion</th>
<th>717</th>
<th>14BR</th>
<th>Significance b</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rootstock</td>
<td>717</td>
<td>14BR</td>
<td>scion root s x r</td>
</tr>
<tr>
<td></td>
<td>wt/wt</td>
<td>a/wt</td>
<td>a/a</td>
</tr>
<tr>
<td>Internode length (cm)</td>
<td>2.54</td>
<td>2.53</td>
<td>2.87</td>
</tr>
<tr>
<td>Leaf:stem ratio (dw)</td>
<td>1.37</td>
<td>1.39</td>
<td>1.67</td>
</tr>
<tr>
<td>Leaf area* (cm²/leaf)</td>
<td>148</td>
<td>123</td>
<td>166</td>
</tr>
<tr>
<td>Leaf mass (g/lf)</td>
<td>0.374</td>
<td>0.304</td>
<td>0.419</td>
</tr>
<tr>
<td>Height (m)</td>
<td>1.40</td>
<td>1.29</td>
<td>1.22</td>
</tr>
<tr>
<td>Scion biomass (g)</td>
<td>19.2</td>
<td>14.8</td>
<td>17.0</td>
</tr>
<tr>
<td>Rootstock biomass (g)</td>
<td>3.82</td>
<td>3.84</td>
<td>3.17</td>
</tr>
</tbody>
</table>

*Leaf area values are means of six youngest fully expanded leaves (LPI10-15). bMain and interaction effects of 2 x 2 factorial treatment structure (scion x rootstock) *P < 0.05, **P < 0.01, ***P < 0.001.

Tissue N concentration of leaves, bark and wood were also a function of
scion genotype, with BSP-antisense tissues having reduced N concentrations
compared to wt scions (Table 2-VI) regardless of rootstock. As with the growth
and partitioning study, root N concentrations did not vary significantly among
genotypes or genotype graft combinations (data not shown).

Nitrate uptake

Clonal differences in nitrate uptake rate, as determined by measuring
disappearance of nitrate from hydroponic solutions, varied with N availability. At
the lower hydroponic nitrate concentration, rate of NO₃⁻ uptake by the two BSP-antisense clones did not significantly differ from that of the wt (3 mM; Figure 2.4). However, wt plants on 7.5 mM NO₃⁻ showed a 2-fold greater uptake rate compared to the 3.0 mM treatment, while the antisense lines did not show a similar dose-dependent increase in uptake rate.

Table 2-VI. Nitrogen content in reciprocally grafted *wt* (717) and BSP-antisense (14BR) plants after 8 weeks of growth in the greenhouse. Scions from 14BR were grafted to *wt* rootstocks and the reciprocal grafts were made. For controls, both *wt* and BSP-antisense plants were grafted back to their own roots.

<table>
<thead>
<tr>
<th>Scion</th>
<th>717</th>
<th>14BR</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rootstock</td>
<td>717</td>
<td>14BR</td>
<td>717</td>
</tr>
<tr>
<td>Leaf N concentration (mg/g)</td>
<td>32.1</td>
<td>31.9</td>
<td>28.8</td>
</tr>
<tr>
<td>Leaf N content (g/m²)</td>
<td>0.932</td>
<td>0.869</td>
<td>0.833</td>
</tr>
<tr>
<td>Bark N (mg/g)</td>
<td>15.8</td>
<td>15.6</td>
<td>14.9</td>
</tr>
<tr>
<td>Wood N (mg/g)</td>
<td>6.76</td>
<td>6.20</td>
<td>5.73</td>
</tr>
</tbody>
</table>

Leaf values are means of determination on LPI 12 and LPI 18. Stem samples from nodes corresponding to LPI 10-30. Main and interaction effects of 2 x 2 factorial treatment structure (scion x rootstock). *P < 0.05, **P < 0.01, ***P < 0.001.

**Significance**

Expression of BSP-antisense cDNA results in altered biomass partitioning and reduced tissue N concentration. It was previously observed that poplar BSP gene expression occurs during long-day growth and is altered by N availability (Coleman et al., 1994; Davis et al., 1993; Zhu and Coleman, in review). The BSP gene expression patterns and the altered growth pattern of BSP-antisense plants both provide evidence for a role of poplar BSP in long day growth and development. The altered N content and nitrate uptake observed in the BSP-antisense plants suggest a function in N uptake and partitioning.
Figure 2.4. Nitrate uptake rate of *wt* (717) and BSP-antisense plants at two levels of nitrogen availability. Uptake rates (nitrate per leaf area per day) were determined by measuring disappearance of nitrate from hydroponic solutions.

There are several possible models for the role of BSP in long day growth. Under LD conditions, BSP gene expression is higher in the stem than in the leaves (Zhu and Coleman, in review) and BSP is found primarily in the stem and shoot tip tissue (Coleman et al., 1994; Davis et al., 1993). BSP may act as a short-term N storage site in the stem. The growing point of the plant requires assimilated N for both stem growth and leaf development. BSP synthesis in the young stem may act to increase stem demand for carbon and N relative to the developing leaves. This would explain how reduced BSP expression results in a shift in partitioning from leaves to stems. However, it is difficult to see how this explanation accounts for the significant reduction in tissue N concentration and the altered nitrate uptake observed in BSP-antsense plants.
The relationship between N availability and biomass partitioning has long been recognized. A number of models have been proposed to explain this relationship (Ägren, 1985; Ägren and Ingestad, 1987; Chapin, 1980; Cohen and Pastor, 1996; Hilbert, 1990; Ingestad and Ägren, 1991; Thornley, 1972). These models all focus on the observation that reduced N uptake (affecting internal N concentration) stimulates increased root growth, resulting in increased capacity for N uptake. Conversely, increased N supply results in increased leaf biomass, thereby increasing capacity for carbon acquisition (leaf area). These adjustments in root-versus-shoot partitioning tend towards maintaining an equilibrium in the internal carbon/nitrogen balance (for reviews see Grace, 1997; Linder and Rook, 1984). However, in each of these models, differences in partitioning are between shoot and root. In some cases, increased N supply may result in increased leaf area per total biomass without altering total root : shoot ratio (McConnaughay and Coleman, 1998). Studies with Pinus seedlings have shown N availability is positively correlated with biomass partitioned to the needles, negatively correlated with total root biomass, but not significantly correlated with stem biomass (Tan and Hogan, 1998).

The altered growth and N partitioning of BSP-antisense plants seems to contradict these models since BSP-antisense plants have lower N concentration in above ground tissues but increased leaf size and a higher proportion of total biomass invested in leaves. However, it is important to recognize that these models were developed based on responses to external N availability and uptake efficiency. In the case of BSP-antisense plants, the internal mechanism for regulating N partitioning may be affected.

Models for the role of vegetative storage proteins in annual seed plants may explain the altered growth and partitioning of BSP-antisense plants. VSPs may play an integral role in N accumulation and partitioning according to a model proposed for soybean (Glycine max) (Imsande and Touraine, 1994). According to
this model, nitrate reduction and assimilation in the leaves produces organic and amino acids. The organic acids are transported to the roots, decarboxylated, and the HCO$_3^-$ ions are excreted into the soil at a rate proportional to nitrate uptake in order to maintain pH homeostasis (Touraine et al., 1988, 1992). Newly synthesized amino acids are incorporated into chlorophyll, and rubisco, VSPs and other leaf proteins. Alternatively, amino acids not utilized in the leaf are exported from the leaf to the growing shoot tip or roots (Cooper and Clarkson, 1989). The uptake of nitrate in the roots is dependent upon an adequate organic acid supply (Touraine et al., 1992), but perhaps more importantly, is feedback inhibited by the concentration of free amino acids (Muller and Touraine, 1992). According to the model proposed by Imsande and Touraine (1994), VSPs may act to regulate the free amino acid levels by supplementing demand for assimilated N (Staswick et al., 1991).

Several components of this model can be related to evidence from poplar and related tree species. First, it has been suggested that this model relies on either shoot-localized nitrate reduction, or the presence of a relatively rapid circulation of reduced carbon and nitrogen, or both of these conditions (Lexa and Cheeseman, 1997). Although root-localized nitrate reduction appears more common in tree species (Andrews, 1986; Gojon et al., 1991), poplar (Chapter 3) and several other temperate hardwoods (Friemann et al., 1992; Stadler and Gebauer, 1992) have higher nitrate reductase activity in the leaves. Further, leaf-localized nitrate assimilation is tightly light regulated (Friemann et al., 1992), with amino acids synthesized only during the light period. Diurnal fluctuations in organic and amino acids have been observed in poplar leaves (Dickson, 1987) which may be further evidence of leaf-localized nitrate reduction and assimilation. Labeled nitrogen studies ($^{15}$N-labeled NH$_4^+$) in beech (Fagus sylvatica) have shown that trees also may have a rapid-turnover ‘transport pool’ of amino acids (Martin and Ben Driss Amraoui, 1989).
Poplar BSP may act as a short-term sink for newly-assimilated N at the predominant site of N reduction (young leaves) or in the nearby stem. Without this supplemental N sink, BSP-antisense plants may have inflated levels of free amino acids which would act to down-regulate enzymes involved in nitrate uptake (Muller and Touraine, 1992) and assimilation (Crawford, 1995) resulting in reduced total N content. Further, such an increase in free amino acid levels may be sensed as high N availability, signaling a shift of carbon partitioning towards leaf growth. In practice, BSP may function both to regulate amino acid levels and to direct partitioning to the stem.

The altered growth pattern, dry weight partitioning and N content of BSP-antisense plants provide additional evidence for a long-day function in growth pattern and biomass partitioning. This function also appears to be related to nitrogen uptake and partitioning.
LITERATURE CITED


CHAPTER 3: Nitrate Reductase Activity in Leaf, Stem and Root Tissue of Hybrid Poplar

ABSTRACT

Plants differ in the site of nitrate reduction and assimilation, with some species reducing nitrate primarily in the leaves and others localizing nitrate reduction and assimilation in the roots. Nitrate reductase activity (NRA) was measured by in vivo assay in leaves, stems and roots of hydroponically grown hybrid poplar (Populus tremula x P. alba) supplied with different concentrations of nitrate. Expressed as a function of tissue fresh or dry weight, NRA of leaves was more than 10x higher than that of roots at all levels of nitrate availability. Both leaf and root NRA increased with increasing nitrate supply, while stem NRA remained constant.

INTRODUCTION

Nitrate reductase (NR) catalyzes the first step in the energy-intensive process of nitrate assimilation. Nitrate assimilation and allocation patterns have been studied in a number of genera representing temperate annuals, perennials and tropical plants (For reviews see Andrews, 1986a; Beevers, 1981; Pate, 1980). Among non-legume temperate annuals, the partitioning of NR activity (NRA) between leaves and roots varies greatly, with some genera reducing NO$_3^-$ primarily in the leaves and others classified as ‘root reducers’ (Andrews, 1986a). Roots have traditionally been considered the predominant site of nitrate reduction in temperate perennials (Andrews, 1986a), but significant NRA has been detected in the leaves of a number of tree species (Smirnoff et al., 1984).
Biochemical advantages of shoot- versus root-localized NRA have been proposed (see Lexa and Cheesman, 1997). Leaf reduction of NO$_3^-$ under high light requires less energy than root-localized NO$_3^-$ reduction (Stewart et al., 1988). Under low light conditions however, root-localized N reduction may prevent NO$_3^-$ accumulation in the leaves (Andrews, 1986b). The site of NO$_3^-$ reduction and assimilation may affect N and C partitioning with root-localized N assimilation driving partitioning to roots (Chapin, 1980; Lexa and Cheeseman, 1997).

The genus *Populus* (aspen and cottonwoods) has long been an important model system for the study of forest-tree breeding (Pauley, 1949). Methods developed for its genetic transformation (Leple et al., 1992) have led to the use of *Populus* as a model system for studying woody plant molecular physiology (Boerjan et al., 1997; Heuchelin et al., 1997; Weigel and Nilsson, 1995). Poplar has also been used to study the seasonal internal redistribution of N (Coleman, 1997). NR activity in poplar leaves has been compared between different clones, at different levels of N availability (Dykstra, 1974) and at different stages of leaf development (Pokhyriyal and Raturi, 1985). These studies showed that NRA was higher in young leaves than in mature leaves and that leaf NRA initially increased with N availability. However neither of these studies measured NRA in the roots or stems. Diurnal fluctuations in organic and amino acids have been observed in poplar leaves (Dickson, 1987), which may be evidence of leaf-localized nitrate reduction and assimilation. However, there are no published studies on leaf : root partitioning of NR activity in poplar. Since the site of N reduction varies with species and may affect N and C partitioning, studies were conducted to determine whether nitrate reductase activity in poplar is leaf or root localized. In this paper we show that the majority of NRA is in the leaf. Furthermore, both leaf and root NRA increased with increased NO$_3^-$ availability.
MATERIALS AND METHODS

Plant material

Plants of the hybrid aspen *Populus tremula* x *P. alba* (INRA clone 717 1-B4; Leplé et al., 1992) were propagated *in vitro*, and established in hydroponic culture as previously described (Chapter 2). Plants were transferred to hydroponic culture (0.5 x modified Hoagland’s solution; Hoagland and Arnon, 1950) when they reached a height of approximately 15 cm. Hydroponic solution in each bottle was replenished daily with distilled water and replaced biweekly with fresh hydroponic solution. Plants were maintained in this hydroponic system in a growth chamber (200 pmol m⁻² s⁻¹ light, 23°C) until an additional 12-15 leaves had been produced.

Nitrate reductase assay

NR activity was determined by an *in vivo* assay similar to that first described by Jaworski (1971), with some modifications (Truax et al., 1994). Leaf disks, stem sections or root sections were rinsed in distilled water, blotted dry with paper towels and divided among 4 20-mL plastic scintillation vials. Assay buffer (200 mM KNO₃, and 5% propanol in 100 mM potassium phosphate buffer, pH 7.5) was added to the vials which were then capped and placed in the dark on an orbital shaker set at 175 rpm. Two replicate reaction vials were removed from the shaker after 10 and 90 minutes, and placed in a boiling water bath for 15 minutes. Reaction vials were then cooled to room temperature and aliquots of buffer were collected and stored at -80°C for later determination of nitrite concentration.

Nitrite concentrations in the buffer aliquots were determined colorimetrically. Equal volumes of 1% sulfanilamide in 3 N HCl, and 0.02% N-naphthyl-ethylene-diamine hydrochloride in water were added to the thawed aliquots, color was developed in the dark at room temperature for 20 min and
absorbance at 540 nm measured by spectrophotometer (Lambda Bio, Perkin Elmer). Nitrite concentrations were corrected for buffer volume and tissue amount (either fresh or dry weights) and enzyme activity calculated based on differences in nitrite amounts between samples incubated for 10 or 90 minutes. Rates were averaged across two replicate determinations. Preliminary experiments determined that the nitrite levels increased linearly with time beyond the 90 min incubation period. These preliminary experiments also found that propanol in the assay buffer resulted in sufficient wetting, without the need for vacuum infiltration (Downs et al., 1993; Gebauer et al., 1984).

**Tissue comparison**

In the first experiment, NRA were compared among roots, stem, and leaves from plants grown on hydroponic solution containing 7.5 mM NO$_3$-. Leaves of different ages were also compared based on leaf plastichron index (LPI; Dickson, 1986). Leaves were numbered sequentially from the growing point basipetaly, with the youngest leaf longer than 2 cm assigned an LPI of 1. Leaves of LPI 3, 6, 9 and 12, and stem and root sections were assayed. For the youngest leaf (LPI 3), 11.6 mm diameter leaf disks were collected using a #8 cork borer. Three disks per reaction vial were incubated in 2.5 mL assay buffer. For the remaining leaves, 5 14.2-mm (#10 cork borer) leaf disks were assayed in 2.5 mL buffer. Stem sections were taken corresponding to LPI 6-9 and root sections were taken from healthy young (<3 mm diam.) roots, with 0.4 - 0.6 g (fresh weight) of stem or root tissue assayed in 2.5 mL of buffer. After incubation and buffer collection, tissue samples were oven dried on aluminum foil at 65°C for tissue dry weight determinations. Leaf, stem and root NRA were determined on 4 replicate plants.
Nitrate reductase induction

NR activity is substrate inducible (Beever and Hageman, 1980; Friemann et al., 1992). To determine the predominant site of substrate-inducible NR, enzyme activity was assayed after plants had been subjected to different hydroponic NO₃⁻ concentrations. Plants were established in hydroponic culture with 0.5x modified Hoagland's solution as described above, and grown until 12 to 15 new leaves had been produced. Plants were then selected for uniformity and assigned to one of five treatments and four replications. The five experimental treatments consisted of NO₃⁻ concentrations ranging from 0 to 15.0 mM, with Cl⁻ substituted for NO₃⁻ in the first four treatments (see Table 3-I). After 7 to 10 days on treatment solutions, NRA was determined for leaves of 3 developmental stages (LPI 4, 8 and 12) and for stem and root tissue as described above.

Trends in enzyme activity relative to NO₃⁻ supply were determined for each tissue by orthogonal contrast analysis using coefficients for regularly spaced treatments (Gill, 1978). Statistical significance and parameter estimates were determined using the GLM procedure of the SAS program package (SAS Institute, Cary, North Carolina).

Table 3-I. Source and concentration of nitrate in hydroponic solutions used for NR induction study. K⁺ and Ca⁺⁺ concentrations were held constant across the first 4 treatments by the addition of KCl and CaCl₂.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>NO₃⁻</th>
<th>KNO₃</th>
<th>Ca(NO₃)₂</th>
<th>KCl</th>
<th>CaCl₂</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>2.5</td>
<td>2.5</td>
</tr>
<tr>
<td>2</td>
<td>1.875</td>
<td>0.625</td>
<td>0.625</td>
<td>1.875</td>
<td>1.875</td>
</tr>
<tr>
<td>3</td>
<td>3.75</td>
<td>1.25</td>
<td>1.25</td>
<td>1.25</td>
<td>1.25</td>
</tr>
<tr>
<td>4</td>
<td>7.50</td>
<td>2.50</td>
<td>2.50</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>5</td>
<td>15.0</td>
<td>5.00</td>
<td>5.00</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>
BSP-antisense plants

Plants of *P. tremula* x *P. alba* transformed with a BSP-antisense gene show altered growth pattern and N partitioning (see Chapter 2). Plants from one BSP-antisense clone (14BR) and the untransformed wt were grown hydroponically and NRA of the leaves was assayed as described above. Because of differences in leaf mass per unit area (LMA), enzyme activity was compared both on a leaf-area and on a fresh-weight basis.

**RESULTS AND DISCUSSION**

**Tissue comparison**

NRA per tissue dry weight was compared among root, stem and 4 different leaf ages by *in vivo* assay (Table 3-II). NRA expressed as a function of tissue dry weight was negatively correlated with leaf age, where the highest activity was found in LPI 3 (5.16 μmol g(dw)^{-1} h^{-1}) and the lowest activity (0.83 μmol g(dw)^{-1} h^{-1}) found in LPI 12 (Table 3-II). For *P. tremula* x *P. alba* (clone 717) under most environmental conditions, the youngest fully expanded leaf generally corresponds to an LPI of 10. Therefore, these results confirm previous observations that the highest leaf NRA is found in young expanding leaves (Pokhriyal and Raturi, 1985), which are also nitrogen sinks. NRA activity of leaves was similar to rates previously reported for poplar (Dykstra, 1974), and was 10 to 50x higher in leaves than in the stem or roots.

**Nitrate reductase induction**

To determine the substrate inducibility of the various tissues, NRA was measured in leaves, stems and roots at different levels of N availability (Table 3-I). NRA increased significantly with N supply from 0 to 7.5 mM hydroponic nitrate
concentration in both leaves and roots. Stem NRA did not change with nitrate supply (Figures 3.1 and Table 3-III). NRA did not significantly differ between LPI 4 and LPI 8, while activity of LPI 12 was lower than LPI 4 and LPI 8 at all levels of NO$_3^-$ availability up to 7.5 mM. Root NRA increased with hydroponic NO$_3^-$ concentration up to 7.5 mM ($P = 0.004$; Table 3-III), and was >20x lower than the NRA of LPI 8 for all hydroponic NO$_3^-$ concentrations (Figure 3.1).

Table 3-II. Comparison of Nitrate reductase activity (NRA) among different tissues of poplar (P. tremula x P. alba). Plants were grown hydroponically and supplied with 7.5 mM NO$_3^-$. NRA was determined by in vivo assay as described in material and methods. Values are based on tissue dry weights ($\mu$mol g$^{-1}$ h$^{-1}$) and are the means of 4 replicate plants.

<table>
<thead>
<tr>
<th>Leaf</th>
<th>mean ± se</th>
</tr>
</thead>
<tbody>
<tr>
<td>LPI 3</td>
<td>5.16 ± 0.73</td>
</tr>
<tr>
<td>LPI 6</td>
<td>2.39 ± 0.42</td>
</tr>
<tr>
<td>LPI 9</td>
<td>1.82 ± 0.43</td>
</tr>
<tr>
<td>LPI 12</td>
<td>0.83 ± 0.17</td>
</tr>
<tr>
<td>Stem</td>
<td>0.14 ± 0.01</td>
</tr>
<tr>
<td>Root</td>
<td>0.14 ± 0.04</td>
</tr>
</tbody>
</table>

Trends in NRA with N availability were less consistent between 7.5 and 15 mM than from 0 to 7.5 mM. For example, NRA of both root and LPI 4 appeared to decline with N supply between the two highest treatments, while activity of LPI 12 continued to increase. These apparent differences may be due to super-optimal NO$_3^-$ availability, since 0.5x modified Hoagland’s solution gives optimum growth of poplar. Alternatively, differences above 7.5 mM may be due to differing ionic properties of the hydroponic solutions.
Figure 3.1. Nitrate reductase activity in the leaf, stem and root tissue of poplar plants grown at different levels of nitrate availability. Four replicate plants were subjected to five different hydroponic nitrate concentrations. Three different leaf ages, as indexed by leaf plastichron index (LPI) were assayed as described in materials and methods. Values are mean±SE of four replicate plants, on a tissue fresh weight basis.
Ionic strength and ionic activities were held constant across the first four treatments by directly substituting Cl\textsuperscript{-} for NO\textsubscript{3}\textsuperscript{-} (Table 3-I). However, the highest treatment had a higher ionic concentration with 2x higher K\textsuperscript{+} and Ca\textsuperscript{2+}. This increase, particularly in the concentration of a divalent cation, may have affected nutrient uptake by changing the ionic properties of the hydroponic solution (Adams, 1971). Trend analysis and regressions were therefore calculated from data for the first four treatments (Table 3-III).

<table>
<thead>
<tr>
<th>AOV term</th>
<th>Leaf</th>
<th>stem</th>
<th>root</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Leaf</td>
<td>stem</td>
<td>root</td>
</tr>
<tr>
<td>Linear</td>
<td>0.004</td>
<td>0.055</td>
<td>0.67</td>
</tr>
<tr>
<td>Quadratic</td>
<td>0.032</td>
<td>0.057</td>
<td>0.86</td>
</tr>
</tbody>
</table>

Orthogonal contrast analysis of leaf NRA compared to N availability (from 0 to 7.5 mM) was significant for both linear and quadratic terms (Table 3-III) and represented a typical saturation curve. For example, the inverse of NRA for LPI 8 plotted against the inverse of hydroponic N concentration gave a linear relationship (R\textsuperscript{2} = 0.970). This inverse linear relationship predicted a maximum enzyme activity of 2.2 \mu mol NO\textsubscript{2}\textsuperscript{-} produced per mg leaf fresh weight per hour (Stryer, 1988). The observed maximum may have been due to limitations in nitrate uptake in low light conditions, since light levels in the growth chamber were well below saturation, and nitrate uptake is an active process requiring a source of fixed carbon (Touraine et al., 1988). However, root NRA continued to increase as leaf NRA approached saturation. Alternatively, the saturation may have been an artifact of
the *in vivo* assay resulting when enzyme activity exceeded available reductant. *In vitro* assays generally involve adding saturating levels of NAD(P)H to the assay buffer, but *in vivo* assays relying on endogenous reductant supply are generally considered a better determinant of a tissue's capacity to reduce and assimilate NO$_3^-$ (Andrews, 1986a). Exogenous reductant was not included in these studies. The observed saturation may be a property of NR induction in the leaf, since LPI 12 appeared to reach a saturation point lower than that of LPI 8 and LPI 4, which may argue against an assay limitation. A similar saturation in N-inducible enzyme activity has previously been reported for poplar leaves (Dykstra, 1974).

**Significance**

Roots are usually considered the predominant site of NO$_3^-$ reduction and assimilation in temperate perennial species (Andrews, 1986a). This conclusion was made based on measurements of xylem sap constituents of *Malus* (Bollard, 1956; 1960). However, significant levels of NRA have been observed in the leaves of a number of woody species (Smirnoff et al., 1984). Measurements of NR activity and N pools of leaves and roots have shown differences in site of N reduction among temperate woody perennials. The predominant site of NR activities is in the roots of *Malus* (Lee and Titus, 1992), *Pinus resinosa, Pinus rigida, Pinus strobus* (Downs et al., 1993) *Pinus sylvestris* (Sarjala et al., 1987), and *Prunus persica* (Gojon et al., 1991), and the leaves of *Acer rubrum* (Downs et al., 1993), *Fraxinus excelsior* (Gebauer and Stadler, 1990; Stadler and Gebauer, 1992) *Fraxinus pennsylvanica*, and *Quercus rubra* (Traux et al., 1994).

The ecological significance of root versus shoot NO$_3^-$ reduction and assimilation is not clear. According to one model (Chapin, 1980), crop plants and other rapidly growing species reduce N primarily in the leaves when N is not limiting. When N becomes limiting for these species, root-localized reduction and assimilation effectively shifts dry weight partitioning from stem to roots, increasing
N interception and consequently overcoming N limitations (Chapin, 1980). In slower growing species, root-localized N reduction and assimilation ensures ongoing dry-weight partitioning to the roots (Chapin, 1980). In studying forest succession, Stewart et al. (1988, 1992) reported that fast-growing pioneer species assimilate N primarily in the leaves, while climax species assimilate N in the roots. These differences may also be related to differences in shade tolerance, where leaf-localized N assimilation is only favored when light is not limiting (Aslam, 1976; Stewart et al., 1988).

Traux et al. (1994) compared NRA and NR distribution in two temperate deciduous species. Under low N availability, both red oak (Quercus rubra), and red ash (Fraxinus pennsylvanica) have slightly higher NRA in leaves than in roots. However high N supply resulted in a leaf : root NRA ratio of 1 in red oak and 4 in red ash. Root NRA increased with N supply in both species, while leaf NRA increased by 2x in red oak and by 12x in red ash. These results apparently support the forest succession studies (Stewart et al., 1988, 1992) in that red ash is an invasive pioneer species with low shade tolerance (Kennedy, 1990), unlike red oak (Crow, 1988).

Lexa and Cheeseman (1997) investigated the effects of NR localization on growth and partitioning. Leaf:root NRA was experimentally manipulated by making reciprocal grafts between NR-deficient and wild-type pea (Pisum sativum) mutants. They found that growth rate and root : shoot biomass partitioning did not differ between root and shoot reducers. Although the site of N reduction alone does not determine N allocation and growth strategies, it may be indicative of broader differences in ecological adaptation.

It is also interesting to note that a number of species which have prominent seasonal vegetative storage proteins (Wetzel and Greenwood, 1991) are also predominantly leaf reducers (Betula, Fraxinus, Populus), although there are notable exceptions (Prunus persica). It has been proposed that VSPs are an important
supplemental sink for leaf-reduced N (Imsande and Touraine, 1994). However, the extent to which leaf-localized N assimilation correlates with the presence of VSPs has not been determined. Also, plants expressing an BSP-antisense gene which are altered in long day growth and partitioning, did not have significantly different in vivo NRA compared to the wt (Table 3-IV).

We report here that both leaf and root NRA is substrate inducible in poplar, but that leaves are the predominant site of NRA in hybrid poplar trees. This is consistent with observed leaf : root NRA partitioning of other forest hardwoods.

Table 3-IV. Nitrate reductase activity in the leaves of BSP-antisense and wt (717) plants. Values are means and standard errors from six replicate plants. Enzyme activities are corrected for leaf fresh weight or leaf area.

<table>
<thead>
<tr>
<th></th>
<th>wt</th>
<th>14BR</th>
</tr>
</thead>
<tbody>
<tr>
<td>mmol g(^{-1}) h(^{-1})</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LPI 3</td>
<td>19.7 ± 3.02</td>
<td>18.2 ± 3.79</td>
</tr>
<tr>
<td>LPI 6</td>
<td>15.0 ± 4.01</td>
<td>13.0 ± 1.90</td>
</tr>
<tr>
<td>mmol m(^{-2}) h(^{-1})</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LPI 3</td>
<td>2.58 ± 0.392</td>
<td>2.74 ± 0.566</td>
</tr>
<tr>
<td>LPI 6</td>
<td>1.34 ± 0.312</td>
<td>1.37 ± 0.190</td>
</tr>
</tbody>
</table>
LITERATURE CITED


CHAPTER 4: Effect of Antisense BSP Expression on Nitrogen Resorption from Autumn-Senescent Leaves of Hybrid Poplar

ABSTRACT

Bark storage proteins (BSP) are considered an important storage form of reduced nitrogen (N) recovered from autumn-senescent leaves. Plants of *Populus tremula x P. alba* were transformed with a poplar BSP-antisense cDNA. Transformed lines were selected for reduced short-day BSP accumulation. Leaf senescence and N resorption in two independent BSP-antisense lines were compared to that of the untransformed wild type. In the first study, leaf senescence was induced in a controlled environment by 8 weeks of short-day (SD) photoperiod followed by SD and low temperature exposure (10°C-day and 4°C-night). In the second study, leaf senescence progressed under natural conditions in an outdoor nursery located at College Park, Maryland. N resorption was calculated from leaf N content determined on periodic samples taken under both of these experimental conditions. A larger fraction of the pre-senescent N levels remained in the abscised leaves of the BSP-antisense lines in both the growth chamber and outdoor studies. This reduced resorption efficiency in BSP-antisense plants provides additional evidence for the importance of BSP in autumn N resorption.

INTRODUCTION

During leaf senescence, plants hydrolyze leaf proteins and redistribute the amino acids to other parts of the plant (Feller and Fischer, 1994). In deciduous perennials, this reduced nitrogen (N) from autumn-senescing leaves is stored as protein in the overwintering stems (Titus and Kang, 1982). During autumn N
resorption, protein levels in leaves decline by 50% while stem protein levels increase by over 200% (Kang and Titus, 1980). In several poplar species, about 75% of total tree nitrogen is found in the leaves during summer growth (Pregitzer et al., 1990), 40 - 80% of which is retained by the tree after leaf abscission (Killingbeck et al., 1990; Pastor and Bockheim, 1984; Pregitzer et al., 1990). Spring remobilization of these stored reserves provides reduced N for spring growth (Coleman et al., 1993; Sauter and van Cleve, 1992).

Seasonal vegetative storage proteins (VSPs) have been identified in temperate deciduous tree species based on: prominence in overwintering stems, accumulation during autumn leaf senescence, and disappearance at the onset of spring growth (O'Kennedy and Titus, 1979). A 32-kilodalton bark storage protein (BSP) has been identified in poplar which accumulates in the fall in response to short day (SD) photoperiod, and which disappears as spring growth resumes (Coleman et al., 1992; Coleman et al., 1993; Sauter et al., 1988; Wetzel et al., 1989a). The accumulation pattern (Sauter et al., 1988; Wetzel et al., 1989a), subcellular location (Sauter et al., 1988; Sauter et al., 1989; Sauter and van Cleve, 1990; Wetzel et al., 1989b), biochemical characteristics (Langheinrich and Tischner, 1991; Stepień and Martin 1992), and degradation (Banados, 1992; Coleman et al., 1993) of poplar BSP have been characterized. The sequence, regulation and expression patterns of the BSP gene have also been documented (Clausen and Apel, 1991; Coleman and Chen, 1993; Coleman et al., 1992; Davis et al., 1993; Stepień and Sauter, 1994). Despite this extensive work (for reviews see Coleman 1997; Stepień et al., 1994) the physiological function and significance of seasonal VSPs like poplar BSP is not well understood (Stepień et al., 1994).

Antisense RNA can inhibit gene expression of the complementary mRNA (Green et al., 1986) providing a useful method for studying the function of a specific gene product (van der Krol et al, 1988; Stockhaus et al., 1990). Several transformed lines of hybrid poplar (Populus tremula x P. alba) have been
regenerated, which have an antisense copy of the BSP cDNA (see Chapter 2). These lines show altered growth pattern, dry weight partitioning and nitrate uptake under LD growth conditions. In this study, N resorption from autumn senescent leaves was measured in the wild type (wt) and BSP-antisense lines to determine whether or not BSP plays a role in N resorption.

MATERIALS AND METHODS

Growth chamber experiment

Two lines of *Populus tremula* x *P. alba* (INRA clone 717 1-B4; Leple et al., 1992) transformed with BSP cDNA in antisense orientation, and selected for reduced short-day BSP accumulation (see Chapter 2), were used to study autumn N resorption. Plants of these BSP-antisense lines (14BR and RT5) and of the non-transformed wild type (wt) were propagated *in vitro* as described before (see Chapter 2). Plants were then transplanted to 1.4-L pots and grown in the greenhouse under supplemental light until approximately 15 to 20 new nodes had been produced. Plants were then sorted for uniformity and one plant from each clone assigned to one of 18 blocks. These plants were then moved to a large growth chamber programmed with 16-h photoperiod (light level 390 μE) and maintained at 20°C. After 2 weeks in the growth chamber, 3 replicate blocks were destructively harvested. After this initial sampling the growth chamber was programmed to an 8-h photoperiod, and plants from 3 replicate blocks were destructively sampled after 2, 4, 6, and 8 weeks. After 8 weeks of SD treatment, the remaining 3 blocks of plants were moved to a smaller growth chamber programmed to an 8 h photoperiod with 10°C-day and 4°C-night temperature. Leaves began to abscise approximately 5 weeks after the beginning of the low
temperature (LT) treatment. Abscising leaves were sampled, and stem samples were collected after leaf abscission was complete.

For destructive sampling, individual leaf blades were collected and fresh weight and leaf area determined before rapid freezing in liquid nitrogen. 10-node stem samples were collected corresponding to fully expanded leaves (leaf plastochron index or LPI of 10 to 20), bark and wood was separated, and fresh weight for each was determined. Stem samples were then frozen in liquid nitrogen, stored at -80°C, and later lyophilized and milled. Lyophilized leaf, bark and wood samples from both experiments were milled with a high speed mill equipped with a 1.0 mm screen (Tecator Cyclotec 1903 Sample Mill). Total N was determined on triplicate sub-samples of milled tissue by Dumas combustion analysis using an automated C/N analyzer (NC 2100 - Soil, CEInstruments, Italy). For determining changes in N content over time, data were analyzed as repeated measures analysis of variance (Gill, 1978).

Outdoor leaf senescence

Plants of two BSP-antisense lines (14BR and RT5) and of the wt were grown to a height of ≈30 cm in 1.4 L pots in the greenhouse. Plants were then transplanted to 21-L pots containing ProGro 300S soil-less potting media, and moved to an outdoor nursery (≈ 20 May) located on the campus of the University of Maryland (College Park, Maryland). Plants were fertilized at 2-week intervals with 5 mM NH₄NO₃ through the summer. When >50% of plants had set terminal buds (26-Aug, 13 h 15 min photoperiod), fertilization was discontinued and five replicate plants of each clone were destructively sampled. Sampling was as described above with leaf blades from every 5th node (LPI 5, 10, 15 . . .) collected and analyzed as described above. Five plants of each clone were destructively sampled at 2-week intervals until ≈50% of leaves had abscised (20-Nov).
In late October, five plants of each clone were selected for an end-point sampling. Starting on 6-Nov, abscising leaves were collected at 2 to 3-day intervals and measured and stored as described above. When all leaves had abscised (4-Jan), bark and wood samples were collected and stored as described above.

The amount of nitrogen present in leaves can be expressed as a function of leaf weight (McColl, 1980; Tew, 1970) or leaf area (Alban, 1985; Killingbeck et al., 1990). For the purpose of the present discussion, N per dry weight is defined as N concentration, and N per leaf area as N content. Leaf dry weight per unit area (LMA) changes during leaf expansion and maturation and again during leaf senescence (Oland, 1963). In poplar, shoot tip growth ceases within ≈2 weeks of exposure to SD photoperiod with the appearance of a morphologically distinct terminal bud by 3 to 4 weeks SD. For these studies, leaf N content was compared at the same relative distance from the shoot tip, based on leaf plastichron index (LPI). Since terminal growth had ceased, comparisons at the same LPI over time also reflect changing leaf age and consequently, changing LMA. Therefore, calculations of N resorption based on leaf area (N content) are most meaningful (Alban, 1985). Resorption efficiency (RE) was calculated as the difference between pre-senescent (N_i) and post-abscission (N_f) leaf N content divided by pre-senescent leaf N (Killingbeck et al., 1990).

\[
RE = \frac{N_i - N_f}{N_i}
\]

Daily minimum and maximum temperatures were obtained from the nearest weather station (≈3 miles from the nursery site at Beltsville, Maryland). From these, 12-d running averages for minimum and maximum temperature were calculated and compared to the progression of leaf senescence.
RESULTS AND DISCUSSION

Controlled environment

Although some reports suggest that leaf senescence of deciduous trees is a SD photoperiodic response (Spencer and Titus, 1972; Stoddart and Thomas, 1982), low temperatures are generally considered requisite for autumn senescence (Smart, 1994). To study N resorption during autumn leaf senescence, poplar plants were treated with 8 weeks of SD photoperiod followed by low temperature until leaf abscission was complete. Leaf abscission occurred between 4.5 and 7 weeks after the beginning of low temperature (LT) exposure. There was little visible evidence of leaf senescence (color change) prior to LT treatment, and the most dramatic changes in leaf and stem N concentration occurred during LT exposure. Comparisons of N content for this experiment were made among leaves of LPI 15, since these represented fully expanded leaves at the beginning of sampling.

N concentration of wt poplar leaves (represented by LPI 15) increased during the first 2 weeks of SD exposure, then decreased steadily from 2 to 8 weeks-SD (Figure 4.1). However, these changes in N concentration prior to LT treatment were due to changes in LMA. Analysis of variance showed that leaf N content from 0 to 8 weeks of SD exposure did not change significantly ($P = 0.18$). Therefore, data for these sampling dates were averaged to represent pre-senescent N in calculating RE. The most dramatic changes in tissue N concentration occurred during LT exposure where N content of wt leaves dropped from 0.495 to 0.151 g/m² representing a 69% decrease in leaf N during senescence and abscission (RE of 0.692; Table 4-l). During the LT exposure, wt stem N concentration increased from 4.30 to 7.93 mg/g in the bark and from 1.48 to 2.93 mg/g in the wood (Figure 4.1).
Figure 4.1. Nitrogen content of leaves (LPI 15, ◦), bark (■) and wood (▲) of wild-type (clone 717) plants grown for 8 weeks in SD photoperiod, followed by low temperature (10° day, 4° night) with SD photoperiod. Final data points represent end-point (EP) N content of abscised leaves and stem samples taken after all leaves had abscised.
Table 4-I. Nitrogen content of pre-senescent and abscised leaves of growth chamber-grown wt (717) and BSP-antisense leaves (LPI 15). Values for pre-senescent leaves are mean of 3 sampling dates prior to LT exposure (4-8 week SD). N content was determined on lyophilized tissue samples by combustion analysis, and is expressed on the basis of leaf dry weight (concentration) and area (content). Resorption efficiency was calculated from N content of pre-senescent and abscised leaves.

<table>
<thead>
<tr>
<th></th>
<th>wt</th>
<th>14BR</th>
<th>RT5</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>N concentration (mg/g)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pre-senescent</td>
<td>18.5</td>
<td>15.3*</td>
<td>14.2*</td>
</tr>
<tr>
<td>Abscised</td>
<td>5.69</td>
<td>4.71**</td>
<td>5.14*</td>
</tr>
<tr>
<td><strong>N content (g/m²)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pre-senescent</td>
<td>0.495</td>
<td>0.419</td>
<td>0.403+</td>
</tr>
<tr>
<td>Abscised</td>
<td>0.151</td>
<td>0.151</td>
<td>0.159</td>
</tr>
<tr>
<td><strong>Resorption efficiency</strong></td>
<td>0.692</td>
<td>0.636</td>
<td>0.604*</td>
</tr>
</tbody>
</table>

Means are significantly different from the wt at *P < 0.10, *P < 0.05, **P < 0.01.

Time course of N resorption in BSP-antisense plants did not differ significantly from that of the wt, as determined by analysis of variance. Therefore, clonal differences were compared on the basis of mean pre-senescent leaf N. BSP-antisense leaves had significantly lower N concentration both before and after LT exposure (Table 4-I). However, due to differences in LMA between BSP-antisense and wt, N content did not show the same degree of clonal differences (Table 4-I). N content of BSP-antisense leaves prior to LT exposure was 16 to 19% lower than that of the wt, whereas N content of the abscised leaves was the same as, or slightly higher than that of the wt. Resorption efficiency (RE) for 14BR and RT5 was 9% and 13% less than the wt, respectively. However, only RT5 was significantly different (*P < 0.05) from the wt (Table 4-I), perhaps due to variation among individual plants and low replication number. Leaves of 14BR and RT5 abscised an average of 4.92±2.72 and 6.29±2.13 days earlier than the wt. Stem N content
was not significantly different between BSP-antisense lines and the \textit{wt} for any sampling point in this experiment (data not shown).

\textbf{Outdoor experiment}

Since BSP-antisense plants have a longer plastichron (time interval between node production; see Chapter 2), comparisons among leaves of the same LPI do not accurately reflect leaf age, particularly among basal leaves. For this study, comparisons were made among mature leaves based on approximated leaf age. At the time of first sampling, \textit{wt} plants had an average of 95 leaf-bearing nodes, compared to \textasciitilde 75 for both BSP-antisense lines. N content was compared among leaves from the lower one-third (LPI 60, 65 for \textit{wt}, and LPI 45, 50 for antisense), and from the upper one-third (LPI 25, 30 for \textit{wt}, and LPI 20, 25 for antisense) of the plant. Comparisons among younger leaves (LPI 5, 10) were made at equal LPI.

To study N resorption under natural conditions, nitrogen content of BSP-antisense and \textit{wt} plants was measured periodically from terminal bud set until all leaves had abscised. The first destructive samples were taken when \textasciitilde 50\% of trees had set terminal buds (26-Aug). The sampling date of 19-Nov corresponded with the point at which \textasciitilde 50\% of leaves had abscised from all remaining trees. Between the first sampling and that of 4-Nov, no visible signs of leaf senescence (color changes) or any appreciable leaf abscission occurred, except in a few of the most basal leaves. Prior to the 4-Nov sampling, leaf N of mature leaves did not change significantly ($P = 0.83$ for N concentration and $P = 0.74$ for N content, based on repeated measures analysis of variance). During this period, bark- and wood-N content of the \textit{wt} plants declined slightly (Figure 4.2).
Figure 4.2. Nitrogen content of upper (●) and lower (■) mature leaves, bark (▲) and wood (◆) of wild-type poplar during autumn leaf senescence of plants grown in an outdoor nursery at College Park Maryland. Last data points are for endpoint (EP) N content of abscised leaves and of stem samples taken at the completion of leaf abscission. Broken lines represent 12-d running average of daily high and low temperature recorded at a nearby weather station (Beltsville, Maryland).
Table 4-II. Nitrogen content of leaves from outdoor-grown BSP-antisense and wt (717) plants prior to leaf abscission. Plants were sampled at two week intervals from terminal bud set (26-Aug) until just prior to leaf abscission (4-Nov). Leaf N levels are expressed as N content (N per leaf area) and as N concentration (N per dry weight). Data were analyzed as repeated measures (split-plot) with means reported below. See text for explanation of leaf positions.

<table>
<thead>
<tr>
<th>Leaf position</th>
<th>wt</th>
<th>I4BR</th>
<th>RT5</th>
</tr>
</thead>
<tbody>
<tr>
<td>N concentration (mg/g)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LPI 5</td>
<td>20.8</td>
<td>18.6**</td>
<td>19.7</td>
</tr>
<tr>
<td>LPI 10</td>
<td>18.4</td>
<td>16.4***</td>
<td>17.2*</td>
</tr>
<tr>
<td>Upper</td>
<td>15.9</td>
<td>14.1***</td>
<td>15.1</td>
</tr>
<tr>
<td>Lower</td>
<td>12.8</td>
<td>11.7**</td>
<td>11.9**</td>
</tr>
<tr>
<td>N content (g/m²)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LPI 5</td>
<td>1.37</td>
<td>1.45</td>
<td>1.55*</td>
</tr>
<tr>
<td>LPI 10</td>
<td>1.40</td>
<td>1.35</td>
<td>1.44</td>
</tr>
<tr>
<td>Upper</td>
<td>1.27</td>
<td>1.15**</td>
<td>1.22</td>
</tr>
<tr>
<td>Lower</td>
<td>0.914</td>
<td>0.995***</td>
<td>1.01***</td>
</tr>
</tbody>
</table>

Means are significantly different from the wt at *P < 0.05, **P < 0.01, ***P < 0.001.

Visible leaf senescence and leaf abscission began with basal leaves and progressed acropetaly. Leaf abscission progressed rapidly after 6-Nov. Based on collection date of abscising leaves, average abscission date for all wt leaves was 18-Nov, approximately 5 to 6 weeks after average daily-minimum temperatures dropped below 10°C (Figure 4.2). The major shift in N content from leaves to stem was closely associated with this period of rapid leaf senescence and abscission. Mean leaf N content decreased from 1.27 g/m² prior to 4-Nov to 0.431 g/m² in abscised leaves (Table 4-II and 4-III). Stem N content increased from 4.63 to 13.2 mg/g in bark and from 1.52 to 3.21 mg/g between 21-Oct and the endpoint sampling (Figure 4.2 and Table 4-V). Since changes in leaf and stem N primarily
occurred after 4-Nov, means of 26-Aug through 4-Nov sampling dates were used to represent pre-senescent N levels in clonal comparisons and RE calculations.

Table 4-III. Nitrogen content of abscised leaves from outdoor-grown BSP-antisense and wt (717) poplar. Abscising leaves were collected at 2- to 3-day intervals after the start of leaf abscission (from 6-Nov). N content of four leaf positions are expressed as concentration and content. Upper and lower mature leaves were collected from one-third and two-thirds down the stem.

<table>
<thead>
<tr>
<th>Leaf position</th>
<th>wt</th>
<th>14BR</th>
<th>RT5</th>
</tr>
</thead>
<tbody>
<tr>
<td>N concentration (mg/g)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LPI 5</td>
<td>6.38</td>
<td>8.96*</td>
<td>6.14</td>
</tr>
<tr>
<td>LPI 10</td>
<td>6.06</td>
<td>6.40</td>
<td>6.26</td>
</tr>
<tr>
<td>Upper mature</td>
<td>7.18</td>
<td>7.66</td>
<td>9.42+</td>
</tr>
<tr>
<td>Lower mature</td>
<td>6.26</td>
<td>6.24</td>
<td>6.88</td>
</tr>
<tr>
<td>Mean</td>
<td>6.33</td>
<td>6.95+</td>
<td>7.16*</td>
</tr>
<tr>
<td>N content (g/m²)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LPI 5</td>
<td>0.184</td>
<td>0.414**</td>
<td>0.186</td>
</tr>
<tr>
<td>LPI 10</td>
<td>0.469</td>
<td>0.522</td>
<td>0.514</td>
</tr>
<tr>
<td>Upper</td>
<td>0.612</td>
<td>0.641</td>
<td>0.911+</td>
</tr>
<tr>
<td>Lower</td>
<td>0.483</td>
<td>0.532</td>
<td>0.624</td>
</tr>
<tr>
<td>Mean</td>
<td>0.431</td>
<td>0.522*</td>
<td>0.534*</td>
</tr>
</tbody>
</table>

Means are significantly different from the wt at *P < 0.10, *P < 0.05, **P < 0.01.

As with the controlled environment study, time course of N resorption did not differ from that of wt as determined by analysis of variance. Therefore, clonal comparisons were made for mean pre-senescent and abscised leaf N levels. Prior to abscission, leaves of BSP-antisense clones had lower N concentration than the wt regardless of leaf position (Table 4-II). LMA varies with LPI (leaf age) and
BSP-antisense plants have a higher LMA than wt (see Chapter 2). Clonal differences in LMA are greatest in expanding leaves and again in the oldest leaves (data not shown). The result of these LMA differences is that pre-senescent leaf N content was higher in BSP-antisense lines than in the wt among the youngest (LPI 5) and the oldest leaves measured (Table 4-II).

Leaves of BSP-antisense clones abscised earlier with a higher end-point N content than leaves of the wt. Average time of leaf abscission of 14BR and RT5 was 3.4±0.35 and 4.3±0.82 days earlier than that of the wt, respectively. Although clonal differences in endpoint N content varied with leaf position, BSP-antisense leaves abscised with an average of 10 - 13% more N on a dry weight basis, and 21 - 24% more N per leaf area than the wt (Table 4-III). The RE was 0.566 for 14BR and 0.564 for RT5 compared to 0.644 for the wt (Table 4-IV), a 13% reduction. Alternatively stated, an average of 35% of pre-senescent N levels remained in the wt leaves after abscission, compared to 44% for BSP-antisense leaves.

Table 4-IV. Resorption efficiency of outdoor-grown BSP-antisense and wt (717) poplar. Resorption efficiency was calculated as difference between pre-senescent and post abscission N content divided by pre-senescent N content. Means of the first six measurements were used to represent pre-senescent N.

<table>
<thead>
<tr>
<th>Leaf position</th>
<th>wt</th>
<th>14BR</th>
<th>RT5</th>
</tr>
</thead>
<tbody>
<tr>
<td>LPI 5</td>
<td>0.865</td>
<td>0.717**</td>
<td>0.880</td>
</tr>
<tr>
<td>LPI 10</td>
<td>0.662</td>
<td>0.610</td>
<td>0.643</td>
</tr>
<tr>
<td>Upper</td>
<td>0.512</td>
<td>0.440</td>
<td>0.242*</td>
</tr>
<tr>
<td>Lower</td>
<td>0.477</td>
<td>0.461</td>
<td>0.384</td>
</tr>
<tr>
<td>Mean</td>
<td>0.644</td>
<td>0.566*</td>
<td>0.564*</td>
</tr>
</tbody>
</table>

Means are significantly different from the wt at
*P < 0.05, **P < 0.01.
Bark and wood N concentrations were consistently lower in BSP-antisense lines at all sampling dates (Table 4-V). N content of stem components in all lines reached a minimum at the 21-Oct sampling (Figure 4.2). During leaf senescence and abscission, \textit{wt} bark N content increased from 4.63 to 13.2 mg/g, or 285% (Table 4-V), compared to 276% and 278% for 14BR and RT5, respectively. Wood N content increased by 211% in the \textit{wt} and 195% and 198% for 14BR and RT5, respectively.

Table 4-V. Stem N content of outdoor-grown BSP-antisense and \textit{wt} (717) poplar stems before and after leaf senescence.

<table>
<thead>
<tr>
<th></th>
<th>\textit{wt}</th>
<th>14BR</th>
<th>RT5</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Bark (mg/g)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>26-Aug</td>
<td>7.62</td>
<td>6.37</td>
<td>6.30</td>
</tr>
<tr>
<td>21-Oct</td>
<td>4.63</td>
<td>4.27</td>
<td>4.61</td>
</tr>
<tr>
<td>Pre-senescent mean</td>
<td>5.71</td>
<td>4.86*</td>
<td>5.10*</td>
</tr>
<tr>
<td>Endpoint</td>
<td>13.2</td>
<td>11.8*</td>
<td>12.8</td>
</tr>
<tr>
<td><strong>Wood</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>26-Aug</td>
<td>1.93</td>
<td>1.66</td>
<td>1.71</td>
</tr>
<tr>
<td>21-Oct</td>
<td>1.52</td>
<td>1.30*</td>
<td>1.36</td>
</tr>
<tr>
<td>Pre-senescent mean</td>
<td>1.68</td>
<td>1.41**</td>
<td>1.48**</td>
</tr>
<tr>
<td>Endpoint</td>
<td>3.21</td>
<td>2.53*</td>
<td>2.69*</td>
</tr>
</tbody>
</table>

Means are significantly different from the \textit{wt} at \(^*P < 0.10\), \(^*P < 0.05\), \(^*P < 0.01\).

**Significance**

In plants which assimilate N in the leaves (Chapter 3), nitrate enters leaves in the transpiration stream and is exported as amino acids through the phloem (Feller and Fischer, 1994). In mature pre-senescent leaves, these N fluxes are in equilibrium. Chlorophyll breakdown and color changes normally associated with leaf senescence are closely associated with the rapid degradation of proteins and
export of amino acids (Feller and Keist, 1986; Peoples and Dalling, 1988). During the senescence process, leaves become net N exporters. In deciduous perennials, nitrogen use efficiency is dependent, in part, on the amount of N which can be resorbed from autumn senescing leaves (Killingbeck et al., 1990). Time courses presented here indicate that N resorption occurs only during leaf senescence and during the few weeks prior to leaf abscission. This observed timing of N resorption agrees with previous reports for *P. tremuloides* and *Quercus rubra* (Collier and Thibodeau, 1995)

The objective of the present study was to determine if BSP-antisense plants had reduced N resorption. Resorption efficiency may be determined by "physiological potential and ecological reality" (Killingbeck et al., 1990). Physiological potential may be influenced by the form and subcellular location of leaf N (source; Feller and Fischer, 1994), and by demand for N in developing seeds, fruit, and storage organs (sink; Thomas and Stoddart, 1980). The "ecological reality" of resorption is influenced by light, temperature, wind, and N and water availability (Killingbeck et al., 1990; Thomas and Stoddart, 1982). These environmental factors were minimized in one of the present studies by determining resorption efficiency in a controlled environment.

Resorption efficiency of the wt poplar was 69.2% under controlled environment, and 64.4% in an outdoor nursery. Similarly, RE of 66% has been reported for independent determinations of *Populus* species grown at two different locations (Pastor and Bockheim, 1984; Verry and Timmons, 1976). Collier and Thibodeau (1995) reported 79% RE for *P. tremuloides* growing in a managed landscape. Killingbeck et al. (1990) measured RE of individual trees in a naturally occurring population of clonal *P. tremuloides* and found RE ranging from 24 to 69% over the course of three seasons. RE within a single season ranged from 32 to 51% with seasonal averages from 24 to 56%.
In both the growth-chamber and greenhouse studies, RE of BSP-antisense clones was lower than RE of the wt. However, RE is a function of both pre-senescent and post-abscission leaf N content, and we have reported previously that pre-senescent N content may be altered in antisense BSP plants (Chapter 2). In the controlled-environment study presented here, calculated REs appeared to be a function of pre-senescent N levels, as N content of abscised leaves was remarkable consistent among clones (ranging from 0.151 to 0.159 g/m²; Table 4-I). The lowest post-abscission N levels were measured in the controlled environment study. This suggests a possible limit in N available for resorption. By some accounts, reduced N for resorption comes only from hydrolysis of specific proteins in the chloroplast and cytosol, with chlorophyll N remaining in the senesced leaves (Feller and Fischer, 1994). N resorption in a growth chamber may represent idealized conditions (absence of wind, rain and freezing temperatures). Under these controlled environment conditions, all of the N available for translocation may have been resorbed.

In the outdoor study, BSP-antisense clones had higher pre-senescent N content relative to the wt (Table 4-II), with the reduced resorption efficiencies due to increased N in the abscised leaves (Table 4-III). Tree to tree variations in resorption efficiency due to differences in abscised-leaf N content were reported to be inversely correlated with time of leaf abscission (Killingbeck et al., 1990). In both the studies reported here, leaves of BSP-antisense clones abscised earlier than the wt (see text for data).

Alternatively, clonal differences in resorption efficiency may be relatively constant, with the similarities and differences in the pre-senescent and post-abscission N levels described above being coincidental. RT5 in the growth chamber study, and both 14BR and RT5 in the outdoor study had resorption efficiencies which were 12 - 13% lower than that of the respective wt. The reduced resorption in BSP-antisense lines may represent differences in
physiological potential for storing resorbed N. Antisense technology is used to reduce normal expression of the target gene. In BSP-antisense plants this reduction in gene expression could result in less N storage per stem tissue which was observed here (Table 4-V). In addition, capacity for N resorption could be reduced because of the altered leaf : stem ratio of BSP-antisense plants (see Chapter 2). Since the BSP-antisense lines have a significantly higher leaf:stem ratio, RE may be limited by amount of stem per leaf as much as by the amount of storage per stem tissue. The relative importance of these changes could be addressed in future studies by physical manipulation of the source and sink sizes.

We report here that two independent poplar lines expressing antisense BSP cDNA and selected for reduced SD accumulation of BSP, resorb less N from autumn-senescent leaves than the untransformed wild-type poplar. This provides strong evidence for the proposed function of BSP as a sink for autumn N resorption.
LITERATURE CITED


CHAPTER 5: Ecotypic and Genetic Variation in Poplar BSP

ABSTRACT

Bark storage proteins (BSP) store nitrogen (N) translocated from senescing leaves in autumn, and supply reduced N for spring growth. BSP accumulation and the expression of the BSP gene are associated with short-day photoperiod. Plants of *Populus deltoides* originating from six locations within the mid-western United States were grown under natural conditions at a common location, and BSP mRNA levels were measured at 2-week intervals from 7-Aug to 16-Oct. The time of maximum BSP mRNA accumulation was inversely correlated with latitude of origin. This inverse correlation is consistent with photoperiodic responses of plants native to temperate climates. Amount of short-day BSP accumulation was compared among *Populus trichocarpa* x *P. deltoides* clones representing six full-sib families. After 6 weeks of short-day photoperiod, there were significant differences in BSP levels among clones within full-sib families. BSP levels were positively correlated with total bark nitrogen content within full-sib family. These full-sib families which vary in SD BSP accumulation will provide an important resource for future studies investigating the ecological costs and benefits of BSP accumulation. The work presented here demonstrates genetic variation in both the time and amount of seasonal BSP accumulation.

INTRODUCTION

Nitrogen translocation from senescing leaves to overwintering storage sites is a common feature of temperate deciduous trees (Ryan and Bormann, 1982). In
poplar it is estimated that approximately 75% of total tree nitrogen is contained in the leaves in late summer and that 70-80% of the nitrogen is still retained within the tree after autumn leaf senescence (Pregitzer et al., 1990). During the winter >50% of the nitrogen contained in the stems of poplar is protein nitrogen (Höllwarth, 1976).

A 32-kD bark storage protein (BSP) has been identified in the bark of overwintering poplars (Wetzel et al., 1989a). This protein is sequestered in protein storage vacuoles of the inner bark parenchyma and xylem ray cells during autumn and winter (Wetzel et al., 1989b). The accumulation of BSP is associated with photoperiod, where short day (SD) stimulates and long day (LD) inhibits accumulation (Coleman et al., 1991). BSP accumulation under SD is correlated with large increases in the steady-state levels of poplar BSP mRNA (Coleman et al., 1992).

Photoperiodic responses in woody plants native to temperate climates include the cessation of shoot growth, development of cold hardiness, and the initiation of bud dormancy. (Vince-Prue, 1975; Weiser 1970). Generally trees from higher latitudes cease growth and cold acclimate at shorter night lengths than plants from lower latitudes (Vaartaja, 1954). In poplar, the critical photoperiod required for induction of growth cessation and bud dormancy is inversely correlated with the latitude of origin (Pauley and Perry, 1954; Vaartaja, 1960). The seasonal pattern of BSP accumulation corresponds temporally with other seasonal responses such as bud dormancy (Coleman et al., 1991), and the expression of BSP genes is associated with photoperiod (Coleman et al., 1992). However, ecotypic patterns in BSP gene expression have not been reported.

Several reports have documented clonal differences in total BSP accumulation. Langheinrich (1993) found clonal differences among different poplar species and interspecific hybrids. Coleman et al. (1991) documented differences in mid-winter BSP levels in the bark of 14 P. deltoides ecotypes grown
at two different field sites. Functioning as a storage protein, BSP accumulation is related to N availability (Coleman et al., 1994). Consequently, it is not clear from previous studies whether clonal differences in BSP accumulation represent genetic or environmental variation. No direct analysis of genetic variation in BSP accumulation has been reported. The results presented here demonstrate genetic variation in both the time of seasonal BSP induction, and the amount of BSP accumulated under SD photoperiod.

MATERIALS AND METHODS

Ecotypic variation

Poplar plants (*Populus deltoides* Bart. Ex Marsh) were established from dormant stem cuttings. Cuttings were rooted and grown in individual pots. Poplar plants of clones native to 44.33° N (clone 172-2; Central Minnesota), 41.92°N (284-3; Northern Illinois), 40.50°N (52-2; Central Ohio), 38.83° N (235-2; Northern Missouri), 33.50°N (108, Southern Oklahoma), and 30.5° N (S7C4; Central Texas) were placed outdoors and grown in the natural day length of Corvallis, OR (44.63°N, 122.20°W). Bark samples were collected from replicate plants at 2-week intervals beginning August 7 (day length 14h, 27 min) and ending October 16 (day length 11h, 30 min). The tissue samples were immediately frozen in liquid nitrogen and stored at -70°C until RNA extraction.

Total RNA was isolated as described previously (Coleman et al., 1992), and 10 µg/lane total RNA was separated in agarose gels containing formaldehyde (Sambrook et al., 1989). Equal loading of RNA per lane was confirmed by comparing the intensities of the rRNAs in an ethidium bromide-stained agarose gel. RNA gel blots were performed on nylon membranes (Zeta-Probe GT, Bio-Rad Corp., Richmond, CA). A gel purified 1.2 kb BSP cDNA (Coleman et al., 1992)
was labeled by random priming (Feinberg and Vogelstein, 1984) and was used as a probe. All prehybridizations were done at 65°C in 0.25 M Na₂HPO₄, pH 7.2 and 7% SDS (Church and Gilbert, 1984). Hybridizations were carried out in the same buffer overnight at 65°C. RNA blots were washed once in 1x SSC and 0.1% SDS at room temperature for 20 min, three times for 20 min each in 0.2x SSC and 0.1% SDS at 65°C, and once in 0.1x SSC and 0.1% SDS at room temperature for 20 min. After verifying that the BSP cDNA hybridized to the appropriately sized mRNA, total RNA (2 μg) was slot blotted to nylon membranes and hybridized to ³²P-labeled BSP cDNA. All prehybridizations, hybridizations and washes were as described for the RNA gel blots. Slot-blots were then autoradiographed, the individual slots cut from the membrane, and the amount of bound radioactivity was determined by liquid scintillation spectroscopy. At least three replicate slot blots were quantified for each clone and sample date, and the means were used to calculate mRNA (expressed as relative percent).

Genotypic variation

Hardwood cuttings of hybrid poplar (Populus trichocarpa x P. deltoides) were obtained from the breeding program at the Poplar Molecular Genetics Cooperative, University of Washington. These clones consist of six full-sib families with 7 to 10 full sibs per family. Plants were propagated from cuttings and planted in 1.4-L pots containing a commercial soil-less media (ProGro 300S), and fertilized (10.7 mM N; Excel 21-5-20) at approximately two-week intervals. At a height of ≈60 cm, plants from each clone were moved to growth chambers maintained at 25°C with SD photoperiod (8 h day, 16 h night) provided by metal halide lamps (470 μmol m⁻² s⁻¹ ppf), and fertilization was discontinued. After six weeks, bark samples from internodes corresponding to LPI 8-10 were collected for chemical analysis. Bark was peeled from wood, immediately frozen in liquid nitrogen, and stored at -80°C until lyophilization. Where possible, SD treatment
and analysis was performed on four replicate plants. However, clones from family 331 are more difficult to propagate, and only 2 - 3 replicate plants were available for some clones from this family.

Lyophilized bark samples were analyzed for total N and total extractable protein. Total N was determined by Dumas combustion on triplicate sub-samples from each plant using an automated Carbon/Nitrogen analyzer (NC 2100 Soil, CE Instruments, Italy). For total bark protein, 30 μL of extraction buffer (62.5 mM Tris-HCl - pH 6.8, 2% SDS, 10% glycerol and 5% 2-mercaptoethanol; Laemmli, 1970) was added per mg of lyophilized ground bark. Samples were then vortexed, held on ice for 15 to 20 min, and then moved to a boiling water bath for 15 min. After boiling, samples were cooled to room temperature, centrifuged at 14,000 rpm (Eppendorf model 5417R centrifuge, Brinkmann Instruments, Westbury NY) and the supernatant collected and stored at -20°C.

Protein concentration was determined using the bicinchoninic acid (BCA) colorimetric assay (Brown et al., 1989). Interfering substances were removed by diluting aliquots 100x in distilled water and then precipitating protein with deoxycholate and trichloroacetic acid followed by centrifugation. Proteins were resuspended in 5% SDS and 0.1 N NaOH and concentration determined using the BCA colorimetric assay kit (Pierce, Rockford IL). The BCA procedure was as per manufacturer recommendations, with bovine serum albumin as a standard and absorbance measured at 570 nm (Benchmark Microplate Reader, BioRad).

BSP levels were compared among clones within full-sib families by western-blot analysis. Protein extract equivalent to 0.33 mg (dry weight) of bark tissue per lane was loaded onto a 12% polyacrylamide gel (Mini-Protein II, BioRad) and electrophoresed according to manufacturer recommendations (45 min at 200 volts). After electrophoresis gels were blotted to nitrocellulose (NitroBind, Micron Separations Inc., Westborough MA) using standard blotting procedures. After blotting, membranes were washed for 10 min in Tris-buffered saline (TBS, 20 mM
Tris-HCl - pH 7.5 and 150 mM NaCl) and blocked overnight at 4°C in TBS + 3% Gelatin. After blocking, blots were warmed at 30°C for 1 h, washed in TBS at room temperature for 15 min, and then incubated in primary antibody solution for 1 h at 30°C. The primary antibody was raised in rabbit against a 20-amino acid residue BSP synthetic peptide (antibody and peptide prepared by Research Genetics, Huntsville, Alabama). The oligopeptide (TEA LKD MKL RKC YSD ECL PG) was selected from the deduced amino acid sequence of BSP cDNA (Coleman et al., 1992). Serum containing the primary antibody (8-week bleed) was diluted (1,000x) in TBS containing 0.05% Tween (TTBS) and 1% Gelatin. After incubation in primary antibody solution, blots were washed three times for 15 min each in TTBS at room temperature, and then incubated in secondary antibody solution (goat-anti-rabbit linked to alkaline phosphatase, diluted 25,000x in TTBS + 1% gelatin) at 30°C for 30 min. Blots were then washed 3 times for 15 min in TTBS, once in TBS and color developed as described by Sambrook et al. (1989). Developed blots were imaged and bands quantified using a digital imaging system (Alpha Imager 2000, Alpha Innotech Corp.). Data were analyzed as a Randomized Block design where blocking was by growth chamber, and by western blot.

RESULTS AND DISCUSSION

Ecotypic variation

To determine if photoperiod-associated accumulation of BSP mRNA differed in poplars native to different latitudes, RNA was isolated from the bark of three different ecotypes which had been grown in the natural day length of Corvallis, OR. RNA was isolated from bark collected at 2-week intervals beginning on 7-Aug and ending on 16-Oct, and analyzed by RNA slot blots. The percent relative abundance of BSP mRNA was determined for each sampling date.
for each poplar clone by liquid scintillation counting of radioactivity recovered from individual slot blots. As shown in Figure 5.1, maximum mRNA accumulation of the northern-most clone (172-2) was measured for 4-Sep, while maximum BSP mRNA accumulation of the southern-most clone (S7C4) was at least 6 weeks later (16-Oct). The remaining four clones showed maximum mRNA accumulation dates intermediate to these (Figure 5.1). With the exception of clone 52-2 (41.92°N), the date of maximum mRNA levels was closely correlated with latitude of origin (Figure 5.2). Including all six ecotypes, this negative correlation had an R² of 0.68.

Photoperiod responses such as growth cessation, cold hardiness and initiation of dormancy have previously been shown to differ in poplars from different latitudes (Pauley and Perry, 1954; Vaartaja 1960). The results presented for BSP mRNA abundance are consistent with that observed for previously documented SD-induced whole-plant physiological changes, and further support the role of photoperiod in poplar BSP gene expression (Coleman et al., 1992; Langheinrich and Tischner, 1991).

**Genetic variation**

To determine the degree of genetic variability in BSP accumulation, we compared SD BSP levels among closely related poplar clones. After six weeks of SD photoperiod, bark samples were collected and levels of BSP (Figure 5.3, Table 5-I), total extractable protein, and total nitrogen (Figure 5.4) were compared among clones within six full-sib families. Within-family differences in BSP accumulation were readily apparent in five of the six families, ranging from relatively high amounts of BSP, to nearly undetectable levels (Figure 5.3). In family 545 for example, 3 of the 7 clones shown in Figure 5.3 had high amounts of BSP (lanes 3-5), one clone had nearly undetectable levels (lane 1), and BSP levels in the remaining three were intermediate (lanes 2, 6 and 7). Clones of family 555 showed the least degree of variation in BSP accumulation (Figure 5.3).
Figure 5.1. Relative abundance of BSP transcript (expressed as a percent of maximum) in the bark of different poplar ecotypes grown under natural day length of Corvallis, Oregon. Poplar clone number and latitude of origin are as follows: 172-2 (44.33° N latitude), 284-3 (41.92°N latitude), 52-2 (40.50°N latitude), 235-2 (38.83° N latitude), 108 (clone 33.50°N latitude), and S7C4 (30.5° N latitude). See materials and methods for mRNA extraction and quantification procedures.
To quantify BSP levels, within-rep comparisons for each family were quantified by spot densitometry. Analysis of variance on the densitometric analysis showed that clonal differences in BSP accumulation were statistically significant in five of the six families. Although clones from family 555 showed the least degree of variation in BSP levels (Figure 5.3), differences were consistent among replications and statistically significant ($P = 0.047$; Table 5-I). Clones from family 331 are more difficult to propagate and grow more slowly than clones from the remaining five families. As a result, only 2 or 3 plants were available from each clone for this experiment. Although differences in BSP levels among family 331 were apparent (Figure 5.3), these differences were not statistically significant due to the reduced replication number (Table 5-I).
Figure 5.3. BSP accumulation in the bark of full-sib *P. trichocarpa* x *P. deltoides* clones after 6 weeks of SD treatment. Total proteins (loaded on an equal-tissue basis) were separated by SDS-PAGE and transferred to nitrocellulose membranes. BSP was detected with an anti-BSP peptide antiserum.
Table 5-I. Relative BSP polypeptide levels in the bark of full-sib clones after SD treatment. Total proteins (loaded on an equal-tissue basis) were separated by SDS-PAGE and transferred to nitrocellulose membranes where BSP was detected with an anti-BSP peptide antiserum. Protein gel blots were quantified by spot densitometry. Corrected integrated density values (CIDV) were determined for replicate plants and compared by analysis of variance. Mean CIDV for each clone is shown below, and statistical differences of clones within a family are presented as probability values (P). Except in the case of family 331, 4 replicate plants were analyzed for each clone.

<table>
<thead>
<tr>
<th>Family</th>
<th>331</th>
<th>432</th>
<th>433</th>
<th>545</th>
<th>555</th>
<th>808</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clone</td>
<td>BSP</td>
<td>Clone</td>
<td>BSP</td>
<td>Clone</td>
<td>BSP</td>
<td>Clone</td>
</tr>
<tr>
<td>1806</td>
<td>25.9</td>
<td>3653</td>
<td>5.6</td>
<td>3985</td>
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<td>1813</td>
<td>13.8</td>
<td>3675</td>
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<td>3987</td>
<td>33.9</td>
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<tr>
<td>1815</td>
<td>9.0</td>
<td>3761</td>
<td>24.2</td>
<td>4027</td>
<td>5.9</td>
<td>5175</td>
</tr>
<tr>
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<td>3809</td>
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<td>33.0</td>
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<td></td>
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<td>50.8</td>
<td>4295</td>
<td>22.0</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>3923</td>
<td>9.1</td>
<td>4332</td>
<td>13.0</td>
<td>5960</td>
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</tbody>
</table>

P = 0.248 0.0007 0.006 0.011 0.047 0.0327

Total nitrogen content also varied significantly among full sib clones (Figure 5.4). In family 808 for example, clone 13847 had a bark N content of 6.13 mg/g which was 96% higher than that of clone 12825 (3.12 mg/g). Family 433 had the least variation in bark N content ranging from a low of 3.36 mg/g to a high of 4.32 mg/g (Figure 5.4). With the exception of family 433, total stem N content was significantly correlated with BSP, with correlation coefficients from 0.819 to 0.941 (Table 5-II).
Figure 5.4. Nitrogen content of the bark of full-sib *P. trichocarpa* x *P. deltoides* clones after 6 weeks of SD photoperiod. Total N was determined by Dumas combustion using an automated carbon-nitrogen analyzer. Values are means and standard errors for replicate plants. Clones within each family are in ascending order. See Table 5-I for a list of clone names.

Alternatively, BSP content was not significantly correlated with amount of extracted protein (Table 5-II). These experiments were designed to screen genetically diverse clones for natural variation in BSP accumulation and not to develop a cause-effect relationship between nitrogen status and BSP levels. However, the correlation between total N and BSP levels is not unexpected since at peak accumulation, BSP represents as much as 70% of total soluble protein (Coleman et al., 1991; Langheinrich and Tischner, 1991).
Table 5-II. Correlation between BSP level (CIDV) and total N or total protein content of clones within six full-sib families. Values presented are correlation coefficients and statistical significance (P).

<table>
<thead>
<tr>
<th>Family</th>
<th>331</th>
<th>432</th>
<th>433</th>
<th>545</th>
<th>555</th>
<th>808</th>
</tr>
</thead>
<tbody>
<tr>
<td>BSP - %N</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Coefficient</td>
<td>0.941</td>
<td>0.819</td>
<td>0.494</td>
<td>0.829</td>
<td>0.890</td>
<td>0.850</td>
</tr>
<tr>
<td>P</td>
<td>0.002</td>
<td>0.004</td>
<td>0.147</td>
<td>0.011</td>
<td>&lt;0.001</td>
<td>0.002</td>
</tr>
<tr>
<td>BSP - protein</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Coefficient</td>
<td>0.934</td>
<td>0.695</td>
<td>-0.029</td>
<td>0.161</td>
<td>0.544</td>
<td>0.599</td>
</tr>
<tr>
<td>P</td>
<td>0.002</td>
<td>0.026</td>
<td>0.937</td>
<td>0.703</td>
<td>0.104</td>
<td>0.067</td>
</tr>
</tbody>
</table>

The results presented here clearly indicate genetic variation in SD BSP accumulation. However the physiological basis for this variation is not clear. It has previously been established that seasonal BSP accumulation requires a SD photoperiod to induce BSP gene expression (Coleman et al., 1991; Langheinrich and Tischner, 1991) and a source for reduced N (Coleman et al., 1994). Several physiologically distinct sources may supply reduced N for BSP synthesis. These include new N assimilation, and N resorbed from either seasonal or developmental leaf senescence. During BSP accumulation in autumn, there may be temporal separation in the different sources of reduced N. Although the seasonal burst in BSP gene expression and initial BSP accumulation are photoperiodic responses (Coleman et al., 1992), resorption of leaf N does not occur until seasonal leaf senescence and abscission (see Chapter 4 and Collier and Thibodeau, 1995) which follows low temperatures. Prior to autumn leaf senescence, the sources of reduced N for BSP synthesis would most likely be newly assimilated N and N resorbed from developmentally senescent leaves. Plants used in this study were relatively uniform in size, age and N supply, and were kept above 20°C. Terminal buds had formed
on all plants by 4 weeks of SD exposure, but only a few basal leaves showed visible signs of senescence at the sampling time (after 6 weeks of SD exposure). Therefore, genetic variation observed here may reflect differences in ability to continue nitrate uptake during and after SD-induced changes in growth. SD-induced BSP accumulation may replace the growing shoot tip as a sink for newly assimilated N (see Chapter 2, see also Imsande and Touraine, 1994). Alternatively, variation observed here may be due to differences in resorption efficiency from developmentally senescent leaves.

Seasonal storage proteins like poplar BSP have been identified in a number of genera (Stepien et al., 1994). The accumulation, degradation, and gene expression of seasonal storage proteins have been extensively studied (For reviews see Coleman, 1997; Stepien et al., 1994). However, little is known about the ecological costs and benefits of accumulating high levels of a specific storage protein. The Populus trichocarpa x P. deltoides clones described here provide plant material which is closely related genetically, but differs in amount of SD BSP accumulation. This material will provide an important research tool for studying the ecological advantages and disadvantages of seasonal BSP accumulation.
LITERATURE CITED


CHAPTER 6: Summary and Conclusions

Nitrogen uptake, assimilation and internal cycling were studied using poplar as a model system. The function of poplar bark storage protein (BSP) was investigated during long-day (LD) growth, as well as during short-day (SD) growth and autumn leaf senescence. Hybrid poplar plants \( (P. \text{tremula} \times P. \text{alba}) \) transformed with a BSP-antisense gene were selected for reduced SD BSP accumulation. Two of these lines were characterized. Additional studies using \( P. \text{deltoides} \) and \( P. \text{trichocarpa} \times P. \text{deltoides} \) investigated the genetic variation in time and amount of BSP accumulation.

**Long-day growth and partitioning**

BSP-antisense plants have larger leaves, longer internodes and slower stem growth rate (as indexed by plastichron). Overall, dry-matter partitioning in the BSP-antisense plants is shifted from stem to leaf. Under LD conditions, greenhouse-grown BSP-antisense lines also showed reduced N content in leaf and stem tissue, and altered nitrate uptake. Grafting studies show that effects on leaf and stem partitioning and N content are determined by the shoot genotype (Chapter 2). Under LD conditions, BSP expression is controlled by N supply and found primarily in the young stem (Coleman et al., 1994). Taken together, these results suggest that BSP has an important function in LD growth and partitioning.

The relationship between N and the partitioning of carbon is well established. In the presence of adequate nitrogen, plants preferentially partition resources to the leaves, thereby increasing capacity to assimilate carbon. When N becomes limiting, plants partition resources to the roots, resulting in increased capacity for N uptake (Chapin, 1980; Cohen and Pastor, 1996). However, BSP-
antisense lines have lower above-ground tissue N concentration in both leaves and stems, but partition a larger portion of their total dry weight to the leaves (leaf : stem ratio) than does the wt. Synthesis of BSP in the growing shoot tip under LD conditions may act to increase the sink strength of the developing stem relative to the maturing leaves.

Some have suggested that the site of nitrate assimilation may regulate N- and consequently C-partitioning (Chapin, 1980). By preferentially retaining reduced N at the expense of amino acid export, the plant organ responsible for reduction and assimilation would effectively direct partitioning of resources, although direct manipulation of the site of N reduction failed to substantiate this hypothesis (Lexa and Cheeseman, 1990). In vivo assays of Nitrate reductase activity in poplar indicate that N reduction occurs primarily in the leaves (Chapter 3), with the highest NR activity found in developing leaves. Synthesis of BSP in the developing stem may act as a sink to draw reduced N out of the developing leaves thereby shifting the balance in resource partitioning between leaf and stem.

Alternatively, the function of BSP under LD conditions may be analogous to that of herbaceous-plant vegetative storage proteins (VSPs). According to a model proposed for soybean (Glycine max), nitrate uptake is regulated in part, by levels of free amino acids in the phloem (Imsande and Touraine, 1994). Synthesis of Rubisco and other proteins in young leaves and growing shoot tips act as a sink for free amino acids. According to this model, VSPs in the leaves and stems of Glycine supplement demand for amino acids, and in so doing, may be involved in regulating levels of free amino acids. In effect, synthesis of excess Rubisco and of VSPs may drive nitrate uptake by preventing the feed-back inhibition of the nitrate transporter (Muller and Touraine, 1992). Experiments conducted in hydroponic culture show that, unlike the wild type, BSP-antisense plants do not increase nitrate uptake rate with increased N supply (Chapter 2). Whether or not this results from feedback inhibition by increased free amino acid levels was not determined.
Stepien et al. (1994) reviewed the literature on woody-plant VSPs. They concluded that the genetic regulation of VSP biosynthesis is complex, and suggested “that VSPs could play several physiological roles within the cell.” Previous efforts have focused only on the seasonal storage role of BSP. The work presented here provides evidence for an important function in long-day growth and biomass partitioning which appears to be related to nitrate uptake and N partitioning.

**Autumn growth transition**

BSP and other woody plant VSPs were first identified based on the close association between their accumulation pattern and the seasonal N movement between leaves and stem (O’Kennedy and Titus, 1979). During spring growth, storage proteins are hydrolyzed concurrently with the movement of N from the stem to the expanding leaves and elongating stems of the new vegetative growth. This spring N mobilization appears to be sink regulated, since degradation of storage proteins depends on the presence of growing shoots (Coleman et al., 1993). Conversely, accumulation of storage proteins under natural conditions is temporally associated with autumn leaf senescence. In addition, induction of BSP-gene expression has been associated with SD photoperiod (Coleman et al., 1991; Langheinrich and Tischner, 1991), which is an important environmental signal in a number of seasonal responses (Vince-Prue, 1975). However, the source-sink relationship in autumn N resorption has not been as clearly defined.

The work presented here examined leaf and stem N content during the autumn growth transition. Shortened photoperiod resulted in terminal bud set, but no concurrent net export of N from leaves. Rapid decline in leaf N content and associated increase in stem N occurred only during the phase of rapid leaf senescence and abscission following low temperature treatment (Chapter 4). SD BSP induction is closely correlated with the formation of a terminal bud. This
suggests that autumn N resorption is dependent upon leaf senescence (i.e. source regulated) and not directed by BSP gene expression and protein synthesis. The SD-associated burst in BSP gene expression (Coleman et al., 1992) may serve to drive continued nitrate uptake after there is no growing shoot tip to act as a sink for newly-assimilated N.

BSP-antisense plants showed decreased N resorption calculated from pre-senescent and post-abscission leaf N content (Chapter 4). Reduced BSP accumulation per sink tissue could result in sink-limited N resorption. At the end of autumn leaf senescence, stem N content was significantly lower in BSP-antisense plants than in the wt (Chapter 4). A sink limitation could also result simply from bulk differences in the amount of source tissue compared to sink tissue, since overall leaf : stem ratio is altered in BSP-antisense lines (Chapter 2). Both BSP accumulation per storage tissue, and storage tissue per source tissue (stem : leaf) could result in limited storage capacity in the BSP-antisense plants.

Little is known about the role of seasonal BSP accumulation in environmental adaptation or the ecological costs and benefits associated with accumulating large amounts of a single storage protein. Results presented here indicate genetic variation in both the time of BSP induction and amount of BSP accumulation. Six different P. deltoides ecotypes grown at a common site differed in the time of maximum BSP mRNA accumulation (Chapter 5), which was inversely correlated with latitude of origin. These results were not unexpected since previous work showed that other photoperiodic responses in poplars such as growth cessation, cold hardiness, and initiation of dormancy, are similarly related to latitude of origin (Pauley and Perry, 1954; Vaartaja 1960).

Clones from six full-sib families, grown under controlled environment conditions, showed significant differences in the amount of BSP accumulated during SD exposure. These results suggest that there is also genetic variation in amount of total BSP accumulation. These full-sib clones with defined differences
in BSP accumulation will provide an important tool for future research efforts in understanding the ecological costs and benefits of BSP accumulation. A better understanding of these costs and benefits may result in an increased understanding of the physiological function of BSP in conserving available N in the plant.
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supply, internal N status and development stage in Jack Pine (*Pinus


