ABSTRACT

Title of Document: NITROGEN REMOBILIZATION AND THE NUCLEOSIDE PHOSPHORYLASE-LIKE VEGETATIVE STORAGE PROTEIN FAMILY IN POPULUS: CHARACTERIZATION, REGULATION AND TRANSGENES

Emily Ann Pettengill, Doctor of Philosophy, and 2012

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Nutrient remobilization and storage allow plants to direct resources toward growth, maintenance and reproduction and redirect nutrients in response to environmental conditions or stresses. Particularly for perennial plants, these capabilities are critical to surviving periods of unfavorable growth such as winter and nutrient limited environments. In *Populus*, bark storage proteins (BSPs) have a dominant role in seasonal storage, and proteins related to BSPs, known as nucleoside phosphorylase-like (NP-like) proteins, can also participate in short-term storage. This research presents a comprehensive examination of the *NP-like* gene family by characterizing their expression, exploring evolutionary relationships within the plant kingdom and investigating metabolic regulation. I also developed and tested a set of qPCR reference genes to use for data normalization in two *Populus* species and four

tissue-types. Lastly, transgenic trees were created to investigate the developmental or physiological functions of altered levels of BSP.

Experiments characterizing the spatial and temporal expression of *NP-like* genes implicated a functional role for all members. Those results also support the phylogenetic analyses demonstrating the expansion of the gene family, which may have occurred through subfunctionalization. I also examined the regulation of carbon (C) and nitrogen (N) metabolites on the *NP-like* gene family expression and observed that amino acids, N compounds and gamma-aminobutyric acid (GABA) treatments modulate expression and likely have a role in regulatory pathways. By investigating transgenic trees with altered BSP levels, I present preliminary evidence that BSPs may have a role in nutrient signaling capable of modulating photosynthesis in young leaves.

The results of this work deepen our understanding of nutrient remobilization and storage in *Populus* on regulatory, evolutionary and functional levels. Practically, the results can advance efforts to increase N use efficiency for sustainable biomass increases in *Populus* for use in agro-forestry, as biofuel feedstock, in phytoremediation and for carbon sequestration.

NITROGEN REMOBILIZATION AND THE NUCLEOSIDE PHOSPHORYLASE-LIKE VEGETATIVE STORAGE PROTEIN FAMILY IN POPULUS: CHARACTERIZATION, REGULATION AND TRANSGENES

By

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Preface

This dissertation is composed of an introductory overview, four chapters, a general conclusion and three appendices. Each chapter is structured in manuscript format with an abstract, introduction, materials and methods, results, discussion and conclusion. As such, descriptions of some methods are repeated. Ideas and facts presented in introduction sections may be expressed in a similar manner. Supplemental material is presented in the corresponding appendices and indicated with syntax for each appendix (i.e. supplemental material in chapter 1 is designated as Table A-1, chapter two, Fig. B-1, etc.). A comprehensive bibliography is located at the end of document.

Acknowledgements

This work would not have been possible without the support of Cécile Partmentier-Line, James B. Pettengill and Gary Coleman. All three have been a source of guidance and ideas. Cécile helped me develop technical laboratory abilities, organizational skills and how to work sustainably. More than anything, she has been a good friend. Gary's ability to generate ideas and experimental systems is a gift, which has been instrumental for developing critical thinking skills, independence and confidence in my own scientific ability. James has been an example of hard work and dedication to the scientific path from the time he worked across the hall to this day as my husband. I am grateful for his support, in my work and in my life. Lastly, I would like to acknowledge my parents, sister, brother and my dog, Gajanana.

Table of Contents

List of Tables

List of Figures

Overview

Plants have evolved mechanisms to redistribute and store nutrients to meet the demands of growth and reproduction as well as to conserve nutrients in response to stress or conditions unsuitable for growth [1]. Given how critical these mechanisms could be to survival, it is worthwhile to investigate the mechanisms underlying their regulation and the ways in which they contribute to growth and development. Redistribution and storage of nitrogen (N) are particularly significant adaptations due to the fact that many plants are N-limited, requiring more N than any other mineral [2, 3]. Thus, N use efficiency can impact growth and represents an ideal target to elucidate the details associated with nutrient storage and remobilization.

In *Populus*, N storage occurs seasonally and in response to N availability and biotic and abiotic stresses within a growing season [4-6]. A family of related proteins with homology to nucleoside phosphorylases (NP) are involved in both inter- and intra-seasonal N storage in *Populus* [7-9]. These NP-like proteins include three bark storage proteins (BSPs) and proteins termed WIN4 and PNI 288 [9, 10]. BSPs are short day (SD) induced in autumn in phloem parenchyma and xylem ray cells [11-13]. They are also found in shoot apices [9]. WIN4 and PNI 288 are expressed in leaves and shoot apices and expression declines in SD conditions [9, 10, 14]. However, all five genes are expressed following wounding and with increasing N availability [9, 10, 14]. Along these lines, BSP gene expression has also been observed following drought and exposure to methyl jasmonate [6, 15].

The stress responses and SD-induced accumulation of the BSPs, which effectively alter metabolic homeostasis, suggest underlying regulation by changes in

metabolism [16]. Indeed, this is supported by study examining the effect of calcium, phosphorylase inhibitors, ammonium nitrate, glutamine, gibberellic acid and sucrose on BSP expression [17]. Based on this prior research, we can conjecture as to the possible signals and pathways involved in storage and remobilization of N. The goal of this dissertation is to provide a more comprehensive examination of *NP-like* genes to advance our understanding of the signals and pathways involved in N storage and remobilization and how these factors might interact.

The objectives of chapter one were to determine the expression of the complete family of *NP-like* genes under long-day (LD) and short-day (SD) conditions in various tissues, examine the evolutionary relationships of NP-like proteins in the plant kingdom and gain information about gene regulation through comparative promoter sequence analyses. Chapter two is focused on establishing a set of stable qPCR reference genes for normalization of qPCR data in studies of gene expression involving in two genotypes of *Populus* that are extensively used in molecular/genomics research. These reference genes represent a valuable resource for use in qPCR analyses for other *Populus* studies. With the results from these chapters, the third chapter investigated the regulation of *NP-like* genes by carbon (C) and N metabolites using a unique feeding assay with excised *Populus* shoots. Lastly, chapter four examined the role of BSPs in growth and development under LD conditions by assessing changes in growth, photosynthesis and gas exchange in transgenic Poplars with either RNAi knock-down of BSP or over-expressing *BSP A*.

The overarching goal of the work is to illuminate the contribution of storage, N use efficiency and growth conferred by the *NP-like* genes family within *Populus*.

With this knowledge and future research, it may be possible to identify characteristics related to N use efficiency that, in turn, can assist plant breeding and selection efforts.

Chapter 1: Comparative analysis and characterization of vegetative storage protein/nucleoside phosphorylase paralogs in *Populus* and distribution of orthologs in the plant kingdom

Abstract

Nucleoside phosphorylases (NPs) have been extensively investigated in human and bacterial systems for their role in metabolic nucleotide salvaging and links to oncogenesis. In plants, NP-like proteins have not been comprehensively studied, likely because there is no evidence of a metabolic function in nucleoside salvage. However, in the forest trees genus *Populus* a family of NP-like proteins function as an important ecophysiological adaptation for inter- and intra-seasonal nitrogen storage and cycling. We conducted phylogenetic analyses to determine the distribution and evolution of NP-like proteins in plants. Additionally, we conducted an evaluation of NP-like genes in *Populus* by examining the transcript abundance of the 13 *NP-like* genes found in *Populus* genome in various tissues of plants exposed to long-day (LD) and short-day (SD) photoperiods. Furthermore, the organization of *cis*-elements in the promoters of these genes was compared. Phylogenetic analysis of higher plant NP-like proteins revealed two major clades designated Class I and II. Proteins encoded by Class I genes were dominated by species belonging to the order Malpighiales and included the *Populus* Bark Storage Protein (BSP) and WIN4-like

proteins while Class II proteins were encoded by genes across a wide range of plant taxa. Expression analyses in *Populus* revealed that members of both Class I and II *NP-like* gene subfamilies are expressed in various tissues in both LD and SD conditions. Analyses of expression of the *Populus* genes suggest that divergence in gene expression may have occurred recently during *Populus* evolution, which supports the adaptive maintenance models. We also found evidence of divergent natural selection in specific *Bark Storage Protein* (*BSP*) and *WIN4-like* genes. Promoter analyses of the 13 *NP-like* genes revealed common regulatory elements known to be involved in light regulation, stress/pathogenesis, and phytohormone responses. Taken together, our findings suggest that NP-like proteins likely play a central role in N sensing and/or signaling.

Introduction

Nucleotides, nucleotide precursors and derivatives are essential components for life. They are components of nucleic acids, act as signaling molecules, intercellular energy transporters, and can be converted to essential enzymatic cofactors. Nucleotide metabolism is therefore a necessary cellular function [18, 19]. In mammalian and bacterial systems, nucleoside phosphorylases salvage nucleosides by cleaving the glycosidic bond of (deoxy-) ribonucleosides in the presence of inorganic phosphate (Pi) to yield (deoxy-) ribose-1-phosphate and a nucleobase [18, 20]. The free nucleobase can be synthesized into organic molecules or degraded, and the (deoxy-) ribose-1-phosphate can be utilized by the Pentose Phosphate Pathway and glycolysis [20]. The most widely studied NPs are purine nucleoside phosphorylases (PNPs), which are a focus in clinical and cancer research. PNPs have

been investigated for their role in mutation-related immunodeficiency diseases, prostate cancer, leukemia and periodontal disease [21-24].

The ability to salvage purines is particularly important for N-limited plants in the context of retaining and remobilizing nitrogen (N) [25, 26]. It is generally believed that the physiological role of purine degradation in plants promotes nitrogen-use efficiency (NUE) through mobilization from source to sink [25]. However, there is no evidence that nucleoside phosphorylases are involved in purine nucleoside salvage in plants; instead hydrolysis of nucleosides occurs by nucleosidases (EC 3.2.2x) [27-35]. Notably, genes in purine salvage and degradation pathways are induced by wounding, drought, abscisic acid (ABA), dark conditions and dark-induced senescence consistent with a role in NUE [36-38]. Although purine salvage appears to be a component of NUE, the role of NP-like proteins in plants in relation to nucleoside salvaging and NUE is not known.

There is a large body of research on a group of proteins that share sequence similarity with purine nucleoside phosphorylases in *Populus* (reviewed by [5]). In this genus, these proteins have been studied for their eco-physiological role in seasonal and short-term nitrogen storage. Bark Storage Proteins (BSPs) accumulate in bark tissues in response to short day (SD) exposure in autumn. BSP abundance declines when growth resumes in spring [7, 12, 39]. Both BSPs and related Vegetative Storage Proteins (VSPs) accumulated following wounding, high nitrogen and drought stress suggesting a role in short-term storage [4, 6, 9, 10]. Seasonal storage facilitated by BSPs is an important evolutionary adaptation that facilitates perennial growth in low nutrient forest systems while short-term storage helps conserve nutrients in response

to stresses. *Populus* is an ideal model species to study NP-like proteins and genes not only because of their importance in seasonal and short-term storage but also because *Populus* possesses more NP-like proteins than any other known plant genera which was discovered in this investigation.

The expansion and functional evolution of protein and gene families provides innovation for adaptation and speciation [40, 41]. Gene duplication is a mechanism for such innovation and occurs through whole genome duplications (WGD) or small-scale genome duplications such as tandem duplications (TD) [42]. Following duplication, genes can have many fates: duplicates may amplify or buffer original function [43-47], randomly gain a novel function (neofunctionalization) [48, 49], accumulate mutations that subdivide the original function (subfunctionalization) [50, 51] or become non-functional (pseudogenization) [48]. The main models of adaptive maintenance are positive dosage, neofunctionalization, subfunctionalization, diversification of multifunctional genes and, in the case of WGD and TD events, the dosage balance model (reviewed by [52]). Positive or increased dosage describes the retention of duplicate genes that contribute to increased fitness [43, 46, 53, 54]. Similarly, positive dosage may result in buffering or functional redundancy that is likewise beneficial [45]. Neofunctionalization is the gain of new function in the duplicate genes through neutral mutations followed by positive selection while preserving the parent copy [48, 49]. The subfunctionalization model, like neofunctionalization, suggests that duplicate genes accumulate neutral mutations, however, these mutations weaken or alter the original function so that both copies must be maintained to perform the original function [50-52]. Under the diversification

of multifunctional genes model, a multifunctional parent gene is uncoupled among gene duplicates [55, 56].

The dosage balance hypothesis is useful for explaining the retention of genes following the type of duplication event. It posits that duplicate gene retention rates are influenced by the duplicate maintaining the stoichiometric balance of protein complexes, favoring high retention rates for genes and proteins with many interactions [57-60]. As predicted by the gene balance model, experimental support suggests that the retention of certain classes of gene functions differ between WGD and TD, having inverse retention relationships. Gene duplicates retained following WGD tend to be involved in many protein-protein interactions or transcriptional regulation and signaling [61-64]. On the other hand, gene duplicates retained following TD have less protein-to-protein or regulatory interactions, such as genes associated with disease resistance [64-66]. This complimentary retention of gene functions associated either with WGD or TD has been experimentally demonstrated in *Populus* [63, 64, 67, 68]. The limits of this model are that only genes sensitive to dosage imbalance would be retained, hence making duplication of genes that are not involved in large networks unlikely [52]. While this model helps explain potential functions following WGD and TD events it does not explain adaptive functionalization of duplicates.

The aims of this research were to investigate NP-like proteins in *Populus* and the plant kingdom to further our understanding of their functional evolution and the extent to which they may be involved in nutrient salvaging and in particular N cycling and NUE. We first constructed the evolutionary relationships among 13 *NP-like*

genes in *Populus*, examined transcript abundance in four tissue-types under long day (LD) and short day (SD) conditions and compared the structure of promoter regions of these genes to gain insight into gene expression regulation. We further examined the functional divergence of the genes by testing whether any of the genes are under divergent natural selection and when gene expression diverged. Finally, we conducted phylogenetic analyses of NP-like proteins across the plant kingdom. Our analyses of natural selection and the evolution of gene expression patterns were then considered in light of this broader phylogenetic context to draw conclusions about the evolution and possible gene fates of *NP-like* genes and proteins within *Populus*.

Materials and Methods

Plant material

Populus trichocarpa (Torr. and Gray) genotype 'Nisqually-1' cuttings were grown in 2.2 L pots in controlled environmental chambers at 18°C with a PAR range of 310-470 μmol m^{-2} s^{-1}. Plants were fertilized one week after transplanting with 5 g of controlled release fertilizer (18-3-3) (Nutricote, Florikan, Sarasota, FL, USA). Tissue samples were collected after 8 weeks under LD conditions (16 h light/8 h dark) and after 3, 6, 8 and 12 weeks under SD conditions (8 h light/16 h dark). The temperature was lowered to 10°C day/4°C night for the last 4 weeks of SD (i.e. from 8 to 12 weeks SD). Shoot tips or apical buds, young leaves (leaf plastochron index (LPI 3), mature leaves (LPI 8) and bark (between LPI 8-9) were harvested and frozen in liquid N_2 and then stored at -80°C until RNA extraction. One replicate was composed of pooled tissues from three plants. Two replicates were used for gene expression analysis.

Identification and phylogenetic analysis of NP-like genes in the Populus genome

Amino acid sequences of NP-like proteins were retrieved from the *Populus* genome using the Phytozome database (http://www.phytozome.net) by performing a homolog search for the *P. trichocarpa BSP A* (locus name: POPTR_0013s10380).

QPCR primer design and validation

Primers for the NP-like gene family and reference genes were designed with MacVector v10 (MacVector Inc., Cary, NC, USA). All primers were synthesized by Invitrogen (Carlsbad, CA, USA). Optimum annealing temperatures were determined by a temperature gradient and amplification efficiencies calculated from a five-point calibration curve of ten-fold serial dilutions. To confirm a single amplification product, melt curves were performed for all qPCR reactions.

RNA extraction, cDNA synthesis, qPCR detection and statistical analyses

Total RNA was purified using RNeasy plant mini kits and the automated QIAcube (Qiagen, Valencia, CA, USA). Tissues were ground in liquid nitrogen and added to RLT extraction buffer containing 1% polyvinylpyrrolidone, and 1% beta-mercaptoethanol. Samples were vortexed and 0.4 volumes of 5 M potassium acetate, pH 6.5 was added to the sample, mixed and incubated on ice for 15 min. Samples were centrifuged at 15,000 g for 15 min at 4°C. The supernatant was transferred to the QIAcube for RNA extraction with on-column DNase I digestion (Qiagen, Valencia, CA, USA). Microfluidic analyses using the Experion^{TM} automated electrophoresis system and RNA StdSens Chips were used to determined RNA quality and quantity (Bio-Rad, Hercules, CA, USA). First strand cDNA was synthesized in triplicate reactions from 1µg of total RNA using the RevertAid First

Strand cDNA Synthesis Kit (Thermo Scientific Inc., Rockford, IL, USA) and oligo dT primers. CDNA was pooled and used as template for qPCR detection. Triplicate amplification reactions were performed with the iQ5 Real-Time Detection System (Bio-Rad, Hercules, CA, USA) using Maxima SYBR green qPCR master mix (Thermo Scientific Inc., Rockford, IL, USA). Amplification reactions consisted of 10 min at 95°C followed by 40 cycles of 15 sec at 95°C and 1 min at the optimum annealing temperature. Stable reference genes were determined for each tissue type by geNorm^{PLUS} in qbase^{PLUS} version 3 (www.qbaseplus.com). (Additional file 1, Table S1) and relative expression analyses were performed with qbase^{PLUS}. All relative expression graphs are scaled to the average relative expression units of each tissue.

Analysis of 5' promoter structure

Promoter regions 500 bp upstream from the transcription start site (ATG codons) were retrieved from Phytozome (http://www.phytozome.net/) for the *Populus NP-like* genes and these regions were used in searches against the PLACE and PlantCARE databases [69, 70]. Elements were manually mapped to the sequences and assigned to categories based on database designated function and/or references.

NP-like sequence identification and motif prediction

For constructing the evolutionary relationships among NP-like genes across the plant kingdom, we retrieved amino acid sequences from the Phytozome database (http://www.phytozome.net) using a BLAST search using default parameters and the *P. trichocarpa BSP A* (locus name: POPTR_0013s10380) protein sequence for the

query. For species absent in Phytozome, a BLASTP search with default parameters was performed at NCBI using the "non-redundant protein sequence" database (http://www.ncbi.nlm.nih.gov/). Sequences for *Malus* were retrieved from the Rosaceae Database (http://www.rosaceae.org/). For all searches, only hits below an E-value of 10^{-4} were used. Sequences were manually inspected for annotation errors and duplicate sequences were removed. A total of 118 sequences were used in the analysis. To confirm that all plant sequences could be classified as NPs, a batch CD-search of the Conserved Domain Database (CDD) using all 118 plant sequences was performed with default setting and the E-value set to 0.01 with all sequences identified as members of the PNP_UDP_1 superfamily (Pfam:01048) [71]. The conserved region was identified with the Gapped Local Alignment of Motifs (GLAM2) program, version 4.8.1, for all sequences with default parameters and 8,000 iterations (http://meme.nbcr.net/meme/cgi-bin/glam2.cgi) [72]. Predicted motifs were cross-referenced with GLAM2SCAN using the NCBI non-redundant protein database with closest matching motifs in proteins within PNP_UDP_1 superfamily (Pfam:01048). To validate GLAM2 predicted motifs, 57 non-plant sequences from the NP family (COG0775, superfamily cl00303) were retrieved from CDD (http://www.ncbi.nlm.nih.gov/Structure/cdd/cdd.shtml) and motifs were predicted for this set of sequences by GLAM2. We then constructed alignments of the predicted motifs from each sequence set (plant and non-plant) with MUSCLE set to default settings to validate the NP-like region in plants [73]. The validated NP-like motif in plants was used to remove regions outside the NP-like motif sequence and the resulting sequences were used for phylogenetic tree construction.

Phylogenetic analyses and protein family evolution

Phylogenetic hypotheses were constructed for each alignment using both a Bayesian (MrBayes v3.0; [74]) and maximum-likelihood based method (Genetic Algorithm for Rapid Likelihood Inference; GARLI; [75]). We used the WAG+I+G amino acid substitution model, determined by ProtTest [76], for all analyses (including the phylogeny of NP-like proteins in *Populus* with full length amino acid sequences). We present the best topology based on 1000 replicate GARLI analyses on the observed dataset and assessed the statistical support for topological relationships from 1000 bootstrap replicates. All GARLI analyses were performed using the computing resources associated with the LATTICE project [77]. For the Bayesian analyses, we used the default settings (two concurrently running independent analyses of four chains, three of which were heated). We used a threshold of 0.01 for the standard deviation of the split frequencies as a measure of sufficient convergence and mixing. Analyses of the smaller 13-protein dataset and the larger 118-gene dataset were run for 10^6 and 10^7 generations, respectively. In both analyses, 25% of the generations served as burn-in.

Tests of natural selection and gene expression evolution

We tested for recombination in our 13-gene dataset, which can adversely affect analyses of natural selection, using the Genetic Algorithm for Recombination Detection (GARD) method [78] implemented in the package HyPhy [79]. The results from GARD did not identify any statistically significant evidence for recombination breakpoints. Thus, we used the Branch-site REL (Random Effects Likelihood) method [80] implemented in the package HyPhy to determine whether any of the

gene duplicates within the 13 gene dataset showed evidence for being under diversifying selection. We chose this method of detecting selection since our primary interest was to determine which gene duplicates, rather than sites, are under selection. This does not make assumptions as to what branches may be under selection, which can increase the incidence of false positives [80]. In all analyses, the codon sequences were used.

We performed two different tests to assess the evolutionary history of gene expression patterns within each of the four tissue types. First, we estimated the parameter λ under which 0 represents a complete lack of phylogenetic signal and 1 indicates a strong phylogenetic signal (i.e., similarity due to shared ancestry). Second, we tested whether the evolution of gene expression levels has increased, decreased, or been constant over time by estimating the parameter δ; $\delta < 1$ indicates that the evolution of differences in gene expression patterns occurred early in gene divergence and $\delta > 1$ signifies that evolutionary differences occurred relatively recently. To assess the statistical fit of λ and δ models, we performed a likelihood ratio test (LRT) with respect to the results under a Brownian motion model of character evolution. All analyses were conducted using the GEIGER package [81] within R [82].

Results

Protein phylogeny and chromosome location of NP-like gene family in Populus

To better understand the relationship between the NP-like proteins within *Populus* we constructed an evolutionary tree based on full-length protein sequences retrieved from the *Populus* genome through Phytozome (Table 1-1). This tree indicates three subfamilies of clustered proteins with strong support, all posterior

probabilities of one and bootstrap support over 99% (Fig. 1-1). The first subfamily is comprised of BSP A, BSP B and BSP C and is designated as the BSP subfamily. The second cluster of proteins includes WIN4 and WIN4-like proteins and is designated as the WIN4-like subfamily. A third subfamily includes four uncharacterized NP-like proteins that we designated as the NP-like subfamily. PNI 288 clustered within the clade composed of WIN4 and BSP subfamilies, but based on this analysis the protein is distinct from the WIN4 and BSP subfamilies.

Table 1-1. List and description of NP-like genes within Populus trichocarpa used for quantitative gene expression analyses and qPCR primer information.

Gene Symbol	Gene name	Locus name Phytozome v2.2	Primers (5'-3')	Product size (bp)	Annealing temp (°C)	PCR efficiency
			F: TGGAGAGAACTTGTTGGGGAC			
BSP A	Bark Storage Protein A	POPTR_0013s10380	R: CAGAAAACTTCCTTGGGCG	81	55	1.981
			F: ATGTTCTCTCCAAGTGAAGCAC			
BSP B	Bark Storage Protein B	POPTR_0013s10370	R: CGGGCAGGCATTTATCTG	130	57.6	1.981
			F: TTCGTGGTGTTCCAAGGTG			
BSP C	Bark Storage Protein C	POPTR_0013s10350	R: AGGCGTTGTAGGAGGCTAAG	85	54	1.936
			F: AGGATTTTCGCCTGCTGG			
WIN4	Wound Induced 4	POPTR_0423s00200	R: AATGAACTTGGCTGCGGC	64	62.6	2.032
	Poplar nitrogen-		F: TGCCAATAGATTCAATGCCAC			
PNI 288	regulated cDNA 288	POPTR_0019s07690	R: GAAGCCAAAGCAACAGCAG	60	55	1.976
			F: TGAACGGAGAGAACTTGTTGGC			
VSP 87A	WIN-like VSP 87A	POPTR_0013s07850	R: AGGATGTGGTGCTGGGAAGC	105	54	1.912
			F: CAAATGTAGCAGGTGAAGCAAG			
VSP 425	WIN-like VSP 425	POPTR_0013s07810	R: TCAAACGACTCAGAAGCAGATAC	154	54	1.983
			F: TCCAGGATTATCGCCTGCTA			
VSP XIII	WIN-like VSP XIII	POPTR_0013s07800	R: AATCCCATCACTCACAAGCC	110	55	2.040
			F: CCTCCTACAATGCTTTCCTTGCTG			
VSP 840	WIN-like VSP 840	POPTR_0013s07840	R:GCAGATACAAAATCCCATCACTCAC	91	55	1.96
			F: GCTGTAGATGCTTCACTTAGGTTC			
NP 157	NP-like 157	POPTR_0006s16610	R: GCCTTATTCGGTAGTTCCAAC	65	54.4	1.986
			F: TCAAACGGGTATCCTGTGATTGTC			
NP 860	NP-like 860	POPTR_0008s02860	R: TGCTAAGGGTCCAAATGTCTGG	96	61	2.07
			F: AGGGGATGGAACTGGAGAAGTG			
NP 870	NP-like 870	POPTR_0008s02870	R: CCACGAAAATGTCTGCGGTTG	102	60	2.01
			F: GCTACCAGGATACAACTCTCCATTG			
NP 880	NP-like 880	POPTR_0008s02880	R:GCTGAAGAACCCCTAAAGATGTCTC	127	55	1.99

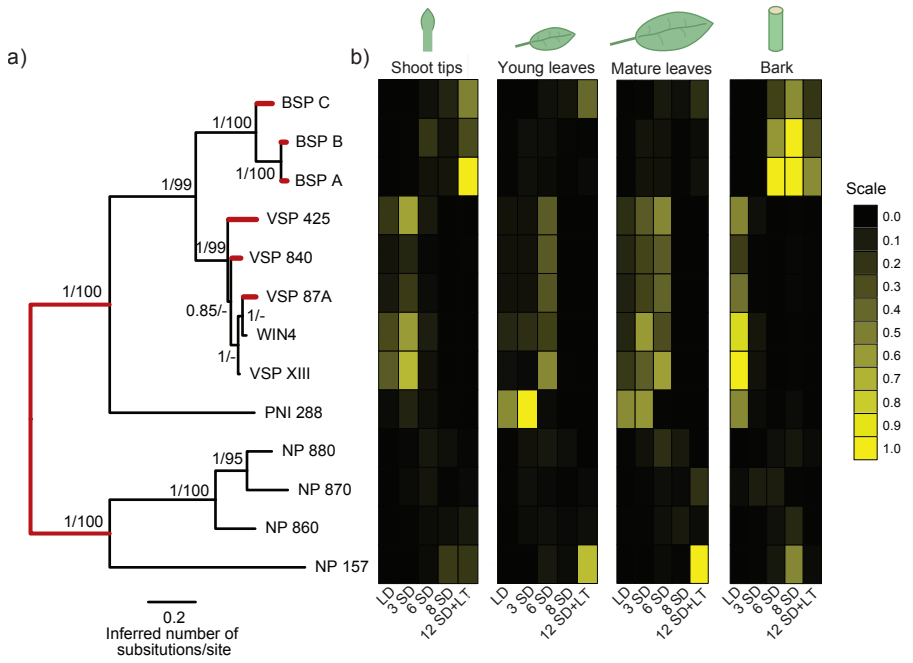

Figure 1-1. NP-like protein family in *Populus trichocarpa* and gene expression. A) Phylogenetic relationship of 13 NP-like proteins in *Populus*. Numbers at branches indicate posterior probabilities and bootstrap percentages based on 1000 replicates, respectively. Branches in blue indicate significant evidence for experiencing episodic diversifying selection based on the branch-site REL test implemented in HyPhy. b) Heat map representing the relative transcript expression of *NP-like* genes in shoot tips, young leaves, mature leaves and bark after 8 weeks long-day (LD) conditions and after 3, 6, 8 and 12 weeks short-day (SD) conditions. Temperatures were lowered between 8 and 12 weeks SD conditions. The 12 week SD treatment was combined with low temperatures for the final 4 weeks of SD (i.e. after 8 weeks SD). Values were rescaled between 0 and 1 with 1 indicating high expression levels.

The chromosome locations of the genes revealed that the *Populus NP-like* genes comprising the three subfamilies reside on four chromosomes (Fig. 1-2). There are two clusters of *NP-like* genes on chromosome XIII that includes one cluster of all the *BSP* subfamily members and a second cluster that includes the *WIN4-like* subfamily gene members. The location of *WIN4* has not been assigned to a

chromosome but is contained on scaffold 423. The *NP-like* subfamily genes are clustered together on chromosome VIII. Only *PNI 288* and *NP 157* were found to not be clustered with other members of the *NP-like* genes with *PNI 288* located on chromosome XIX while *NP 157* is on chromosome VI.

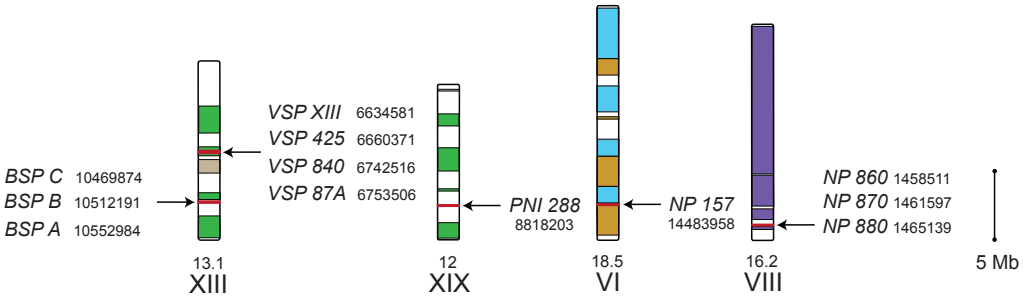

Figure 1-2. Chromosome location of *NP-like* genes in *Populus trichocarpa*. Arrows indicate approximate location of genes. Roman numerals indicate chromosome designation and numbers reflect start site location according to Phytozome (www.phytozome.com). *WIN4* has not been assigned a location. Colored blocks indicate known syntentic regions retrieved from PopGenIE (www.popgenie.org).

Expression patterns within the Populus NP-like gene family

Gene expression analysis of the *NP-like* gene family in *P. trichocarpa* was assessed in four tissue types (shoot tips, young leaves, mature leaves and bark) of plants treated with LD, SD and SD combined with low-temperatures (Table 1-1). Overall, expression of genes within each subfamily was associated with a particular type of tissue and environmental treatment. Expression of all three *BSP*s increased during SD treatment in shoot tips and bark (Fig. 1-1b). Although expression of all three *BSPs* was associated with SD, some differences in expression were observed, notably the induction of *BSP C* in young and mature leaves after 12 weeks SD when the last 4 weeks of SD were combined with low-temperatures. It is interesting to note

that the expression of *BSP A* and *BSP B* were very similar and these two genes are more closely related to each other while *BSP C* is more distant.

In contrast to the *BSP* subfamily, expression of *WIN4-like* subfamily members was greater in LD or during the early stages of SD photoperiod (3 and 6 weeks) treatment for all tissues studied. For all the *WIN-4-like* genes, continued exposure to SD resulted in a decline in the steady state abundance of mRNA. This decline in expression of *WIN4-like* genes in plants treated with SD was most dramatic in bark. These patterns of gene expression show that expression of *BSP* subfamily members are closely associated with SD in perennial tissues such as shoot tips and bark while members of the *WIN4*-subfamily are associated with LD in these same perennial tissues. Furthermore, compared to the *BSP* subfamily the *WIN4*-subfamily members are also expressed to a greater extent in both young and mature leaves. *PNI 288* expression was similar to members of the *WIN4-like* subfamily and was detected in all tissues with expression declining during SD treatment.

Except for *NP 157*, expression of the *NP-like* genes (*NP 880*, *NP 870*, *NP 860*) was observed to occur at lower levels in shoot-tips, young leaves, matures leaves and bark when compared to members of the *BSP* and *WIN4-like* subfamilies. Expression of the *NP-like* subfamily genes also tended to be associated with SD conditions. *NP 157* was expressed to a greater level than other members of the *NP-like* subfamily and expression was associated with SD in all tissues. The greatest levels of expression for *NP 157* were observed in both young and mature leaves after 12 weeks of SD with the last 4 weeks SD combined with low temperature.

In summary, members of each of the three *NP-like* subfamilies (*BSP*, *WIN4-like* and *NP-like*) were observed to have similar expression patterns that were associated with each subfamily. Members of the *BSP* subfamily are expressed in SD and tend to be associated with perennial tissues. Members of the *WIN4-like* subfamily are found in both perennial and deciduous tissues but are repressed by SD. The *NP-like* subfamily is found in both perennial and deciduous tissues but only one member, *NP 157*, appears to show a SD response. Combined with the phylogenetic analysis, our analysis of gene expression demonstrates a correlation between phylogenetically defined *NP-like* subfamilies and gene expression.

Natural selection and continuous character evolution

Based on the Branch-site REL method, we found statistically significant evidence for episodic diversifying selection within *VSP 425*, *VSP 840*, *VSP 87A*, *BSP A*, and *BSP C* (Holm-Bonferroni corrected p-value < 0.05) (Fig. 1-1a and Table 1-2). We also found evidence for diversifying selection at the base of the gene tree corresponding to the initial gene duplication that gave rise to phylogenetic clades referred to as Class I and Class II NP-like proteins (Fig. 1-1a and Table 1-2).

Table 1-2. Results of the tests for episodic diversifying selection among the 13 *NP-like* genes assayed within *Populus trichocarpa.*

Branch	$\overline{\omega}$	ω^-	Pr[ω=ω⁻]	ω^N	Pr[ω=ω^N]	ω^+	Pr[ω=ω⁺]	LRT	*p*-value	Corrected *p*-value
VSP425	0.911	1.0	0.888	1.0	0.009	10000.0	0.102	120.182	0.0	0.0
VSP840	1.078	0.607	0.968	1.0	0.004	72.780	0.028	12.596	0.0	0.004
VSP87A	10.0	0.0	0.748	0.0	0.071	10000.0	0.180	8.721	0.002	0.033
Node3	1.855	0.0	0.653	0.0	0.052	21.384	0.296	8.193	0.002	0.042
BSPA	0.394	0.279	0.996	0.281	0.0	10000.0	0.004	8.079	0.002	0.043
BSPC	0.899	0.0	0.673	0.0	0.236	12.791	0.091	8.013	0.002	0.042
Node2	0.324	0.202	0.934	1.0	0.002	24.413	0.064	5.244	0.011	0.187
BSPB	0.595	0.059	0.959	0.197	0.001	21.161	0.040	3.666	0.028	0.444
NP880	0.155	0.094	0.959	0.841	0.0	593.764	0.041	2.912	0.044	0.660
NP157	0.545	0.0	0.499	0.857	0.0	2.940	0.501	0.754	0.193	1.0
NP860	0.174	0.108	0.855	0.730	0.120	684.793	0.026	0.587	0.222	1.0
Node12	0.446	0.302	0.881	0.650	0.0	3.380	0.119	0.277	0.299	1.0
Node9	10.0	0.0	0.240	0.0	0.111	10000.0	0.649	0.245	0.310	1.0
Node14	0.375	0.150	0.832	0.993	0.0	2.068	0.168	0.186	0.333	1.0
Node5	0.163	0.0	0.404	0.321	0.554	3333.11z	0.042	0.172	0.339	1.0
PNI288	0.540	0.0	0.428	0.999	0.0	1.192	0.572	0.108	0.371	1.0
VSPXIII	0.973	0.986	0.0	0.984	0.0	1.040	1.0	0.001	0.486	1.0
NP870	0.301	0.165	0.867	1.0	0.060	3.224	0.073	-0.380	0.500	1.0
Node7	0.0	0.0	0.885	0.996	0.0	2.106	0.115	-0.001	0.500	1.0
Node1	10.0	0.093	0.971	0.894	0.028	8.630	0.001	-0.001	0.500	1.0
Node21	10.0	0.095	0.009	0.897	0.010	8.819	0.980	-0.001	0.500	1.0
Node17	10.0	0.054	0.370	0.492	0.232	0.293	0.398	0.0	1.0	1.0
WIN4	0.545	0.714	0.0	0.722	1.0	1.578	0.0	0.0	1.0	1.0

Branch = The name of the branch (see tree plot on the main analysis page for the location of automatically named internal branches).

$\overline{\omega}$ = The ω ratio inferred under the MG94xREV model that permits lineage-to-lineage but no site- to-site ω variation.

ω^- = The maximum likelihood estimate (MLE) of the first rate class with $\omega \leq 1$

Pr[ω=ω⁻] = The MLE of the proportion of sites evolving at ω-.

ω^N = The MLE of the second rate class with $\omega \leq 1$

Pr[ω=ω^N] =The MLE of the proportion of sites evolving at ωN.

ω^+ = The MLE of the rate class with unconstrained ω

Pr[ω=ω+] = The MLE of the proportion of sites evolving at ω+.

LRT = Likelihood ratio test statistic for ω+ = 1 (null) versus ω+ unrestricted (alternative)

p-value = The uncorrected *p*-value for the LRT test.

Corrected *p*-value = The *p*-value corrected for multiple testing using the Holm-Bonferroni method

Tests for whether there is a phylogenetic signal, λ, to gene expression patterns were significantly different than the alternative of no signal (i.e., Brownian motion model of character evolution) (Table 1-3). Additionally, the tests of evolutionary differences between the NP-like proteins within *Populus* illustrated that expression divergence was concentrated late in the evolutionary process. Specifically, δ was greater than 1 which is indicative of evolutionary changes occurring recently and that a model raising all branches to the power δ was a better fit than a Brownian motion model (Table 1-3).

Table 1-3. Results of tests for a significant phylogenetic signal of the expression data as measured by Pagel's lambda transformation. A value of 0 represents no signal with a value of 1 signifying complete phylogenetic patterning. *P*-values denoted as *P*-value < 0.05, ** *P*-value < 0.01 and *** *P*-value < 0.001.

Condition	λ_{Bark}	$\lambda_{Shoot\ tips}$	$\lambda_{Mature\ leaves}$	$\lambda_{Young\ leaves}$
LD	0.658	0.414	0.972**	0.974**
SD 3	0.000	0.502	0.845*	0.986***
SD 6	0.901	0.000	0.792*	0.815*
SD 8	1.000*	1.000*	1.000*	0.000
SD 12	0.936*	0.667	1.000*	1.000*

Promoter sequence analyses of the NP gene family in Populus

We identified 139 *cis*-regulatory elements (CREs) in the 13 promoter regions. These CREs were assigned to general functional categories based on database annotations and literature support (Fig. 1-3). For the 13 promoter regions, there were a total of 94 core promoter elements, 269 light responsive elements, 33 low frequency elements, 130 phytohormone responsive elements, 19 storage related elements, 153 stress or defense responsive elements, 27 sugar responsive elements and 219 tissue

specific elements (Appendix A, Fig. A-1). Core promoter elements include TATA and CAAT boxes and poly-A signals.

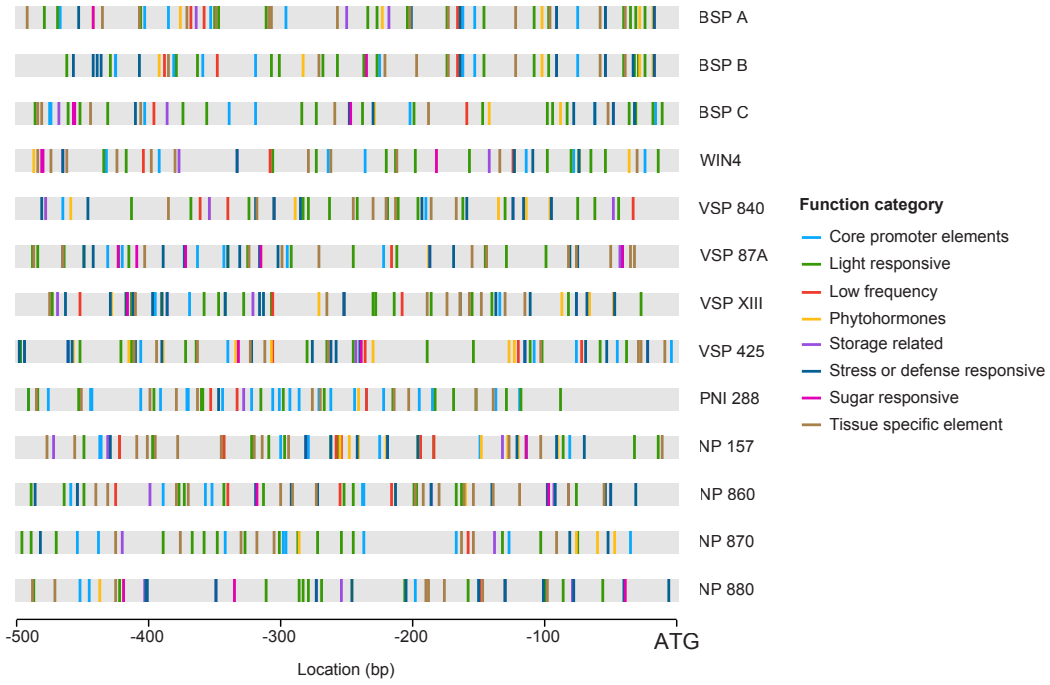

Figure 1-3. *Cis*-regulatory element distribution in the promoter regions of *NP-like* genes in *Populus trichocarpa*.

In each *NP-like* promoter region there was between 12-29 light-responsive elements and between 5-32 stress and defense responsive elements detected. All promoter regions contained between 2-15 ABA responsive elements and 2-15 GA responsive elements. Between 6-23 meristem specific and 1-8 root specific were identified in all promoter regions. Specific elements identified in 10 or more of the *NP-like* promoter regions were: ARR1AT, BIHD1OS, CAATBOX1, CACTFTPPCA1, DOFCOREZM, EBOXBNNAPA, GATABOX, GTGANTG10, MYCCONSENSUSAT, POLLEN1LELAT52, ROOTMOTIFTAPOX1 and TAAAGSTKST1 (Table 1-2).

Table 1-4. *Cis*-regulatory elements identified in the promoter regions of 10 or more *NP-like* genes within *Populus trichocarpa*.

	BSP subfamily				WIN-like subfamily					NP-like subfamily			
Element name	*BSP A*	*BSP B*	*BSP C*	*WIN4*	*VSP 840*	*VSP 87A*	*VSP XII*	*VSP 425*	*PNI 288*	*NP 157*	*NP 860*	*NP 870*	*NP 880*
ARR1AT	•	•	•	•	•	•	•	•			•	•	•
CAATBOX1	•	•	•	•	•	•	•	•	•	•	•	•	•
CACTFTPPCA1	•	•	•		•	•	•	•	•		•	•	•
CARGCW8GAT*	•	•	•		•		•	•		•		•	
DOFCOREZM	•	•	•	•	•	•	•	•	•	•	•	•	•
EBOXBNNAPA	•	•	•	•	•	•	•	•		•	•		•
GATABOX	•	•	•	•	•	•	•	•				•	•
GTGANTG10	•	•		•	•		•	•	•	•	•		•
MYCCONSENSUSAT	•	•	•	•	•	•	•	•		•	•		•
POLLEN1LELAT52	•		•	•	•	•	•		•	•	•		•
ROOTMOTIFTAPOX1	•	•	•	•	•	•	•	•	•	•	•	•	•
TAAAGSTKST1	•	•	•	•	•	•	•	•	•	•			

* Indicates element found in less than 10 promoter regions of *NP-like* genes but found in all *BSP* promoter regions

Phylogenetic distribution in the plant kingdom

To investigate the diversity of plant NP-like proteins, we retrieved 118 complete and non-redundant amino acid sequences across the plant kingdom (Appendix A, Table A-2) with NP sequence homology based on BLASTP searches. We used a gapped alignment motif prediction program to identify a long motif corresponding to the NP-like region for performing phylogenetic analyses (see Materials and Methods). We found that *Populus* has the largest number of NP-like proteins of the genera examined. Phylogenetic analyses based on alignments of the predicted NP-like region for 33 genera revealed 2 general classes of NP-like proteins (Fig. 1-4). Class I and II have strong support, with posterior probabilities of 1.0 as well as bootstrap support of 81%. Class I includes NP-like proteins from all represented genera having complete genome sequences with subclasses that generally cluster according to Rosid and monocot lineages [83, 84]. Class I also includes *Populus* proteins that are not known to be involved in storage (the NP subfamily members).

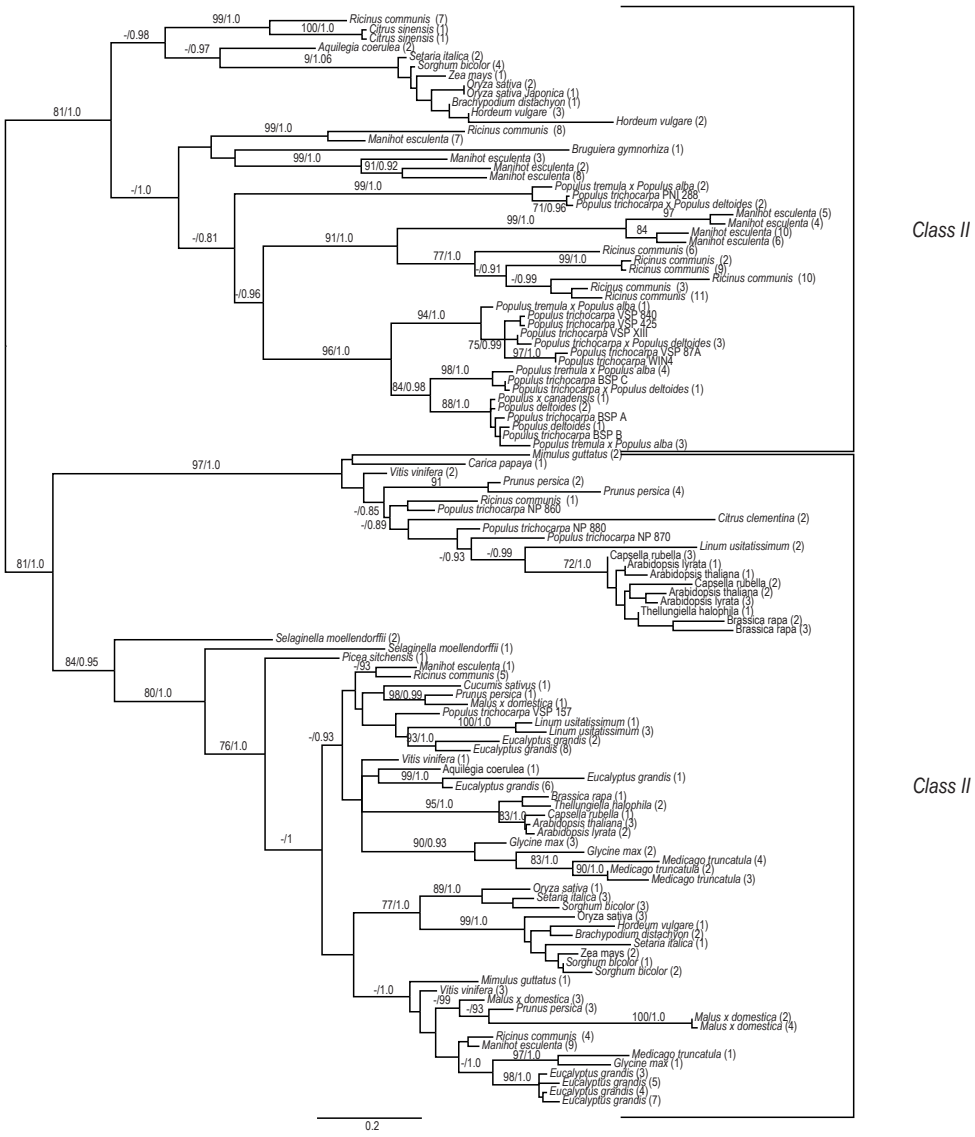

Figure 1-4. Phylogenetic analyses of NP-like proteins in the plant kingdom. Phylogenetic relationships were constructed using Bayesian and maximum-likelihood methods. Numbers at branches indicate posterior probabilities and bootstrap percentages based on 1000 replicates, respectively. Phytozome loci or NCBI sequence identifiers can be found in Table A-2.

Class II is mainly composed of proteins from *Populus*, *Manihot esculenta* and *Ricinus communis.* There is a smaller clade of proteins from monocot genera and 2 citrus proteins. The *Populus* proteins in Class II include the BSPs, WIN4-like proteins and PNI 288 (Fig 1-4).

Discussion

Protein phylogeny and chromosomal distribution of NP-like gene family in Populus

The protein phylogeny of the evolutionary relationships among NP-like proteins across the plant kingdom and the chromosomal distribution indicates that the *NP-like* gene family in *Populus* expanded through whole genome duplication (WGD) and tandem duplication (TD) events. This is consistent with previous studies investigating the evolutionary history of the *Populus* genome [67, 84-86]. A WGD event occurred in *Populus* approximately 65 Mya or earlier, followed by genome wide reorganization that resulted in, among other changes, paralogous sets of chromosomes of which XIII and XIX are a pair (where *PNI 288* and the *BSP*/*WIN4-like* subfamilies reside, respectively) and VI and VIII are a pair (where *NP 157* and the *NP* subfamily reside, respectively) [67, 85, 86]. The members of each gene subfamily are located within a 100 Kb region and are likely the result of TD (Fig. 1-2). This proximity of the genes to each other is generally a good indication of TD [64, 87, 88]. The type of duplication event (i.e. TD or WGD) has implications on gene function: genes families in *Populus* that have expanded through TD are enriched for functions involving defense responses, apoptosis and protein kinases [64].

Promoter analyses of the NP gene family in Populus

Promoter analyses are an important component to investigating the functional evolution of genes particularly in cases where genes are created by TD. This is because TD mainly occurs through unequal recombination, which can result in subfunctionalization if regulatory regions are not duplicated [57, 89, 90]. These analyses also provide information regarding possible regulation and tissue-specific expression—crucial for determining function. The most widely distributed CREs identified in the promoter regions of the *NP-like* gene family have been associated with light, phytohormone and stress and defense responses as well as tissue-specific expression (Table 1-2). The presence of these CREs suggests the possibility of NP-like genes might be associated or involved in light, phytohormone and stress or defense. ARR1AT is the binding site for a transcription factor (TF) involved in cytokinin signaling [91, 92] and salt stress responses [93]. BIHD1OS may be involved in defense responses [94] and seed-specific expression [95]. The CAATBOX1 is a motif frequently found in promoters of eukaryotic genes [96]. It is the target sequence of Nuclear-Factor Y (NF-Y) which interacts with other TFs [97, 98]. The element CACTFTPPCA1 could contribute to mesophyll- and sperm cell-specific expression [99]. DOFCOREZM and TAAGSTKST1 are elements with nearly identical sequences. DOFCOREZM is the target of Dof (DNA-binding with one finger) domain proteins which function in response to light, phytohormone, biotic defense and tissue specific expression [100]. The TAAAG motif is associated with expression in guard cells and rhizome tips [101-103]. The clustering of 3 (A/T)AAAG motifs within 100 bp on the same strand may direct guard-cell specific

expression [102, 104]. This pattern was observed in *BSP A* and *BSP B* promoter regions at positions -39, -58, and -96 bp and observed in the *BSP C* promoter regions at positions -36, -50 and -83 bp.

EBOXBNNAPA and MYCCONSENSUSAT share a motif targeting basic helix-loop-helix (bHLH) TFs that direct cell-specific expression [105] and may be involved in ABA signaling under drought and salt stress [106]. The common GATABOX element is associated with N and light responses [107, 108]. GTGANTG10 may be pollen-specific [109]. The ROOTMOTIFTAPOX1 (5'-ATATT-3') was identified in all promoter regions and could direct root-specific expression [69]. The CARGCW8GAT motif which targets MADS TFs involved in GA metabolism, was found in *BSP* promoters [110, 111]. In all *WIN4-like* promoters, the ABA responsive element ACGTATERD1 was present. This motif is associated with photosynthesis and stress genes and with ABA responses [112, 113]. Additional ABA responsive motifs are located in *WIN4-like* promoters.

Taken together, the large number of CREs involved in light, stress and defense and hormone responses highlight the potential regulation of these genes and gives additional information about the signaling and tissue-specificity of *NP-like* genes. In particular, an important finding is the possible regulatory roles of ABA and GA pathways on *NP-like* genes.

Expression divergence and natural selection analyses

Our evaluation of selection pressure indicates diversifying selection at the initial duplication event and more recent divergent selection of *BSP* and *WIN4-like* subfamily members. This succession of duplication events is consistent with

phylogenetic studies in plants [63, 84, 114-116]. *Populus* specific duplication events have also been evaluated [63, 67, 84-86, 115, 116]. In *Populus* there are reports that correlate tandemly duplicated genes to the expansion of plant defense and stress gene families, consistent with the gene dosage model [64, 65, 68, 87, 117]. Strong patterns between opposing functional gene groups retained following either WGD or TD events have been observed in *Populus* [63, 64]. This fits well with observations of BSPs and VSPs accumulating following wounding and drought stress [6, 9, 10, 14].

Our results show that *BSP* and *WIN4-like* subfamilies gene expression patterns evolved relatively recently and members of these subfamilies are also under selection pressure; this suggests they are undergoing subfunctionalization or diversification of multifunctional genes. This is supported by the expression data showing differential co-expression between the *BSP* and *WIN4-like* gene subfamilies (Fig. 1-1b). Perhaps the data reflect amplification, buffering or "near" subfunctionalization, all of which can facilitate functional redundancy that enables organisms to respond to a greater range of cellular, environmental and/or genetic perturbations [118, 119]. This positive dosage model is particularly applicable to genes involved in stress and environmental responses [43, 45, 46, 53, 54]. Such a strategy would functionally promote optimum nutrient cycling and storage pathways [1, 120]. The amplification model describes the potential functional evolution and fixation of genes from unstable (i.e. tandem) duplications [47]. The high degree of nucleic acid similarity between *BSP* genes (*BSP A* vs. *BSP B*: 98.2%; *BSPA+B* vs. *BSP C* ~94%) also supports the amplification model since sequence divergence is loosely correlated with expression divergence [64].

We qualify our predictions of the functional gene fates, acknowledging that classification is restricted to the current gene duplication models, which are limited by a poor understanding of the role of population size, selection pressure and fixation preceding and following duplication [52, 121, 122]. Research is trending toward developing a more general model of adaptive maintenance duplication [121-123]. Without such information it is particularly difficult to distinguish the subfunctionalization models and the diversification of a multifunctional genes [122]. Another complexity involves multiple types of functionalization occurring over the course of evolution [124].

NP-like gene family expansion order within Populus trichocarpa

Our data suggest that following the split from bacteria and animal lineages NP-like proteins were conserved in higher plants (Fig. 1-4). A hypothetical origin of *NP-like* genes may have occurred as depicted in Fig. 1-5. The *NP-like* parent gene was retained following an ancient WGD event after which the *NP-like* parent gene and the duplicate became the progenitors of Class I and II NP-like proteins, respectively. These genes could have been retained if they had a function involvement in signaling networks or pathways, the dosage balance model. The progenitor of Class II NP-like genes may have undergone a functionalization process that conferred fitness or became advantageous following a change (i.e. environmental) followed by stabilizing selection pressure. Another WGD event occurred, specific to the *Populus* lineage and was followed by tandem duplications where upon the amplification of this function would result in immediate stabilizing selection pressure, the positive dosage model.

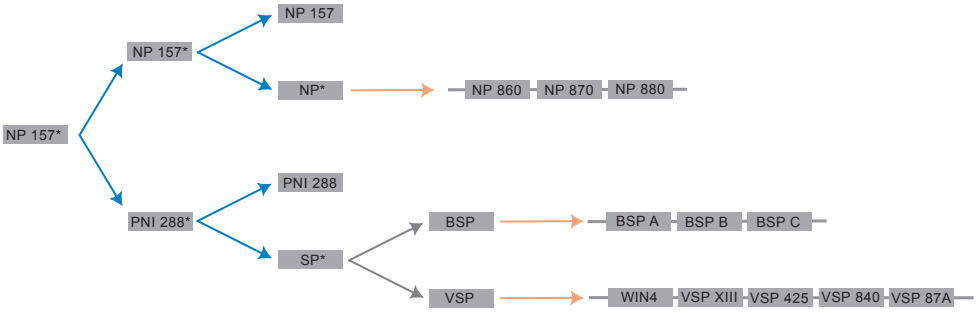

Figure 1-5. Hypothetical origin of the *NP-like* gene family in *Populus trichocarpa*. Blue arrows represent whole genome duplication events and orange arrows represent tandem duplication events. Asterisks denote ancestral genes with unknown sequence similarity to present-day genes.

Our selection and phylogenetic analyses suggest *NP 157* is the likely progenitor gene. Further, the expression patterns of *NP 157* were distinctly different from those of the *BSP* and *WIN4-like* gene subfamilies. It seems unlikely that resources would be directed toward transcription of high levels of *NP 157* transcripts, and presumably the translation of proteins, of a nonfunctional or pseudogene in senescing leaves. Two explanations are that NP 157 is scavenging nucleosides prior to senescence or has retained regulatory elements similar to those of other genes involved in purine salvage and degradation. Increased expression of other genes involved in purine salvage and degradation has been observed under dark-induced senescence conditions [1]. Additionally, knocking-down the enzyme considered to be the key bottleneck in purine degradation, xanthine dehydrogenase, resulted in reduced growth, early senescence and infertility [36, 125]. Yet another explanation could be that NP 157 acts as transient storage protein or is otherwise involved in nutrient signaling. At the very least, *NP 157* as well as *BSP* and *WIN4-like* genes may be responding to changes in nutrient signals induced by SD photoperiod and/or the onset of senescence. This

seems plausible when compared to NP expression in other systems. In the bacteria *Bacillus subtilis*, *PNP* transcription is induced by nucleosides in growth media and repressed by glucose, pointing to modulation of *PNP* by carbon and energy availability [126, 127]. In humans, *PNP*s are up-regulated in diseased and cancerous tissues where metabolic shifts occur [21-23]. While more research is needed to conclusively define the function of NP 157 we posit that NP-like proteins may still be involved in nutrient signaling in plants.

Phylogenic distribution

The phylogeny of NP-like proteins across the plant kingdom reveals that the *P. trichocarpa* subfamily designations are consistent with those found in the tree of the 13 NP-like proteins in *Populus* (Fig. 1-1, 1-4). The BSP and WIN4-like subfamilies and PNI 288 cluster within Class II and the NP-like subfamily belong to the Class I. If NP-like proteins functioned as storage proteins in all plants, we would expect that NP-like proteins from other woody perennial genera would cluster near *Populus* storage proteins yet *Prunus*, *Picea*, *Thuja* and *Malus* NP-like proteins did not cluster near *Populus* storage proteins. Instead, proteins from these genera cluster in Class I near proteins from the NP-like subfamily members in *Populus*. This suggests that NP-like proteins are not storage proteins throughout the plant kingdom.

In addition to the conclusions based on our phylogenetic analyses, the finding that NP-like proteins are not general storage proteins is consistent with previous reports on seasonal storage proteins in other woody plant genera. For example, *Prunus persica* and *Picea sitchensis* are both woody perennials. In the bark of *Prunus persica*, the predominant storage proteins are a 60 kDa dehydrin, a 19 kDa allergen-

related protein and a 16 kDa protein with no known homology [128]. For *Picea*, the predominant storage proteins were 20 and 27 kDa, loosely suggesting that 32 kDa BSPs are not the dominant storage proteins in *Picea* [129].

Our results point to an expansion of the *NP-like* gene family in a common ancestor of the order *Malpighiales* of which *Populus, Manihot, Ricinus* and *Linum* are representatives [83, 84, 130]. While proteins from *Populus, Manihot* and *Ricinus* often clustered together in both Classes, *Linum* has only 3 NP-like proteins that cluster in Class I. We propose that *Linum* lost class II NP-like proteins. The recent assembly of the *Linum* genome revealed that *Linum* has experienced a recent WGD followed by the loss of one or some chromosomes [131]. The many proteins found in *Manihot* and *Ricinus* that cluster near the *Populus* storage proteins suggest a storage function in these genera.

Conclusions

Our investigation into the evolutionary history of NP-like proteins and genes within *Populus* and across the plant kingdom illustrates the importance of both microevolutionary (e.g., natural selection) and macroevolutionary (e.g., genome duplication and tandem duplications) forces in shaping patterns of diversity within this protein family. Of particular note is that we found evidence that hitherto uncharacterized *NP-like* genes might serve a functional role within *Populus* based on expression data; the functional significance of such proteins is unknown. Further, analyses of promoter regions that showed a preponderance of motifs associated with light responses, phytohormone responses (notably ABA and GA), stress and defense and tissue-specificity that also support a functional role for NP-like proteins. We also

found significant evidence for episodic diversifying selection acting on the NP-like proteins within *Populus* and that changes in gene expression levels had occurred relatively recently in the evolution of this gene family. Consistent with the other findings, our inferred phylogeny implicates both historical genome duplication events and more recent taxon specific independent duplication events as mechanism that gave rise to the extant diversity of NP-like proteins within *Populus.* In conclusion, our results are an exciting discovery in plant biology upon which future studies can build and further elucidate the functional significance of NP-like proteins within plants.

The current consensus related to purine nucleoside salvaging in plants suggest that only nucleosidases are involved while purine phosphorylase activity has not be found in plants [27-35]. Although our results did not measure enzyme activity, the expression of the potential purine phosphorylase, NP 157, during leaf senescence is consistent with what would be predicted for nucleoside salvaging. Further research is needed to determine if NP 157 does indeed possess enzymatic activity. If it is determined that NP 157 does possess purine phosphorylase activity, this would be the first demonstration of this enzyme activity in plants and would provide new insight into the nature of nucleoside salvage pathways in plants.

Chapter 2: Evaluation of qPCR reference genes in two genotypes of *Populus* for use in photoperiod and low-temperature studies

Abstract

Quantitative PCR (qPCR) is a widely used technique for gene expression analysis. A common normalization method for accurate qPCR data analysis involves stable reference genes to determine relative gene expression. Despite extensive research in the forest tree species *Populus*, there is not a resource for reference genes that meet the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) standards for qPCR techniques and analysis. Since *Populus* is a woody perennial species, studies of seasonal changes in gene expression are important towards advancing knowledge of this important developmental and physiological trait. The objective of this study was to evaluate reference gene expression stability in various tissues and growth conditions in two important *Populus* genotypes (*P. trichocarpa* "Nisqually 1" and *P. tremula X P. alba* 717 1-B4) following MIQE guidelines. Gene expression stability was evaluated in shoot tips, young leaves, mature leaves and bark tissues from *P. trichocarpa* and *P. tremula. x P. alba* grown under long-day (LD), short-day (SD) or SD plus low-temperatures conditions. Gene expression data were analyzed for stable reference genes among *18S rRNA*, *ACT2*, *CDC2*, *CYC063*, *TIP4-like*, *UBQ7*, *PT1* and *ANT* using two software packages, geNorm^{PLUS} and BestKeeper. GeNorm^{PLUS} ranked *TIP4-like* and *PT1* among the most stable genes in most genotype/tissue combinations while BestKeeper

ranked *CDC2* and *ACT2* among the most stable genes. This is the first comprehensive evaluation of reference genes in two important *Populus* genotypes and the only study in *Populus* that meets MIQE standards. Both analysis programs identified stable reference genes in both genotypes and all tissues grown under different photoperiods. This set of reference genes was found to be suitable for either genotype considered here and may potentially be suitable for other *Populus* species and genotypes. These results provide a valuable resource for the *Populus* research community.

Introduction

Grown for timber, paper and bioenergy, the forest tree genus *Populus* is one of the most widely cultivated tree genera and has become a model for tree research [132]. Within this genus, two genotypes, *P. trichocarpa* and the hybrid *P. tremula x P. alba* are frequently used in molecular and genomic research. *P. trichocarpa* (Torr. and Gray) genotype 'Nisqually-1' has become a vital resource since completion of genome sequence [67] while *P. tremula x P. alba* clone INRA no. 717-1B4 is widely used for molecular biology research because of the ease and efficiency of *in vitro* shoot regeneration and genetic transformation methods [133]. These two genotypes have been extensively used to study seasonal nitrogen cycling and storage, SD associated growth cessation, leaf senescence, bud development and dormancy [14, 17, 134-139]. Identifying stable reference genes in various tissues in plants grown in both SD and LD conditions will help facilitate future research of seasonal traits in *Populus* using qPCR.

Results from qPCR assays and the conclusions based on qPCR data, have been an invaluable source for studying gene expression yet the broad application of

qPCR methods requires standards that promote accuracy, reproducibility and transparency. There has been rapid adoption of a specific set of standards termed the Minimum Information for the Publication of Real-time Quantitative PCR Experiments (MIQE) [140-142]. The MIQE guidelines are a set of ideal practices for qPCR experiments that aim to reduce the publication of inaccurate data that could be interpreted to make incorrect or misleading scientific conclusions. The scope of the guidelines is extensive and includes stipulations for experimental design, sample acquisition, preparation and quality control, reverse transcription and qPCR reactions and data analysis. The guidelines also encompass rules related to nomenclature, particularly using the term quantification cycle (Cq) instead of threshold cycle (Ct) and the term reference genes as opposed to housekeeping genes [140]. Despite the wide acceptance of the need for experimental and publication standards, Gutierrez et al. [143] and Guenin et al. [144] note that plant biology research has been slow to adopt these standards and these guidelines are often ignored in publications.

An important component of the MIQE guidelines is the appropriate analysis of raw fluorescence data to normalize technical variation. A routine method incorporates data from stable reference genes to calculate relative gene expression. Stable reference genes are generally defined as genes with uniform transcript abundance across all samples that is above background fluorescence levels [145]. This is determined by statistical analyses that estimate gene expression stability for a set of candidate reference genes. Data for stable reference genes can then be included in normalization analyses [144]. QPCR validation is crucial for accurate data analysis and involves techniques that test if fluorescence data are a direct measure of gene

expression in experimental samples [140]. This concept is illustrated by PCR amplification efficiencies (E), which are calculated by quantifying the increase of amplified product after each thermocycle in samples with a range of transcript abundance [140, 146]. For example, aberrant product synthesis due to enzymatic inhibitors or secondary structures of the primers may not reflect the actual transcript quantity [146, 147]. PCR efficiency values for each primer pair are included in calculations for stability and relative gene expression analyses [148, 149].

Two reports that fail to conform to the publication standards outlined in the MIQE guidelines have been published evaluating reference genes for qPCR analysis in *Populus* [150, 151]. The first report by Brunner et al. [150], omits the PCR efficiencies for each primer pair as well as the size of the amplification product. This work used ANOVA and linear regression techniques that have been supplanted by the availability of advanced statistical programs that rank reference gene stability [148, 149]. In the second report by Xu et al. [151], all efficiencies are outside of the range of acceptable efficiencies (E = 1.9-2.1), indicative of possible unreliable product amplification that questions the validity of the findings [146, 152]. Besides the technical aspects of these previous studies, both studies also used interspecific hybrids *(P. deltoides x P. nigra* or *P. trichocarpa x P. deltoides*) to conduct the analysis. Because of the lack of a detailed report of qPCR reference genes that conform to MIQE guidelines in poplar we conducted a MIQE compliant examination of reference genes in two poplar genotypes that are extensively used in genomic and transgenic studies, *P. trichocarpa* (Nisqually-1) and *P. tremula x P. alba* clone 717 1-B4.

In this study we report on the gene expression stability of 8 candidate reference genes (*18S rRNA*, *ACT2*, *CDC2*, *CYC063*, *TIP4-like*, *ANT*, *UBQ7*, and *PT1*) in 4 different tissues from plants grown under various photoperiodic conditions. Analyses were performed with the software packages geNorm^{PLUS} and BestKeeper. The results of this study provide a resource for *Populus* researchers and demonstrates the use of MIQE guidelines to the study of poplar gene expression.

Materials and Methods

Plant material

P. trichocarpa (Nisqually-1) plants were grown from cuttings prepared from greenhouse grown plants. *P. tremula x P. alba* clone (717 1-B4) plants were propagated using *in vitro* shoot cultures and rooted plantlets. Plants of both genotypes were grown in 2.2 L pots containing a commercial potting mix (Sunshine LC1) and fertilized with approximately 5 g of the slow release fertilizer (Nutricote, 18-3-3; Florikan, Sarasota, FL, USA). All photoperiod studies were conducted in controlled environment chambers (Conviron Inc., Winnipeg, Manitoba, Canada) at 18°C with a PAR at 50 cm above the surface of pots, ranging from 310–470 μmol m^{-2} s^{-1}.

To study the effect of changing photoperiods, plants were grown for 8 weeks in long-days (LD; 16 h light/8 h dark) followed by short-days (SD; 8 h light/16 h dark) for an additional 12 weeks. During the last 4 weeks in SD, the temperature was lowered to 10°C day/4°C night. Various tissues were collected at 5 time points: 8 weeks LD and after 3, 6, 8 and 12 weeks SD. The tissues included apical shoot tips/buds, bark (between leaf plastochron index 8 and 9 [LPI 8–9]), young leaves (LPI

3) and mature leaves (LPI 9) [153]. Samples were immediately frozen in liquid N_2 and stored at −80°C until used for RNA extraction. Triplicate biological samples were composed of the pooled tissues from 4 individuals (total of 12 plants).

Design and validation of qPCR primers

Primers were designed using MacVector version 11 (MacVector Inc., Cary, NC, USA) based on the following criteria: 18–25 nucleotides in length, GC content of 40-60%, product length ~60-150 bp, and designed to amplify products within 500 bp of the 3' end [147, 152]. Primers were tested for optimum annealing temperature using a temperature gradient and for specificity with a melt curve. PCR amplification efficiencies for all primer pairs were calculated by the iQ5 software (Bio-Rad, Hercules, CA, USA) from a five-point calibration curve of ten-fold serial dilutions. Melt curves were performed for every run to confirm amplification of a single product.

RNA extraction, cDNA synthesis and qPCR detection

Total RNA was extracted using the RNeasy plant mini kit with the automated QIAcube (Qiagen, Valencia, CA, USA). Samples were ground in liquid N_2 with a mortar and pestle. RLT buffer containing 1% beta-mercaptoethanol and 1% polyvinylpyrrolidone was added to 50 mL tubes containing ground tissue and vortexed thoroughly. Following suspension in the modified RLT buffer, 0.4 volumes 5 M potassium acetate, pH 6.5 was added to the buffer, mixed by inverting and incubated for 15 min on ice. Samples were centrifuged for 15 min at 15,000 *g* at 4°C. Supernatant was then loaded into the QIAcube and RNA extraction was performed

with an on-column DNAse I (Qiagen, Valencia, CA, USA) digestion. RNA quality and quantity was assessed with microfluidics using the Experion™ automated electrophoresis system and RNA StdSens chips (Bio-Rad, Hercules, CA, USA). CDNA synthesis reactions were performed with 1 µg of total RNA and oligo dT primers according to manufacturer's instructions (RevertAid, Thermo Scientific Inc., Rockford, IL, USA). Separate reactions were performed for *18S rRNA* using random primers instead of oligo dT primers. The cDNA from triplicate first strand cDNA reactions was pooled and served as the template for triplicate technical qPCR reactions with the Maxima SYBR green qPCR master mix (Thermo Scientific Inc., Rockford, IL, USA) and detected with the iQ5 Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA). Cycling conditions consisted of 10 min at 95°C followed by 40 cycles of 15 sec at 95°C and 1 min at the optimum annealing temperature (Table 1-2).

Statistical analyses

Data from the iQ5 Real-Time Detection System (Bio-Rad, Hercules, CA, USA) were analyzed with geNorm^{PLUS} in qbase^{PLUS} version 3 (http://www.qbaseplus.com) and BestKeeper version 1 (http://gene-quantification.com/bestkeeper.html [1].

Results

Candidate reference genes selection, PCR efficiency and expression profiles

To evaluate candidate reference genes for gene expression studies in *P. trichocarpa* and *P. tremula x P. alba*, qPCR assays were performed on triplicate biological samples from shoot tips, young leaves, mature leaves and bark at 5 time points under long day or short day photoperiods and short day photoperiods supplemented with low-temperatures. Reference genes were selected from existing literature on *Populus* (Table 1-1).

Table 2-1. List and description of candidate reference genes for qPCR. Gene symbol, *Populus* locus name (Phytozome), NCBI *Arabidopsis* ortholog accession number, gene name, function and reference for each gene.

Symbol	Locus name Phytozome v2.2	At ortholog accession no.	Gene name	Function	Reference Literature
TIP4-like	POPTR_0009s09620	NM_119592	TIP4-like	Putative cytoskeletal protein	[15]
CYC063	POPTR_0005s26170	AY652862[a]	Cyclophilin	Peptidylprolyl isomerase, protein folding	[37]
PT1	POPTR_0014s03160	NM_119492	Unknown protein	Unknown function, expressed in pollen tube cells	[15]
CDC2	POPTR_0004s14080	NM_114734	Cell division control protein 2	Cyclin-dependent kinase 2	[38][b]
ACT2	POPTR_0001s31700	AB067722	Actin 2	Formation of filaments, component of cytoskeleton	[38][b]
18S rRNA	Scaffold 17	AY652861[a]	18S ribosomal RNA	Constituent of ribosome	[37, 39]
ANT	POPTR_0014s01260	AY117207	AINTEGUMENTA	Putative ovule development protein	[15][b]
UBQ7	POPTR_0005s09940	NM_129118	Ubiquitin	Protein modification, ubiquitin-dependent protein catabolism	[22]

[a] accession number for *Populus*

[b] primers were not redesigned

PCR efficiencies were calculated from the slopes of standard curves for all primer pairs and were found to be within the acceptable range of E = 1.9-2.1 for both *P. trichocarpa* and *P. tremula x P. alba* (Table 2-2). Comparison of the same primer pairs between each genotype showed that the efficiencies were similar. The largest difference in PCR efficiencies between genotypes was 0.049 (or 4.9%) for *TIP4-like* and the smallest was 0.002 (or 0.2%) for *CYC063*. Expression levels of the candidate reference genes, presented in quantification cycle (Cq) values, showed that transcripts for all reference genes were detected in all samples for all tissues (Fig. 2-1). Cq values are the number of cycles when fluorescence crosses a threshold above background levels [140]. As shown in Fig. 2-1, the mean Cq values of all reference genes clustered together, around 20 cycles, except for *18S rRNA* where very low mean Cq values were observed around 5 cycles, indicating large transcript abundance. Furthermore, the Cq values for *ANT* tended to show greater variance than the other candidate genes, which is particularly evident in young and mature leaves of SD treated plants (Fig. 1-1 C, D, E, F). Shoot tips/buds and bark samples exhibited the least variation in mean Cq values of all genes amongst all the tissues.

Table 2-2. Characteristics of qPCR primers pairs for candidate reference genes. Primer sequences, PCR product sizes, annealing temperatures, PCR amplification efficiencies in *P. trichocarpa* and *P. tremula x P. alba* for each candidate reference gene.

Gene	Primers (5'-3')	Product size (bp)	Annealing temp (°C)	*P. trichocarpa* PCR efficiency	*P. tremula x P. alba* PCR efficiency
TIP4-like	F: GCTGATAATGGGGTGTCG R: CAACTCTAAGCCAGAATCGC	88	57	1.969	2.018
CYC063	F: CCTGGCACTAATGGGTCTCAG R: CACAACTCTTCCGAACACCAC	87	52	1.98	1.978
PT1	F: GCGGAAAGAAAAACTGCAAG R: TGACAGCACAGCCCAATAAG	126	57	2.025	2.083
CDC2	F: ATTCCCCAAGTGGCCTTCTAAG R: TATTCATGCTCCAAAGCACTCC	137	57	2.04	2.035
ACT2	F: TTCTACAAGTGCTTTGATGGTGAGTTC R: CTATTCGATACATAGAAGATCAGAATGTTC	159	52	1.935	1.951
18S rRNA	F: GATTCTATGGGTGGTGGTGC R: CAGGCTGAGGTCTCGTTCG	87	60	1.951	1.965
ANT	F: TCTGTCTGTTATGCCCCTCA R: CCACCTAGGAAGTCCTCCAGT	119	55	2.062	2.033
UBQ7	F: GGAACGGGTTGAGGAGAAAGAAG R: GCAAGAACAAGATGAAGCACAGAGC	135	55	2.028	2.016

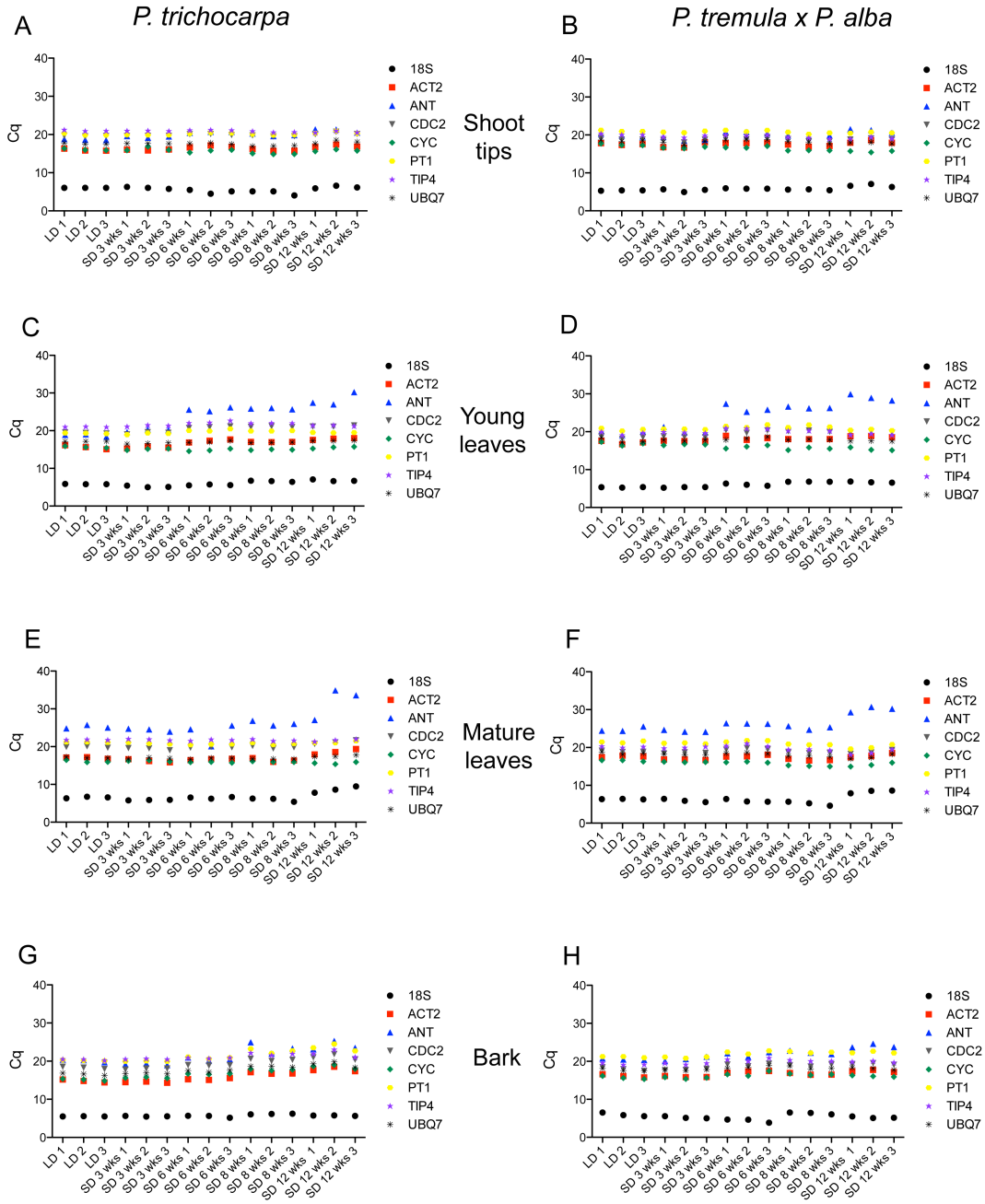

Figure 2-1. Cq distributions for each candidate reference gene.
Expression data for reference genes where each graphed point represents the mean of the technical replicates. Each graph shows the quantification cycle (Cq) distribution for candidate reference genes in shoot tips/buds, young leaves, mature leaves and bark of both genotypes (*P. trichocarpa* and *P. tremula x P. alba*). LD, long day photoperiod; SD, short day photoperiod.

GeNorm^{PLUS} analyses

We determined the expression stability of the candidate reference genes using the geNorm^{PLUS} program within qbase^{PLUS} version 3. In these analyses we assumed that none of the selected genes were co-regulated since this is a prerequisite for geNorm^{PLUS} analysis. GeNorm^{PLUS} calculates the average gene expression stability (M) from the variation of the expression ratios of each pair of reference genes. This is based on the theory that two stable genes should share an identical expression ratio in all samples [149, 154]. Lower M values indicate more stable gene expression with an upper threshold of M = 0.5, above which the reference genes are not considered stable. GeNorm^{PLUS} ranked the candidate reference genes according to their M values, from least stable to most stable (Fig. 2-2) *PT1* was ranked within the top three most stable genes for 7 out of the 8 genotype/tissue combinations and *TIP4-like* was ranked within the top three most stable genes for 5 out of the 8 genotype/tissue combinations. *ANT* and *18S rRNA* were ranked as the least stable genes in 6 out of the 8 genotype/tissue combinations. Ranking profiles differed for the same tissues between the two *Populus* genotypes. Genes were ranked at the same position in only 12 instances when comparing the two genotypes. Compared to other tissues, bark showed the greatest variation in stability ranking between the two genotypes of the reference genes. In contrast, young leaves showed the most similarities with 5 genes ranking at the same position for both genotypes: *PT1* and *TIP4-like* as the most stable and *18S rRNA, CYC063* and *ANT* as the least stable.

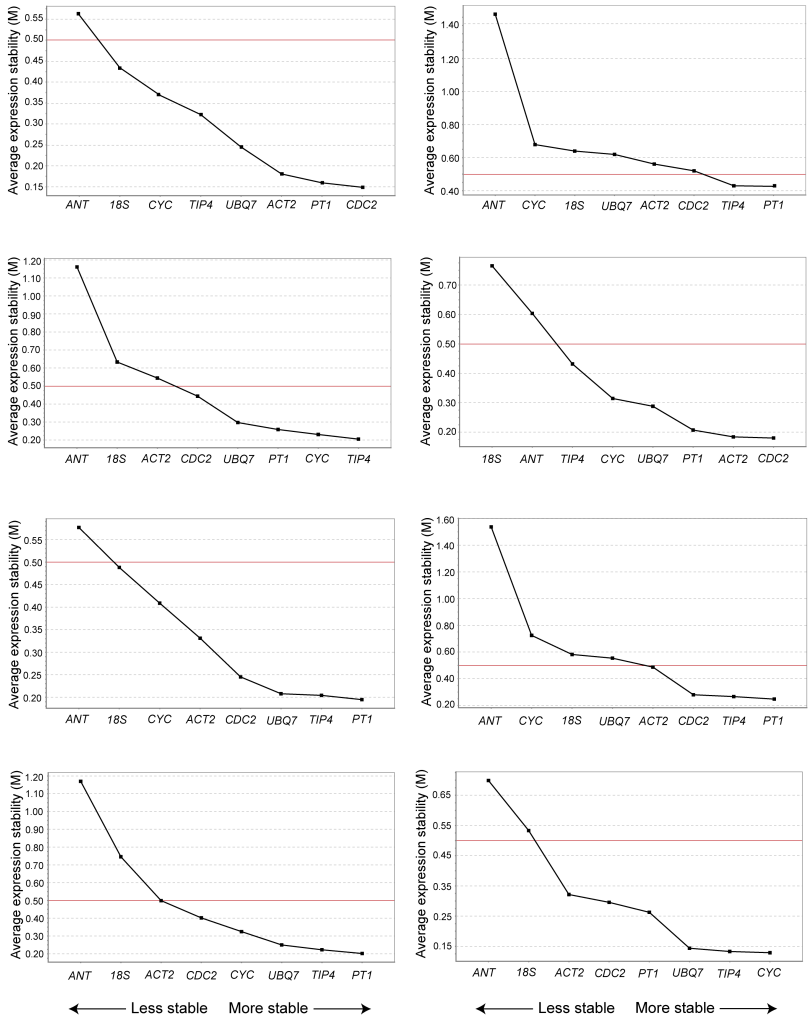

Figure 2-2. Average expression stability values (M) and ranking of candidate reference genes determined by geNorm^{PLUS}.

Candidate reference genes ordered from least stable (left) to most stable (right) in shoot tips/buds, young leaves, mature leaves and bark of both genotypes (*P. trichocarpa* and *P. tremula x P. alba*). The red line indicates the limit above which genes are considered non-stable (M=0.5).

GeNorm^{PLUS} also determines the minimum number of reference genes to include in normalization analysis by calculating the average pairwise variation (V) of normalization factors which is determined by the two most stable genes and the addition of the next most stable gene until all genes have been added [154]. It has a cut-off value of 0.15, below which the addition of another reference gene has no significant effect and is not required. For samples of young leaves from *P. trichocarpa*, pairwise variation analysis showed that normalization should be performed with 3 reference genes since the V2/3 value was higher than 0.15 (Fig. 2-3). For all other tissues, the two most stable reference genes were sufficient to give a V value below 0.15.

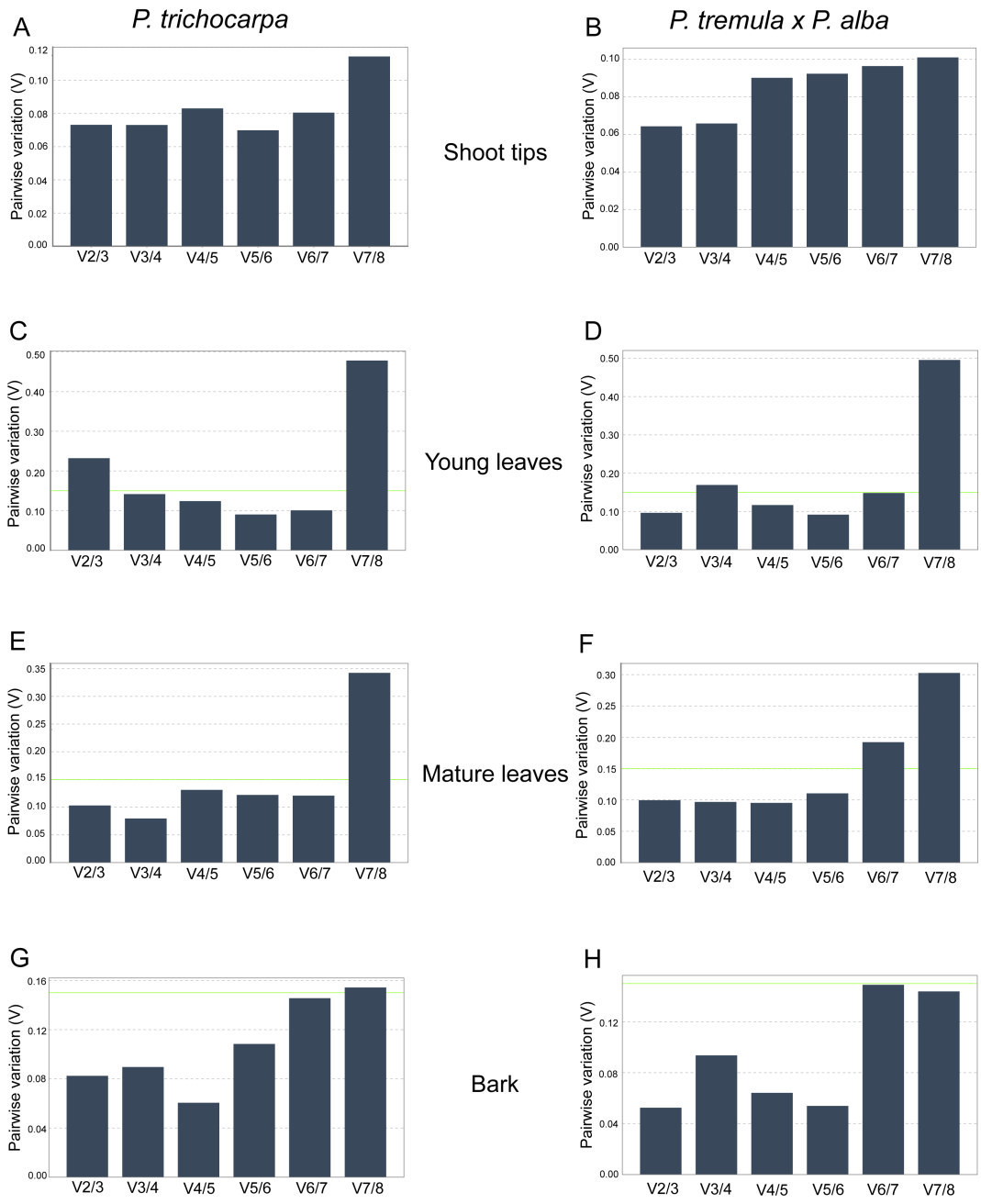

Figure 2-3. Determination of optimal number of reference genes by geNorm^{PLUS}. Pairwise variation (V) analyses were performed to determine the optimum number of reference genes for normalization. V2/3 is the pairwise variation between the 2 most stable genes and the 3 most stable genes. V3/4 compares the 3 most stable genes with the 4 most stable genes, etc. The green line indicates the variation cut-off (V= 0.15) below which additional genes are not required for adequate normalization.

BestKeeper analyses

BestKeeper determines stable expression by first calculating descriptive statistics for each reference gene using the mean Cq data of the technical replicates for each sample. Then, using pairwise correlation analysis, the program compares each reference gene to the *BestKeeper Index* (*BKI*) and calculates a Pearson's correlation coefficient (r) and p-value [148]. Higher correlation coefficients suggest more stable expression. Table 2-3 shows the ranking of reference genes with corresponding (r) and p-values as determined by BestKeeper. *CDC2* was one of the 3 most stable genes in 7 of the 8 genotype/tissue combinations ($r \geq 0.718$, p-value = 0.001). *ACT2* was ranked as one of the 3 most stable genes in 5 of the 8 genotype/tissue combinations ($r \geq 0.862$, p-value = 0.001). Conversely, BestKeeper ranked *TIP4-like* as the least or second least stable gene in 6 out of 8 genotype/tissue combinations with the lowest correlation values of $r = 0.057$ (p-value = 0.837) in mature leaves of *P. tremula x P. alba*. Despite a low ranking in the bark of *P. trichocarpa TIP4-like* expression had a high correlation coefficient and significant p-value ($r = 0.957$, p-value = 0.001) when compared to the *BKI*. Rankings between the different tissues of the two genotypes were very distinct. Pfaffl et al. [148] recommend that if the standard deviation of the mean Cq values for replicates for a reference gene is greater than 1 then the data is considered inconsistent and calculations should be performed again without these genes. We observed standard deviations greater than 1 in all genotype/tissue combinations except shoot tips (Appendix B, Table B-1). These reference genes were removed and the data was

reanalyzed. Removing these genes did not change the overall rankings for the remaining genes but resulted in increased correlation coefficients (*r*) for most of the remaining reference genes (Appendix B, Table B-1).

Table 2-3. Pairwise correlation analyses using BestKeeper.
Each candidate reference gene was compared to the *BestKeeper Index* to calculate a Pearson's coefficient of correlation (*r*) and a *p*-value. Larger correlation coefficients indicate greater correlation with the index and are evidence of higher gene expression stability.

Populus trichocarpa

Shoot tips/buds	Coeff. of corr. (*r*)	*p*-value	Young leaves	Coeff. of corr. (*r*)	*p*-value	Mature leaves	Coeff. of corr. (*r*)	*p*-value	Bark	Coeff. of corr. (*r*)	*p*-value
UBQ7	0.892	0.001	*CDC2*	0.970	0.001	*18S rRNA*	0.964	0.001	*CDC2*	0.993	0.001
18S rRNA	0.810	0.001	*ANT*	0.963	0.001	*ACT2*	0.962	0.001	*PT1*	0.993	0.001
CDC2	0.718	0.003	*ACT2*	0.961	0.001	*CDC2*	0.943	0.001	*ACT2*	0.983	0.001
PT1	0.660	0.007	*18S rRNA*	0.799	0.001	*ANT*	0.917	0.001	*UBQ7*	0.980	0.001
ACT2	0.655	0.008	*PT1*	0.610	0.016	*UBQ7*	0.845	0.001	*ANT*	0.970	0.001
CYC063	0.639	0.010	*UBQ7*	0.532	0.041	*PT1*	0.663	0.007	*CYC063*	0.969	0.001
ANT	0.360	0.188	*TIP4-like*	0.517	0.048	*TIP4-like*	0.088	0.754	*TIP4-like*	0.957	0.001
TIP4-like	0.223	0.427	*CYC063*	-0.008	0.977	*CYC063*	-0.477	0.072	*18S rRNA*	0.650	0.009

Populus tremula x Populus alba

Shoot tips/buds	Coeff. of corr. (*r*)	*p*-value	Young leaves	Coeff. of corr. (*r*)	*p*-value	Mature leaves	Coeff. of corr. (*r*)	*p*-value	Bark	Coeff. of corr. (*r*)	*p*-value
CDC2	0.905	0.001	*CDC2*	0.977	0.001	*ACT2*	0.894	0.001	*PT1*	0.812	0.001
ACT2	0.862	0.001	*ANT*	0.944	0.001	*18S rRNA*	0.816	0.001	*CDC2*	0.793	0.001
ANT	0.788	0.001	*18S rRNA*	0.886	0.001	*ANT*	0.653	0.008	*ANT*	0.738	0.002
18S rRNA	0.659	0.008	*ACT2*	0.881	0.001	*CDC2*	0.569	0.027	*ACT2*	0.650	0.009
UBQ7	0.651	0.009	*PT1*	0.562	0.029	*CYC063*	0.420	0.119	*CYC063*	0.647	0.009
PT1	0.562	0.029	*TIP4-like*	0.416	0.123	*UBQ7*	0.323	0.240	*UBQ7*	0.644	0.009
TIP4-like	0.457	0.087	*UBQ7*	0.358	0.191	*TIP4-like*	0.057	0.837	*TIP4-like*	0.633	0.011
CYC063	0.129	0.646	*CYC063*	-0.717	0.003	*PT1*	0.050	0.860	*18S rRNA*	0.389	0.152

GeNorm^{PLUS} versus BestKeeper

In comparing candidate reference gene stability rankings produced by geNorm^{PLUS} and BestKeeper, we found that these two programs ranked the reference genes differently (Table 2-4). For instance, BestKeeper frequently assigned *ANT* a middle ranking and even ranked it as the second most stable gene in young leaves of both genotypes. On the other hand, geNorm^{PLUS} consistently ranked *ANT* as the least or second least stable gene in all tissues. The rankings of *18S rRNA* by geNorm^{PLUS} and BestKeeper also showed differences. *18S rRNA* ranked as the least or second least stable gene when analyzed by geNorm^{PLUS} while BestKeeper assigned *18S rRNA* a high or middle ranking except for bark tissues where it was ranked as one of the least stable genes. There were only 4 occurrences in *P. trichocarpa* where the two programs gave the same ranking for a gene: *CYC063* in shoot tips and *CDC2*, *UBQ7* and *18S rRNA* in bark. In all tissues of *P. tremula x P. alba* and in mature leaves of *P. trichocarpa*, the genes recommended by geNorm^{PLUS} for normalization calculations were ranked amongst the 4 least stable genes by BestKeeper.

Table 2-4. Comparison of stability rankings between geNorm^{PLUS} and BestKeeper.
Candidate reference genes ordered from most stable (top) to least stable (bottom) for each tissue by geNorm^{PLUS} and BestKeeper in *P. trichocarpa* and *P. tremula x P. alba*. Reference genes identified by geNorm^{PLUS} for inclusion in normalization calculations are indicated by * symbol.

Populus trichocarpa

Shoot tips/buds		**Young leaves**		**Mature leaves**		**Bark**	
geNorm^{PLUS}	BestKeeper	geNorm^{PLUS}	BestKeeper	geNorm^{PLUS}	BestKeeper	geNorm^{PLUS}	BestKeeper
*CDC2**	*UBQ7*	*PT1**	*CDC2*	*TIP4-like**	*18S rRNA*	*CDC2**	*CDC2*
*PT1**	*18S rRNA*	*TIP4-like**	*ANT*	*CYC063**	*ACT2*	*ACT2**	*PT1*
ACT2	*CDC2*	*CDC2**	*ACT2*	*PT1*	*CDC2*	*PT1*	*ACT2*
UBQ7	*PT1*	*ACT2*	*18S rRNA*	*UBQ7*	*ANT*	*UBQ7*	*UBQ7*
TIP4-like	*ACT2*	*UBQ7*	*PT1*	*CDC2*	*UBQ7*	*CYC063*	*ANT*
CYC063	*CYC063*	*18S rRNA*	*UBQ7*	*ACT2*	*PT1*	*TIP4-like*	*CYC063*
18S rRNA	*ANT*	*CYC063*	*TIP4-like*	*18S rRNA*	*TIP4-like*	*ANT*	*TIP4-like*
ANT	*TIP4-like*	*ANT*	*CYC063*	*ANT*	*CYC063*	*18S rRNA*	*18S rRNA*

Populus tremula x Populus alba

Shoot tips/buds		**Young leaves**		**Mature leaves**		**Bark**	
geNorm^{PLUS}	BestKeeper	geNorm^{PLUS}	BestKeeper	geNorm^{PLUS}	BestKeeper	geNorm^{PLUS}	BestKeeper
*PT1**	*CDC2*	*PT1**	*CDC2*	*PT1**	*ACT2*	*CYC063**	*PT1*
*TIP4-like**	*ACT2*	*TIP4-like**	*ANT*	*TIP4-like**	*18S rRNA*	*TIP4-like**	*CDC2*
UBQ7	*ANT*	*UBQ7*	*18S rRNA*	*UBQ7*	*ANT*	*UBQ7*	*ANT*
CDC2	*18S rRNA*	*CDC2*	*ACT2*	*CYC063*	*CDC2*	*PT1*	*ACT2*
ACT2	*UBQ7*	*ACT2*	*PT1*	*CDC2*	*CYC063*	*CDC2*	*CYC063*
CYC063	*PT1*	*18S rRNA*	*TIP4-like*	*ACT2*	*UBQ7*	*ACT2*	*UBQ7*
18S rRNA	*TIP4-like*	*CYC063*	*UBQ7*	*18S rRNA*	*TIP4-like*	*18S rRNA*	*TIP4-like*
ANT	*CYC063*	*ANT*	*CYC063*	*ANT*	*PT1*	*ANT*	*18S rRNA*

Discussion

Recent reports have questioned the validity of selecting reference genes for qPCR analysis of gene expression based on results from other species or different experimental regimes [144, 155-158]. In this report we undertook a stability analysis of 8 reference genes expressed in various tissues of two genotypes of *Populus* grown in LD and SD conditions. The stability of the reference genes was then determined using two different programs: geNorm^{PLUS} and BestKeeper. In addition to these two programs, NormFinder is another program that measures reference gene expression stability [159]. Together, these are the three widely cited programs used for stability analysis. GeNorm^{PLUS} has been cited over 4,000 times, followed by NormFinder with over 650 citations and BestKeeper with over 500 citations (determined by Google Scholar search). In contrast to geNorm^{PLUS} and BestKeeper, NormFinder requires defining two or more groups of samples composed of at least eight samples per group for accurate analysis [159]. Since our experimental design did not meet these requirements NormFinder was not included in this study.

Irrespective of the analysis program used to determine reference gene stability, the most stable reference genes vary among tissues of both genotypes. Besides variation in gene expression stability between tissues within a genotype, it was also found that reference gene stability also varies between genotypes within a given tissue. For example, in shoot tips/buds *CDC2* was ranked by geNorm^{PLUS} as the most stable reference gene in *P. trichocarpa* but ranked as the fourth most stable reference gene in *P. tremula x P. alba* (Table 2-4). BestKeeper ranked *UBQ*7 as the most stable reference gene in shoot tips/buds in *P. trichocarpa* and as the fifth most

stable gene in *P. tremula x P. alba*. This difference of ranking in the same tissues of the two genotypes occurred regardless of the program used. Although, geNorm^{PLUS} rankings between genotypes of the least stable reference genes were more consistent than rankings of the most stable genes. Previous studies on coffee and petunia [160, 161] also concluded that reference genes were different in different tissues for a single genotype and also for the same tissue between different genotypes. This variation in reference gene stability underscores the importance of empirically testing all samples in an experiment to validate reference gene stability.

This report is a rigorous evaluation of reference gene stability in *Populus* and a valuable resource when compared to previous reports in *Populus* [150, 151]. Beside our adherence to the MIQE guidelines, there are additional distinctions between this report and previous reports. Brunner et al. [150] determined reference gene stability using ANOVA and linear regression analyses while we used currently available methods capable of more refined statistics. An additional difference between the current study and Brunner et al. [150] is that primers used in their study were designed from a limited number of ESTs, whereas we designed primers using sequences from the *Populus* genome, which is a more complete resource. Xu et al. [151] used the same programs we used to evaluate stable reference genes in bark and root tissues during adventitious root formation. However, the reported amplification efficiencies were outside the range suggested by the MIQE guidelines making it difficult to determine the accuracy of their stability rankings. Finally, in this report we performed reference gene evaluations using the two important *Populus* genotypes, *P. trichocarpa* "Nisqually 1" and *P. tremula* x *P. alba* 717 1-B4.

Consistent with prior reports, our results found that stability rankings were not consistent amongst geNorm^{PLUS} and BestKeeper programs [151, 162, 163]. These discrepancies are a consequence of the different statistical methods that the programs are based. BestKeeper performs pairwise correlation analysis using Cq values compared to an index value while geNorm^{PLUS} calculates the ratio of variation between pairs of reference genes. *ANT* is a good example of the differences between stability rankings. While *ANT* is not generally considered to be a reference gene, it was included in this study as a gene with documented variable expression in cambium [143]. The mean Cq distributions of *ANT* clearly confirm expression in all genotype/tissue combinations we studied making *ANT* a suitable candidate reference gene to test. The mean Cq distributions show that *ANT* expression is variable (Fig. 2-1). Consistent with the report of variable *ANT* expression, geNorm^{PLUS} ranked *ANT* as the overall least stable gene in all genotype/tissue combinations. Yet BestKeeper assigned, in most cases, a high rank to *ANT.* Although geNorm^{PLUS} ranked *ANT* as one of the least stable reference genes in both genotypes and range of tissues, there may be unique conditions in which *ANT* could be used as a reference gene. For example, the Cq distributions in bark in *P. trichocarpa* indicate that *ANT* appears stable in samples up to 6 weeks of SD exposure (Fig. 2-1, G). This could account for the high correlation coefficients of *ANT* ($r = 0.970$, p-value = 0.001) in this tissue type as calculated by BestKeeper (Table 2-3). Additionally, geNorm^{PLUS} generally ranked the expression of *18S rRNA* as unstable in all genotype/tissue combinations while BestKeeper tended to rank this gene unstable in bark and more stable in the other tissues. The graphs of Cq distributions show that Cq values for *18S rRNA* do not

appear to be as stable compared to the other reference genes (Fig. 2-1) and the Cq distributions more closely agree with the assigned rankings by geNorm^{PLUS} than by BestKeeper. This calls attention to the importance of reviewing the Cq distributions in conjunction with the ranking profiles by expression stability programs for confirmation of stability. Regardless of its stability, inclusion of *18S rRNA* as a reference gene for qPCR assays requires cDNA synthesized with random primers instead of oligo dT primers. It is common to synthesize cDNA with oligo dT primers to limit sample complexity when investigating differential expression by qPCR. Therefore, omitting *18S rRNA* as a reference gene would allow a researcher to maintain a low sample complexity when synthesizing cDNA. For those reasons we do not recommend *18S rRNA*.

The purpose of this study was not to provide specific reference gene recommendations but to offer a set of rigorously tested reference genes that are potentially suitable as reference genes for expression analyses in *Populus*. Testing the PCR efficiencies of primer pairs in both genotypes revealed that PCR efficiencies were similar although not identical yet within the acceptable range. It is probable that these primers may also be suitable for use in other *Populus* species provided that adequate PCR efficiencies are validated [164].

Researchers should carefully choose a gene stability analysis program that fits their experimental needs. Each program has limitations and specific requirements for analyses. For example, NormFinder requires at least 2 groups of 8 or more samples for accurate analyses [159]. This is significant because it can be difficult to define logical groups that comprise an adequate number of samples within a group. There

are reports in which samples are grouped in multiple ways, which affected the calculations and rankings [165, 166]. Results from BestKeeper can be difficult to interpret, as illustrated in this paper. High correlation coefficients and significant *p*-values can be calculated even for unstable reference genes. When considering geNorm^{PLUS}, researchers should take into account that the program currently does not perform analyses for a reference gene if the Cq data were collected from more than one plate, which may be impractical for large studies. Therefore, the choice of analysis program must be appropriate for the experimental design.

The importance of using multiple reference genes for normalization analyses has long been established and including multiple reference genes for normalization is a component of MIQE guidelines [140, 154]. One of the unique features of geNorm^{PLUS} is the ability to calculate the minimum number of reference genes to include in normalization analyses. In this study, analysis with geNorm^{PLUS} indicates that the 2 most stable reference genes were adequate for normalization analyses except for one case where 3 reference genes were recommended. This offers an advantage in accurate normalization calculations compared to analysis with NormFinder or BestKeeper. If using these programs, including 3 or more stable reference genes is suggested as a "universally applicable method" [145]. In this study, geNorm^{PLUS} is the program that best fits our experimental needs. It differentiates between biological and technical replicates and calculates the best number of reference genes needed for normalization. More practically, it is the most user-friendly program with clear indications of the most stable reference genes as well as integrated alerts that inform users of data errors or omissions.

Conclusions

In this study it was possible to identify stable reference genes that can be employed to investigate changes in differential gene expression in *Populus* under controlled environments including LD, SD and SD with low temperatures. Rigorous testing of candidate reference genes can be time and energy intensive but it is crucial to obtaining valuable scientific conclusions. Here we provide a set of established reference genes for which we tested the normalization potential in a study of their expression stability in two poplar genotypes. We also conclude that geNorm^{PLUS} is the most useful program to determine the stability of reference genes. It calculates stability based on rigorous statistical methods, and integrates calculations to determine the appropriate number of reference genes for normalization and it is user-friendly. This report emphasizes the importance of the MIQE recommendations and promotes the continued adoption of the recommendations by researchers studying *Populus.*

Chapter 3: Carbon and nitrogen regulation of the nucleoside phosphorylase-like vegetative storage protein gene family in *Populus trichocarpa*

Abstract

Vegetative storage proteins (VSPs) in *Populus* function in seasonal and short-term N storage and, thus, represent an important adaptive mechanism. These proteins share homology with nucleoside phosphorylases and can be described as nucleoside phosphorylase-like proteins (NP-like). Despite their significance in N storage and cycling little is known about the metabolic regulation of these genes. In theory, growth, nutrient remobilization and storage should be tightly regulated based upon the availability of nutrients within a plant or organ. The availability and relative amounts of carbon (C) and nitrogen (N), known as the C/N balance, can regulate plant growth and metabolism. This research investigates the effect of feeding C and N metabolites on *NP-like* gene expression in shoot tips and bark tissue of *Populus trichocarpa*. The objective was to provide insight into the regulatory and signaling networks involved in nutrient storage and remobilization by feeding *Populus* shoots solutions containing sucrose, amino acids, tricarboxylic acid cycle (TCA) intermediates, gamma-aminobutryic acid (GABA) and N compounds. Data show that *NP-like* gene expression is induced by metabolites of primary N metabolism and amino acids. Additionally, this study establishes a potential role for GABA in nutrient remobilization and storage. These results offer greater insight into the processes governing remobilization and storage.

Introduction

Nutrient remobilization and storage are fundamental adaptations that contribute to plant growth [1, 120]. From an eco-physiological perspective, this adaptation contributes to a plant's ability to respond to developmental requirements, environmental changes and stresses by uncoupling supply from demand [5, 167]. Ultimately, these processes are regulated by the availability of carbon (C) and nitrogen (N) and their relative amounts (i.e., C/N balance), which regulate growth, metabolism and cellular function [168-172]. C and N signals such as inorganic nitrate and ammonium, N-rich amino acids, CO_2 and carbohydrates induce changes in gene expression and enzyme activity that coordinate the metabolic processes of N assimilation, photosynthesis, photorespiration and respiration [172-175]. The overall effect of C/N balance on metabolism can, for example, dynamically influence growth in the form of altered seedling size and fresh weight [169], growth rate [176], root architecture [177] and flowering [178, 179]. Despite advances in the basic understanding of C/N balance, the regulatory pathways between C and N metabolism, remobilization and storage are unclear.

Populus is a model system appropriate to examine remobilization and storage given the extensive research investigating short-term and seasonal nutrient storage [5]. The majority of this research involves the subfamily of nucleoside phosphorylase-like (NP-like) proteins termed bark storage proteins (BSPs) which are known to accumulate seasonally in the bark tissues of *Populus* trees following the transition to short-day (SD) conditions in autumn [7, 11, 180]. In spring, BSPs are catabolized to support re-growth [7, 180, 181]. During active growth, BSPs and

additional members of the NP-like protein family, WIN4 and PNI 288, act as short-term storage proteins in response to N availability [4, 9, 10]. NP-like proteins accumulate following stresses, such as mechanical wounding and drought [4, 6, 10, 14]. Exposure to methyl jasmonate can also induce accumulation of these storage proteins likely due to the metabolic shifts in response to, or caused by, stress [15, 16]. A major distinction between the *BSP* and *WIN4* is that transcripts are expressed in different tissues. Gene expression of *WIN4* and *PNI 288* is found in young leaves and shoot apices (i.e., shoot tips) while *BSP* genes are expressed in bark and shoot apices [4, 9, 10]. Therefore, shoot apices are an ideal tissue-type to examine regulatory differences among the family of *NP-like* genes while bark tissues are an ideal choice to investigate *BSP* expression.

There is preliminary evidence that *BSP* and *WIN4* gene expression are regulated by N and may be regulated by the C/N balance within a tissue. *Populus* trees fertilized with increasing concentrations of ammonium nitrate (NH_4NO_3) accumulated mRNA transcripts of both *BSP* and *WIN4* genes proportional to the amount of N supplied [4, 9, 10]. Additionally, organic N in the form of glutamine (Gln) activated the promoter of *BSP A* in bark tissues of shoots incubated in Gln solutions [17]. Along these lines, promoter deletions of *BSP A,* identified an N-responsive region [134]. In the case of C, there is little evidence that BSPs are C-responsive, illustrated by feeding shoots with sucrose (Suc) which did not activate the *BSPA* promoter [17].

To gain a better understanding of the metabolic regulatory and signaling mechanism governing storage proteins in *Populus trichocarpa*, I focused on a critical

junction between C and N pathways involving the tricarboxylic acid (TCA) cycle and N assimilation where C-rich organic acids and inorganic ammonium are converted to amino acids [182, 183]. The TCA cycle is an important metabolic cycle that generates energy containing metabolites by oxidizing respiratory substrates, supplies C skeletons for amino acid biosynthesis and converts organic acids from processes such as glycolysis, amino acid catabolism and lipid breakdown [184, 185]. Gamma-aminobutyric acid (GABA) made from amino acids can also contribute C to the TCA cycle [186-188]. To investigate metabolic regulation, I incubated shoots in solutions containing amino acids, TCA intermediates, N sources and GABA and determined that *NP-like* gene expression was associated with these metabolites and their pathways, using qPCR.

Materials and Methods

Plant material and excised shoot assays

The excised shoot assays were designed to test the effect of metabolites and chemical compounds in *Populus* shoots [17]. Shoots between 25 and 40 cm in length were excised from greenhouse grown *Populus trichocarpa* (Nisqually-1) stock plants grown under long-day conditions (LD; 16 h light/8 h dark). The basal ends of the excised shoots were pre-incubated in water for 24 h to reduce confounding wound responses. Following the water pre-incubation, excised shoots were then transferred to solutions containing metabolites as described below for each assay. All incubations were performed in environmental chambers (Conviron Inc., Winnipeg, Manitoba, Canada) under LD conditions at 18°C with approximately 400 μmol m^{-2} s^{-1} light. Shoot tips and bark tissue from shoots between leaf plastochron index (LPI) 5-8, were

collected after incubation in the respective metabolite solution after 24 h or 48 h, flash frozen in liquid N_2 and stored at -80°C until extraction [1]. Data represent two replicates, each composed of tissues from three shoots.

QPCR primer design and validation

Primers were designed for qPCR detection of *NP-like* genes using MacVector v10 (MacVector Inc., Cary, NC, USA) and synthesized by Invitrogen (Invitrogen Corporation, Carlsbad, CA, USA). Optimum annealing temperatures were determined using a temperature gradient. PCR amplification efficiencies for all primer pairs were calculated from a five-point calibration curve of ten-fold serial dilutions. To confirm a single amplification product, melt curves were performed on all reactions.

RNA extraction and qPCR analysis

RNA was extracted using the Plant RNeasy Mini Kit (Qiagen, Valencia, CA, USA) with minor modifications. The RLT extraction buffer was augmented with 1% polyvinylpyrrolidone (PVP), and 1% beta-mercaptoethanol. Following the addition of ground plant tissue to the buffer, 0.4 volumes of 5 M potassium acetate (pH 6.5) were added. Samples were incubated on ice for 15 min and then centrifuged at 15,0000 *g* for 15 mins at 4°C before extraction using the QIAcube (Qiagen, Valencia, CA, USA). The procedure was performed with on-column DNase digestions with DNase I (Qiagen, Valencia, CA, USA) according to manufacturers instructions. Experion RNA StdSens Chips (Bio-Rad, Hercules, CA, USA) were used to determine RNA quality and quantity.

For each sample, triplicate reverse-transcription reactions were performed with the RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific Inc.,

Rockford, IL, USA) using 1µg of total RNA per reaction and oligo dT primers. The resulting cDNA was pooled and used for triplicate qPCR reactions using Maxima SYBR green qPCR master mix (Thermo Scientific Inc., Rockford, IL, USA). Fluorescence was detected with the iQ5 Real-Time Detection System (Bio-Rad, Hercules, CA, USA). Reaction conditions consisted of 95°C for 10 min, 40 cycles of 95°C for 15 sec followed by 1 min at the annealing temperature determined for each primer set (found in Table 2-2, Table 1-1).

Normalization and relative expression analyses

Reference gene stability was calculated for each experiment and tissue type by geNorm^{PLUS} in the program qbase^{PLUS} v3 (http://www.qbaseplus.com). The qbase^{PLUS} program was also used to determined relative gene expression levels. The stable reference genes used for expression normalization for each experiment can be found in Appendix C (Table C-1).

Amino acid excised shoot assay

To examine the regulation of *NP-like* gene expression by amino acids, a 25 mM solution was prepared for 19 proteinogenic amino acids (excluding isoleucine and tyrosine). All solutions including the water control treatment were adjusted to pH 6.1 ± 0.6. The solutions were replenished after 24 h and samples were collected after 48 h from treatments that had healthy, curled or mildly necrotic leaves (for the condition of leaves after 48 h refer to Table C-2 in Appendix C). Samples were collected from the following treatments: alanine (Ala), arginine (Arg), asparagine (Asn), aspartic acid (Asp), glutamine (Gln), glycine (Gly), serine (Ser), threonine

(Thr), valine (Val) and H_2O.

TCA metabolites excised shoot assay

The regulation of *NP-like* genes by metabolites associated with the TCA cycle was tested using the following metabolite solutions and concentrations: 10 mM sodium pyruvate (pyruvate or Pyr), 7 mM di-methyl(s)-(-)-malate (malate or Mal), 7 mM alpha-ketoglutaric acid sodium salt (2-oxoglutarate or 2OG), 15 mM gamma-aminobutyric acid (GABA) and 7 mM citric acid (citrate or Cit). All chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA). Concentrations were determined from preliminary excised shoot assays that identified the maximum solution concentrations that did not result in foliar necrosis after 72 h incubation (data not shown). Treatments also included solutions of 25 mM Gln and 25 mM Suc. All solutions including water were pH adjusted to an average of 6.12 ± 0.09. Samples were collected after 24 h and 48 h.

Nitrogen compounds and metabolites excised shoot assay

To determine if N sources differentially regulate *NP-like* genes, excised shoots were incubated for 48 h in the following solutions: 10 mM ammonium nitrate (NH_4NO_3), 5 mM ammonium sulfate ($(NH_4)_2SO_4$), 10 mM potassium nitrate (KNO_3), 5 mM potassium sulfate (K_2SO_4), 25 mM Gln, 25 mM Gln plus 10 mM NH_4NO_3, 25 mM Suc, 25 mM Suc plus 10 mM NH_4NO_3, 17 mM GABA, 17 mM GABA plus 10 mM NH_4NO_3, 17 mM GABA and 25 mM Suc, and H_2O. Solutions were adjusted to an average pH of 6.12 ± 0.17.

Results

Amino acid excised shoot assay

To assess the effect of amino acids on expression of the *NP-like* gene family, samples from excised shoots that displayed healthy to mildly necrotic leaves after 48 h of incubation in 25 mM amino acid solutions were collected. Amino acid treatments toxic to the shoots at concentrations of 25 mM were composed of cysteine, glutamic acid, histidine, leucine, lysine, methionine, phenylalanine, proline and tryptophan. In shoot tips, Arg treatments strongly induced *BSP* expression and lower expression was induced by Gln, Thr and Val treatments (Fig. 3-1 A, B). The Arg treatments resulted in greater *BSP C* expression than *BSP A* and *BSP B* (Fig. 3-1 B). Ser and Val treatments appear to repress the *WIN4-like* gene subfamily (*WIN4*, *VSP 87A*, *VSP XIII*, *VSP 840*, *VSP 840*) as their expression levels are lower than in the H_2O sample (Fig. 3-1 A). On the other hand, *NP 157* was induced the most in Thr and Val.

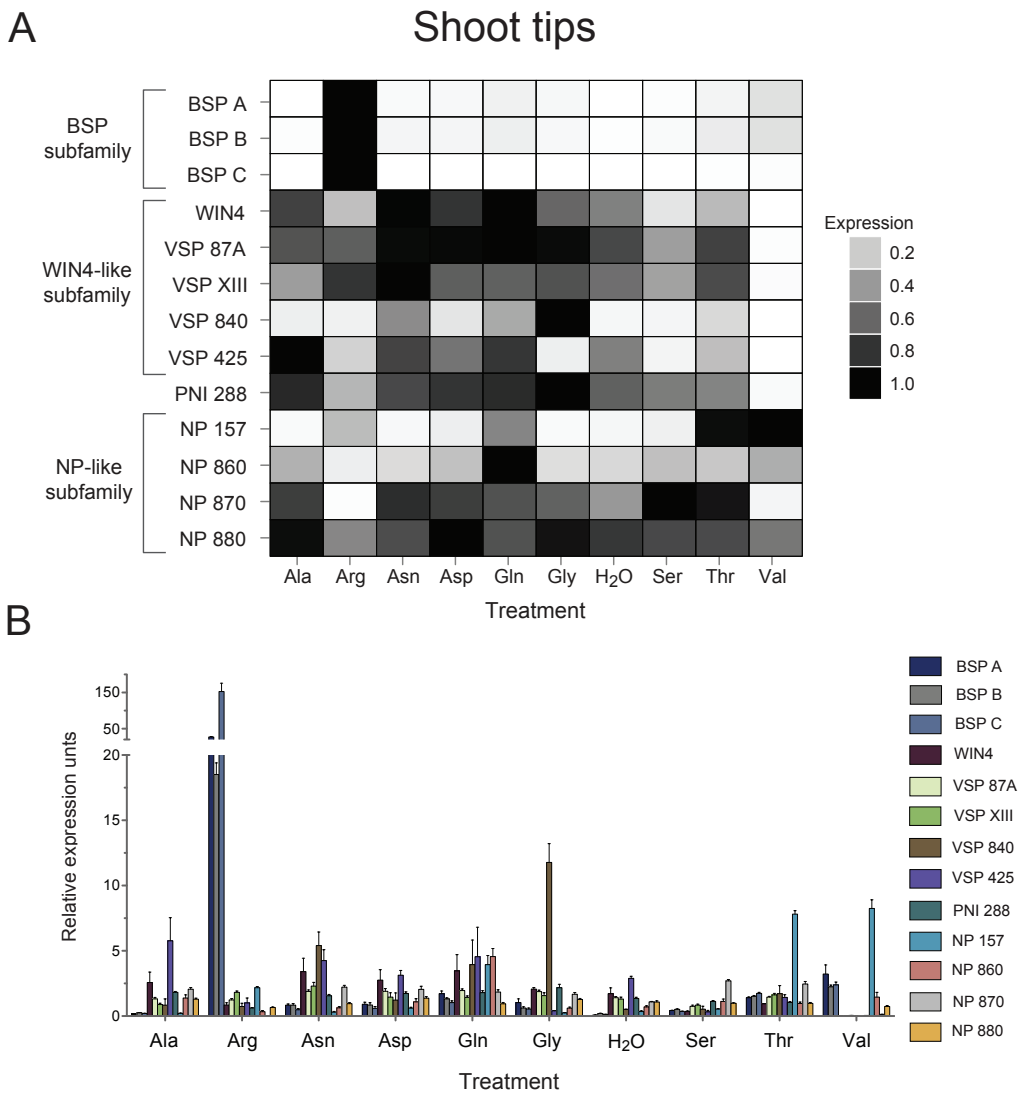

Figure 3-1. Relative gene expression of the *NP-like* gene family in shoot tips of excised *Populus trichocarpa* shoots incubated in 25 mM amino acid solutions for 48 hours.
A) Data represented as a heat map scaled to maximum. B) Data represented as a bar graph scaled to the average relative expression of the experiment.

In bark tissues, Arg, Asn, Asp, Gln and Gly treatments induce high *BSP* expression, Ala and Ser treatments induce moderate *BSP* expression, and H_2O, Thr and Val treatments did not induce *BSP* expression (Fig. 3-2 A, B). Arg treatments strongly induced *BSP C* above transcript levels of *BSP A* and *BSP B* (Fig. 3-2 B).

Arg was the only amino acid that strongly induced *BSP* genes in both tissues, with *BSP C* exhibiting greater transcript levels than *BSP A* and *BSP B* (Fig. 3-1 B, Fig. 3-2 B). Tissue specific amino acid induction of *BSPs* expression was observed. Indeed, Val induced *BSP* expression only in shoot tips (Fig. 3-1) while Ala, Asn, Asp, Gln, Gly and Ser induced *BSP* expression only in bark tissues (Fig. 3-2).

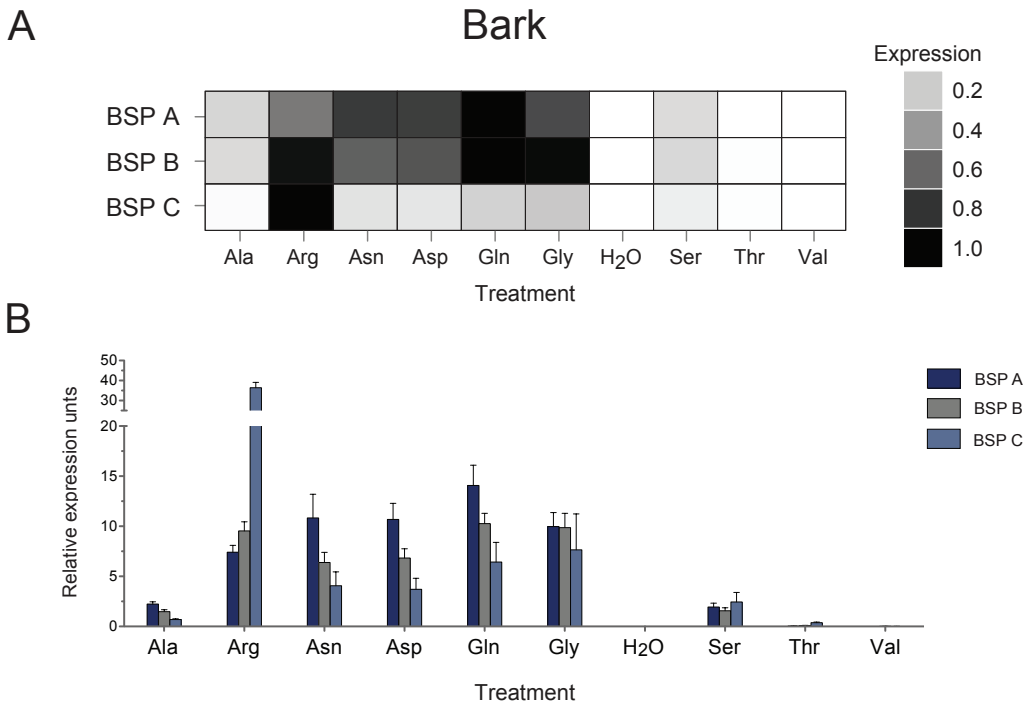

Figure 3-2. Relative gene expression of the *BSP* genes in bark tissues of excised *Populus trichocarpa* shoots incubated in 25 mM amino acid solutions for 48 hours. A) Data represented as a heat map scaled to maximum. B) Data represented as a bar graph scaled to the average relative expression of the experiment.

TCA metabolites excised shoot assay

To examine the effect of C metabolites on *NP-like* gene expression, solutions containing 2OG, Cit, Mal and Pyr along with GABA, Gln and Suc were tested individually. In shoot tips, *BSP* genes and *NP 157* were strongly induced following incubation in GABA after 24 and 48 h (Fig. 3-3 B). Gln treatments also induced *BSP* expression but to a much lower level compared to GABA (Fig. 3-3 B). Expression of

the *WIN4-like* gene *VSP 425* was also observed in Gln treatments after 24 h and water treatments after 24 h and 48 h while high transcript levels of *WIN4* and *PNI 288* were detected in Suc treatments after 48 h (Fig. 3-3 A, B).

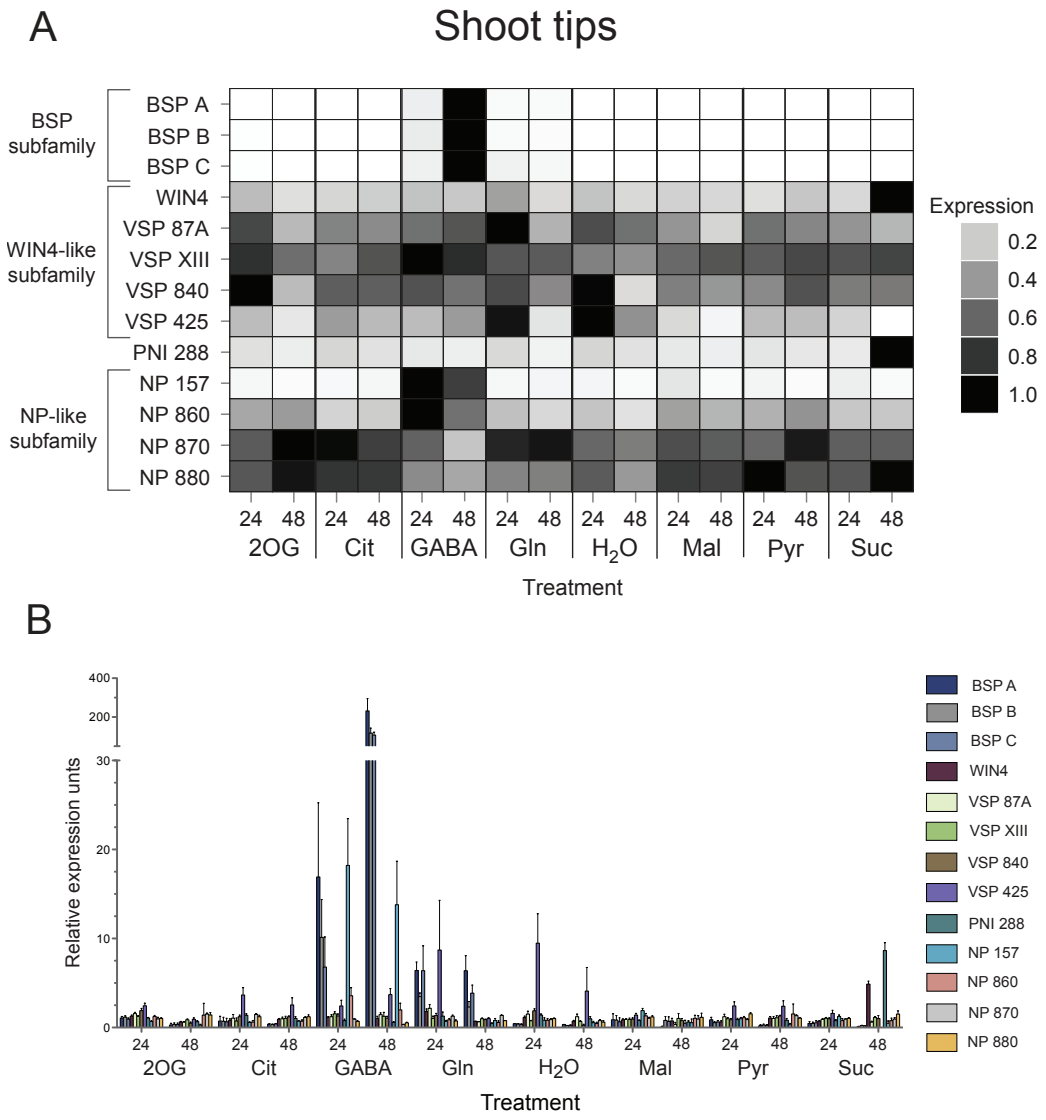

Figure 3-3. Relative gene expression of the *NP-like* gene family in shoot tips of excised *Populus trichocarpa* shoots incubated solutions containing TCA intermediates for 24 and 48 hours. Treatments were composed of 7 mM 2-oxoglutarate (2OG), 7 mM citrate (Cit), 15 mM gamma-aminobutyric acid (GABA), 25 mM glutamine (Gln), 7 mM malate (Mal), 10 pyruvate (Pyr) and 25 mM sucrose (Suc). A) Data represented as a heat map scaled to maximum. B) Data represented as a bar graph scaled to the average relative expression of the experiment.

In bark tissues, GABA treatments induced *BSP* expression after 48 h with *BSP C* expression higher than that of *BSP A* and *BSP B* (Fig. 3-4 B). Gln treatments strongly induced *BSP* expression after 24 h and 48 h (Fig. 3-4 A, B). *BSP* expression also increased following Cit treatments of 24 h and 48 h but at a lower level compared to Gln (Fig. 3-4 B). Across tissues, Gln and GABA treatments induced *BSP* expression although in shoot tips GABA treatments appears to induce higher *BSP* expression than Gln treatments while in bark tissues this relationship is reversed where Gln treatments result in higher *BSP* expression than GABA treatments (Fig. 3-3 B, Fig. 3-4 B).

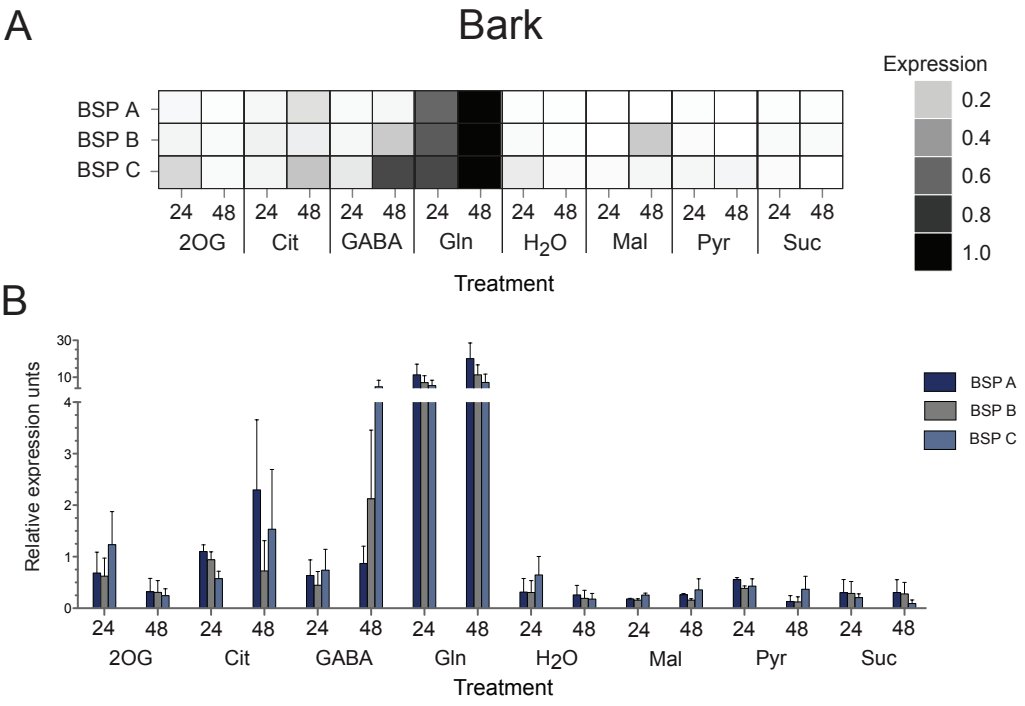

Figure 3-4. Relative gene expression of the *BSP* genes in bark-tissues of excised *Populus trichocarpa* shoots incubated solutions containing TCA intermediates for 24 and 48 hours. Treatments were composed of 7 mM 2-oxoglutarate (2OG), 7 mM citrate (Cit), 15 mM gamma-aminobutyric acid (GABA), 25 mM glutamine (Gln), 7 mM malate (Mal), 10 pyruvate (Pyr) and 25 mM sucrose (Suc). A) Data represented as a heat map scaled to maximum. B) Data represented as a bar graph scaled to the average relative expression of the experiment.

N compounds and metabolites with excised shoot assay

To examine the transcriptional responses of the *NP-like* gene family by different N sources (i.e. organic such as Gln or GABA or inorganic such as NH_4NO_3 or KNO_3) shoots were treated with solutions containing GABA, Gln, NH_4NO_3, K_2SO_4, KNO_3, $(NH_4)_2SO_4$ and Suc and combinations of GABA plus Gln, GABA plus Suc, Gln plus NH_4NO_3 and Suc plus NH_4NO_3. In the shoot tips, treatments comprising GABA and Gln, alone or combined with other compounds, induced the highest expression levels of the *BSP* genes (Fig. 3-5 A, B). GABA treatments generally induced all *NP-like* genes except *NP 870* and *NP 880* and strongly induced *NP 157* and *NP 860* (Fig. 3-5 A). Gln appeared to be more specific in inducing *BSP* expression in shoot tips (Fig. 3-5 A, B). Suc treatments did not induce any *NP-like* gene expression (Fig. 3-5 A, B).

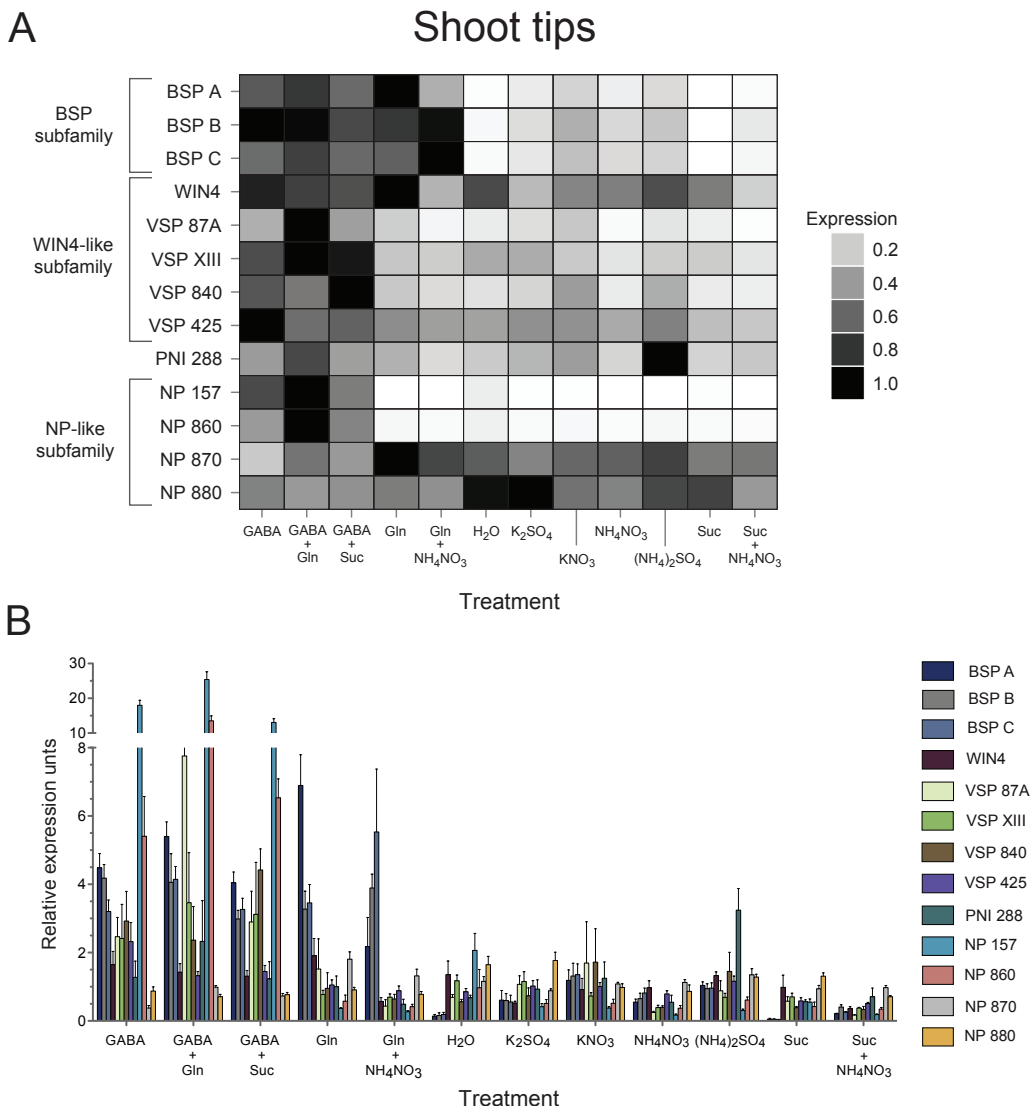

Figure 3-5. Relative gene expression of the *NP-like* gene family in shoot tips of excised *Populus trichocarpa* shoots incubated in solutions composed of N compounds and metabolites for 48 hours. Metabolite solutions include 17 mM gamma-aminobutyric acid (GABA), 17 mM GABA and 25 mM glutamine (Gln), 17 mM GABA and 25 mM sucrose (Suc), 25 mM Gln, 25 mM Gln and 10 mM ammonium nitrate (NH_4NO_3), 5 mM potassium sulfate (K_2SO_4), 10 mM potassium nitrate (KNO_3), 10 mM NH_4NO_3, 5 mM ammonium sulfate $(NH_4)_2SO_4$, 25 mM Suc, and 25 mM Suc and 10 mM NH_4NO_3. A) Data represented as a heat map scaled to maximum. B) Data represented as a bar graph scaled to the average relative expression of experiment.

In bark tissues, *BSP* expression was strongly induced by Gln treatments and any treatment containing ammonium (either NH_4NO_3 or $(NH_4)_2SO_4$) while KNO_3 did

not induce *BSP* expression (Fig. 3-6 A, B). GABA, alone or combined with Gln or Suc, induced *BSP C* expression to greater levels than *BSP A* and *BSP B* (Fig. 3-6 B). There was no transcriptional response of BSP genes to water, Suc or the treatments K_2SO_4 and KNO_3, which were included to determine an effect of K^+ or SO_4-^{2} (Fig. 3-6 A, B). Expression data in both tissues show that GABA and Gln treatments induce *BSP* genes irrespective of the tissue assayed.

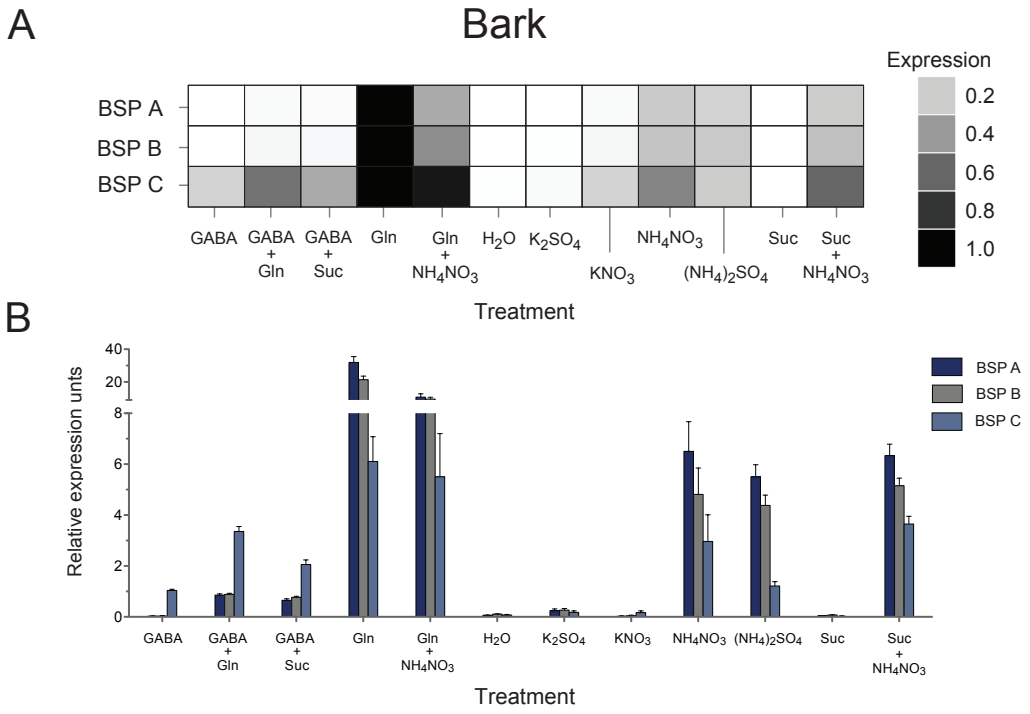

Figure 3-6. Relative gene expression of the *BSP* gene family in bark-tissues of excised *Populus trichocarpa* shoots incubated in solutions composed of N compounds and metabolites for 48 hours. Metabolite solutions include 17 mM gamma-aminobutyric acid (GABA), 17 mM GABA and 25 mM glutamine (Gln), 17 mM GABA and 25 mM sucrose (Suc), 25 mM Gln, 25 mM Gln and 10 mM ammonium nitrate (NH_4NO_3), 5 mM potassium sulfate (K_2SO_4), 10 mM potassium nitrate (KNO_3), 10 mM NH_4NO_3, 5 mM ammonium sulfate $(NH_4)_2SO_4$, 25 mM Suc, and 25 mM Suc and 10 mM NH_4NO_3. A) Data represented as a heat map scaled to maximum. B) Data represented as a bar graph scaled to the average relative expression of experiment.

Comparison of results across experiments

H_2O, Gln, Suc and GABA treatments were included across multiple experiments and provide the basis for conclusions regarding the effect these nutrients have on the expression of *NP-like* genes. In shoot tips, *BSP* transcripts were consistently low or undetected in H_2O treated shoots and, conversely, Gln induced *BSP* expression in all experiments (Fig. 3-1, 3-3, 3-5). In the H_2O treatment included in the amino acid (Fig. 3-1) and TCA metabolites (Fig. 3-3) experiments, *VSP 425* transcript levels were the highest of the *NP-like* transcripts detected but not in the N experiment (Fig. 3-5). In the Gln treatments of the TCA metabolites (Fig. 3-3) and N experiments (Fig. 3-5), *BSP* expression was higher than those of other *NP-like* genes but this relationship was not observed in the amino acid experiment (Fig. 3-1). The data show that Suc treatments performed in the TCA metabolites (Fig. 3-3) and N experiments (Fig. 3-5) resulted in low or undetectable *BSP* expression while increased *WIN4* transcripts were detected after 48 h. Also in the TCA metabolites (Fig. 3-3) and N experiments (Fig. 3-5), the relative expression levels of *NP-like* genes induced by GABA treatments were slightly different between experiments but the overall pattern is the same: strong induction of *BSP* genes and *NP 157* and induction of *NP 860* by GABA treatments.

Expression patterns across experiments in bark tissues were generally consistent. In H_2O treatments, *BSP* transcripts were absent or undetectable while Gln treatments induced *BSP* expression in all experiments (Fig. 3-2, 3-4, 3-6). Furthermore, the data show that Suc treatments performed within the TCA

metabolites (Fig. 3-4) and N experiments (Fig. 3-6) did not induce *BSP* expression while GABA treatments in these experiments show that *BSP C* was strongly induced.

Discussion

Plant growth and metabolism are governed by the availability and balance of resources. Understanding the regulation and signaling of storage proteins meant to conserve nutrients is integral to understanding growth and the balance of resources [120, 167]. To examine how the balance of resources is coordinated, I investigated the regulatory effect of C and N metabolites and compounds on *NP-like* gene expression in *Populus* shoot tips and bark. One major finding was that there was a tissue-specific effect of metabolites on the expression of *BSP* genes. For example, a range of amino acids induced *BSP* expression in bark but only Arg appeared to have a major role in *BSP* expression in shoot tips. The TCA intermediates tested had little effect on *NP-like* gene expression in shoot tips while Cit treatment induced *BSP* expression in bark tissues. A similar pattern was observed with inorganic N compounds, which had little impact on *NP-like* transcript levels in shoot tips. Whereas, inorganic ammonium sources induced *BSP* expression in bark tissues. Gln and GABA treatments consistently induced *BSP* expression but to different levels in each tissue. These results make it clear that there are tissue-specific responses of *BSP* genes, underscoring the importance of storage location and perhaps storage type (i.e. short- vs. long-term).

Another major finding is that *NP-like* gene subfamilies and genes within subfamilies are differentially regulated by metabolites. For example, in shoot tips, the *BSP* and *WIN4-like* genes generally show expression patterns similar to other genes in

their respective subfamily. However, this was not the case for the *NP-like* gene subfamily where expression of *NP 157* and *NP 860* is different from *NP 870* and *NP 880*. In the majority of the treatments, levels of expression of the three *BSP* genes were very close or *BSP C* expression was reduced compared to *BSP A* and *BSP B*. However, in shoot tips of shoots treated with Arg, Thr or Gln combined with NH_4NO_3 and in bark tissues from Arg and GABA treatments, *BSP C* expression levels were greater than *BSP A* and *BSP B*. The observed differences in *NP-like* gene expression between subfamilies and genes within respective subfamilies is consistent with subfunctionalization theories and suggests positive selection pressure acting upon these genes [51, 59, 64].

In shoot tips, relative expression results were not consistent across the three excised shoot experiments performed (amino acid, TCA metabolites and N experiments). For example, *VSP 425* transcripts levels were higher than the levels of all the other genes in shoot tips of the H_2O treatments for the amino acid and TCA metabolites experiments but not in the H_2O treatment of the N experiment. Also, in Gln treated shoot tips from the amino acid experiment *BSP* transcript levels were lower than the other *NP-like* genes. The opposite effect was observed in Gln treated shoot tips from the TCA and N experiments. It is common that the sensitivity of qPCR detection identifies inherent biological variation in samples and across experiments which can, on occasion, mask treatment effects [189]. Aside from statistical corrections which may not be included in the software used for expression analyses, increasing sample size could help distinguish significant differences [140]. In fact, expression discrepancies across experiments is likely due to biological

variation which can be common in differentiating tissues such as shoot tips, where most of expression discrepancies were observed, or due to slight variations in growing conditions of the stock plants [189].

In plants, amino acids are an important nutrient currency transported from source to sink tissues [167, 190-192]. It follows that amino acids would contribute to regulating storage processes and, by extension, *NP-like* genes. The importance of amino acids to N remobilization and storage is supported by the observation that Arg treatments induced *BSP* expression in shoot tips and bark tissues. In addition, in bark tissues, Asn, Asp and Gly also strongly induced *BSP* expression. Gln was also a potent inducer of *BSP* expression, which confirms earlier results by Zhu and Coleman [17] which found that Gln feeding activates the *BSP* promoter in bark tissues from excised shoot assays. For the other *NP-like* subfamilies, Arg, Asn and Gln treatment of excised stems only slightly induced expression of *WIN4-like* and *NP-like* genes. Arg, Asn and Gln are the major transport amino acids in *Populus* and in senescing leaves of *P. trichocarpa*, Gln and Asn are the predominant transport amino acids associated with N transport from senescing leaves to perennial tissue [192]. Through the winter Arg is the prominent free amino acid found in bark and xylem tissue [193, 194] whereas regrowth is characterized by Glu and Gln in the xylem [13, 193, 195]. While Asp is not considered a major transport amino acid in *Populus*, it can be transported from source to sink tissues in *Arabidopsis* [196, 197] and *Picea abies* (spruce) [198] and like other transport amino acids, Asp induced *BSP* expression in bark tissues. The data from this study clearly shows that transport amino acids in *Populus* regulate storage protein gene expression in bark.

Other amino acids can also serve as metabolic signals and are regulated by C and N availability as well as stress. For example, high C/N ratios found under light conditions or supplemental sucrose inhibits Asn biosynthesis while low C/N ratios resulting from dark exposure or supplemental N promote Asn biosynthesis [199]. Knocking-down asparagine synthetase in *Arabidopsis* affects N assimilation and distribution [200]. Gly and Ser can supply a large source of C into central plant metabolism under photorespiratory conditions [201, 202]. Furthermore, Gly levels can reflect changes in photorespiration and could possibly negatively feed-back to photosynthesis as a form of stress signaling [202-205]. In relation to these studies, Gly feeding could induce changes in metabolic processes and/or signal stress, which resulted in *BSP* gene induction shoot tips and bark along with a slight induction of *VSP 840*. It is not known if Ser is involved in stress signaling or metabolic regulation but Ser treatments showed low induction of *BSPs* and suggests a more limited role for potential signaling by Ser.

Stress signaling by amino acids may also explain the observed *BSP* induction by Ala, Thr and Val treatments [206]. High Ala concentrations frequently co-accumulate with GABA following stresses like low temperatures, mechanical and hypoxia stress [207-210]. Enzymes for Ala conversion, Ala aminotransferases, and perhaps Ala accumulation, may also have a role in photorespiration and hypoxia [211-214]. Similarly, Thr along with methionine and isoleucine, accumulate following osmotic stress such as drought, flooding, salt or heat [215]. Their accumulation is hypothesized to function as an additional C source under stress conditions or as signaling molecules in a similar manner to Gly and Ser [201, 202,

215]. Lastly, elevated Val levels were observed in drought stressed leaves of *Brassica* [216]. Taken together, these studies suggest that high concentrations of Ala, Thr and Val in tissues may be involved in stress responses and/or signaling and this could explain why these treatments induced *BSP* expression in bark and slightly modulated expression of *VSP 840*, *VSP 425*, *PNI 288* and *NP 870* in shoot tips.

The TCA cycle has important metabolic functions including oxidizing respiratory substrates, providing carbon skeletons for N assimilation and converting organic acids from other processes [185]. Organic acid accumulation and secretion occurs in response to osmotic and micronutrient stress and appears to be an important mechanism for adapting to stresses [217-219]. For example, *Arabidopsis* and wheat secrete malate in roots in response to high Al^{3+} levels while in soybean Cit is secreted [220, 221] Additionally, Cit is important for chelating and transporting iron in the xylem [219]. Therefore, the Cit treatments that induced *BSP* expression could reflect that Cit may signal a nutrient imbalance or stress in bark tissues. Other organic acids 2OG, Mal and Pyr had no effect on *NP-like* expression and these treatments are likely not a signal or do not induce metabolic changes in shoot tips or bark.

Treatments containing the non-protein amino acid GABA strongly induced *NP-like* genes but interpreting what GABA treatments were signaling in these experiments is complicated since GABA is involved in C and N metabolism, amino acid pathways, stress signaling and utilized directly as a N source [187, 188]. GABA is synthesized from glutamate (Glu) and converted to succinate where it enters the TCA cycle, connecting amino acids, N assimilation to C metabolism [187]. Labeling experiments confirm that GABA provides a significant amount of succinate to the

mitochondria and the GABA shunt is considered as part of the TCA cycle by some researchers [186-188]. Yet GABA can also be an N source and regulate N pathways, illustrated by studies that show that *Arabidopsis* can grow on media with GABA as the sole source of N and that GABA application to *Arabidopsis* seedlings regulates N uptake and utilization [222, 223]. This larger metabolic context of the GABA shunt helps to explain the large volume of studies finding GABA related to or involved in abiotic stresses, defense, oxidative stress, cytosolic pH regulation, osmoregulation, glutamate homeostasis and pollen tube development [224-227]. Results from this study indicate another potential role for GABA that involves the regulation of *NP-like* genes. Although, further studies are needed to determine if elevated GABA levels are acting as a stress, a signal of stress or source of N that induces *NP-like* genes.

N is a major limiting growth factor and its availability can impact general plant metabolism and overall plant growth [176, 228-230] While large portions of the *Arabidopsis* genome is N-responsive, less is known about more nuanced responses to various N compounds and regulatory pathways, particularly surrounding ammonium regulatory and signaling pathways [111, 170, 231-235]. In this study, *BSP* expression was induced in bark tissues by treatments containing ammonium which may reflect that *Populus* is adapted to utilizing ammonium and amino acids which are the most available form of N in forest ecosystems [236, 237]. Ammonium transporters have been studied in *Arabidopsis*, which is adapted to nitrate available soils and to a limited degree in *Oryza* which has evolved to take up ammonium, the data here suggest that *Populus* and bark tissues may be a good model to examine ammonium regulation and signaling in a woody ammonium adapted species [238-240].

Conclusions

Metabolism has a central role for coordinating remobilization and storage in plants. This study provides the first evidence of the regulatory capability of amino acids, N sources and GABA on *NP-like* genes in *Populus*. The tissue-specific regulation of *NP-like* genes by metabolites illustrates the possibility of storage strategies suited for plant organs. Importantly, this work identified that GABA could be an essential metabolite involved in remobilization and storage. The expression patterns of *NP-like* genes supports storage functions that overlap but are not entirely redundant. In whole, this work offers future investigations information useful for identifying candidate genes and signaling mechanisms involved in the adaptation to remobilize and store nutrients in *Populus*.

Chapter 4: Effects of altered BSP levels in transgenic P. tremula x alba trees

Abstract

Allocating resources by nutrient remobilization and storage facilitates optimal growth in plants. Although these processes are components of nitrogen use efficiency (NUE), their regulation is unclear. In *Populus*, bark storage proteins (BSPs) function as seasonal and intra-seasonal reserves. Investigating BSPs provides an opportunity to expand our knowledge of the mechanisms of these ecophysiological adaptations in trees. By extension, I assessed the role of storage proteins in growth and development by engineering *Populus* trees that express high and low *BSP* transcript levels. I specifically examined the effect of altered BSP accumulation on growth parameters, photosynthesis and gas exchange. The data show changes in photosynthetic rates of young leaves, suggesting a role for BSPs in nutrient signaling. Additionally, the lack of strong knock-down lines points to importance of BSPs in survival or regeneration. This research provides a hopeful direction for future efforts to increase biomass and assist the development of more sustainable *Populus* plants.

Introduction

Throughout the life of a plant, nutrient demand shifts to support growth, reproduction and defense by internally distributing and mobilizing nutrients to meet those demands [1, 241]. Additionally, remobilization and storage promote efficient utilization of limiting nutrients such as N [1, 25, 242, 243]. This capacity is known as nitrogen use efficiency (NUE) and it describes the biomass produced by a unit of N

and how long N resides in the plant [120, 241, 244]. Many of the molecular mechanisms and regulatory pathways are still unknown but involve interactions of genetic, environmental and adaptive trade-offs (i.e. biomass production versus residence time of N) [120, 240, 245, 246]. None the less, improving NUE is imperative to developing sustainable agriculture and agroforestry in the face of over-fertilization and the rising cost of fertilizers [247]. In fact, improving NUE, remobilization and storage capacity are particularly important for sustainable increases in yield and biomass of *Populus* used for biofuels [248, 249]. While *Populus* is already used as fast-growing perennial feed-stock for fiber and biofuels, increases in biomass would make *Populus* a more cost effective choice [248, 249]. Therefore, advancing our understanding of N remobilization and storage can assist in selection and breeding of more productive trees that are more sustainably managed [249]. Sustainable increases in yield and biomass could also support the suitability of *Populus* for phytoremediation and carbon (C) sequestration [250, 251].

A significant advantage of using *Populus* to examine remobilization and storage is that these processes have been studied on a seasonal level for over 20 years [5, 7]. This body of research has provided insight into some of the physiological and regulatory aspects of N remobilization and storage. In *Populus* and many other perennial species, proteins accumulate in bark tissues following the onset of short-day conditions in fall [252]. These proteins were designated bark storage proteins (BSPs) in *Populus* [7]. The change in photoperiod induces large-scale metabolic shifts from leaf anabolism to catabolism in *Populus*, characterized by transcriptional decreases in genes involved in the Calvin cycle, the tricarboxylic acid (TCA) cycle, energy related

processes, starch degradation and a myriad other processes [135-139]. These changes are followed by, or concurrent with, leaf senescence, which is the coordinated process of cell death that involves the degradation of leaf proteins [135, 136, 191, 253]. Leaf proteins are catabolized into amino acids that are then converted to stable transport amino acids by transaminases [183, 192, 241, 254]. The amino acids are translocated to bark tissues by amino acid transporters where BSPs are synthesized [7, 192, 255]. BSPs accumulate in the storage vacuoles of bark parenchyma and xylem ray cells to be stored through the winter [11, 12, 181, 256]. Following dormancy and a chilling period, protein levels of BSP gradually decline to near undetectable levels in the summer [11, 180, 181]. The scale and efficiency of remobilization and storage are staggering. During senescence, *Populus* can mobilize up to 80% of the total leaf N [253]. This N is transported to bark and wood tissues where BSPs account for 60-70% of the total protein present in these tissues in young trees over winter [5, 257]. To illustrate the scale of abundance, it is estimated that Rubisco constitutes up to 50% of the total soluble proteins and up to 30% of total protein in C3 leaves [258].

BSPs can also accumulate during active growth under long-day (LD) conditions in response to stress and changes in N availability. Drought, wounding and methyl jasmonate application induce BSP accumulation [6, 10, 14, 15]. Regulation of BSPs by methyl jasmonate is possibly due to the change in N metabolism induced by methyl jasmonate treatment [15, 259, 260]. Increased N availability (under LD conditions) corresponds with increased BSP accumulation [17, 261, 262]. Along the same line, the *BSP A* promoter was shown to have distinct regulatory regions specifically related to N responses and SD responses [134]. Regulation by N could

indicate that BSPs are involved in N metabolism or pathways which may explain why gibberellic acid, calcium and phosphorylation also have roles in regulating the *BSP A* promoter [134].

The role of BSPs in development and physiology was explored by Black and his work suggested a link between BSPs, biomass partitioning and nitrate uptake [263]. Plants transformed with antisense-*BSP A* constructs had reduced BSP accumulation that resulted in notable differences in physiology and nutrient uptake. Trees from the knock-down lines had increased leaf area and internode length in conjunction with decreased stem biomass and nitrate uptake. The results were interpreted to mean that BSPs contribute to biomass partitioning from stems to leaves [263]. The hypothetical mechanism behind this conclusion is that trees with reduced storage ability have a high concentration of amino acids that signals and/or regulates nitrate uptake. This is a plausible mechanism and is consistent with several studies that have established a correlation between amino acid cycling and the regulation of nitrate uptake [241, 264, 265].

In this study I examine remobilization and storage of N by manipulating storage proteins with the aim of better understanding the ways in which NUE can influence bioenergy and bioremediation. To achieve this, I will build on research connecting NUE and photosynthetic rates by examining photosynthesis and gas exchange in transgenic trees that have elevated or knocked-down levels of BSPs in two independent experiments. I also expand the current understanding of the physiological importance of BSPs in growth and development to provide greater

clarity into the ecophysiological adaptive mechanisms that enables perennial species, such as *Populus*, to inhabit nutrient-limiting forest environments.

Materials and Methods

Plant Material and Transformation

Stem sections from the hybrid poplar (*Populus tremula x Populus alba*) clone 717 were transformed by co-cultivation with *Agrobacterium tumefaciens* strain C58/pMP90 according to Leple *et al.* [133]. *BSP A* PCR products were cloned into the entry vector with pENTR/D-TOPO® (Invitrogen, Calsbad, CA, USA). The entry vectors were recombined with the destination vectors using Gateway® LR Clonase® (Invitrogen, Calsbad, CA, USA). Two over-expression vectors were constructed: pB7WG2 that contains CaMV 35S-*BSPA* and pEarleyGate103 containing CaMV 35S-*BSPA*-*GFP* (Table 4-1) [266, 267]. Two constructs for the vector pB7GWIWG2(II) containing different small cloned DNA segments of the *BSP A* gene from *P. trichocarpa* were used to express dsRNA for post-translational silencing via the RNAi pathway (Table 4-1) [266]. All vectors confer resistance to glufosinate ammonia. Transformed plants were selected on Murashige and Skoog (MS) media containing 5 mg/L glufonsinate ammonia [268]. Plants were maintained *in vitro.*

Wild type and transgenic plants were grown under LD conditions (16 h light/8 h dark) at 18°C for approximately 8 weeks (at which plants were approximately 30 cm tall) in controlled environment chambers (Conviron Inc., Winnipeg, Manitoba, Canada) with a PAR of 400 μmol $m^{-2}s^{-1}$ at 50 cm above the surface of pots. Plants were grown in 2.2 L pots using a professional potting mix (Sunshine LC1, Sun Gro Horticulture Canada Ltd., Bellevue, WA, USA) and fertilized with approximately 5 g

of the slow release fertilizer Nutricote 18-3-3 (Florikan, Sarasota, FL, USA).

Table 4-1. Destination vector names, description, function, selection gene, insert description and primers.

Vector name	Description	Function	Insert description	Primers (5'-3')
pB7GWIWG2(II)	p35S-*BSP B*-T35S	Hairpin RNA expression for silencing	103 nt *BSP B*	F: CACCTCGCTTAGGGCTTGTTTTTACG R: GATGACTCCGTGAATGCTGAATC
pB7GWIWG2(II)	p35S-*BSP A*-T35S	Hairpin RNA expression for silencing	229 nt *BSP A*	F: CACCCCAGAGAATGGAGAGAACTTG R: TGGTGATGGGAAGCCAGAAAAC
pB7WG2	p35S-*BSP A*-T35S	Over-expression	*BSP A*	F: CACCATGTCTACAGTCAATTTGGCAGCAC R: TCACTCACAAGCAAGTCGTG
pEarleyGate103	p35S-*BSP A*-GFP-6xHis-OCS	Over-expression fused to reporter	*BSP A*	F: CACCATGTCTACAGTCAATTTGGCAGCAC R: CTCACAAGCAAGTCGTGGCGTAGG

Abbreviations: OCS, Octopine synthase terminator; 6xHis, Histidine tag.
Gene Phytozome locus: *BSP A,* POPTR_0013s10380; *BSP B*, POPTR_0013s10370

Screening

Two rounds of screening for altered BSP levels were conducted. To identify specific lines, the first round of screening examined bark protein levels in individuals from random lines. From these lines, between 5 to 7 individuals were sampled during the second round of screening. Bark from trees transformed with RNAi (knock-down) constructs was collected after 4 weeks of SD conditions. Over-expression and over-expression-*GFP* trees were harvested after 8 weeks LD and after 4 weeks SD in both screening rounds. In total, 575 plants were screened. Of these, 40 RNAi (knock-down) lines, 29 over-expression and 18 over-expression-GFP lines were screened. Three lines for each construct were selected based on protein accumulation relative to wild type plants. Bark between leaf plastochron index (LPI) 9 and LPI 11 was collected and flash frozen in liquid nitrogen [153]. Tissue was stored at -80°C until protein extraction.

Protein Extraction and SDS-PAGE

Proteins were extracted as described by Coleman *et al.* [11]. Briefly, approximately 200 mg of tissue ground in liquid nitrogen was transferred to 1 mL of extraction buffer (50 mM sodium borate, 50 mM ascorbic acid, 1% beta-mercaptoethanol and 1 mM PMSF at pH 9). The mixture was homogenized using Power Gen 125 tissue homogenizer (Thermo Fisher Scientific, Waltham, MA, USA) at full speed for 30 sec and then centrifuged for 30 min at 35,000 *g* at 4°C. Proteins were precipitated overnight with 0.1 M ammonium acetate at -20°C. The proteins were then centrifuged for 20 min at 10,000 *g* at 4°C then washed two times with

ammonium acetate and once with acetone. The protein pellets were air-dried and re-suspended in 10 µl Laemmli sample buffer per 30 mg of tissue. Protein concentrations were determined by the bicinchoninic acid assay (Pierce BCA Protein Assay Kit, Thermo Scientific Inc., Rockford, IL, USA). 4 µg of proteins were loaded in each well and electrophoresed using precast mini-gels at 200V (12% Mini-Protean TGX Precast Gel, Bio-Rad Laboratories). Gels were stained with Coomassie blue (GelCode Blue Stain Reagent, Thermo Scientific Inc., Rockford, IL, USA) and imaged with the VersaDoc (Bio-Rad Laboratories, Hercules, CA, USA).

Western Blotting

Proteins were transferred from mini-gels with the Genie Electrophoretic Blotter (Idea Scientific Company, Minneapolis, MN) according to the manufacture's instructions onto nitrocellulose membranes. The blotting time was 30 min at 24 volts in a standard Tris-glycine buffer in 20% v/v methanol. Membranes were washed for 10 min in TBS (20 mM Tris-HCl, pH 7.5, 150 mM NaCl) and blocked overnight at 4°C with 3% Bovine Serum Albumin (BSA) in TTBS (TBS, 1% Tween-20). The membranes were washed 15 min in TBS. Primary antibodies were diluted in 1% BSA in TTBS. GFP antibodies were diluted to 1:500 (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA). BSP antibodies were diluted 1:2000 (Research Genetics, Inc., Huntsville, AL, USA). Next, the membranes were washed three times for five minutes in TTBS before incubating in a secondary antibody solution (goat anti-rabbit conjugated alkaline phosphatase, diluted 1:5000) (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA). Again the membranes were washed three times in TTBS and once in TBS. The membranes were stained with nitro-blue tetrazolium (NBT)

chloride and 5-bromo-4-chloro-3'indolylphosphate p-toluidine salt (BCIP) for 30 minutes. Membranes were imaged with the VersaDoc (Bio-Rad Laboratories, Hercules, CA, USA). All washes, incubations and staining were conducted at room temperature, except for the blocking step which was at 4°C.

In vitro growth experiments

Assessing *in vitro* growth of transgenic lines was performed by growing 45 explants for 8 weeks in Magenta GA-7 boxes containing approximately 30 mL half-strength Linsmaier and Skoog (LS) [269]. After this time the following measurements were taken: plant height (m), fresh weight (g), length of longest shoot and root (mm), total number of shoots and roots and performance rating. Performance was determined by rating plants on a six-point scale: (6) actively growing with roots > 5 mm, (5) actively growing with roots < 5 mm, (4) shoot growth but no root growth, (3) no shoot or root growth, (2) explant nearly dead, (1) dead explant.

Growth, photosynthesis and gas exchange measurements

Six characteristics were measured to assess growth of transgenic lines: plant height, plastochron, fresh weight, internode length, leaf area and stem diameter. Leaf area was determined using a LI-3100 leaf area meter (LI-COR Inc., Lincoln, Nebraska).

Two independent experiments, conducted in 2011 and 2012, measuring photosynthesis and gas exchange were performed using a LI-6400 Portable Photosynthesis System (LI-COR Inc., Lincoln, NE, USA). Measurements were carried out between 2-8 hours after the lights were turned on with a light intensity in

the chambers of 400 μmol photon m^{-2} s^{-1}. The air flow in the sample chamber was set at 500 μmol $s^{-1,}$, the chamber was set to a block temperature of 19°C and the CO_2 concentration in the sample chamber was set to 360 μmol mol^{-1}.

Statistical analysis

Significance was determined by one-way ANOVA tests followed by Dunnett's post-test with alpha set at 0.05. I calculated Pearson's correlation coefficients between transgenic lines and wild-type plants, the significance of which was assessed using a two-tailed test and 95% confidence. All analyses were performed using the Prism program (GraphPad Software, Inc., La Jolla, CA, USA).

Results

Screening

To confirm BSP levels of transgenic plants, western blots were performed with BSP antibodies using proteins from bark samples of individuals from knock-down (Fig. 4-1 A) and over-expression (Fig. 4-1 B) lines. The knock-down line A81 was the only surviving line screened that had no observable BSP accumulation after 4 weeks under SD conditions (Fig. 4-1 A).

While over-expression constructs were driven by the constitutive 35S promoter, in trees grown under LD conditions there was little to no accumulation of BSPs in samples from bark tissues (Fig. 4-1B). Following 4 weeks SD conditions, BSP accumulation was detected at greater abundance than in a wild-type representative sample. No BSP accumulation was detected in samples from bark tissues of trees over-expression lines with *BSP A* fused to *GFP* grown under LD

conditions and low accumulation was observed after 4 weeks SD conditions (Fig. 4-1B). A western blot using GFP antibodies was performed and showed GFP accumulation under LD conditions and decreased GFP accumulation under SD conditions (Fig. 4-2).

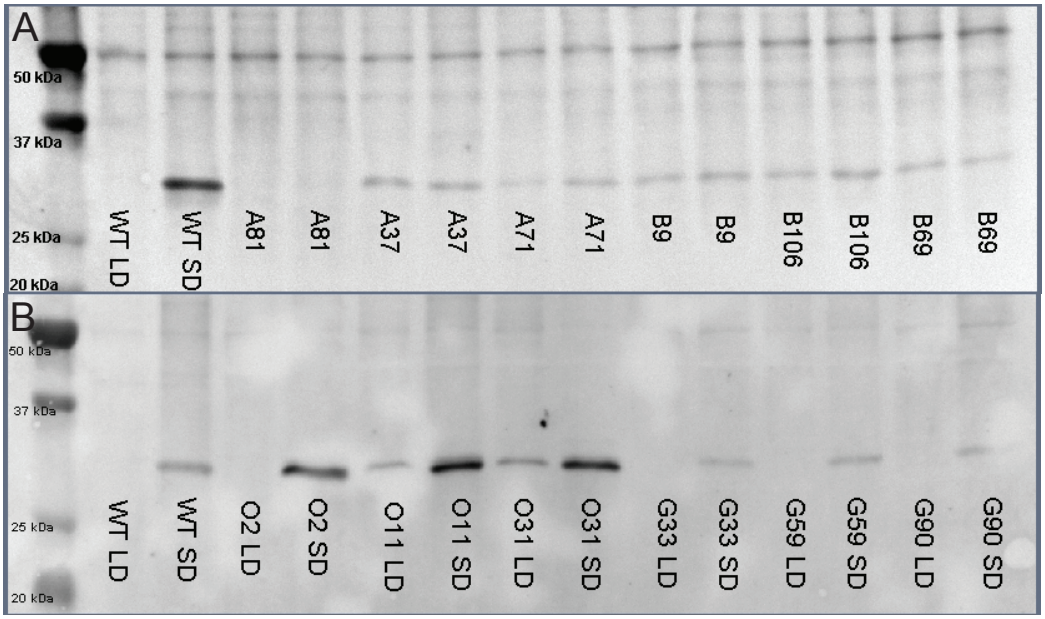

Figure 4-1. Western blots of bark samples using BSP antibodies from wild-type (WT) plants and transgenic *Populus* lines grown under long-day (LD) and short-day (SD) conditions. A) *BSP* knock-down lines. B) *BSP* over-expression and over-expression *BSP-GFP* lines.

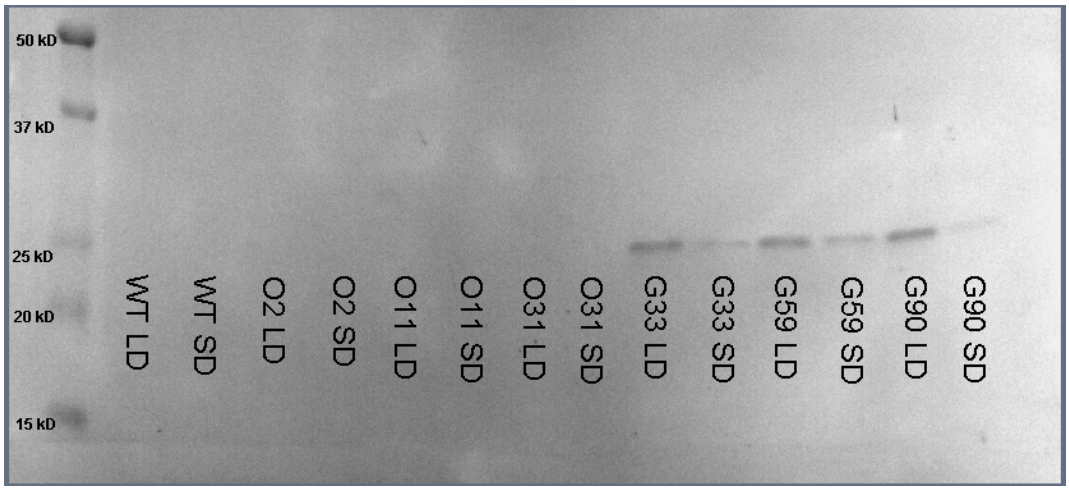

Figure 4-2. Western blot of bark samples using GFP antibodies from BSP over-expression and over-expression *BSP-GFP* lines of transgenic *Populus* lines grown long-day (LD) and short-day (SD) conditions.

In vitro growth assessment

To investigate the possibility of altered BSP levels having an effect on growth and physiology early in development, explants were propagated from *in vitro* individuals of *BSP* knock-down and over-expression lines as well as wild-type plants. After 8 weeks under LD conditions, the knock-down line B106 had significantly lower fresh weight compared to wild-type explants (Table 4-2). Line A71 displayed significantly greater height than wild-type explants. Other than this, there were no significant mean values of measured growth parameters for knock-down or over-expression lines in relation to wild-type means (Table 4-2, 4-3).

Table 4-2. *In vitro* assessment of growth and performance after 8 weeks of wild-type plants and *BSP* knock-down transgenic lines of *Populus tremula x Populus alba*. 45 explants were evaluated for each line.
Performance was rated on a 6-point scale: (6) actively growing with roots > 5 mm; (5) actively growing with roots < 5 mm; (4) shoot growth but no root growth; (3) no shoot or root growth; (2) explant nearly dead; (1) dead explant. Significance determined by ANOVA followed by Dunnett's multiple comparison test ($\alpha = 0.05$) and denoted by * *P*-value < 0.05, ** *P*-value < 0.01, *** *P*-value < 0.001.

	Wild-type		Knock-down (RNAi) lines											
	Control		B69		B106		B9		A71		A81		A37	
	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
Performance	4.69	0.27	4.84	0.28	4.53	0.27	4.20	0.29	4.91	0.24	5.03	0.22	4.20	0.28
Height (cm)	58.59	3.70	50.72	3.36	58.61	4.13	72.81	5.89	74.9*	4.26	58.13	5.03	58.38	6.14
Fresh weight (g)	0.83	0.08	0.57	0.07	0.44**	0.05	0.89	0.11	0.72	0.08	0.54	0.07	0.57	0.11
Longest root (cm)	87.48	9.45	62.92	10.92	61.26	8.76	81.32	9.98	88.26	8.40	68.31	13.15	75.06	15.47
Longest shoot (cm)	45.15	2.04	39.48	3.19	44.7	3.55	54.59	5.08	60.52	4.12	43.69	5.52	44.19	5.64
Number of roots	1.44	0.11	1.62	0.16	1.61	0.16	1.41	0.13	1.41	0.11	1.44	0.13	1.69	0.15
Number of shoots	1.85	0.13	2.00	0.12	1.44	0.19	2.09	0.17	2.00	0.18	1.69	0.20	1.75	0.25
Fresh weight/day (g day^{-1})	2.50	0.27	1.74	0.31	1.79	0.26	2.40	0.29	2.52	0.24	1.95	0.38	2.15	0.44

Table 4-3. In vitro assessment of growth and performance after 8 weeks of wild-type plants and *BSP* over-expression transgenic lines of *Populus tremula x Populus alba*.
45 explants were evaluated for each line. Performance was rated on a 6-point scale: (6) actively growing with roots > 5 mm; (5) actively growing with roots < 5 mm; (4) shoot growth but no root growth; (3) no shoot or root growth; (2) explant nearly dead; (1) dead explant. Significance determined by ANOVA followed by Dunnett's multiple comparison test ($\alpha = 0.05$) and denoted by * P-value < 0.05, ** P-value < 0.01, *** P-value < 0.001.

	Wild-type		Over-expression lines											
	Control		O11		O31		O2		G33		G59		G90	
	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
Performance	4.69	0.27	5.29	0.16	3.89	0.28	3.87	0.28	4.18	0.27	4.18	0.28	4.58	0.28
Height (cm)	58.59	3.70	68.9	4.85	73.00	7.67	47.61	3.75	57.94	4.41	54.82	3.90	53.25	3.95
Fresh weight (g)	0.83	0.08	0.98	0.08	0.75	0.13	0.56	0.07	0.62	0.09	0.64	0.11	0.62	0.08
Longest root (cm)	87.48	9.45	88.0	8.61	81.9	16.3	80.78	9.83	78.0	11.08	73.89	13.0	68.67	9.72
Longest shoot (cm)	45.15	2.04	52.72	4.46	51.18	7.04	35.82	3.47	45.15	7.46	40.72	4.33	40.88	4.08
Number of roots	1.44	0.11	1.59	0.14	1.73	0.24	1.39	0.14	1.45	0.11	1.63	0.24	1.58	0.17
Number of shoots	1.85	0.13	2.24	0.27	1.73	0.24	1.78	0.19	1.85	0.13	1.95	0.18	1.88	0.14
Fresh weight/day (g day^{-1})	2.50	0.27	2.67	0.24	2.14	0.46	2.42	0.27	2.18	0.33	2.13	0.39	1.96	0.28

Growth, photosynthesis and gas exchange measurements

Growth parameters were assessed in transgenic and wild-type plants after 8 weeks of growth under LD conditions. Among knock-down lines, growth parameters in the lines B9 and A37 were significantly different than wild-type plants (Table 4-4). Plants of the line B9 had smaller stem diameters near the petiole of LPI 7. Plants of the line A37 exhibited shorter internodes between LPI 10-11 and LPI 11-12. These plants also had lower fresh weights of the total bark tissues.

The two over-expression lines O31 and O2 had growth parameters significantly different than those of wild-type plants (Table 4-5). The line O31 had smaller stem diameters near the petiole of LPI 7. There were eight significantly different parameters for line O2. Plants of the O2 line had smaller stem diameters at LPI 14 and 22, decreased leaf area for LPI 14, lower fresh weight of LPI 14 and total leaves, as well as lower fresh weight of the bark and pith between LPI 12-13 and 13-14.

Comparing 95% confidence intervals (CIs) of the stem diameters of the transgenic lines to those from wild-type plants, the transgenic lines had intervals that extend below the CI for wild-type plants of stem diameters measured at LPI 7, 14 and 22 (Fig. 4-3). For stem sections near LPI 7, CIs lower than wild-type plants were observed for A37, B69, B9, O2, O31 and G59 (Fig. 4-3 A). Stem diameters at LPI 14 and LPI 22, lower CIs than wild-type plants were observed for the lines A37, A81, B106, B69, B9, O2, O31 and G59 (Fig. 4-3 B, C). Lower CIs correspond with significantly different diameter means for B9 and O31 at LPI 7 (Fig. 4-3A, Table 4-

4), O2 at LPI 14 (Fig. 4-3 B, Table 4-5) and O2 at LPI 22 (Fig. 4-3 C, Table 4-5).

Table 4-4. Growth assessment of wild-type plants and *BSP* knock-down transgenic lines of *Populus tremula x Populus alba* grown for 8 weeks under long day conditions. Significance determined by ANOVA followed by Dunnett's multiple comparison test ($\alpha = 0.05$) and denoted by * *P*-value < 0.05, ** *P*-value < 0.01, *** *P*-value < 0.001.

				Knockdown (RNAi) lines											
		Wild-type		B69		B106		B9		A71		A81		A37	
	LPI	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
Height (m)		1.09	0.05	1.00	0.09	1.09	0.03	1.07	0.03	1.09	0.04	1.06	0.04	1.08	0.04
Plastochron (days/node)		1.27	0.04	1.43	0.10	1.36	0.03	1.25	0.08	1.29	0.04	1.32	0.03	1.23	0.03
Stem diameter (cm)	7	0.58	0.03	0.48	0.03	0.53	0.02	0.45**	0.02	0.56	0.01	0.54	0.02	0.52	0.03
	14	0.85	0.04	0.69	0.04	0.76	0.02	0.71	0.03	0.75	0.05	0.75	0.03	0.70	0.07
	22	0.99	0.05	0.90	0.02	0.91	0.01	0.87	0.03	0.98	0.04	0.89	0.04	0.93	0.03
Internode length (mm)	7-8	34.4	1.84	33.7	2.18	34.7	0.09	34.2	1.39	38.1	2.86	31.8	1.73	29.3	1.79
	8-9	37.1	2.53	32.7	2.27	32.8	1.59	35.8	1.97	35.1	2.95	36.0	2.24	30.4	1.23
	9-10	35.6	2.27	35.0	1.98	34.3	0.09	39.5	1.29	35.4	2.65	33.2	1.54	29.3	2.42
	10-11	39.6	2.32	36.0	1.35	38.6	2.06	37.4	1.22	37.3	2.10	38.1	1.38	30.8*	1.59
	11-12	38.8	1.32	36.0	0.82	36.4	1.40	37.1	1.64	36.9	1.93	36.3	2.03	31.7*	1.80
	12-13	39.2	2.30	35.7	2.90	39.4	0.97	34.5	1.10	37.7	1.21	38.1	1.50	33.2	1.77
Leaf area (cm_2/leaf)	7	71.4	6.87	50.2	4.48	57.2	9.46	62.2	3.85	74.3	9.50	68.5	6.85	68.4	10.02
	14	203	22.2	156	10.5	192	15.8	183	14.2	210	16.4	186	13.7	180	14.1
	22	212	17.4	182	13.1	221	16.6	216	19.5	218	11.1	203	12.6	204	15.0
Fresh weight (FW) (g)	7	1.23	0.10	0.92	0.09	1.14	0.14	1.08	0.05	1.45	0.17	1.21	0.14	1.17	0.14
	14	3.43	0.42	2.64	0.16	3.28	0.23	2.93	0.21	3.37	0.24	3.06	0.28	3.15	0.22
	22	3.69	0.33	3.06	0.12	3.87	0.31	3.51	0.27	3.84	0.29	3.54	0.27	3.45	0.22
FW Total leaves (g)		93.9	10.0	73.4	11.0	92.0	2.18	87.1	6.10	92.4	8.44	81.6	9.00	98.4	4.77
FW Bark (g)	12-13	0.64	0.06	0.45	0.03	0.57	0.05	0.58	0.04	0.67	0.05	0.61	0.06	0.49	0.05
FW Pith (g)	12-13	0.87	0.06	0.60	0.05	0.85	0.06	0.82	0.07	0.94	0.07	0.85	0.09	0.63	0.08
FW Bark (g)	13-14	0.65	0.05	0.52	0.06	0.66	0.05	0.60	0.05	0.65	0.05	0.55	0.06	0.43*	0.06
FW Pith (g)	13-14	0.90	0.08	0.75	0.10	0.98	0.06	0.90	0.08	0.98	0.10	0.82	0.08	0.65	0.10
FW Total bark + pith (g)		51.9	5.60	40.3	6.37	51.6	1.87	50.3	3.87	57.7	5.86	47.9	5.71	56.9	2.67

Table 4-5. Growth assessment of wild-type plants and *BSP* over-expression transgenic lines of *Populus tremula x Populus alba* grown for 8 weeks under long day conditions.
Significance determined by ANOVA followed by Dunnett's multiple comparison test ($\alpha = 0.05$) and denoted by * *P*-value < 0.05, ** *P*-value < 0.01, *** *P*-value < 0.001.

				Over-expression lines											
		Wild-type		O11		O31		O2		G33		G59		G90	
	LPI	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
Height (m)		1.09	0.05	1.01	0.04	1.05	0.07	0.95	0.04	1.16	0.04	1.14	0.05	1.10	0.03
Plastochron (days/node)		1.27	0.04	1.29	0.06	1.40	0.08	1.33	0.03	1.22	0.03	1.35	0.03	1.27	0.01
Stem diameter (cm)	7	0.58	0.03	0.57	0.04	0.47*	0.03	0.48	0.02	0.56	0.01	0.51	0.04	0.61	0.03
	14	0.85	0.04	0.79	0.04	0.73	0.05	0.64**	0.03	0.80	0.02	0.72	0.04	0.85	0.01
	22	0.99	0.05	0.93	0.06	0.91	0.05	0.79*	0.04	0.98	0.03	1.03	0.09	1.01	0.03
Internode length (mm)	7-8	34.4	1.84	32.3	0.60	32.9	2.00	32.7	2.38	33.2	2.40	34.1	2.50	33.6	1.37
	8-9	37.1	2.53	37.4	2.03	33.00	1.56	33.0	1.92	33.4	1.68	31.3	2.04	35.8	1.91
	9-10	35.6	2.27	33.7	1.16	32.9	1.25	34.3	2.21	34.4	2.39	35.0	2.14	34.8	1.91
	10-11	39.6	2.32	34.6	1.60	35.2	1.58	33.8	2.09	34.2	1.20	34.1	1.71	35.0	1.98
	11-12	38.8	1.32	35.2	1.69	35.9	1.07	33.8	1.63	35.9	1.85	37.6	2.08	37.8	1.23
	12-13	39.2	2.30	36.4	1.30	36.3	1.53	36.0	1.76	40.9	1.91	41.6	1.55	38.2	1.78
Leaf area (cm_2/leaf)	7	71.4	6.87	73.0	4.65	57.0	4.16	53.2	4.23	70.5	5.83	57.6	8.27	69.9	8.78
	14	203	22.2	196	17.1	151	16.5	118**	16.8	186	10.9	189	14.8	199	18.4
	22	212	17.4	185	16.7	185	14.2	151	10.6	232	17.0	228	18.3	223	11.0
Fresh weight (FW) (g)	7	1.23	0.10	1.27	0.10	1.00	0.10	0.83	0.11	1.31	0.08	1.09	0.13	1.29	0.14
	14	3.43	0.42	3.73	0.59	2.70	0.38	2.10*	0.19	3.38	0.14	3.18	0.30	3.60	0.33
	22	3.69	0.33	3.36	0.31	3.06	0.39	2.70	0.23	4.08	0.31	4.26	0.28	3.95	0.22
FW Total leaves (g)		93.9	10.0	87.3	11.1	76.6	12.4	53.8**	6.32	107.9	6.81	92.3	9.38	105	5.03
FW Bark (g)	12-13	0.64	0.06	0.59	0.06	0.43	0.05*	0.40**	0.03	0.74	0.06	0.57	0.06	0.67	0.03
FW Pith (g)	12-13	0.87	0.06	0.79	0.07	0.64	0.09	0.49**	0.05	0.98	0.08	0.75	0.10	1.00	0.05
FW Bark (g)	13-14	0.65	0.05	0.61	0.05	0.51	0.07	0.39*	0.04	0.72	0.06	0.59	0.06	0.61	0.05
FW Pith (g)	13-14	0.90	0.08	0.85	0.09	0.75	0.10	0.57	0.06	1.07	0.09	0.88	0.09	0.90	0.08
FW Total bark + pith (g)		51.9	5.60	50.0	6.86	47.7	8.28	31.8	4.16	67.9	5.05	56.9	6.88	61.0	4.08

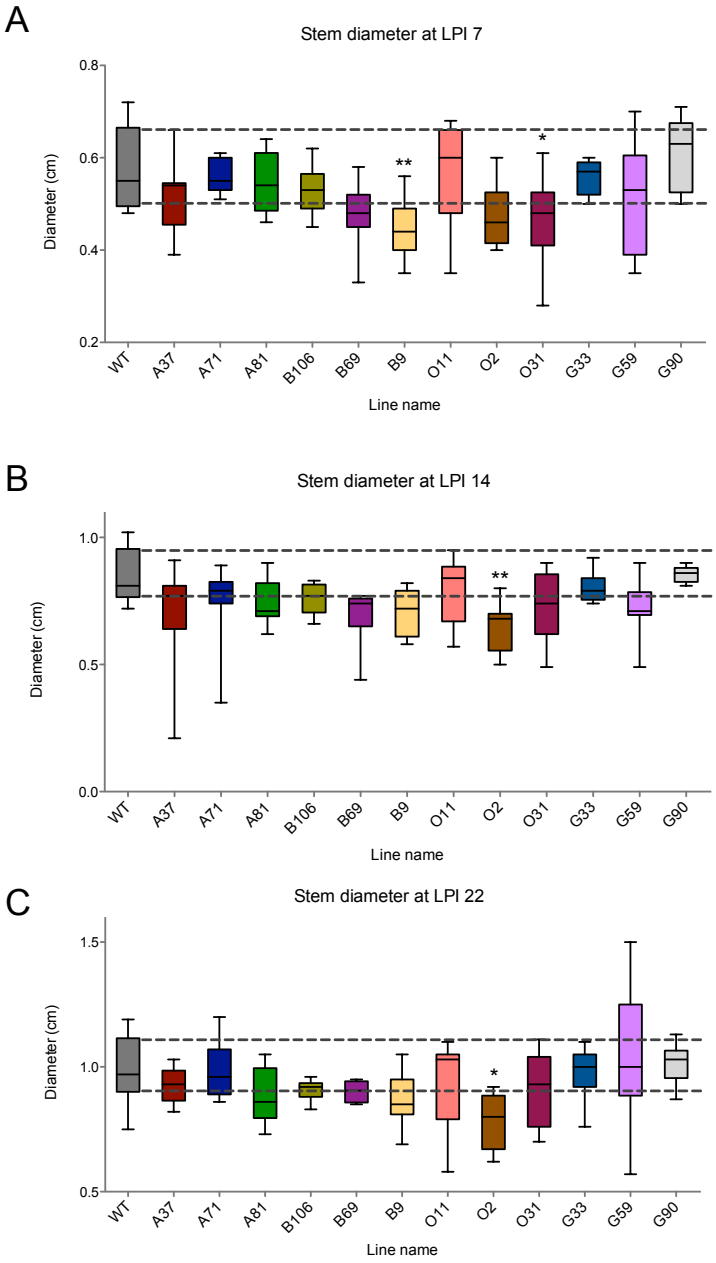

Figure 4-3. Box and whisker plots of stem diameters in wild-type (WT) and *BSP* knock-down and over-expression lines of *Populus* after 8 weeks of growth under LD conditions.
Stem diameters at A) LPI 7, B) LPI 14 and C) LPI 22. Boxes represent 95% confidence intervals (CI) and error bars represent the SE. Upper and lower limits of the 95% CIs for WT plants are represented by the dotted gray line. Significance determined by ANOVA followed by Dunnett's multiple comparison test ($\alpha = 0.05$) and denoted by * *P*-value < 0.05, ** *P*-value < 0.01, *** *P*-value < 0.001.

Photosynthesis and gas exchange measurements were performed in two independent experiments in the years 2011 and 2012 using a Li-Cor portable photosynthesis system (LI-6400). Each experiment is referred to by the year it was performed. For both experiments, plants were grown in a controlled chamber at 19°C under LD conditions. In 2011, measurements were taken at LPI 7, 14 and 22. In 2012, measurements were taken at LPI 7 and 22 to sample a greater number of individuals.

Experiments performed in 2011

For the experiment performed in 2011, leaves of the line B69 had significantly lower photosynthetic rates at LPI 22 than wild-type leaves (Table 4-6). Leaves of the line B106 had higher photosynthetic rates at LPI 7 and lower photosynthetic rates at LPI 22 than wild-type leaves. In leaves of the line A81, conductance was higher at LPI 7 than those of wild-type plants. For over-expression lines, significant differences were not detected in photosynthetic rates, conductance, intercellular CO_2 concentrations and transpiration rates (Table 4-7). Correlation analyses were performed using the means of the measurements in young leaves (LPI 7) and mature leaves (LPI 22) (Fig. 4-4). From these analyses, a significant correlation was detected in photosynthetic rates of young and mature leaves of knock-down lines and wild-type plants with $r = -0.949$, *P-value* = 0.001 and $r^2 = 0.901$ (Fig. 4-4 A). There was also a correlation detected in conductance means in the young and mature leaves of knock-down lines with $r = -0.791$, *P*-value = 0.034 and $r^2 = 0.626$ (Fig. 4-4 B). While not significant, there may be a correlation in transpiration rates for both knock-down lines ($r = 0.715$, *P*-value = 0.071, $r^2 = 0.511$; Fig. 4-4 D) and over-expression lines (*r*

= 0.595, *P*-value = 0.159, r^2 = 0.348; Fig. 4-4 H).

Table 4-6. Photosynthesis and gas exchange measurements of wild-type plants and *BSP* knock-down transgenic lines of *Populus tremula x Populus alba* in 2011.
Significance determined by ANOVA followed by Dunnett's multiple comparison test (α = 0.05) and denoted by * *P*-value < 0.05, ** *P*-value < 0.01, *** *P*-value < 0.001.

				Knock-down (RNAi) lines											
		Wild-type		B69		B106		B9		A71		A81		A37	
	LPI	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
Photosynthetic rate	7	4.74	0.51	7.06	1.25	9.53***	1.00	6.41	0.86	5.14	0.60	6.44	0.40	5.15	0.62
(μmol CO_2 $m^{-2}s^{-1}$)	14	11.1	0.50	9.26	0.99	9.08	0.39	10.0	0.67	9.23	0.88	10.3	0.60	10.8	0.47
	22	10.8	0.49	7.16**	0.62	5.53***	0.23	8.42	0.57	8.91	0.68	8.50	0.94	9.31	0.47
Conductance	7	0.29	0.02	0.35	0.02	0.30	0.01	0.36	0.03	0.29	0.03	0.39*	0.03	0.29	0.02
(mol H_2O $m^{-2}s^{-1}$)	14	0.62	0.02	0.61	0.03	0.63	0.02	0.65	0.03	0.60	0.03	0.61	0.03	0.57	0.03
	22	0.58	0.02	0.43	0.04	0.62	0.05	0.54	0.03	0.57	0.04	0.46	0.07	0.61	0.05
Intercellular CO_2 conc.	7	279.9	4.30	272	3.12	273	7.58	283.9	5.21	275.7	4.92	272.4	4.78	288.7	8.42
(μmol CO_2 mol^{-1})	14	264.4	3.78	275	6.08	273	6.42	283.4	5.15	270.9	7.87	257.3	3.08	275	5.46
	22	265	4.69	276	8.45	281	8.52	284.3	5.32	267.4	5.81	265.4	7.92	277.4	7.30
Transpiration rate	7	1.86	0.21	1.70	0.13	2.05	0.24	2.01	0.27	1.83	0.34	2.20	0.29	2.34	0.34
(mmol H_2O $m^{-2}s^{-1}$)	14	2.91	0.19	2.85	0.38	3.23	0.29	2.99	0.25	3.05	0.31	3.15	0.35	3.50	0.49
	22	2.80	0.19	2.35	0.49	3.17	0.30	2.68	0.30	3.06	0.38	2.79	0.40	3.72	0.48

Table 4-7. Photosynthesis and gas exchange measurements of wild-type plants and *BSP* over-expression transgenic lines of *Populus tremula x Populus alba* in 2011.
Significance determined by ANOVA followed by Dunnett's multiple comparison test (α = 0.05) and denoted by * *P*-value < 0.05, ** *P*-value < 0.01, *** *P*-value < 0.001.

		Over-expression lines															
		Wild-type		O11		O31		O2		G33		G59		G90	
	LPI	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
Photosynthetic rate (μmol CO_2 $m^{-2}s^{-1}$)	7	4.74	0.51	5.91	0.57	5.46	0.59	5.65	0.63	5.91	0.64	4.29	0.41	6.06	0.88
	14	11.1	0.50	9.98	0.70	11.4	0.78	9.32	0.44	11.0	0.66	10.5	0.69	11.7	0.48
	22	10.8	0.49	11.0	1.24	8.44	0.71	8.91	0.86	10.9	0.66	10.4	0.76	11.2	1.12
Conductance (mol H_2O $m^{-2}s^{-1}$)	7	0.29	0.02	0.34	0.03	0.29	0.03	0.35	0.05	0.27	0.02	0.28	0.03	0.33	0.04
	14	0.62	0.02	0.60	0.04	0.63	0.03	0.62	0.02	0.63	0.02	0.63	0.02	0.64	0.01
	22	0.58	0.02	0.62	0.05	0.65	0.04	0.59	0.04	0.60	0.04	0.63	0.04	0.51	0.04
Intercellular CO_2 conc. (μmol CO_2 mol^{-1})	7	279.9	4.30	283	5.62	269	8.04	278.7	7.02	275.3	5.44	281.6	8.39	269.5	4.04
	14	264.4	3.78	274	6.69	273	4.79	276.6	7.90	267.4	7.39	275.8	5.02	265.2	6.55
	22	265	4.69	273	6.86	278	6.67	271.8	7.18	267.4	6.93	274.5	6.46	257.9	3.92
Transpiration rate (mmol H_2O $m^{-2}s^{-1}$)	7	1.86	0.21	2.14	0.36	1.71	0.24	2.42	0.48	1.60	0.22	1.73	0.24	2.02	0.30
	14	2.91	0.19	2.91	0.36	2.88	0.24	3.56	0.35	2.95	0.26	2.92	0.20	2.87	0.22
	22	2.80	0.19	3.38	0.45	2.95	0.26	3.22	0.47	2.70	0.33	3.09	0.34	2.71	0.28

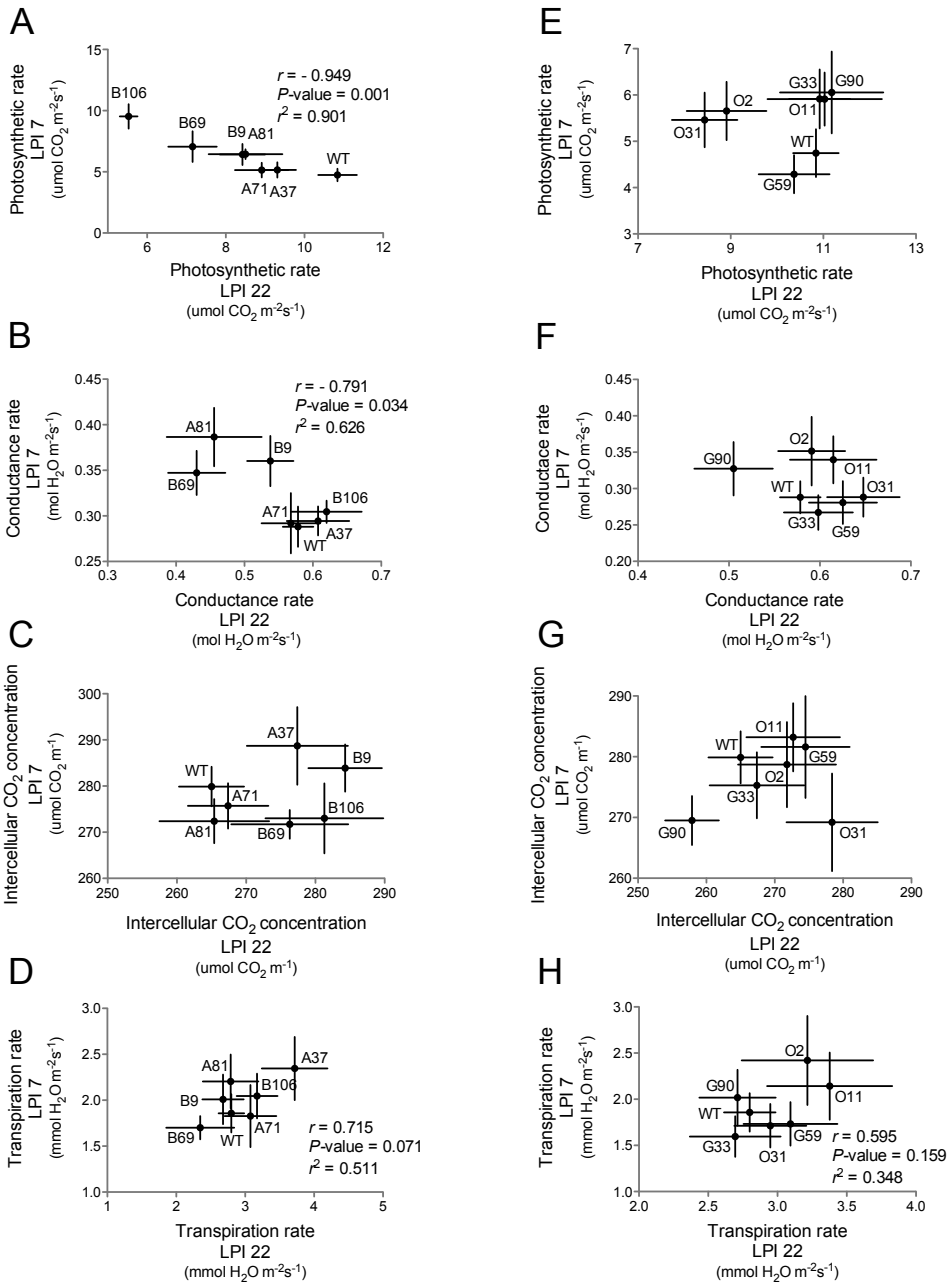

Figure 4-4. Means and standard errors of photosynthesis and gas exchange parameters from wild-type (WT) and transgenic *Populus* lines measured at LPI 7 and LPI 22 in an independent experiment conducted in 2011.
Pearson's correlation coefficient (*r*), *P*-value and r^2 indicated for significant and non-significant correlations below *P*-value 0.16. Means of photosynthetic rate, conductance, intercellular CO_2 concentration and transpiration rate for *BSP* knock-down lines (A-D) and *BSP* over-expression lines (E-H).

Experiments performed in 2012

For the experiment performed in 2012, leaves of the line B106 had significantly higher photosynthetic rates and lower intercellular CO_2 concentrations at LPI 22 compared to wild-type leaves (Table 4-8). Leaves of the line A71 had lower intercellular CO_2 concentrations at LPI 7 and leaves of the line A81 had higher photosynthetic rates at LPI 22 (Table 4-8). For over-expression lines, significant differences in photosynthesis and gas exchange measurements were not detected (Table 4-9). Correlation analyses of measurements taken in young leaves and mature leaves show a significant correlation for intercellular CO_2 concentrations in the knock-down lines (Fig. 4-5 C) as well as conductance and transpiration rates in over-expression lines (Fig. 4-5F, H). Notably, non-significant correlations with low *P*-values were calculated for photosynthetic rates (*P*-value = 0.124) and transpiration rates (*P*-value = 0.055) (Fig. 4-5 A, D).

Table 4-8. Photosynthesis and gas exchange measurements of wild-type plants and *BSP* knock-down transgenic lines of *Populus tremula x Populus alba* in 2012.
Significance determined by ANOVA followed by Dunnett's multiple comparison test ($\alpha = 0.05$) and denoted by * *P*-value < 0.05, ** *P*-value < 0.01, *** *P*-value < 0.001.

		Knock-down (RNAi) lines													
		Wild-type		B69		B106		B9		A71		A81		A37	
	LPI	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
Photosynthetic rate	7	6.17	0.35	7.44	0.24	7.14	0.37	5.73	0.66	7.22	0.47	7.44	0.33	6.87	0.17
(μmol CO_2 $m^{-2}s^{-1}$)	22	8.28	0.28	8.24	0.33	9.39*	0.33	8.55	0.39	8.99	0.37	9.47*	0.28	8.14	0.25
Conductance	7	0.21	0.02	0.18	0.03	0.16	0.02	0.18	0.03	0.17	0.03	0.17	0.03	0.21	0.03
(mol H_2O $m^{-2}s^{-1}$)	22	0.35	0.03	0.30	0.05	0.27	0.04	0.28	0.03	0.28	0.03	0.34	0.04	0.28	0.04
Intercellular CO_2 conc.	7	294	2.77	263	14.7	265	9.95	278	16.3	252*	18.6	262	10.4	277	11.4
(μmol CO_2 mol^{-1})	22	296	3.49	289	9.01	264*	9.77	283	7.22	275	9.39	281	7.77	278	10.2
Transpiration rate	7	0.98	0.06	0.83	0.10	0.93	0.14	0.87	0.10	0.91	0.16	1.00	0.18	0.96	0.10
(mmol H_2O $m^{-2}s^{-1}$)	22	1.31	0.07	1.15	0.13	1.26	0.18	1.16	0.08	1.22	0.14	1.47	0.19	1.15	0.12

Table 4-9. Photosynthesis and gas exchange measurements of wild-type plants and *BSP* over-expression transgenic lines of *Populus tremula x Populus alba* in 2012.
Significance determined by ANOVA followed by Dunnett's multiple comparison test (α = 0.05) and denoted by * *P*-value < 0.05, ** *P*-value < 0.01, *** *P*-value < 0.001.

				Over-expression lines											
		Wild-type		O11		O31		O2		O57		O66		O73	
	LPI	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
Photosynthetic rate	7	6.60	0.89	6.66	0.91	4.01	0.46	6.53	1.09	7.38	0.77	5.52	0.79	5.74	0.95
(μmol CO_2 $m^{-2}s^{-1}$)	22	10.5	1.58	9.26	0.99	10.3	0.84	10.1	1.40	11.2	0.71	10.4	0.97	10.7	0.87
Conductance	7	0.23	0.01	0.21	0.03	0.24	0.04	0.28	0.04	0.26	0.01	0.23	0.02	0.26	0.02
(mol H_2O $m^{-2}s^{-1}$)	22	0.28	0.03	0.30	0.03	0.29	0.03	0.38	0.04	0.33	0.04	0.33	0.04	0.38	0.01
Intercellular CO_2 conc.	7	304	5.89	296	10.7	316	7.41	312	4.68	309	6.78	320	9.59	316	5.77
(μmol CO_2 mol^{-1})	22	300	12.8	299	1.43	300	10.6	305	7.02	296	5.34	297	6.01	303	4.53
Transpiration rate	7	2.05	0.29	1.60	0.15	1.64	0.17	1.82	0.21	2.21	0.21	1.80	0.16	2.00	0.23
(mmol H_2O $m^{-2}s^{-1}$)	22	2.15	0.31	2.05	0.21	1.91	0.09	2.24	0.18	2.50	0.37	2.30	0.24	2.54	0.23

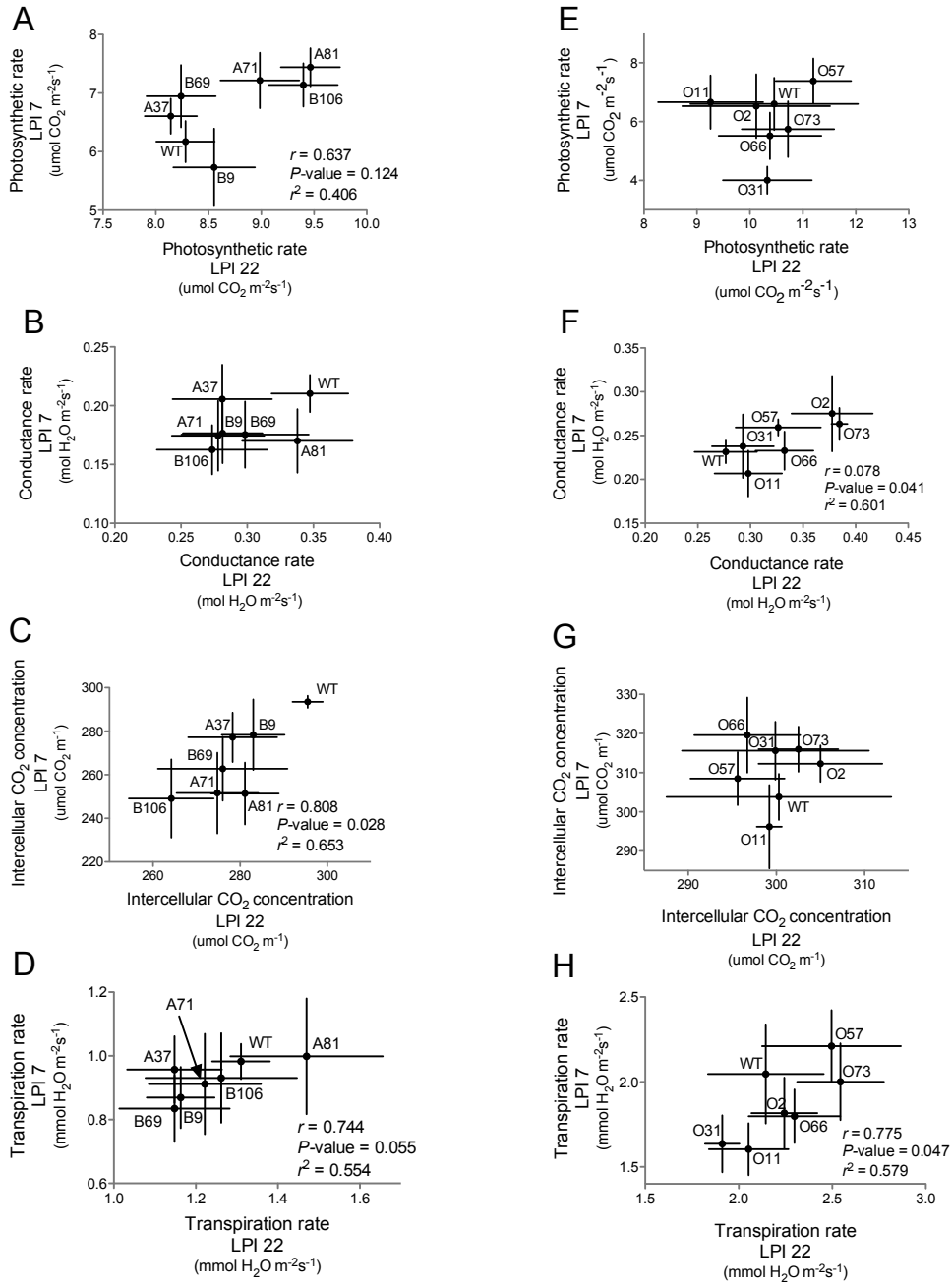

Figure 4-5. Means and standard errors of photosynthesis and gas exchange parameters from wild-type (WT) and transgenic *Populus* lines measured at LPI 7 and LPI 22 in an independent experiment conducted in 2012.
Pearson's correlation coefficient (*r*), *P*-value and r^2 indicated for significant and non-significant correlations below *P*-value 0.13. Means of photosynthetic rate, conductance, intercellular CO_2 concentration and transpiration rate for *BSP* knock-down lines (A-D) and *BSP* over-expression lines (E-H).

Source-sink Ratios

To assess a relationship between young and mature leaves as well as to compare both experiments, photosynthesis and gas exchange measurements were converted to a source-sink ratio of measurements for mature leaves (LPI 22) and young leaves (LPI 7; Table 4-10). Using this method, the significance of the relative change of photosynthesis and gas exchange within a whole plant can be tested. A ratio = 1 indicates that measured parameters are the same for young and mature leaves; a ratio < 1 indicates a parameter is higher in young leaves; and a ratio > 1 indicate a higher parameter in mature leaves. For the experiment conducted in 2011, the source-sink ratios of the photosynthetic rates were significantly lower than wild-type source-sink ratios in lines B69 and A81 (Table 4-10). This ratio reflects an increase in photosynthetic rates in young leaves and a decrease in mature leaves (Table 4-6). The source-sink ratio for intercellular CO_2 concentration was higher in line O31, reflecting a lower concentration in young leaves and a higher concentration in mature leaves (Table 4-10, Table 4-7). In 2012, higher source-sink ratios of intercellular CO_2 concentrations in lines A71 and A81 and higher conductance in A81 (Table 4-10). In both these lines, the change in source-sink ratios is due to lower intercellular CO_2 concentrations in young leaves (Table 4-8). There was a significantly lower source-sink ratio of photosynthetic rates in O31, resulting from lower values in young leaves (Table 4-9).

Table 4-10. Photosynthesis and gas exchange measurements of wild-type plants, *BSP* knock-down and *BSP* over-expression transgenic plants of *Populus tremula x Populus alba* presented as a ratio of values from mature leaves (LPI 22) and young leaves (LPI 7).
Top: Independent experiment performed in 2011; bottom: independent experiment performed in 2012. Significance determined by ANOVA followed by Dunnett's multiple comparison test (α = 0.05) and denoted by * *P*-value < 0.05, ** *P*-value < 0.01, *** *P*-value < 0.001.

2011

			Knock-down (RNAi) lines											
	Wild-type		B69		B106		B9		A71		A81		A37	
Ratio of mature/young leaves	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
Photosynthetic rate (µmol CO_2 $m^{-2}s^{-1}$)	1.83	0.20	1.17*	0.17	1.62	0.14	1.44	0.13	1.62	0.19	1.33*	0.19	1.55	0.09
Conductance to water (mol H_2O $m^{-2}s^{-1}$)	2.08	0.16	1.33	0.18	2.01	0.22	1.62	0.17	1.96	0.30	1.75	0.42	2.00	0.16
Intercellular CO_2 conc. (µmol CO_2 mol^{-1})	0.95	0.02	1.01	0.02	0.98	0.02	0.99	0.02	0.99	0.01	1.00	0.02	0.99	0.02
Transpiration rate (mmol H_2O $m^{-2}s^{-1}$)	1.83	0.20	1.17	0.17	1.62	0.14	1.44	0.13	1.62	0.19	1.33	0.19	1.55	0.09

			Over-expression lines											
	Wild-type		O11		O31		O2		G33		G59		G90	
Ratio of mature/young leaves	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
Photosynthetic rate (µmol CO_2 $m^{-2}s^{-1}$)	1.83	0.20	1.59	0.19	1.88	0.16	1.91	0.39	2.42	0.82	2.21	0.26	2.60	0.58
Conductance to water (mol H_2O $m^{-2}s^{-1}$)	2.08	0.16	1.92	0.25	2.36	0.27	2.46	0.58	2.16	0.42	2.31	0.40	1.72	0.28
Intercellular CO_2 conc. (µmol CO_2 mol^{-1})	0.95	0.02	0.97	0.02	1.15***	0.10	1.01	0.01	0.96	0.02	0.98	0.02	0.96	0.01
Transpiration rate (mmol H_2O $m^{-2}s^{-1}$)	1.83	0.20	1.59	0.19	1.88	0.16	1.91	0.39	1.79	0.30	1.79	0.27	1.46	0.19

2012

			Knock-down (RNAi) lines											
	Wild-type		B69		B106		B9		A71		A81		A37	
Ratio of mature/young leaves	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
Photosynthetic rate (µmol CO_2 $m^{-2}s^{-1}$)	1.32	0.06	1.39	0.09	1.39	0.13	1.41	0.10	1.46	0.10	1.59	0.10	1.17	0.03
Conductance to water (mol H_2O $m^{-2}s^{-1}$)	1.62	0.12	1.70	0.15	1.68	0.17	1.70	0.13	1.74	0.15	2.13*	0.16	1.33	0.07
Intercellular CO_2 conc. (µmol CO_2 mol^{-1})	1.00	0.01	1.05	0.02	0.99	0.04	1.04	0.05	1.13*	0.06	1.14**	0.05	1.00	0.02
Transpiration rate (mmol H_2O $m^{-2}s^{-1}$)	1.32	0.06	1.39	0.09	1.39	0.13	1.41	0.10	1.46	0.10	1.59	0.10	1.17	0.03

			Over-expression lines											
	Wild-type		O11		O31		O2		O57		O66		O73	
Ratio of mature/young leaves	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
Photosynthetic rate (µmol CO_2 $m^{-2}s^{-1}$)	1.32	0.06	1.30	0.16	1.23***	0.15	1.27	0.15	1.41	0.12	2.15*	0.42	2.01	0.27
Conductance to water (mol H_2O $m^{-2}s^{-1}$)	1.62	0.12	1.52	0.28	1.36	0.22	1.47	0.20	1.42	0.11	1.46	0.13	1.48	0.08
Intercellular CO_2 conc. (µmol CO_2 mol^{-1})	1.00	0.01	1.01	0.03	0.95	0.04	0.99	0.02	0.96	0.03	0.93	0.02	0.96	0.01
Transpiration rate (mmol H_2O $m^{-2}s^{-1}$)	1.32	0.06	1.30	0.16	1.23	0.15	1.27	0.15	1.27	0.07	1.28	0.07	1.29	0.05

Standardized comparison of photosynthesis and gas exchange in years 2011 and 2012

Absolute values of parameters were scaled to the maximum value for each year and statistically analyzed as described above (Table 4-11; Fig. 4-6). At LPI 7, the CIs for standardized photosynthetic rates of B106 and B69 for 2011 and 2012 appear higher than wild-type CIs (Fig. 4-6 A). Additionally, the CIs for lines A71 and A81 were higher and the CIs for O31 were lower in 2012 (Fig. 4-6 A). At LPI 22, the CIs for photosynthetic rates that are outside the range of the CI for wild-type plants display opposite trends in independent experiments performed in 2011 and 2012 (Fig. 4-6 B). In this case, the CIs of B106, B69, B9 and O31 are lower than wild-type CIs in 2011 and A81, B106 and O31 are higher in 2012 (Fig. 4-6 B). For the standardized conductance rates, notable differences in CIs are observed at LPI 7, for A71, A81, B106 and O11 in 2012 (Fig. 4-6 C) and, at LPI 22, A81 and B69 in 2011 (Fig. 4-6 D). For intercellular CO_2 concentrations, the data for 2012 show the CIs of all lines are below the wild-type CI at LPI 7 (Fig. 4-6 E) and again in 2012, the line A37, A71, B106, O11 and O31 are below the wild-type CI at LPI 22 (Fig. 4-6 F). Lastly, CIs for transpiration rates at LPI 7 in 2012 were lower for A71, O11 and O31 than CIs for wild-type plants (Fig. 4-6 G). At LPI 22, transpiration rates in 2011 were higher for A37, A71, B106, O11 and O2 and, in 2012, low A37, O11 and O31 (Fig. 4-6 H).

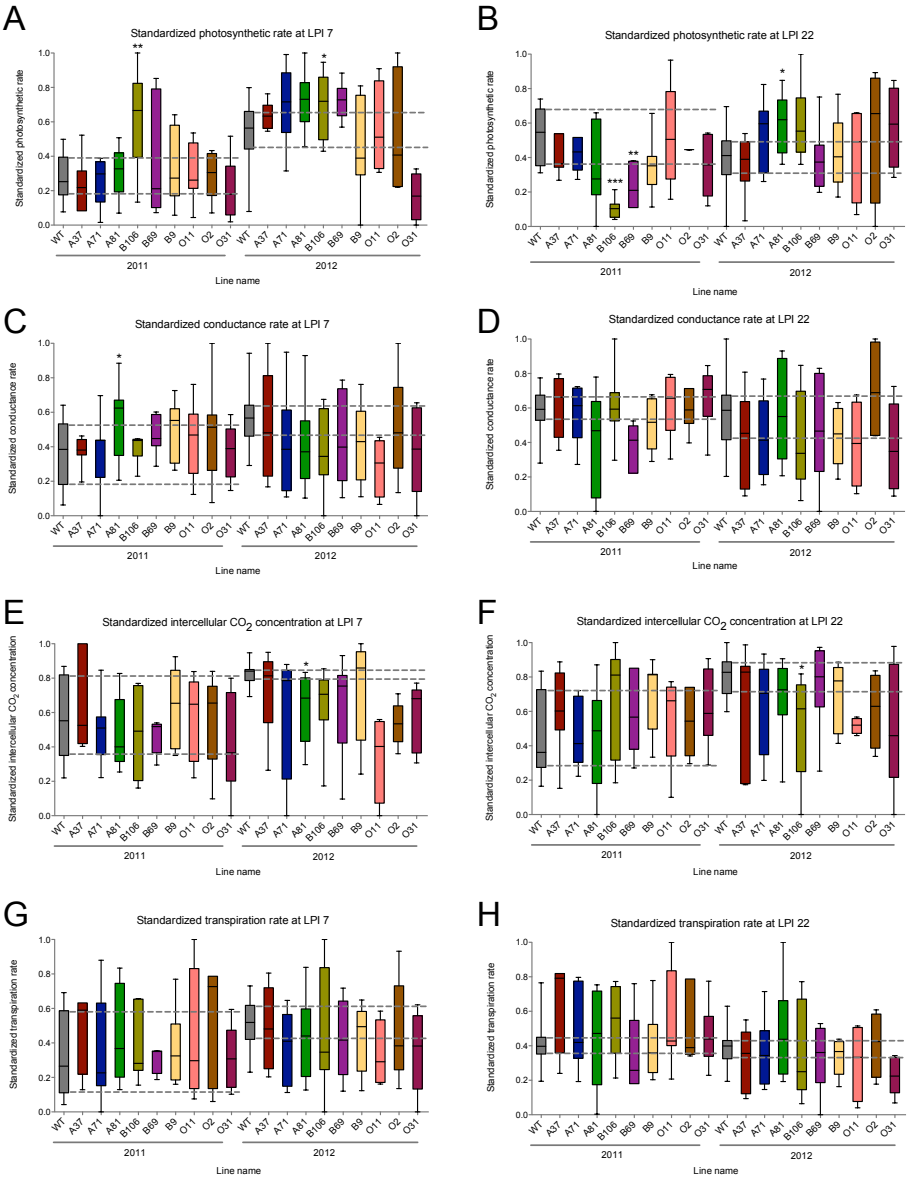

Figure 4-6. Box and whisker plots showing the standardized photosynthesis and gas exchange measurements in wild-type (WT) and *BSP* knock-down and over-expression lines of *Populus* after 8 weeks of growth under LD conditions for experiments performed in 2011 and 2012.
Photosynthetic rate at LPI 7 (A) and LPI 22 (B). Conductance rate at LPI 7 (C) and LPI 22 (D). Intercellular CO_2 concentration at LPI 7 (E) and LPI 22 (F). Transpiration rate at LPI 7 (G) and LPI (H). Boxes represent 95% confidence intervals (CI) and error bars represent the SE. Upper and lower limits of the 95% CIs for WT plants are represented by the dotted gray line. Significance determined by ANOVA followed by Dunnett's multiple comparison test ($\alpha = 0.05$) and denoted by * *P*-value < 0.05, ** *P*-value < 0.01, *** *P*-value < 0.001.

Discussion

The established roles of BSPs are that they store N for short (i.e. intra-seasonally) and long (i.e. inter-seasonally) time scales [5]. In this way, BSPs are part of a broader picture of mechanisms, called nitrogen use efficiency (NUE), that have evolved to optimize utilization of a limiting nutrient, nitrogen. Recent research suggests that aspects of NUE are linked to photosynthesis [171, 246, 270, 271]. To expand our understanding of the relationship between BSPs and NUE, I examined the physiological and photosynthetic performance of transgenic *Populus* knocking-down or over-expressing *BSP*. This work also elucidates the relationship between storage and remobilization of N and photosynthesis. The results indicate various significant differences in growth, photosynthetic rate and gas exchange in specific lines of transgenic plants (Table 4-11). Most notably, in two independent experiments, increased photosynthetic rates were observed in young leaves of lines with knocked-down levels of BSPs, whereas decreased photosynthetic rates were observed for the over-expression line O31. The possibility that storage capacity acts as a nutrient signal that feeds-back to photosynthesis could be a significant consideration for breeding and selection of *Populus* for bioenergy and bioremediation.

Table 4-11. Summary of significantly different means of variables for each transgenic line in all experiments.
Significance determined by ANOVA followed by Dunnett's multiple comparison test ($\alpha = 0.05$) and denoted by • *P*-value < 0.05, •• *P*-value < 0.01, ••• *P*-value < 0.001.

Line	Variable	Sig. level	Experiment	Table
A37	Internode length LPI 10-11	•	Growth assessment knock-down lines	4-4
	Internode length LPI 11-12	•	Growth assessment knock-down lines	4-4
	Fresh weight bark LPI 13-14	•	Growth assessment knock-down lines	4-4
A71	Height	•	*In vitro* growth assessment	4-2
	Intercellular CO_2 conc. LPI 7	•	Photosynthesis/gas exchange 2012 knock-down lines	4-8
	Intercellular CO_2 conc.	•	Photosynthesis/gas exchange ratios 2012	4-10
A81	Conductance LPI 7	•	Photosynthesis/gas exchange 2011 knock-down lines	4-6
	Photosynthetic rate LPI 22	•	Photosynthesis/gas exchange 2012 knock-down lines	4-8
	Photosynthetic rate	•	Photosynthesis/gas exchange ratios 2011	4-10
	Conductance	•	Photosynthesis/gas exchange ratios 2012	4-10
	Intercellular CO_2 conc.	••	Photosynthesis/gas exchange ratios 2012	4-10
B106	Fresh weight	••	*In vitro* growth assessment	4-2
	Photosynthetic rate LPI 22	••	Photosynthesis/gas exchange 2011 knock-down lines	4-6
	Photosynthetic rate LPI 7	••	Photosynthesis/gas exchange 2011 knock-down lines	4-6
	Photosynthetic rate LPI 22	•	Photosynthesis/gas exchange 2012 knock-down lines	4-8
	Intercellular CO_2 conc. LPI 22	•	Photosynthesis/gas exchange 2012 knock-down lines	4-8
B69	Photosynthetic rate LPI 22	••	Photosynthesis/gas exchange 2011 knock-down lines	4-6
	Photosynthetic rate	•	Photosynthesis/gas exchange ratios 2011	4-10
B9	Stem diameter LPI 7	••	Growth assessment knock-down lines	4-4
O2	Stem diameter LPI 14	••	Growth assessment over-expression lines	4-5
	Stem diameter LPI 22	•	Growth assessment over-expression lines	4-5
	Leaf area LPI 14	••	Growth assessment over-expression lines	4-5
	Fresh weight LPI 14	•	Growth assessment over-expression lines	4-5
	Fresh weight total leaves	••	Growth assessment over-expression lines	4-5
	Fresh weight bark LPI 12-13	••	Growth assessment over-expression lines	4-5
	Fresh weight pith LPI 12-13	••	Growth assessment over-expression lines	4-5
	Fresh weight bark LPI 13-14	•	Growth assessment over-expression lines	4-5
O31	Stem diameter LPI 7	•	Growth assessment over-expression lines	4-5
	Conductance	•••	Photosynthesis/gas exchange ratios 2011	4-10
	Photosynthetic rate	•••	Photosynthesis/gas exchange ratios 2012	4-10

The results and conclusions of the experiments conducted depend heavily on the transgenic lines chosen during the screening process. There are relevant considerations regarding both knock-down and over-expression lines that offer context for the difficulty in observing consistent and significant results. In particular, of the 40 knock-down lines screened only a single strong knock-down line survived. Additional lines were identified but died *in vitro* soon after screening. This is strong evidence that BSPs are necessary for survival and/or regeneration. As for weaker knock-down lines, the low levels of BSPs may be sufficient to support wild-type-like growth, photosynthesis and gas exchange under the experimental conditions.

For trees transformed with over-expression constructs, unexpectedly low levels of BSPs under LD conditions could reflect catabolism of BSPs. The accumulation patterns of the over-expression-*GFP* lines support the hypothesis that BSPs are being broken down. GFP was detected using GFP antibodies while no visible BSPs were detected in these samples using BSP antibodies (Fig. 4-1, 4-2). The presence of GFP on the blots indicates that the GFP tagged BSP was indeed produced. Decreased GFP levels following SD conditions suggest a silencing or feedback mechanism. In all, these results point to post-translational regulation of BSP accumulation. The lines in which GFP is cleaved from BSP A could be a useful resource for investigating the transport and breakdown of BSPs [272, 273]. With fluorescence microscopy, it may be possible to determine if the fusion protein is delivered to a storage vacuole then broken down or if the fusion protein has disrupted transport due to the GFP. If cleavage products could be isolated, the fragmented peptides could be identified which may give clues about the types of proteases

involved in BSP catabolism [274].

While significantly different growth parameters were observed for 8 transgenic lines (i.e. internode lengths and fresh weight in A37 plants, height *in vitro* in A71 plants, fresh weight *in vitro* in B106 plants, stem diameter in B9 plants, stem diameter, leaf area and fresh weights in O2 plants and stem diameter in O31 plants) there were no parameters consistently different across all knock-down or over-expression lines. Additional experiments that assess the effect of altered BSP levels are necessary to determine the robustness of the results observed in this study. That said, there is very limited evidence that BSPs play a role in biomass allocation in transgenic lines based upon the observation that both knock-down and over-expression lines had smaller stem diameters at three stem locations (Fig. 4-3). If reduced stem diameter is a true effect of altered BSP levels, then constitutive over-expression of *BSP A* in *Populus* trees results in a phenotype similar to plants with knocked-down expression. Nonetheless, a change in biomass allocation is consistent with observations of an increase in LAs and internode lengths and decrease in stem FWs for 2 anti-sense *BSP* knock-down lines [263]. While the findings by Black are not exactly replicated in the 6 RNAi *BSP* knock-down lines, biomass allocation may be governed by storage ability or signaling thereof [263]. It is important to acknowledge the difference between anti-sense methods with full-length transcripts for creating *BSP* knock-downs and the RNAi technology with regions more specific to *BSPs* used here. It is quite possible that anti-sense *BSP* transgenic plants are knocking down additional *NP-like* proteins, also involved in storage [275]. This would explain the striking differences in biomass allocation and N uptake observed

by Black [263]. The WIN-like proteins and other NP-like proteins could compensate for reduced BSP levels. Future studies should include the effect of BSP levels on other *NP-like* genes and proteins.

Source-sink ratios were computed to test for relative changes in photosynthesis and gas exchange to evaluate dynamics within a whole plant as opposed to significant means at a single location. The source-sink ratios were significantly different for photosynthesis and/or gas exchange parameters for lines A81 and O31 in experiments conducted in both 2011 and 2012 (Table 4-10). Interestingly, these lines represent the strongest knock-down (A81) and over-expressing (O31) lines. This could reflect the importance of relative changes in storage capacity within a plant. Storage capacity in a given tissue or location contributes to the ability to respond to abiotic stresses in evergreen tree species [276].

Comparisons of standardized photosynthetic and gas exchange measurements in 2011 and 2012 show higher photosynthetic rates at LPI 7 of the knock-down lines and lower photosynthetic rates in the over-expression line O31. This establishes a relationship between storage and photosynthetic rates. Observing changes in photosynthetic rates is consistent with other studies that have discovered a connection between N uptake, assimilation and remobilization [171, 246, 271]. The changes in photosynthetic rates were observed in young leaves and not in mature leaves perhaps because young leaves are more dependent on transient storage than mature leaves and/or young leaves are more sensitive to changes in nutrient signaling or metabolism that mature leaves. Young leaves being dependent on transient storage is consistent with the presence of BSP related storage proteins found in younger leaves of *Populus*

where they possibly provide transient storage for sink tissues [10]. An interesting consideration is whether plants with altered *BSP* expression influence the regulation of related NP-like proteins and how this contributes to nutrient signaling. The findings point to a possible link between BSPs and photosynthesis, supporting a role for BSP in nutrient signaling in sink tissues.

Future considerations

The aim of this study was to find evidence of changes in growth, photosynthesis and gas exchange in plants with altered BSP levels, possibly due to perturbed nutrient signaling or nutrient metabolism. Methods of investigation that specifically focus on metabolism, such as identifying metabolites and amino acids in tissues, might be a more telling approach for future investigations. Since amino acids are the N currency within trees and can reflect environmental stresses, this seems particularly suitable [192, 277-279].

In conjunction with other methods, another possibility for future studies is to investigate older plants for differences in signaling and metabolism of transgenic plants as the effects of altered storage may become more distinct over time. For example, a reduction in storage ability over multiple years could impact the resorption efficiency in the fall and resource availability for spring remobilization [5, 257]. For example, in *Quercus* trees, May *et al.* prevented nutrient resorption by defoliation and they reported reduced stem growth, foliar biomass and acorn yield per plant in the years after defoliation [280]. Examining plants at critical developmental stages, like flowering and seed set, is another way of assessing cumulative effects of altered storage capacity. Seed proteins in *Populus* share homology with BSPs and

related proteins and there could be a very direct relationship between storage proteins and seed development [281]. Furthermore, the *BSP A* promoter is activated in developing transgenic tobacco seeds [134].

Stress conditions can be useful events for observing significant differences in growth, metabolism, signaling and/or regulation [282, 283] [282]. Studies implicate a role for BSPs in responding to stress. For example, drought and wounding are conditions known to induce BSP accumulation [6, 10, 14]. Additionally, BSPs are also regulated by methyl jasmonate exposure which is involved in signaling plant defense responses and related metabolic regulation [15, 260, 284].

Storage location may be an important component to understanding BSPs involvement in growth and development. A better understanding of the above- and below-ground dynamics of storage protein accumulation would expand our knowledge of nutrient cycling and storage in whole plants. There has been little work investigating the BSPs in roots although BSPs accumulate in roots when N availability increases [262]. Along these lines, storage location can contribute to the ability to respond to herbivory in evergreen trees and other plant species by remobilizing nutrients away from tissues being eaten [276, 285, 286]. This could be an informative way to study an effect of storage site on growth and development.

Conclusions

In nutrient limiting environments, uncoupling nutrient utilization from nutrient uptake allows for the optimum use or storage of nutrients while promoting the conservation of nutrients in response to stresses. While BSPs play a dominant role in seasonal nutrient storage, their importance in growth, development, photosynthesis

and gas exchange in young trees grown under LD conditions remains obscure. BSPs may be involved in nutrient signaling and this has important ramifications for understanding the mechanisms contributing to NUE and, therefore, tree improvement. There are interesting possibilities for future investigations using transgenic trees with altered BSP levels particularly related to extended growth and stress responses.

Conclusions

The work performed in each chapter advances our understanding of the adaptive mechanisms within plants to recycle and store nutrients in response to environmental fluctuations. Specifically, the advancements involve elucidating the roles of Bark Storage Proteins (BSPs) and nucleoside phosphorylase-like (NP-like) proteins in nutrient cycling using phylogenetic analyses, expression analyses and through the creation of transgenic plants. All experiments were conducted using *Populus*, which is a valuable genus for agro-forestry, biofuels, phytoremediation and C sequestration [132, 249-251]. For these applications, it is crucial to discover ways to increase the sustainability and stress tolerance of *Populus* as fertilizer costs increase and the predicted increases in abiotic and biotic stresses due to climate change [237, 247]. Since BSPs and VSPs are known to contribute to nutrient storage and induced in responses to stresses, NP-like proteins provide an ideal system to investigate nutrient sustainability and stress-induced nutrient storage strategies [5, 6, 15].

The first chapter characterized NP-like proteins in *Populus* and examined the evolution of NP-like proteins in the plant kingdom. Major findings of this chapter were that NP-like proteins are well conserved throughout the plant kingdom with *Populus* having the largest family of these proteins. The expansion of the NP-like protein family in *Populus* is consistent with more recent and ancient duplication events. Expression analysis of *NP-like* genes supports the phylogenetic results, evident from the similar expression patterns observed for each gene subfamily. Both phylogenetic evidence and expression patterns support a role for *NP 157* as a

nucleoside phosphorylase. Based on promoter analyses I was also able to infer that this family of genes is transcriptionally regulated by light, stress/pathogenesis and phytohormones elements.

Chapter two established a set of reference genes in *Populus* for adequate normalization of qPCR data. This was necessary to ensure the accuracy of experiments performed in chapter one and three, yet the utility of the work can be applied to other qPCR studies examining expression in *Populus*. Two widely used analysis programs, BestKeeper and geNorm^{PLUS}, were used to find stable reference genes in two species of *Populus* and in four commonly studied tissue types: shoot tips, young leaves, mature leaves and bark.

The objective of chapter three was to determine the metabolic regulation of *NP-like* genes using excised shoot assays. The studies were predicated on evidence that *BSP* genes are regulated by nitrogen (N) and built upon that work by exploring a critical junction of C and N pathways: the tricarboxylic acid (TCA) cycle and N assimilation. One important implication of this work is that storage protein expression is regulated by metabolites in a tissue-specific manner. Another significant result of this work is that amino acids, N pathways and gamma-aminobutyric acid (GABA) are important regulators of *NP-like* genes. Evidence for differential expression of members within *NP-like* gene subfamilies illustrates that these proteins may have overlapping but not redundant function, which is consistent with analyses in chapter one suggesting that some *NP-like* genes are currently under selection pressure. The differential expression patterns may be evidence of subfunctionalization, also observed from data in chapter one. Lastly, observing high *NP 157* transcript levels in

response to GABA treatments supports a functional role for this storage protein progenitor gene and is consistent with expression of mammalian *NP* genes involved in nucleoside salvage [21-23]. Further, GABA treatments provide a condition in which to more specifically examine any enzymatic activity of NP 157.

The importance of BSPs in growth, development, photosynthesis and gas exchange in transgenic *Populus* trees was examined in chapter four. This study provides tantalizing preliminary evidence that BSPs may have a role in nutrient signaling capable of modulating photosynthesis in young leaves. There is also evidence that BSPs may influence biomass allocation, confirming earlier research. These transgenic plants could provide a useful resource for further studies investigating seasonal storage dynamics.

Until now, there has never been a comprehensive examination of NP-like proteins in any plant species. This is likely because there has been no evidence of their involvement in nucleoside salvaging [29, 31, 33-35]. Yet the phylogeny in chapter one suggests that NP-like proteins are highly conserved in the plant kingdom. While their function in plants is still unknown, it was exciting to find expression patterns (in chapter one) consistent with a salvage function for the progenitor gene *NP 157* and even more exciting to find conditions (GABA treatments performed in chapter three) to further study *NP 157*.

As a whole, the chapters show that the *NP-like* gene family in *Populus* is a model of functional evolution due to the large expansion of the family and their expression divergence. The novel discoveries of my dissertation also represent a foundation on which future studies can build to better understand the evolutionary

history and regulation of *NP-like* proteins. The transgenic trees can also be utilized for broader evaluation of the effect of altered BSP levels. *Populus* may also be an appropriate species to determine the function of NP-like proteins in plants through further study of NP 157. This is because results from this work provide information on where this gene is expressed and under what conditions, which is a prerequisite to tackling larger questions surrounding function. In a broader context, this body of work has revealed greater insight into the regulation of the adaptive remobilization and storage processes.

Appendices

Appendix A: Supplemental Material for Chapter 1

Table A-1. QPCR reference gene symbol, gene name, Phytozome locus name, primer sequences, size of amplification product, annealing temperature, PCR efficiency and the tissue type that the reference gene was used for normalization calculations.

Gene symbol	**Gene name**	**Phytozome locus v2.2**	**Primers (5'-3')**	**Product size (bp)**	**Annealing temp (°C)**	**PCR efficiency**	Tissue type(s)
TIP4-like	TIP4-like	POPTR_0009s09620	F: GCTGATAATGGGGTGTCG R: CAACTCTAAGCCAGAATCGC	88	57	1.969	YL, ML
CYC063	Cyclophilin	POPTR_0005s26170	F: CCTGGCACTAATGGGTCTCAG R: CACAACTCTTCCGAACACCAC	87	52	1.98	ML
PT1	Unknown protein	POPTR_0014s03160	F: GCGGAAAGAAAAACTGCAAG R: TGACAGCACAGCCCAATAAG	126	57	2.025	ST, YL
CDC2	Cell division control protein 2	POPTR_0004s14080	F: ATTCCCCAAGTGGCCTTCTAAG R: TATTCATGCTCCAAAGCACTCC	137	57	2.04	ST, B
ACT2	Actin 2	POPTR_0001s31700	F: TTCTACAAGTGCTTTGATGGTGAGTTC R: CTATTCGATACATAGAAGATCAGAATGTTC	159	52	1.935	YL, B

Tissue types: ST, shoot tips; YL, young leaves; ML, mature leaves; B, bark.

Table A-2. Identifiers from Fig. 1-4 corresponding to the Phytozome locus or NCBI sequence identifier.

Tree identifier	Phytozome or NCBI identifier
Aquilegia coerulea (1)	AcoGoldSmith_v1_007822m
Aquilegia coerulea (2)	AcoGoldSmith_v1_008107m
Arabidopsis lyrata (1)	354336
Arabidopsis lyrata (2)	491863
Arabidopsis lyrata (3)	492412
Arabidopsis thaliana (1)	AT4G24340
Arabidopsis thaliana (2)	AT4G24350
Arabidopsis thaliana (3)	AT4G28940
Brachypodium distachyon (1)	Bradi1g51710
Brachypodium distachyon (2)	Bradi2g07760
Brassica rapa (1)	Bra011070
Brassica rapa (2)	Bra013793
Brassica rapa (3)	Bra013794
Bruguiera gymnorhiza (1)	gi_171451984
Capsella rubella (1)	Carubv10005161m
Capsella rubella (2)	Carubv10007645m
Capsella rubella (3)	Carubv10007687m
Carica papaya (1)	evm_TU_supercontig_128_63
Citrus clementina (1)	clementine0_9_013074m
Citrus clementina (2)	clementine0_9_027744m
Citrus sinensis (1)	orange1_1g019168m
Cucumis sativus (1)	Cucsa_332440
Eucalyptus grandis (1)	Egrandis_v1_0_046643m
Eucalyptus grandis (2)	EucgrC01273
Eucalyptus grandis (3)	EucgrC02910
Eucalyptus grandis (4)	EucgrC02911
Eucalyptus grandis (5)	EucgrC02912
Eucalyptus grandis (6)	EucgrC03808
Eucalyptus grandis (7)	EucgrD02406
Eucalyptus grandis (8)	EucgrL02649
Glycine max (1)	Glyma06g08860
Glycine max (2)	Glyma07g09200
Glycine max (3)	gi_255638967
Hordeum vulgare (1)	gi_326503550
Hordeum vulgare (2)	gi_326512570
Hordeum vulgare (3)	gi_326526309
Linum usitatissimum (1)	Lus10023668
Linum usitatissimum (2)	Lus10028834
Linum usitatissimum (3)	Lus10034939
Malus x domestica (1)	MDP0000319502
Malus x domestica (2)	MDP0000379203
Malus x domestica (3)	MDP0000640395
Malus x domestica (4)	MDP0000720196
Manihot esculenta (1)	cassava4_1_010849m
Manihot esculenta (2)	cassava4_1_011513m
Manihot esculenta (3)	cassava4_1_012024m
Manihot esculenta (4)	cassava4_1_012172m
Manihot esculenta (5)	cassava4_1_012283m
Manihot esculenta (6)	cassava4_1_016574m
Manihot esculenta (7)	cassava4_1_021711m
Manihot esculenta (8)	cassava4_1_022576m
Manihot esculenta (9)	cassava4_1_022755m
Manihot esculenta (10)	cassava4_1_029121m
Medicago truncatula (1)	AC225517_12

Medicago truncatula (2)	AC233783_17
Medicago truncatula (3)	AC233783_19
Medicago truncatula (4)	AC233783_22
Mimulus guttatus (1)	mgv1a009063m
Mimulus guttatus (2)	mgv1a009527ms
Oryza sativa (1)	LOC_Os05g13970
Oryza sativa (2)	LOC_Os06g02210
Oryza sativa (3)	Os01g12940
Oryza sativa Japonica (1)	gi_115465984
Picea sitchensis (1)	gi_148906549
Populus deltoides (1)	gi_728986
Populus deltoides (2)	gi_728987
Populus tremula x Populus alba (1)	gi_284519832
Populus tremula x Populus alba (2)	gi_284519834
Populus tremula x Populus alba (3)	gi_284519844
Populus tremula x Populus alba (4)	gi_284519846
Populus trichocarpa BSP A	POPTR_0013s10380
Populus trichocarpa BSP B	POPTR_0013s10370
Populus trichocarpa BSP C	POPTR_0013s10350
Populus trichocarpa NP 860	POPTR_0008s02860
Populus trichocarpa NP 870	POPTR_0008s02870
Populus trichocarpa NP 880	POPTR_0008s02880
Populus trichocarpa PNI 288	POPTR_0019s07690
Populus trichocarpa VSP 157	POPTR_0006s16610
Populus trichocarpa VSP 425	POPTR_0013s07810
Populus trichocarpa VSP 840	POPTR_0013s07840
Populus trichocarpa VSP 87A	POPTR_0013s07850
Populus trichocarpa VSP XIII	POPTR_0013s07800
Populus trichocarpa WIN4	POPTR_0423s00200
Populus trichocarpa x Populus deltoides (1)	gi_118489218
Populus trichocarpa x Populus deltoides (2)	gi_12658404
Populus trichocarpa x Populus deltoides (3)	gi_309839
Populus x canadensis (1)	gi_4775662
Prunus persica (1)	ppa018203m
Prunus persica (2)	ppa023149m
Prunus persica (3)	ppa023740m
Prunus persica (4)	ppb023416m
Ricinus communis (1)	27428_t000006
Ricinus communis (2)	30073_t000077
Ricinus communis (3)	30073_t000078
Ricinus communis (4)	29794_t000126
Ricinus communis (5)	29912_t000213
Ricinus communis (6)	30073_t000018
Ricinus communis (7)	30073_t000069
Ricinus communis (8)	30073_t000070
Ricinus communis (9)	30073_t000071
Ricinus communis (10)	30073_t000072
Ricinus communis (11)	30073_t000073
Selaginella moellendorffii (1)	431848
Selaginella moellendorffii (2)	441751
Setaria italica (1)	Si003754m
Setaria italica (2)	Si006840m
Setaria italica (3)	Si024797m
Sorghum bicolor (1)	Sb03g000850
Sorghum bicolor (2)	Sb03g000860
Sorghum bicolor (3)	Sb09g007440
Sorghum bicolor (4)	Sb10g000960
Thellungiella halophila (1)	Thhalv10025575m
Thellungiella halophila (2)	Thhalv10025609m

Vitis vinifera (1)	GSVIVT01015354001
Vitis vinifera (2)	GSVIVT01034674001
Vitis vinifera (3)	GSVIVT01035644001
Zea mays (1)	GRMZM2G051949
Zea mays (2)	GRMZM2G099678

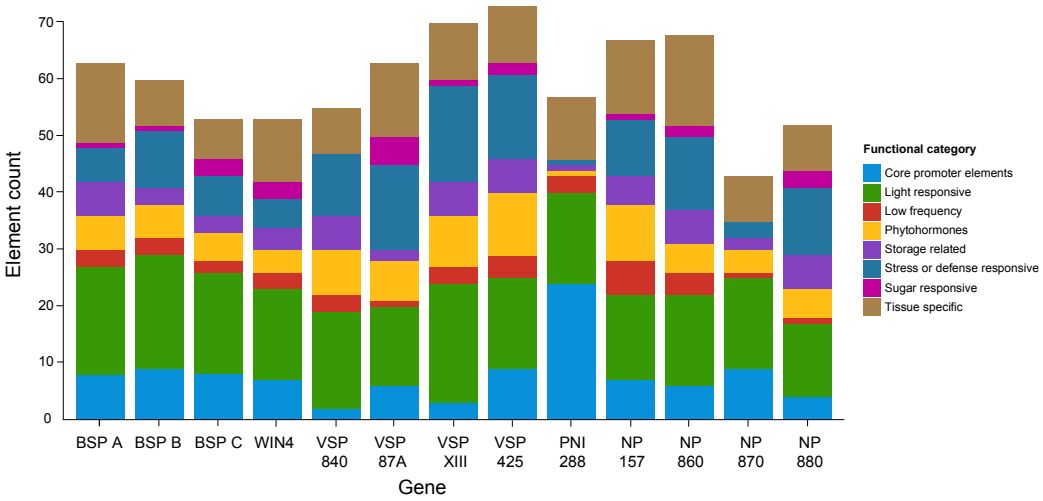

Figure A-1. Bar plot of the distributions of *cis*-regulatory elements in the 500 bp upstream promoter regions of *NP-like* genes in *Populus trichocarpa*.

Appendix B: Supplemental Material for Chapter 2

BestKeeper input and output data

Cq distributions, descriptive statistics, pairwise correlation analysis tables, correlation coefficient values for first and second analyses

Mean Cq of technical replicates for each sample

Populus trichocarpa

Shoot tips

18S rRNA HKG 1	*ACT2* HKG 2	*ANT* HKG 3	*CDC2* HKG 4	*CYC063* HKG 5	*PT1* HKG 6	*TIP4-like* HKG 7	*UBQ7* HKG 8	Sample
6.060	16.330	18.810	19.680	16.360	20.330	21.200	17.800	LD Rep 1
6.040	15.790	18.610	19.510	15.980	19.870	20.860	17.490	LD Rep 2
6.060	15.780	18.560	19.590	15.890	19.870	20.910	17.530	LD Rep 3
6.280	16.030	19.590	19.640	15.860	19.990	20.930	17.650	SD 3 weeks Rep 1
6.050	15.840	19.160	19.630	16.840	19.900	20.960	17.670	SD 3 weeks Rep 2
5.760	16.090	19.520	19.690	15.990	19.930	20.890	17.630	SD 3 weeks Rep 3
5.500	16.620	20.440	20.080	15.290	20.250	21.080	17.610	SD 6 weeks Rep 1
4.520	17.150	20.710	20.150	15.820	20.500	21.190	17.800	SD 6 weeks Rep 2
5.130	16.810	20.710	19.870	15.960	20.410	21.080	17.480	SD 6 weeks Rep 3
5.130	16.230	20.250	19.780	15.050	20.100	20.850	16.930	SD 8 weeks Rep 1
5.110	15.730	19.600	19.510	14.840	19.850	20.520	16.980	SD 8 weeks Rep 2
4.070	15.760	19.830	19.560	14.870	19.960	20.590	17.100	SD 8 weeks Rep 3
5.920	16.690	21.360	20.070	15.600	20.320	20.470	17.630	SD 12 wk + LT Rep 1
6.600	17.390	21.310	20.750	16.130	20.920	20.930	18.530	SD 12 wk + LT Rep 2
6.150	16.860	20.510	20.160	15.760	20.290	20.500	18.070	SD 12 wk + LT Rep 3

Young leaves

18S rRNA HKG 1	*ACT2* HKG 2	*ANT* HKG 3	*CDC2* HKG 4	*CYC063* HKG 5	*PT1* HKG 6	*TIP4-like* HKG 7	*UBQ7* HKG 8	Sample
5.890	16.160	18.900	19.530	15.910	19.400	20.960	17.140	LD Rep 1
5.820	15.650	19.010	19.400	15.600	19.320	21.080	17.160	LD Rep 2
5.800	15.070	18.410	19.270	15.420	19.200	20.950	17.200	LD Rep 3
5.430	15.280	19.640	19.010	14.840	18.960	21.070	16.440	SD 3 weeks Rep 1
5.020	15.760	20.310	19.590	15.140	19.390	21.460	16.640	SD 3 weeks Rep 2
5.100	15.530	20.180	19.430	15.220	19.250	21.320	16.770	SD 3 weeks Rep 3
5.500	16.880	25.580	20.750	14.570	20.050	22.000	16.870	SD 6 weeks Rep 1
5.720	17.340	25.150	20.990	14.770	19.930	22.100	16.980	SD 6 weeks Rep 2
5.550	17.690	26.170	21.14	15.220	20.510	22.640	17.390	SD 6 weeks Rep

			0					3
			21.01					SD 8 weeks Rep
6.720	17.050	25.850	0	14.840	19.910	21.930	16.750	1
			21.17					SD 8 weeks Rep
6.620	16.980	26.000	0	15.060	19.920	22.000	16.870	2
			20.99					SD 8 weeks Rep
6.410	17.030	25.680	0	14.960	20.030	21.960	16.920	3
			21.10					
7.070	17.570	27.420	0	15.240	19.540	21.380	17.290	SD 12 wk + LT Rep 1
			21.08					
6.630	17.890	26.960	0	15.600	19.450	21.460	17.300	SD 12 wk + LT Rep 2
			21.38					
6.690	18.040	30.220	0	15.730	19.510	21.230	17.420	SD 12 wk + LT Rep 3

Mature leaves

18S rRNA	*ACT2*	*ANT*	*CDC2*	*CYC063*	*PT1*	*TIP4-like*	*UBQ7*	
HKG 1	HKG 2	HKG 3	HKG 4	HKG 5	HKG 6	HKG 7	HKG 8	Sample
			19.93					
6.330	17.120	24.820	0	16.440	21.220	21.780	17.290	LD Rep 1
			20.10					
6.740	17.200	25.730	0	15.860	21.220	21.880	16.980	LD Rep 2
			19.70					
6.570	16.750	25.020	0	15.930	21.130	21.740	16.970	LD Rep 3
			19.59					SD 3 weeks Rep
5.780	16.660	24.760	0	16.220	21.120	22.000	16.740	1
			19.53					SD 3 weeks Rep
5.880	16.190	24.530	0	16.370	20.850	21.910	17.090	2
			19.03					SD 3 weeks Rep
5.900	15.830	23.960	0	16.060	20.680	21.640	16.910	3
			19.70					SD 6 weeks Rep
6.510	16.420	24.570	0	15.880	20.490	21.500	16.630	1
			19.94					SD 6 weeks Rep
6.230	16.710	20.080	0	15.940	20.640	21.850	16.970	2
			19.80					SD 6 weeks Rep
6.650	16.780	25.580	0	15.750	20.680	21.700	16.940	3
			20.18					SD 8 weeks Rep
6.250	16.970	26.820	0	16.100	21.060	21.950	16.770	1
			19.52					SD 8 weeks Rep
6.170	15.960	25.580	0	15.900	20.540	21.440	16.730	2
			19.81					SD 8 weeks Rep
5.390	16.320	26.010	0	15.900	20.820	21.590	16.710	3
			20.70					
7.800	17.940	27.050	0	15.630	21.010	21.320	17.330	SD 12 wk + LT Rep 1
			21.10					
8.610	18.530	34.890	0	15.350	21.020	21.690	17.400	SD 12 wk + LT Rep 2
			21.66					
9.460	19.360	33.570	0	15.920	21.540	21.930	17.790	SD 12 wk + LT Rep 3

Bark

18S rRNA	*ACT2*	*ANT*	*CDC2*	*CYC063*	*PT1*	*TIP4-like*	*UBQ7*	
HKG 1	HKG 2	HKG 3	HKG 4	HKG 5	HKG 6	HKG 7	HKG 8	Sample
			18.53					
5.500	15.180	20.380	0	15.360	20.300	20.490	16.880	LD Rep 1
			18.30					
5.560	14.810	20.270	0	15.200	20.140	20.450	16.640	LD Rep 2
			17.93					
5.500	14.450	19.720	0	14.840	20.060	20.160	16.260	LD Rep 3
			17.78					SD 3 weeks Rep
5.650	14.490	19.130	0	15.600	19.990	20.500	16.700	1
			18.07					SD 3 weeks Rep
5.450	14.650	19.380	0	15.800	20.100	20.640	16.910	2
			18.05					SD 3 weeks Rep
5.520	14.320	18.950	0	15.530	19.950	20.450	16.670	3
			18.99					SD 6 weeks Rep
5.660	15.270	20.990	0	16.650	21.070	20.860	17.510	1
5.620	15.080	20.080	18.85	16.530	20.570	20.670	17.120	SD 6 weeks Rep

			0					2
			18.950					SD 6 weeks Rep
5.160	15.580	20.490	0	16.680	20.880	20.880	17.610	3
			20.640					SD 8 weeks Rep
6.040	17.100	24.940	0	17.930	23.230	22.140	18.660	1
			20.040					SD 8 weeks Rep
6.150	16.680	22.010	0	17.540	22.040	21.530	18.680	2
			20.360					SD 8 weeks Rep
6.220	16.760	23.340	0	17.750	22.720	21.890	18.420	3
			21.000					
5.740	17.660	23.150	0	18.650	23.500	21.950	19.290	SD 12 wk + LT Rep 1
			21.850					
5.770	18.530	25.290	0	19.020	24.510	23.010	19.800	SD 12 wk + LT Rep 2
			20.400					
5.630	17.390	23.480	0	17.960	22.690	21.050	18.300	SD 12 wk + LT Rep 3

Populus tremula x Populus alba

Shoot tips

18S rRNA	*ACT2*	*ANT*	*CDC2*	*CYC063*	*PT1*	*TIP4-like*	*UBQ7*	
HKG 1	HKG 2	HKG 3	HKG 4	HKG 5	HKG 6	HKG 7	HKG 8	Sample
			19.260					
5.290	17.840	20.250	0	17.710	21.250	20.330	18.670	LD Rep 1
			18.790					
5.410	17.350	19.870	0	17.300	20.950	20.040	18.340	LD Rep 2
			18.790					
5.380	17.570	19.580	0	17.270	20.950	20.010	18.540	LD Rep 3
			18.650					SD 3 weeks Rep
5.660	16.760	19.460	0	16.640	20.720	19.480	18.090	1
			18.030					SD 3 weeks Rep
4.950	16.700	18.470	0	16.440	20.600	19.280	17.390	2
			18.790					SD 3 weeks Rep
5.540	18.060	18.710	0	16.850	21.000	19.800	18.310	3
			19.220					SD 6 weeks Rep
5.945	17.850	20.850	0	16.700	21.260	19.980	18.680	1
			18.820					SD 6 weeks Rep
5.830	17.810	20.810	0	16.630	20.830	19.790	18.870	2
			19.340					SD 6 weeks Rep
5.835	18.010	20.410	0	17.060	21.230	19.980	18.760	3
			18.910					SD 8 weeks Rep
5.590	17.480	19.860	0	15.880	20.720	19.340	18.270	1
			18.350					SD 8 weeks Rep
5.670	16.930	19.680	0	15.950	20.200	19.200	18.120	2
			18.370					SD 8 weeks Rep
5.435	17.110	19.850	0	15.920	20.540	18.920	17.650	3
			18.770					
6.580	17.960	21.510	0	15.720	20.470	18.910	17.930	SD 12 wk + LT Rep 1
			19.100					
7.060	18.610	21.300	0	15.440	20.760	19.450	18.030	SD 12 wk + LT Rep 2
			18.790					
6.290	17.970	20.550	0	15.770	20.540	19.040	17.670	SD 12 wk + LT Rep 3

Young leaves

18S rRNA	*ACT2*	*ANT*	*CDC2*	*CYC063*	*PT1*	*TIP4-like*	*UBQ7*	
HKG 1	HKG 2	HKG 3	HKG 4	HKG 5	HKG 6	HKG 7	HKG 8	Sample
			18.880					
5.350	17.570	20.120	0	17.320	20.920	19.940	18.440	LD Rep 1
			18.140					
5.260	16.690	19.050	0	16.320	20.200	19.050	17.220	LD Rep 2
			18.560					
5.400	17.160	20.000	0	16.890	20.590	19.670	17.350	LD Rep 3
			18.790					SD 3 weeks Rep
5.227	17.620	21.240	0	16.420	20.700	19.890	17.830	1
			18.640					SD 3 weeks Rep
5.410	17.500	19.780	0	16.700	20.690	19.730	17.550	2
			18.540					SD 3 weeks Rep
5.400	17.530	19.820	0	16.620	20.580	19.620	17.810	3

6.320	18.930	27.390	20.420	15.570	21.410	20.900	17.860	SD 6 weeks Rep 1
6.000	17.860	25.250	19.700	16.070	21.110	20.790	18.250	SD 6 weeks Rep 2
5.750	18.310	25.820	20.370	16.430	21.840	20.740	18.690	SD 6 weeks Rep 3
6.810	18.080	26.620	20.150	15.160	21.120	20.220	17.870	SD 8 weeks Rep 1
6.840	18.020	26.190	20.330	15.840	21.800	20.260	18.020	SD 8 weeks Rep 2
6.810	18.050	26.260	19.970	15.530	21.250	20.180	17.890	SD 8 weeks Rep 3
6.880	18.790	29.920	19.790	15.870	20.390	19.110	17.540	SD 12 wk + LT Rep 1
6.670	18.890	28.910	20.040	15.220	20.650	19.420	17.570	SD 12 wk + LT Rep 2
6.550	18.590	28.250	19.740	15.110	20.300	19.120	17.520	SD 12 wk + LT Rep 3

Maure leaves

18S rRNA HKG 1	*ACT2* HKG 2	*ANT* HKG 3	*CDC2* HKG 4	*CYC063* HKG 5	*PT1* HKG 6	*TIP4-like* HKG 7	*UBQ7* HKG 8	Sample
6.360	17.460	24.420	19.150	16.630	21.460	20.250	18.830	LD Rep 1
6.410	17.860	24.380	18.810	16.610	21.230	19.910	18.180	LD Rep 2
6.290	17.620	25.540	18.860	16.270	21.690	20.160	18.520	LD Rep 3
6.430	16.830	24.630	18.420	16.210	21.100	19.760	18.350	SD 3 weeks Rep 1
5.910	16.870	24.170	18.700	16.090	21.230	19.920	18.060	SD 3 weeks Rep 2
5.550	16.710	24.110	18.550	16.140	20.930	20.180	18.030	SD 3 weeks Rep 3
6.390	17.580	26.370	19.440	16.080	21.450	20.410	18.470	SD 6 weeks Rep 1
5.750	17.660	26.330	19.870	16.260	21.820	21.030	18.640	SD 6 weeks Rep 2
5.670	18.060	26.260	19.500	15.960	21.800	20.290	18.380	SD 6 weeks Rep 3
5.680	17.000	25.650	18.680	15.260	20.910	19.410	17.740	SD 8 weeks Rep 1
5.270	16.570	24.690	18.490	15.090	20.690	19.460	17.680	SD 8 weeks Rep 2
4.610	16.650	25.330	18.500	14.970	20.750	19.220	17.500	SD 8 weeks Rep 3
7.890	17.420	29.310	18.310	14.960	19.620	18.070	17.010	SD 12 wk + LT Rep 1
8.540	17.740	30.700	18.680	15.350	19.940	18.460	17.310	SD 12 wk + LT Rep 2
8.600	18.530	30.220	19.360	15.960	20.760	19.460	18.220	SD 12 wk + LT Rep 3

Bark

18S rRNA HKG 1	*ACT2* HKG 2	*ANT* HKG 3	*CDC2* HKG 4	*CYC063* HKG 5	*PT1* HKG 6	*TIP4-like* HKG 7	*UBQ7* HKG 8	Sample
6.540	16.580	20.770	18.430	16.140	21.260	19.580	18.160	LD Rep 1
5.830	16.130	20.470	17.930	15.610	21.260	19.070	17.550	LD Rep 2
5.570	15.770	20.380	17.420	15.310	20.950	19.120	17.500	LD Rep 3
5.570	16.150	20.060	17.610	15.820	21.130	19.520	17.820	SD 3 weeks Rep 1
5.120	15.830	20.610	17.710	15.350	20.820	19.090	17.590	SD 3 weeks Rep 2

5.020	15.880	21.210	18.030	15.660	21.160	19.480	17.840	SD 3 weeks Rep 3
4.670	17.000	22.120	19.170	16.550	22.450	20.460	18.180	SD 6 weeks Rep 1
4.640	17.390	21.170	18.700	16.190	21.910	20.010	18.420	SD 6 weeks Rep 2
3.860	17.440	22.400	19.370	17.490	22.760	20.890	18.890	SD 6 weeks Rep 3
6.510	16.920	22.790	18.990	16.730	22.570	20.300	18.820	SD 8 weeks Rep 1
6.410	16.470	22.230	18.610	16.350	22.200	20.010	18.010	SD 8 weeks Rep 2
6.060	16.490	21.900	19.150	16.550	22.410	20.060	18.130	SD 8 weeks Rep 3
5.510	17.340	23.670	19.120	16.300	22.240	19.870	18.330	SD 12 wk + LT Rep 1
5.100	17.770	24.600	19.550	16.070	22.650	20.100	18.130	SD 12 wk + LT Rep 2
5.170	17.170	23.740	19.090	15.900	22.210	19.660	17.760	SD 12 wk + LT Rep 3

Descriptive data output

Populus trichocarpa

Shoot tips

	18S HKG 1	ACT2 HKG 2	ANT HKG 3	CDC2 HKG 4	CYC HKG 5	PT1 HKG 6	TIP4 HKG 7	UBQ7 HKG 8
n	15.000	15.000	15.000	15.000	15.000	15.000	15.000	15.000
geo Mean [CP]	5.581	16.332	19.912	19.842	15.740	20.164	20.863	17.589
ar Mean [CP]	5.625	16.340	19.931	19.845	15.749	20.166	20.864	17.593
min [CP]	4.070	15.730	18.560	19.510	14.840	19.850	20.470	16.930
max [CP]	6.600	17.390	21.360	20.750	16.840	20.920	21.200	18.530
std dev [± CP]	0.572	0.464	0.769	0.268	0.413	0.248	0.186	0.273
CV [% CP]	10.173	2.840	3.860	1.352	2.622	1.228	0.891	1.554
min [x-fold]	-2.745	-1.487	-2.660	-1.267	-1.850	-1.248	-1.305	-1.594
max [x-fold]	1.976	2.011	2.852	1.911	2.119	1.705	1.257	1.945
std dev [± x-fold]	1.466	1.364	1.672	1.196	1.318	1.180	1.132	1.200

Young leaves

	18S rRNA HKG 1	*ACT2* HKG 2	*ANT* HKG 3	*CDC2* HKG 4	*CYC063* HKG 5	*PT1* HKG 6	*TIP4-like* HKG 7	*UBQ7* HKG 8
n	15.000	15.000	15.000	15.000	15.000	15.000	15.000	15.000
geo Mean [CP]	5.970	16.630	23.400	20.370	15.200	19.620	21.560	17.010
ar Mean [CP]	6.000	16.660	23.700	20.390	15.210	19.620	21.570	17.010
min [CP]	5.020	15.070	18.410	19.010	14.570	18.960	20.950	16.440
max [CP]	7.070	18.040	30.220	21.380	15.910	20.510	22.640	17.420
std dev [± CP]	0.550	0.870	3.430	0.810	0.300	0.350	0.430	0.240
CV [% CP]	9.230	5.220	14.480	3.990	2.000	1.770	1.990	1.440
min [x-fold]	-1.880	-2.810	-37.050	-2.640	-1.540	-1.590	-1.520	-1.490
max [x-fold]	2.090	2.530	139.000	2.050	1.620	1.870	2.070	1.340

std dev [± x-fold]	1.450	1.790	9.910	1.720	1.220	1.260	1.330	1.180
Mature leaves								
	18S rRNA HKG 1	*ACT2* HKG 2	*ANT* HKG 3	*CDC2* HKG 4	*CYC063* HKG 5	*PT1* HKG 6	*TIP4-like* HKG 7	*UBQ7* HKG 8
n	15.000	15.000	15.000	15.000	15.000	15.000	15.000	15.000
geo Mean [CP]	6.610	16.960	25.980	20.010	15.950	20.930	21.730	17.010
ar Mean [CP]	6.680	16.980	26.200	20.020	15.950	20.930	21.730	17.020
min [CP]	5.390	15.830	20.080	19.030	15.350	20.490	21.320	16.630
max [CP]	9.460	19.360	34.890	21.660	16.440	21.540	22.000	17.790
std dev [± CP]	0.780	0.700	2.340	0.490	0.190	0.250	0.160	0.240
CV [% CP]	11.710	4.110	8.930	2.430	1.200	1.170	0.750	1.420
min [x-fold]	-2.260	-2.110	-71.550	-2.010	-1.500	-1.370	-1.320	-1.310
max [x-fold]	6.730	4.880	630.930	3.240	1.400	1.530	1.200	1.730
std dev [± x-fold]	1.690	1.590	4.770	1.380	1.140	1.180	1.110	1.180
Bark								
	18S rRNA HKG 1	*ACT2* HKG 2	*ANT* HKG 3	*CDC2* HKG 4	*CYC063* HKG 5	*PT1* HKG 6	*TIP4-like* HKG 7	*UBQ7* HKG 8
n	15.000	15.000	15.000	15.000	15.000	15.000	15.000	15.000
geo Mean [CP]	5.670	15.810	21.350	19.280	16.690	21.400	21.100	17.670
ar Mean [CP]	5.680	15.860	21.440	19.320	16.740	21.450	21.110	17.700
min [CP]	5.160	14.320	18.950	17.780	14.840	19.950	20.160	16.260
max [CP]	6.220	18.530	25.290	21.850	19.020	24.510	23.010	19.800
std dev [± CP]	0.200	1.190	1.810	1.120	1.120	1.330	0.660	0.930
CV [% CP]	3.590	7.510	8.440	5.790	6.720	6.210	3.130	5.250
min [x-fold]	-1.410	-2.670	-5.660	-2.910	-3.530	-2.780	-1.890	-2.700
max [x-fold]	1.440	6.020	17.360	6.260	4.920	8.970	3.650	4.520
std dev [± x-fold]	1.150	2.220	3.350	2.110	2.120	2.440	1.560	1.860
Populus tremula x Populus alba								
Shoot tips								
	18S rRNA HKG 1	*ACT2* HKG 2	*ANT* HKG 3	*CDC2* HKG 4	*CYC063* HKG 5	*PT1* HKG 6	*TIP4-like* HKG 7	*UBQ7* HKG 8
n	15.000	15.000	15.000	15.000	15.000	15.000	15.000	15.000
geo Mean [CP]	5.742	17.593	20.060	18.795	16.472	20.799	19.565	18.216
ar Mean [CP]	5.764	17.601	20.077	18.799	16.485	20.801	19.570	18.221
min [CP]	4.950	16.700	18.470	18.030	15.440	20.200	18.910	17.390
max [CP]	7.060	18.610	21.510	19.340	17.710	21.260	20.330	18.870
std dev [±	0.394	0.441	0.685	0.248	0.570	0.248	0.392	0.356

CP]								
CV [% CP]	6.833	2.503	3.413	1.318	3.460	1.193	2.003	1.953
min [x-fold]	-1.704	-1.816	-3.090	-1.723	-2.022	-1.552	-1.584	-1.785
max [x-fold]	2.427	1.974	2.798	1.472	2.326	1.402	1.711	1.581
std dev [± x-fold]	1.303	1.345	1.586	1.181	1.468	1.182	1.302	1.271

Young leaves

	18S rRNA HKG 1	*ACT2* HKG 2	*ANT* HKG 3	*CDC2* HKG 4	*CYC063* HKG 5	*PT1* HKG 6	*TIP4-like* HKG 7	*UBQ7* HKG 8
n	15.000	15.000	15.000	15.000	15.000	15.000	15.000	15.000
geo Mean [CP]	6.010	17.960	24.020	19.460	16.060	20.900	19.900	17.820
ar Mean [CP]	6.050	17.970	24.310	19.470	16.070	20.900	19.910	17.830
min [CP]	5.230	16.690	19.050	18.140	15.110	20.200	19.050	17.220
max [CP]	6.880	18.930	29.920	20.420	17.320	21.840	20.900	18.690
std dev [± CP]	0.610	0.520	3.450	0.700	0.560	0.420	0.490	0.300
CV [% CP]	10.070	2.880	14.170	3.610	3.490	1.990	2.450	1.670
min [x-fold]	-1.690	-2.340	-33.900	-2.550	-1.910	-1.670	-1.820	-1.530
max [x-fold]	1.800	1.910	65.950	1.980	2.360	2.000	2.020	1.840
std dev [± x-fold]	1.510	1.420	10.160	1.610	1.460	1.320	1.390	1.220

Mature leaves

	18S rRNA HKG 1	*ACT2* HKG 2	*ANT* HKG 3	*CDC2* HKG 4	*CYC063* HKG 5	*PT1* HKG 6	*TIP4-like* HKG 7	*UBQ7* HKG 8
n	15.000	15.000	15.000	15.000	15.000	15.000	15.000	15.000
geo Mean [CP]	6.266	17.362	26.059	18.883	15.846	21.016	19.719	18.054
ar Mean [CP]	6.357	17.371	26.141	18.888	15.856	21.025	19.733	18.061
min [CP]	4.610	16.570	24.110	18.310	14.960	19.620	18.070	17.010
max [CP]	8.600	18.530	30.700	19.870	16.630	21.820	21.030	18.830
std dev [± CP]	0.816	0.479	1.646	0.384	0.487	0.477	0.575	0.413
CV [% CP]	12.844	2.759	6.297	2.033	3.069	2.269	2.916	2.288
min [x-fold]	-3.060	-1.697	-3.988	-1.502	-1.830	-2.786	-3.182	-2.080
max [x-fold]	4.839	2.183	26.910	2.017	1.707	1.803	2.511	1.723
std dev [± x-fold]	1.736	1.382	3.040	1.296	1.389	1.380	1.475	1.322

Bark

	18S rRNA HKG 1	*ACT2* HKG 2	*ANT* HKG 3	*CDC2* HKG 4	*CYC063* HKG 5	*PT1* HKG 6	*TIP4-like* HKG 7	*UBQ7* HKG 8
n	15.000	15.000	15.000	15.000	15.000	15.000	15.000	15.000
geo Mean [CP]	5.390	16.680	21.830	18.580	16.130	21.860	19.810	18.070
ar Mean [CP]	5.440	16.690	21.870	18.590	16.130	21.870	19.810	18.080

min [CP]	3.860	15.770	20.060	17.420	15.310	20.820	19.070	17.500
max [CP]	6.540	17.770	24.600	19.550	17.490	22.760	20.890	18.890
std dev [± CP]	0.600	0.560	1.130	0.590	0.430	0.610	0.420	0.330
CV [% CP]	11.010	3.360	5.150	3.170	2.660	2.810	2.140	1.810
min [x-fold]	-2.810	-1.830	-3.520	-2.280	-1.740	-2.140	-1.680	-1.490
max [x-fold]	2.180	2.080	7.110	1.990	2.540	1.940	2.140	1.780
std dev [± x-fold]	1.500	1.460	2.140	1.490	1.340	1.510	1.330	1.250

Pairwise correlation analysis

Populus trichocarpa

Shoot tips

vs.	*18S rRNA*	*ACT2*	*ANT*	*CDC2*	*CYC063*	*PT1*	*TIP4-like*	*UBQ7*
HKG 2	0.108	-	-	-	-	-	-	-
p-value	0.703	-	-	-	-	-	-	-
HKG 3	-0.145	0.814	-	-	-	-	-	-
p-value	0.605	0.001	-	-	-	-	-	-
HKG 4	0.243	0.930	0.805	-	-	-	-	-
p-value	0.384	0.001	0.001	-	-	-	-	-
HKG 5	0.626	0.159	-0.240	0.124	-	-	-	-
p-value	0.013	0.572	0.390	0.660	-	-	-	-
HKG 6	0.141	0.950	0.746	0.916	0.188	-	-	-
p-value	0.619	0.001	0.001	0.001	0.502	-	-	-
HKG 7	0.053	0.236	-0.179	0.088	0.508	0.282	-	-
p-value	0.852	0.395	0.521	0.754	0.053	0.308	-	-
HKG 8	0.630	0.684	0.317	0.746	0.639	0.696	0.260	-
p-value	0.012	0.005	0.252	0.001	0.010	0.004	0.350	-
BestKeeper vs.	*18S rRNA*	*ACT2*	*ANT*	*CDC2*	*CYC063*	*PT1*	*TIP4-like*	*UBQ7*
coeff. of corr. [r]	0.810	0.655	0.360	0.718	0.639	0.660	0.223	0.892
p-value	0.001	0.008	0.188	0.003	0.010	0.007	0.427	0.001

Young leaves

vs.	*18S rRNA*	*ACT2*	*ANT*	*CDC2*	*CYC063*	*PT1*	*TIP4-like*	*UBQ7*
HKG 2	0.645	-	-	-	-	-	-	-
p-value	0.009	-	-	-	-	-	-	-
HKG 3	0.668	0.947	-	-	-	-	-	-
p-value	0.006	0.001	-	-	-	-	-	-
HKG 4	0.669	0.956	0.964	-	-	-	-	-
p-value	0.006	0.001	0.001	-	-	-	-	-
HKG 5	0.200	-0.001	-0.158	-0.178	-	-	-	-
p-value	0.472	1.000	0.572	0.527	-	-	-	-
HKG 6	0.185	0.649	0.605	0.744	-0.397	-	-	-
p-value	0.508	0.009	0.017	0.001	0.143	-	-	-
HKG 7	0.051	0.575	0.564	0.684	-0.579	0.942	-	-
p-value	0.860	0.025	0.029	0.005	0.024	0.001	-	-
HKG 8	0.452	0.542	0.404	0.429	0.640	0.233	0.016	-
p-value	0.090	0.037	0.136	0.111	0.010	0.405	0.953	-
BestKeeper vs.	*18S rRNA*	*ACT2*	*ANT*	*CDC2*	*CYC063*	*PT1*	*TIP4-like*	*UBQ7*
coeff. of corr. [r]	0.799	0.961	0.963	0.970	-0.008	0.610	0.517	0.532
p-value	0.001	0.001	0.001	0.001	0.977	0.016	0.048	0.041

Mature

leaves

vs.	*18S rRNA*	*ACT2*	*ANT*	*CDC2*	*CYC063*	*PT1*	*TIP4-like*	*UBQ7*
HKG 2	0.939	-	-	-	-	-	-	-
p-value	0.001	-	-	-	-	-	-	-
HKG 3	0.823	0.805	-	-	-	-	-	-
p-value	0.001	0.001	-	-	-	-	-	-
HKG 4	0.918	0.976	0.812	-	-	-	-	-
p-value	0.001	0.001	0.001	-	-	-	-	-
HKG 5	-0.563	-0.443	-0.519	-0.492	-	-	-	-
p-value	0.029	0.098	0.047	0.062	-	-	-	-
HKG 6	0.531	0.716	0.531	0.629	0.117	-	-	-
p-value	0.042	0.003	0.042	0.012	0.681	-	-	-
HKG 7	-0.028	0.155	0.014	0.092	0.485	0.524	-	-
p-value	0.922	0.578	0.961	0.747	0.067	0.045	-	-
HKG 8	0.843	0.855	0.653	0.799	-0.194	0.667	0.139	-
p-value	0.001	0.001	0.008	0.001	0.490	0.007	0.619	-
BestKeeper vs.	*18S rRNA*	*ACT2*	*ANT*	*CDC2*	*CYC063*	*PT1*	*TIP4-like*	*UBQ7*
coeff. of corr. [r]	0.964	0.962	0.917	0.943	-0.477	0.663	0.088	0.845
p-value	0.001	0.001	0.001	0.001	0.072	0.007	0.754	0.001

Bark

vs.	*18S rRNA*	*ACT2*	*ANT*	*CDC2*	*CYC063*	*PT1*	*TIP4-like*	*UBQ7*
HKG 2	0.537	-	-	-	-	-	-	-
p-value	0.039	-	-	-	-	-	-	-
HKG 3	0.615	0.955	-	-	-	-	-	-
p-value	0.015	0.001	-	-	-	-	-	-
HKG 4	0.580	0.990	0.962	-	-	-	-	-
p-value	0.023	0.001	0.001	-	-	-	-	-
HKG 5	0.550	0.959	0.901	0.969	-	-	-	-
p-value	0.033	0.000	0.001	0.001	-	-	-	-
HKG 6	0.588	0.985	0.970	0.992	0.963	-	-	-
p-value	0.021	0.001	0.001	0.001	0.001	-	-	-
HKG 7	0.618	0.919	0.917	0.944	0.919	0.954	-	-
p-value	0.014	0.001	0.001	0.001	0.001	0.001	-	-
HKG 8	0.574	0.972	0.912	0.979	0.980	0.973	0.954	-
p-value	0.025	0.001	0.001	0.001	0.001	0.001	0.001	-
BestKeeper vs.	*18S rRNA*	*ACT2*	*ANT*	*CDC2*	*CYC063*	*PT1*	*TIP4-like*	*UBQ7*
coeff. of corr. [r]	0.650	0.983	0.970	0.993	0.969	0.993	0.957	0.980
p-value	0.009	0.001	0.001	0.001	0.001	0.001	0.001	0.001

Populus tremula x Populus alba

Shoot tips

vs.	*18S rRNA*	*ACT2*	*ANT*	*CDC2*	*CYC063*	*PT1*	*TIP4-like*	*UBQ7*
HKG 2	0.696	-	-	-	-	-	-	-
p-value	0.004	-	-	-	-	-	-	-
HKG 3	0.806	0.650	-	-	-	-	-	-
p-value	0.001	0.009	-	-	-	-	-	-
HKG 4	0.400	0.755	0.584	-	-	-	-	-
p-value	0.140	0.001	0.022	-	-	-	-	-
HKG 5	-0.616	-0.082	-0.321	0.321	-	-	-	-
p-value	0.014	0.769	0.244	0.244	-	-	-	-
HKG 6	-0.165	0.420	0.069	0.754	0.747	-	-	-

p-value	0.559	0.119	0.806	0.001	0.001	-	-	-
HKG 7	-0.314	0.248	-0.066	0.611	0.883	0.882	-	-
p-value	0.255	0.374	0.814	0.016	0.001	0.001	-	-
HKG 8	-0.039	0.382	0.272	0.731	0.622	0.721	0.812	-
p-value	0.891	0.160	0.326	0.002	0.013	0.002	0.001	-
BestKeeper vs.	*18S rRNA*	*ACT2*	*ANT*	*CDC2*	*CYC063*	*PT1*	*TIP4-like*	*UBQ7*
coeff. of corr. [r]	0.659	0.862	0.788	0.905	0.129	0.562	0.457	0.651
p-value	0.008	0.001	0.001	0.001	0.646	0.029	0.087	0.009
Young leaves								
vs.	*18S rRNA*	*ACT2*	*ANT*	*CDC2*	*CYC063*	*PT1*	*TIP4-like*	*UBQ7*
HKG 2	0.750	-	-	-	-	-	-	-
p-value	0.001	-	-	-	-	-	-	-
HKG 3	0.906	0.916	-	-	-	-	-	-
p-value	0.001	0.001	-	-	-	-	-	-
HKG 4	0.818	0.836	0.885	-	-	-	-	-
p-value	0.001	0.001	0.001	-	-	-	-	-
HKG 5	-0.845	-0.682	-0.823	-0.691	-	-	-	-
p-value	0.001	0.005	0.001	0.004	-	-	-	-
HKG 6	0.284	0.282	0.278	0.681	-0.084	-	-	-
p-value	0.304	0.308	0.317	0.005	0.769	-	-	-
HKG 7	0.081	0.201	0.146	0.533	-0.004	0.871	-	-
p-value	0.776	0.472	0.605	0.041	0.992	0.001	-	-
HKG 8	-0.011	0.188	0.110	0.437	0.208	0.757	0.740	-
p-value	0.969	0.502	0.696	0.104	0.455	0.001	0.002	-
BestKeeper vs.	*18S rRNA*	*ACT2*	*ANT*	*CDC2*	*CYC063*	*PT1*	*TIP4-like*	*UBQ7*
coeff. of corr. [r]	0.886	0.881	0.944	0.977	-0.717	0.562	0.416	0.358
p-value	0.001	0.001	0.001	0.001	0.003	0.029	0.123	0.191
Mature leaves								
vs.	*18S rRNA*	*ACT2*	*ANT*	*CDC2*	*CYC063*	*PT1*	*TIP4-like*	*UBQ7*
HKG 2	0.639	-	-	-	-	-	-	-
p-value	0.010	-	-	-	-	-	-	-
HKG 3	0.834	0.601	-	-	-	-	-	-
p-value	0.001	0.018	-	-	-	-	-	-
HKG 4	0.039	0.660	0.139	-	-	-	-	-
p-value	0.891	0.007	0.619	-	-	-	-	-
HKG 5	-0.007	0.354	-0.394	0.495	-	-	-	-
p-value	0.977	0.197	0.145	0.061	-	-	-	-
HKG 6	-0.515	0.159	-0.579	0.657	0.722	-	-	-
p-value	0.049	0.572	0.024	0.008	0.002	-	-	-
HKG 7	-0.502	0.094	-0.580	0.681	0.751	0.948	-	-
p-value	0.057	0.739	0.023	0.005	0.001	0.001	-	-
HKG 8	-0.217	0.301	-0.432	0.686	0.874	0.904	0.902	-
p-value	0.438	0.275	0.107	0.005	0.001	0.001	0.001	-
BestKeeper vs.	*18S rRNA*	*ACT2*	*ANT*	*CDC2*	*CYC063*	*PT1*	*TIP4-like*	*UBQ7*
coeff. of corr. [r]	0.816	0.894	0.653	0.569	0.420	0.050	0.057	0.323
p-value	0.001	0.001	0.008	0.027	0.119	0.860	0.837	0.240
Bark								
vs.	*18S rRNA*	*ACT2*	*ANT*	*CDC2*	*CYC063*	*PT1*	*TIP4-like*	*UBQ7*

HKG 2	-0.352	-	-	-	-	-	-	-
p-value	0.197	-	-	-	-	-	-	-
HKG 3	-0.131	0.792	-	-	-	-	-	-
p-value	0.639	0.001	-	-	-	-	-	-
HKG 4	-0.216	0.888	0.854	-	-	-	-	-
p-value	0.438	0.001	0.001	-	-	-	-	-
HKG 5	-0.198	0.651	0.447	0.767	-	-	-	-
p-value	0.478	0.009	0.095	0.001	-	-	-	-
HKG 6	-0.178	0.832	0.814	0.950	0.829	-	-	-
p-value	0.527	0.001	0.001	0.001	0.001	-	-	-
HKG 7	-0.331	0.742	0.562	0.831	0.946	0.892	-	-
p-value	0.226	0.002	0.029	0.001	0.001	0.001	-	-
HKG 8	-0.169	0.698	0.445	0.708	0.905	0.744	0.878	-
p-value	0.546	0.004	0.097	0.003	0.001	0.001	0.001	-
BestKeeper vs.	*18S rRNA*	*ACT2*	*ANT*	*CDC2*	*CYC063*	*PT1*	*TIP4-like*	*UBQ7*
coeff. of corr. [r]	0.389	0.650	0.738	0.793	0.647	0.812	0.633	0.644
p-value	0.152	0.009	0.002	0.001	0.009	0.001	0.011	0.009

Pairwise correlation analyses with (1st analysis) and without (2nd analysis) genes determined to have a st. dev. of Cq values greater than 1. Pearson's coefficient of correlation [r] and p-value.

Populus trichocarpa

1st analysis			**2nd analysis**		
Shoot tips	**Coeff. of corr. [r]**	**p-value**			
UBQ7	0.892	0.001			
18S rRNA	0.810	0.001			
CDC2	0.718	0.003			
PT1	0.660	0.007			
ACT2	0.655	0.008			
CYC063	0.639	0.010			
ANT	0.360	0.188			
TIP4-like	0.223	0.427			

Young leaves	**Coeff. of corr. [r]**	**p-value**	**Young leaves**	**Coeff. of corr. [r]**	**p-value**
CDC2	0.970	0.001	*CDC2*	0.924	0.001
ANT	0.963	0.001	*ACT2*	0.923	0.001
ACT2	0.961	0.001	*18S rRNA*	0.862	0.001
18S rRNA	0.799	0.001	*UBQ7*	0.617	0.014
PT1	0.610	0.016	*PT1*	0.573	0.026
UBQ7	0.532	0.041	*TIP4-like*	0.439	0.102
TIP4-like	0.517	0.048	*CYC063*	0.126	0.653
CYC063	-0.008	0.977			

Mature leaves	**Coeff. of corr. [r]**	**p-value**	**Mature leaves**	**Coeff. of corr. [r]**	**p-value**
18S rRNA	0.964	0.001	*ACT2*	0.982	0.001
ACT2	0.962	0.001	*18S rRNA*	0.976	0.001
CDC2	0.943	0.001	*CDC2*	0.952	0.001

ANT	0.917	0.001	*UBQ7*	0.892	0.001
UBQ7	0.845	0.001	*PT1*	0.679	0.005
PT1	0.663	0.007	*TIP4-like*	0.127	0.653
TIP4-like	0.088	0.754	*CYC063*	-0.429	0.111
CYC063	-0.477	0.072			

Bark	**Coeff. of corr. [r]**	**p-value**	**Bark**	**Coeff. of corr. [r]**	**p-value**
CDC2	0.993	0.001	*TIP4-like*	0.949	0.001
PT1	0.993	0.001	*UBQ7*	0.941	0.001
ACT2	0.983	0.001	*18S rRNA*	0.809	0.001
UBQ7	0.980	0.001			
ANT	0.970	0.001			
CYC063	0.969	0.001			
TIP4-like	0.957	0.001			
18S rRNA	0.650	0.009			

Populus tremula x Populus alba

1st analysis			**2nd analysis**		
Shoot tips	**Coeff. of corr. [r]**	**p-value**			
CDC2	0.905	0.001			
ACT2	0.862	0.001			
ANT	0.788	0.001			
18S rRNA	0.659	0.008			
UBQ7	0.651	0.009			
PT1	0.562	0.029			
TIP4-like	0.457	0.087			
CYC063	0.129	0.646			

Young leaves	**Coeff. of corr. [r]**	**p-value**	**Young leaves**	**Coeff. of corr. [r]**	**p-value**
CDC2	0.977	0.001	*CDC2*	0.970	0.001
ANT	0.944	0.001	*18S rRNA*	0.799	0.001
18S rRNA	0.886	0.001	*ACT2*	0.783	0.001
ACT2	0.881	0.001	*PT1*	0.743	0.002
PT1	0.562	0.029	*TIP4-like*	0.594	0.020
TIP4-like	0.416	0.123	*UBQ7*	0.530	0.042
UBQ7	0.358	0.191	*CYC063*	-0.564	0.029
CYC063	-0.717	0.003			

Mature leaves	**Coeff. of corr. [r]**	**p-value**	**Mature leaves**	**Coeff. of corr. [r]**	**p-value**
ACT2	0.894	0.001	*ACT2*	0.852	0.001
18S rRNA	0.816	0.001	*18S rRNA*	0.677	0.006
ANT	0.653	0.008	*CYC063*	0.649	0.009
CDC2	0.569	0.027	*CDC2*	0.627	0.012
CYC063	0.420	0.119	*UBQ7*	0.543	0.037

UBQ7	0.323	0.240	*TIP4-like*	0.277	0.317
TIP4-like	0.057	0.837	*PT1*	0.268	0.336
PT1	0.050	0.860			

Bark	**Coeff. of corr. [r]**	**p-value**	**Bark**	**Coeff. of corr. [r]**	**p-value**
PT1	0.812	0.001	*PT1*	0.721	0.002
CDC2	0.793	0.001	*CDC2*	0.686	0.005
ANT	0.738	0.002	*CYC063*	0.638	0.010
ACT2	0.650	0.009	*UBQ7*	0.637	0.011
CYC063	0.647	0.009	*TIP4-like*	0.585	0.022
UBQ7	0.644	0.009	*ACT2*	0.533	0.041
TIP4-like	0.633	0.011	*18S rRNA*	0.516	0.049
18S rRNA	0.389	0.152			

Appendix C: Supplemental Material for Chapter 3

Table C-1. Stable reference genes used for expression normalization in excised stem experiments.

Experiment	Tissue-type	Reference Genes
Amino acids	Shoot tips	*TIP4-like* and *ACT2*
	Bark	*CYC063* and *CDC2*
TCA metabolites	Shoot tips	*CDC2* and *PT1*
	Bark	*PT1* and *TIP4-like*
N compounds and metabolites	Shoot tips	*CYC063* and *TIP4-like*
	Bark	*PT1* and *TIP4-like*

Table C-2. Foliar condition of leaves from amino acid cut shoot assay

Treatment	**Condition after 48 h**	**Sampled**	**pH**
Alanine	Curled leaves	•	6.2
Arginine	Mild necrosis	•	5.58
Asparagine	Curled leaves	•	5.00
Aspartic acid	Curled leaves	•	5.70
Cysteine	Dead		6.00
Glutamine	Healthy	•	5.6
Glutamic acid	Necrosis		6.5
Glycine	Curled leaves	•	6.5
Histidine	Severe necrosis		6.00
Leucine	Severe necrosis		6.54
Lysine	Severe necrosis		5.66
Methionine	Dead		6.40
Phenylalanine	Severe necrosis		5.80
Proline	Necrosis		6.65
Serine	Curled leaves	•	6.42
Threonine	Mild necrosis	•	6.46
Tryptophan	Necrosis		6.35
Valine	Curled leaves	•	6.70

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