

Expression of the Scots pine (*Pinus sylvestris*) TIP-like and sucrose synthase genes during two growing seasons and correlation with relative wood densities

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Abstract

Scots pine is one of the dominant tree species in Latvian forests and has great ecological, social and economic importance globally. Research on connecting molecular data with phenotype is still developing for non-model plant species. To study the linkages between gene expression and desirable wood properties in Scots pine and to determine the dynamics of gene expression for organisms growing in field conditions, we investigated the expression of two genes, previously reported to be involved in wood formation processes. Fifty Scots pine individuals with differing relative wood densities were analysed at one time point during early and late wood formation during the first growing season. During the second growing season, a subset of fifteen trees with large gene expression differences detected during the first season were analysed at four time points during early wood formation and five time points during late wood formation. We found that during both growing seasons, sucrose synthase gene expression tended to be higher for trees with higher wood density, while the tonoplast intrinsic protein-like gene was more highly expressed in trees with lower wood density. Both genes showed differential gene expression dynamics during the second growth season, but at some time points there was correlation with gene expression in the first season.

Key words: gene expression dynamics, *Pinus sylvestris*, Scots pine, *SuSy*, *TIP-like*.

Abbreviations: AQP, aquaporin; EST, expressed sequence tag; EW, early wood; HD, high density; LD, low density; LW, late wood; MIP, major intrinsic protein; NIP, NOD26-like intrinsic protein; PIP, plasma membrane intrinsic protein; SAGE, serial analysis of gene expression; SIP, small basic intrinsic protein; *SuSy*, sucrose synthase; *SuSy*, sucrose synthase gene; *TIP*, tonoplast intrinsic protein; *TIPl*, tonoplast intrinsic protein like gene.

Introduction

Scots pine (*Pinus sylvestris* L.) is one of the most widely distributed conifers in the world (Lev-Yadun, Sederoff 2000). Conifer wood formation in temperate regions has an annual pattern of early wood (EW) formation in spring/summer and late wood (LW) formation in late summer and autumn when cell division activity decreases (Uggla et al. 2001). Thus, early wood and late wood tracheids differ in diameter and cell wall thickness (Lev-Yadun, Sederoff 2000). Early wood consists of thin-walled cells with larger cell lumens, whereas late wood tracheids have narrower lumens and thicker cell walls (Uggla et al. 2001). These characteristics are affected by water and sugar resource allocation during the growth season. Early wood cell formation is considered to be highly dependent on the availability of soil water and sugars are used mainly for shoot and needle growth, not xylogenesis. When apical growth ceases, carbohydrates are also used for LW tracheid cell wall construction (Yang, Loopstra 2005). Early wood has higher lignin content, lower hemicellulose and cellulose content, and lower density (Li et al. 2009).

Desirable wood properties vary depending on potential wood/timber usage. Tree breeding efforts have mostly concentrated on rapid radial growth (Wegner et al. 2010). Wood density is one of the most important wood quality parameters, but negative correlations have been reported in some studies between fast growth and wood density (Plomion et al. 2001). The wood density of *P. sylvestris* increases in the juvenile phase up to age 15 and then stabilizes in the mature phase, but there is a strong correlation between wood density in juvenile and mature wood (Hannrup et al. 1998). In Scots pine, wood density strongly correlates with late wood percentage, which is affected by growth rate and tree age (Peltola et al. 2009). High heritability for wood density was detected in *P. sylvestris* progeny trials in Sweden (Hannrup et al. 1998), as well as for other gymnosperm species (Vargas-Hernandes, Adams 1994). In *Pinus radiata*, a correlation was found between wood density and modulus of elasticity ($r = 0.5$; Kumar et al. 2004).

Despite the economic and ecological importance of conifer species, genomic studies and tools have only recently started to be developed due to large genome sizes,

and high-quality genomic information generated by large scale sequencing projects is still not available for many tree species, including Scots pine (Neale et al. 2013). A range of studies have been done in various conifer species examining the correlation between wood formation and properties with gene expression, using microarrays and EST sequences of genes involved in a broad spectrum of biochemical pathways (Allona et al. 1998; Lorenz, Dean 2002; Egertsdotter et al. 2004; Kumar et al. 2009; Li et al. 2010). Aquaporins (AQP) are a large group of channel proteins, which facilitate transport of water, some small solutes (urea, boric acid, silicic acid) and some gases (e.g. CO₂), and even hydrogen peroxide in living organisms (Tyerman et al. 2002; Martinez-Ballesta et al. 2009; Heinen et al. 2009; Yue et al. 2014). AQPs belong to an even larger group of ancient channel proteins, called major intrinsic proteins (MIPs; Chrispeels, Maurel 1994; Schaffner et al. 1998). In plants, MIPs are highly diverse, due to the physiological importance of MIPs or AQPs, and the multiple mechanisms involved in water uptake and evaporation in plants (Zardoya, Villalba 2001; Abascal et al. 2014). Plant AQPs are divided into four subfamilies: TIPs (tonoplast intrinsic proteins), PIPs (plasma membrane intrinsic proteins), SIPs (small basic intrinsic proteins) and NIPs (NOD26-like intrinsic proteins) (Zardoya 2005; Di Giorgio et al. 2014). Some of the most abundant AQPs are PIPs and TIPs, which are involved in rapid water transport and maintenance of water balance in cells, as the gene products (proteins) of this subfamily, mediate stomatal closure (Maurel et al. 2008; Laur, Hacke 2014; Yue et al. 2014). Some of the most broadly expressed TIP paralogs are TIP2:1, TIP1:2 and TIP1:1 (Abascal et al. 2014). In *Picea glauca*, TIP2:1 is detected in roots, stems, needles, reproductive parts, while TIP2:2 is detected only in stems (Laur, Hacke 2014). TIP overexpression in transgenic plants has been associated with increased plant growth rate and biomass production (Rodrigues et al. 2013).

Expression of AQPs is associated with plant growth, especially cell division, elongation and differentiation (Yue et al. 2014; Novikova et al. 2014). Overexpression of some TIPs and PIPs can also enhance tolerance to low temperatures (Yue et al. 2014). Serial analysis of gene expression (SAGE) of a single 10 year old loblolly pine tree revealed that various aquaporins are among the most abundant transcripts in lignifying loblolly pine xylem (Lorenz, Dean 2002), this was further confirmed by Yang et al. (2004). It has been reported that aquaporin-like gene expression positively affects wood maturation, with a positive correlation between aquaporin-like gene expression and wood properties such as LW density, EW cell diameter, EW wall thickness, EW lumen diameter, LW cell diameter, LW wall thickness, LW percentage, and tracheid length (Kumar et al. 2009). In *P. radiata*, a cDNA microarray study comparing transcript abundance during EW and LW formation with wood property data

obtained using Silviscan found that aquaporin and MIP-2 transcripts were more highly accumulated in EW, while a different aquaporin like gene was more abundant in LW, demonstrating the diverse regulation patterns of channel protein coding transcripts (Li et al. 2010).

Sucrose synthase (SuSy) is one of the two main enzymes catalysing sucrose hydrolysis and formation of UDP-glucose and fructose (Coleman et al. 2009). SuSy has been linked with the synthesis of both storage and structural carbohydrates, also with channelling UDP-glucose into the cellulose synthase complex (Delmer, Amor 1995). In Arabidopsis and rice there are six *SuSy* family genes with various expression patterns and localizations (Bieniawska et al. 2007; Hirose et al. 2008). There are also multiple *SuSy* genes in *Pinus taeda*, but only one is highly expressed (Nairn et al. 2008). In poplar, much higher *SuSy* transcript levels were detected in leaves than in xylem (Coleman et al. 2009). Analysing microarray data from *P. taeda* it was found, that *SuSy* transcripts were more abundant in LW compared to EW (Egertsdotter et al. 2004; Yang, Loopstra 2005), and a similar result was found in *P. radiata* (Li et al. 2010). Two different *SuSy* gene transcripts analysed in Egertsdotter et al. (2004) showed fluctuations in transcript abundance during the growth season, with lower values in spring and increased expression in October. Increased activity of SuSy was detected in *P. sylvestris* tracheids undergoing secondary cell wall formation (Uggla et al. 2001).

The aim of this study was to determine the expression of two genes (*TIP1* and *SuSy*), previously reported to be involved in wood formation processes, in Scots pine trees with differing relative wood densities. *TIP1* and *SuSy* gene expression was determined at two time points (during EW and LW formation) for fifty trees during the first growing season. Subsequently, a more detailed analysis of gene expression was undertaken for fifteen trees with significant gene expression differences detected during the first growing season. Gene expression levels were determined at four time points during EW and five time points during LW formation during the second growing season. Correlation of gene expression with relative wood density as a representative wood quality trait as well as changes in gene expression during the second growing season and correlation with expression during the first growing season were determined.

Materials and methods

Candidate gene selection and primer design

Genes analysed in this study were selected based on previously published studies and the availability of gene sequences from pine species. Gene sequences for primer design were obtained from the NCBI database (<http://www.ncbi.nlm.nih.gov/>), *Aqual* Genbank accession number EU301695.1, *SuSy* EF619967.1. Taking in consideration the available aquaporin and aquaporin-like sequences

and current nomenclature, and as the utilised Aqual gene had more than 70 % identity with annotated *TIP*s in other plant species, this gene was designated TIP-like (*TIPl*). Primers for candidate genes were selected using the program Primer3 (<http://frodo.wi.mit.edu/>), FastPCR (<http://primerdigital.com/fastpcr.html>) and checked by NetPrimer (<http://www.premierbiosoft.com/netprimer/>). For each gene, seven primer pairs were tested and the pair with the highest PCR effectivity was selected for further real time PCR analysis. *TIPl* gene primers used for real time PCR were forward 5'-CCTTGGCGGCCAGATCACCA-3', reverse 5'GCCGCCGGTGACGAACTTGA3'; *SuSy*-forward 5'-CAAGACTCGACCGGGTTAAA-3', reverse 5'-TTCGGCCACTTCTTCTCTGT-3'. *GAPDH* primers were utilized as endogenous controls as described previously (Šķipars et al. 2011). A subset of the samples were also analyzed with two other endogenous control genes, β -actin and α -tubulin, but as expression trends were similar, only *GAPDH* was used for further analyses.

Plant material

The plant material for gene expression analysis was collected from a pine plantation consisting of plus tree half-sib families, established in 1982 in central Latvia (Vecumnieki, 56°41'N, 24°26'E). Distance between trees was 2 × 1 m and there was no thinning done in this site. Sampled trees were open pollinated progenies from four populations growing in different regions of Latvia. Individuals with high wood density represented four families, and individuals with low wood density were from six half-sib families.

Relative wood density determination

Relative wood density was measured using the PILODYN 6J-Forest instrument (Hansen 2000). For gene expression analysis, fifty 30 year old trees were selected, from which 25 had higher relative wood density and 25 had lower relative wood density.

Sample collection for RNA extraction

Samples were collected at one time point during first season, in spring (April-May) during early wood (EW) formation and during late wood (LW) formation in late summer/autumn (August-September). Second season samples were gathered from 15 trees that showed large differences in gene expression during the first season. During the second season, samples were gathered at four time points during EW formation and five times during LW formation. Cambium/xylem tissues were obtained at 1 m height above the tree base by removing the bark and scraping off the underlying soft tissue with a scalpel. Tissues were placed into microtubes and immediately stored in liquid nitrogen.

RNA extraction

An adapted CTAB buffer based method was used for RNA extraction, (Chang et al. 1993; Rubio-Piña, Zapata-Pérez

2011). RNA quantity was assessed fluorometrically using a Qubit fluorometer and Quant-iTreatments (Invitrogen) following the manufacturer's instructions. RNA extracts were treated with DNase I (Thermo Fisher) and purified using phenol/chloroform extraction. RNA purity was verified by standard PCR reaction and gel electrophoresis in a 1.2% agarose gel, stained with ethidium bromide. RNA integrity was checked using Bioanalyzer 2100 (Agilent Technologies), following the manufacturer's instructions. For all samples, RNA Integrity Number was higher than seven.

cDNA synthesis

Samples of total RNA (1 µg) were reverse transcribed in a 50 µL reaction using Taqman reverse transcription reagents (Invitrogen) and Oligo(dT)18 primer (Thermo Fisher) according to the manufacturer's instructions. cDNA for standard curves was obtained from 2 µg total RNA per reaction. cDNA quality was checked using a PCR reaction with primers that span an exon-exon junction and visualized by gel electrophoresis.

Real time PCR

Gene expression analysis was performed using relative standard curve real time PCR method and carried out on an Applied Biosystems StepOnePlus 96-well real-time PCR system using a standard three step real time PCR protocol. For real-time PCR, the Maxima SYBR Green PCR kit (Thermo Fisher) in a total volume of 20 µL containing 2 µL of cDNA and 0.15 µM of each primer was used. Each sample was run in three technical replicates for each candidate gene and the endogenous control gene *GAPDH* was run on the same plate. After amplification, dissociation curves were checked for detection of primer dimers and other non-specific PCR products. Standard curves for gene expression quantification were calculated from 1:5 serial dilutions of cDNA used in this study.

Data analysis

Data were analysed using StepOne software v2.2, ANOVA and Pearson correlation. Transcript levels of candidate genes were calculated as a percentage of *GAPDH* gene expression in the same sample. Samples were grouped according to wood density [high density (HD) and low density (LD)] as well as time point [early wood (EW) and late wood (LW)] for further analysis.

Results

TIPl and *SuSy* gene expression during the first growing season

TIPl gene expression during EW formation was more than ten times higher than *SuSy* gene expression. The *TIPl* gene was significantly ($P < 0.05$) overexpressed in trees with lower wood density during EW formation, whereas *SuSy*

had slightly increased expression in trees with higher wood density, but the difference was not statistically significant. During LW formation, no significant differences in *TIPl* and *SuSy* gene expression between trees with high and low wood density were observed. During the first season both gene transcripts were more highly expressed in EW (Table 1).

TIPl and SuSy gene expression during the second growing season

During the second growing season, tissue samples were obtained from fifteen trees that were sampled four times during EW and five times during LW formation. During EW formation, the *TIPl* and *SuSy* genes had differing expression patterns. *SuSy* gene expression during EW formation increased over the course of the growth season, with lower expression found in trees with lower wood density (Fig. 1). *SuSy* expression during EW formation

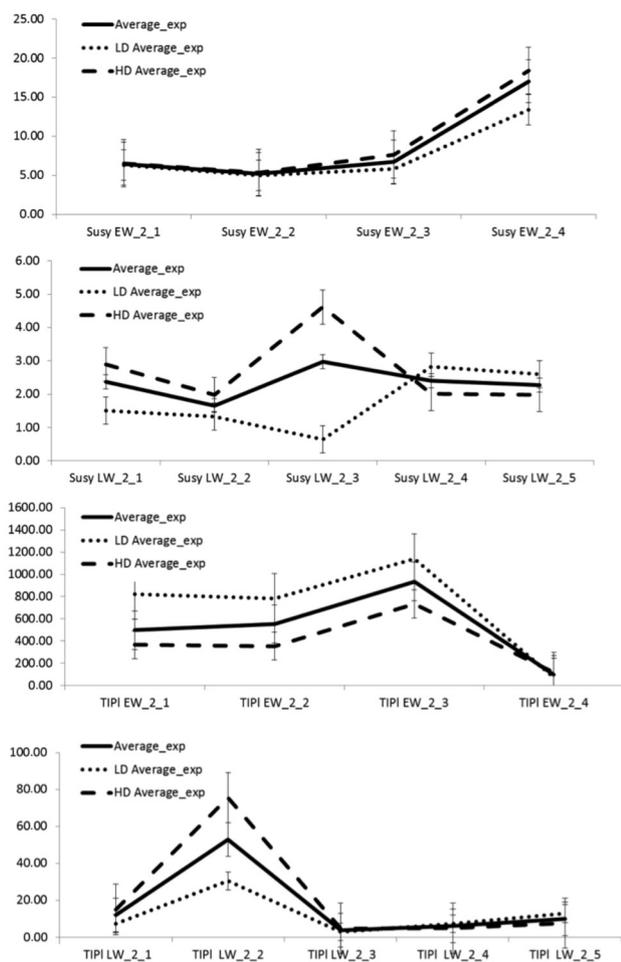


Fig. 1. *TIPl* and *SuSy* gene expression profiles during the second growing season. Panels A and B: *SuSy* gene expression as % of *GAPDH* expression during early (EW) and late (LW) wood formation with standard errors shown. Panels C and D: *TIPl* gene expression as % of *GAPDH* expression during EW and LW formation.

Table 1. *TIPl* and *SuSy* gene expression for fifty trees with high (HD) and low (LD) wood density during early (EW) and late (LW) wood formation during the first growing season. Mean gene expression given as percentage of *GAPDH* gene expression

Gene/wood formation stage	Mean expression	HD mean expression	LD mean expression
<i>SuSy</i> EW	88.50	100.00	77.95
<i>TIPl</i> EW	1043.73	626.43	1462.88
<i>SuSy</i> LW	3.60	4.53	2.53
<i>TIPl</i> LW	70.15	71.66	68.88

was not significantly different between trees with HD and LD. During LW formation, *SuSy* expression was lower for trees with LD and showed a differing expression profile between HD and LD trees at the third time point. During LW formation, *SuSy* expression at the third time point was significantly lower in trees with lower wood density.

During EW development, expression of the *TIPl* gene declined at the final time point. *TIPl* gene expression was higher for trees with lower wood density, with highest expression at the third time point. *TIPl* gene expression was higher for trees with LD at three of four sampling times. During LW formation, *TIPl* gene expression peaked at the second time point, and in contrast to expression during EW time points, the highest *TIPl* expression was found in trees with higher wood density (Fig. 1).

Comparison of TIPl and SuSy gene expression between the two growing seasons

Of the time points sampled during the second growth season, the most similar to the sampling times of the first growing season were the 4th time point for EW formation and the 3rd time point for LW formation. Significant correlations were detected between *SuSy* expression during EW formation in first season and *SuSy* expression at the third and fourth time points in the second growing season (*SuSy*_{EW_2_3} and *SuSy*_{EW_2_4}) points ($r = 0.810$ and $r = 0.909$; $P < 0.01$) (Table 2). During LW formation, a significant correlation was found for *SuSy* expression in the first season and the third sampling point in the second season ($P < 0.01$).

For the *TIPl* gene, significant correlations were also found between gene expression in the first season during EW formation and the first and second time points of the second growing season ($P < 0.01$). A significant correlation was also found between *TIPl* gene expression in the first season during LW formation with the first time point of the second season ($r = 0.738$; $P < 0.01$).

Discussion

The genes investigated were selected based on previous reports indicating their possible significant role in wood formation. AQP_s have been reported to control movement

Table 2. Correlations between *TIP1* and *SuSy* gene expression during the first (s1) and second (s2) growing seasons during early (EW) and late (LW) wood formation

	SuSy_EW_s1	SuSy_EW_s2_tp1	SuSy_EW_s2_tp2	SuSy_EW_s2_tp3	SuSy_EW_s2_tp4
SuSy_EW_s1	1				
SuSy_EW_s2_tp1	0.294	1			
SuSy_EW_s2_tp2	-0.238	0.859	1		
SuSy_EW_s2_tp3	0.810	0.342	-0.042	1	
SuSy_EW_s2_tp4	0.909	0.472	-0.013	0.756	1
	TIP1_EW_s1	TIP1_EW_s2_tp1	TIP1_EW_s2_tp2	TIP1_EW_s2_tp3	TIP1_EW_s2_tp4
TIP1_EW_s1	1				
TIP1_EW_s2_tp1	0.754	1			
TIP1_EW_s2_tp2	0.783	0.907	1		
TIP1_EW_s2_tp3	0.678	0.751	0.891	1	
TIP1_EW_s2_tp4	-0.071	-0.138	-0.135	-0.171	1
	TIP1_LW_s1	TIP1_LW_s2_tp1	TIP1_LW_s2_tp2	TIP1_LW_s2_tp3	TIP1_LW_s2_tp4
TIP1_LW_s1	1				
TIP1_LW_s2_tp1	0.738	1			
TIP1_LW_s2_tp2	-0.153	-0.212	1		
TIP1_LW_s2_tp3	-0.135	-0.317	-0.201	1	
TIP1_LW_s2_tp4	0.607	0.488	0.112	-0.209	1
TIP1_LW_s2_tp5	-0.139	0.126	0.072	-0.146	-0.145
	SuSy_LW_s1	SuSy_LW_s2_tp1	SuSy_LW_s2_tp2	SuSy_LW_s2_tp3	SuSy_LW_s2_tp4
SuSy_LW_s1	1				
SuSy_LW_s2_tp1	-0.114	1			
SuSy_LW_s2_tp2	0.095	-0.066	1		
SuSy_LW_s2_tp3	0.768	0.136	-0.119	1	
SuSy_LW_s2_tp4	0.607	-0.508	0.333	-0.218	1
SuSy_LW_s2_tp5	0.012	-0.467	0.282	-0.040	0.079

of water and other small solutes, and their involvement in plant growth and development has also been reported (Zardoya, Villalba 2001; Zardoya 2005; Abascal et al. 2014; Di Giorgio et al. 2014; Novikova et al. 2014) and they have been reported as one of most abundant transcript families in *P. taeda* xylem (Lorenz, Dean 2002). AQPs are represented by large gene families with various functions in a wide range of species (Yue et al. 2014), but most TIPs are classified as probable or confirmed aquaporins (Beaulieu et al. 2011). In some cases, the gene expression patterns described in his study differed from previously published reports. This may be a result of the analyzed genes being members of gene families, with various members having differing functions, localization or expression profiles, as well as species differences. In addition, the results presented in this report are average expression levels over a number of individuals, while the previous reports were mostly based on analysis of a single individual. Expression levels of three lignin biosynthesis genes were previously investigated in the same plant material (Kanberga-Silina et al. 2015), and large differences in expression levels were detected between individuals. This variation of gene expression between individuals also needs to be considered when comparing gene expression levels.

An aquaporin like gene was reported to be more

abundant during LW formation while a different aquaporin gene and the *MIP-2* gene were reported to be more abundant in EW (Li et. al. 2010). As our primers were based on the *P. radiata* aquaporin-like gene sequence, we expected that the expression pattern in *P. sylvestris* may be similar to that in *P. radiata*, and it would be more abundant during LW formation. However, our results identified the opposite expression pattern, as *TIP1* was more than ten times more highly expressed during EW formation (both for trees with HD and LD) than during LW formation, therefore suggesting a functional role as an aquaporin, facilitating transfer of solutes in the early part of the growing season. During the second season, *TIP1* gene was also more highly expressed during EW formation, corresponding to the result from the first season. One explanation for these differing expression profiles might be that these genes are paralogous between *P. radiata* and *P. sylvestris* and do not share the same function in the two species. *P. radiata* is not also closely phylogenetically related to *P. sylvestris* (Gernandt et al. 2005). It is possible that a different member of this gene family is performing the function of the *P. radiata* *TIP1* gene in *P. sylvestris*. A more thorough phylogenetic analysis of this gene family is necessary to identify all of the functional members in the *P. sylvestris* genome. The results obtained by Li et. al. (2010) were based

on microarray data from *P. radiata* trees of various ages and although the expression of a subset of their identified candidate genes was confirmed using multiple ligation-dependent probe amplification (MLPA), the *TIP1* gene was not, and therefore direct comparison can be made between the two studies.

The *TIP1* gene was significantly overexpressed in trees with lower wood density during EW formation in the first season. As EW wood has wider cell lumens and thinner cell walls (Uggla et al. 2001), then faster growth facilitated by aquaporins can lead to lower wood density. However, there is evidence that expression of some aquaporin-like genes is positively correlated with wood properties such as LW density and EW cell wall thickness (Kumar et al. 2009). *TIP1* expression during EW formation in the second season was higher for trees with low wood density. However, the statistically significant difference in *TIP1* gene expression between trees with high and low wood density was not confirmed by statistically significantly differing results from the second season, possibly due to the smaller number of analyzed individuals.

A sucrose synthase sequence was reported by Li et al. (2009) as one of thirty most abundant EST transcripts in *P. radiata* and Li et al. (2010) reported *SuSy* to be preferentially transcribed in juvenile wood with high stiffness and low microfibril angle. Yang and Loopstra (2005) reported that *SuSy* transcripts were more abundant in LW than EW, however expression of the *SuSy* was not confirmed using real time PCR as was done for some other genes identified in this report. Our results indicated that *SuSy* was more highly expressed during EW formation in both seasons. Fluctuations of *SuSy* transcript levels during the second growing season were slight and not significantly different between trees with HD and LD, except at the third time point during LW formation where in trees with HD, *SuSy* expression was significantly higher. These changes may be related to specific activity of transcriptional factors influencing *SuSy* expression, or, as mentioned previously, multiple members of the *SuSy* gene family may have differentiated to perform differing roles. Nairn et al. (2008) reported that *SuSy* was more highly expressed in xylem than in phloem for three of four analysed *P. taeda* trees and that expression profiles of this gene varied slightly between the analysed individual trees at four sampling points (Apr, Jun, Jul, Aug). From our data, *SuSy* expression did not vary significantly between trees with higher and lower wood density in the first growing season, and differences in mean expression between EW and LW were also not statistically significant. In hybrid poplar, *SuSy* is considered to provide the largest effect on biomass accumulation and height growth due to increased cellulose synthesis linked with *SuSy* overexpression (Coleman et al. 2009). Our findings about *SuSy* gene expression patterns also tend to agree with findings about lack of seasonality for carbohydrate availability and suggestions that the length

of the developmental time period of late wood cell walls results in thicker late wood cell walls, but not the pace of cell wall material deposition (Uggla et al. 2001).

The results obtained in this study contribute to our understanding of the expression patterns of these two genes during early and late wood formation during two growing seasons in trees with differing wood properties growing in an uncontrolled field environment. Expression levels of both analysed genes fluctuate over the course of the growing season, and further phylogenetic analysis of both gene families is required to determine the full functional complement of these gene families in *P. sylvestris*.

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