Expression of three phenylpropanoid pathway genes in Scots pine (*Pinus sylvestris* L.) in open-pollinated families with differing relative wood densities during early and late wood formation

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Abstract

Wood volume and quality are the most important aspects of commercial forestry production, and studies of wood formation are important in order to increase the value and efficiency of forestry production. The phenylpropanoid pathway produces various compounds with diverse functions both for plant defence against biotic and abiotic stress as well as structural development. One of the main roles is monolignol production for lignin biosynthesis, which is a crucial aspect of wood formation. For this study three candidate genes involved in lignin biosynthesis were selected: phenylalanine ammonia-lyase (*PAL1*), cinnamyl alcohol dehydrogenase (*CAD*) and cinnamoyl-CoA reductase (*CCR*). Candidate gene expression was analysed in selected individuals with high and low wood density from open-pollinated Scots pine families during early wood (EW) and late wood (LW) formation and correlation between expression of these genes, total lignin content, and wood density was determined. Wood density values for analysed trees were similar within tree families but differed significantly between families with high and low wood density ($p = 1{,}06E\text{-}20$). Wood density was slightly negatively correlated with lignin content ($r = -0.36$, $p = 0.038$), but only in individuals in the high density wood group. In trees with low wood density, expression of the *CAD* gene was significantly lower in late wood formation compared to early wood ($p = 0.00179$). In trees with high wood density, expression of the *PAL1* gene was five times higher during early wood formation compared to late wood formation. A positive correlation was detected between *PAL1* and *CCR* gene expression during early wood formation ($r = 0.804$) and late wood formation ($r = 0.466$).

Key words: CAD, *CCR*, *PAL1*, Scots pine, *Pinus sylvestris*, wood density, early wood, late wood.

Introduction

Scots pine (*Pinus sylvestris* L.) is one of the most important tree species in northern Europe and is one of most widely distributed conifer species in the world (Nicolov and Helmisaari, 1992). In Latvia, Scots pine covers 35% of the forested area. Despite the economic and ecological importance of this species, it has been less studied at a molecular level than two other economically important pine species – *Pinus radiata* and *Pinus taeda* (Lev-Yadun and Sederoff, 2000).

Wood formation is influenced by exogenous and endogenous factors and their interaction and driven by coordinated expression of structural and regulatory genes (Plomion et al., 2001). Wood properties are controlled by gene and protein activity during xylogenesis (Whetten et al., 2001). Conifer wood formation in temperate regions has an annual pattern of early wood formation in spring/summer and late wood formation, in late summer and autumn when cell division activity decreases (Uggla et al., 2001). As a result, early wood and late wood tracheids differ in diameter and cell wall thickness (Lev-Yadun and Sederoff, 2000). Early wood consists of thin-walled and larger cell lumens whereas late wood tracheids have narrower lumens and thicker cell walls (Uggla et al., 2001). Early wood has more lignin, lower hemicellulose, cellulose and lower density (Li et al., 2009). Wood properties also vary as trees age, with juvenile wood having a higher proportion of early wood (Plomion et al., 2001; Cato et al., 2006). The wood density of *P. sylvestris*

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increases in the juvenile phase up to age 15 and then stabilizes in the mature phase and there is a strong correlation between wood density in juvenile wood and in mature wood. 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 and ADAMS, 1994).

Lignin is a complex biological compound, which lacks a defined structure and is highly heterogeneous. It is one of main biopolymers within plants and is necessary for structural integrity of the cell wall, stiffness and strength of the stem. It waterproofs the cell wall, enabling water and solute transport and plays a role in plant protection against pathogens (BOERJAN et al., 2003). Average lignin content in various *Pinus* species ranges between 25–35% of dry weight (PLOMION et al., 2001). Lignin biosynthesis derives from the general phenylpropanoid pathway (DIXON et al., 2002; VANHOLME et al., 2010). Because of the high heterogeneity of lignin among species and within a single plant, the regulation of the lignin biosynthesis pathway may vary among cell types and species, and the activity of lignin biosynthesis entry-point enzymes are likely to affect metabolite flux in the biosynthesis pathway (CAMPBELL and SEDEROFF, 1996).

The phenylalanine ammonia-lyase (PAL) enzyme catalyses the deamination of phenylalanine to cinnamate which is the first step in phenylpropanoid biosynthesis and provides substrates for the monolignol biosynthesis pathway (WHETTEN and SEDEROFF, 1995). In *Pinus taeda*, a single *PAL* gene was identified in a xylem-specific cDNA library (WHETTEN and SEDEROFF, 1992), while in angiosperm genomes, *PAL* belongs to a multigene family, and it has been suggested that gymnosperm and angiosperm lignin biosynthesis pathways are highly distinct. However, in *Pinus banksiana*, research suggests that *PAL* is encoded by a multigene family (BUTLAND et al., 1998), and it has been reported recently that there are actually five *PAL* genes in the *P. taeda* genome (BAGAL et al., 2012). *PAL* activity increases in *P. taeda* cells treated with plant growth regulators (EBERHARDT et al., 1993), as well as in elicited *P. banksiana* cells (CAMPBELL and ELLIS, 1992). Elevated *PAL* activity may be necessary in order to redirect sufficient phenylalanine towards phenylpropanoid biosynthesis because of the importance of this pathway both for normal development and plant defence (CAMPBELL and SEDEROFF, 1996). Single nucleotide polymorphisms in the *PAL1* gene are associated with wood density in *P. radiata* (DILLON et al., 2010). DVORNYK et al. (2002) found that Scots pine has a low level of within-population diversity at the *pal1* locus which suggests a very high functional constraint on the protein. The cinnamoyl-CoA reductase (CCR) enzyme catalyses the reduction of hydroxycinnamoyl-CoA thioesters to corresponding aldehydes and is hypothesized as a key regulator in lignin biosynthesis as the first committed step in monolignol production from phenylpropanoid metabolites (WHETTEN and SEDEROFF, 1995). Genes encoding CCR in various species belong to families with multiple members. In the *Populus* genome there are 8 CCR-homologs or CCR-like gene sequences (LI et al., 2006), while only two CCR genes were identified in a Norway spruce EST library (KOUTANIEMI et al., 2007). Cinnamyl alcohol dehydrogenase (CAD) catalyses the reduction of hydroxycinnamaldehydes to hydroxycinnamylalcohols and this is final step in the monolignol biosynthesis pathway. The extractability of lignin is increased in plants with reduced CAD activity, making them more suitable for pulp production (WHETTEN and SEDEROFF, 1995; BAUCHER et al., 1996). *P. taeda* trees carrying a mutant *cad-n1* allele have significantly higher wood density compared to wild-type trees because *cad-n1* heterozygotic trees had more late wood than trees with the wild type allele (YU et al., 2005; YU et al., 2006). In *P. taeda*, CAD is encoded by single gene (O'MALLEY et al., 1992). CAD may modulate lignin composition in pine (MACKAY et al., 1997), and LI et al. (2011b) found that CAD was preferentially transcribed in wood with high stiffness (low microfibril angle) in *P. radiata*. Reduction of PAL activity affects lignin content and flux through the lignin biosynthesis pathway more than decreased CAD activity (MACKAY et al., 1997; BATE et al., 1994).

Expression of lignin biosynthesis related genes is influenced by environmental factors and developmental signals, and coordinated by transcription factors such as MYB, and these networks are mostly still unknown (PLOMION et al., 2001). RAHANTAMALALA et al. (2010) reported coordinated transcriptional regulation of CAD and CCR through MYB binding sites, and overexpression of EgMYB1 in Arabidopsis and poplar leads to reduced lignin biosynthesis

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related gene expression (LEGAY et al., 2010). Candidate genes involved in lignin biosynthesis may influence microfibril angle and wood density through their importance in secondary cell wall formation (LI et al., 2011a). Gene expression patterns have been studied in many organisms in response to a wide range of conditions and treatments, and gene expression differences are important for phenotypic variation within species. Gene expression levels can vary between cell types within a single plant and also between individuals (PALLE et al., 2010). Expression of specific candidate genes has been studied as well as a more general profiling of expression using sequencing, microarrays and other techniques. However, gene expression variation and differences in populations growing in natural conditions has not been widely investigated.

Understanding the structure and formation of wood is important for molecular marker assisted tree breeding strategies (PLOMION et al., 2001). UKRAINETZ et al. (2008) suggest that breeding for improved early wood density in Douglas-fir would result in negligible reductions in volume and appears to be suitable for selecting for improved wood density while maintaining growth rates. The main genes of interest are those which affect wood properties such as cell wall thickness, wood density, and the chemical composition of cell wall components such as cellulose, lignin and hemicelluloses. These genes are potential targets for modification of wood properties (YANG and LOOPSTRA, 2005). The aim of this study was to select genes involved in lignin biosynthesis, one of the important processes in wood formation, to study the expression of these genes during early wood and late wood formation in open-pollinated families growing in natural conditions, and to determine whether a correlation exists between expression of these phenylpropanoid biosynthesis genes, total lignin content, and wood density with a future goal of including this knowledge in tree breeding strategies.

Materials and methods

Genes analysed in this study were selected based on previously published studies and availability of gene sequences in pine species. Gene sequences for primer design were obtained from the NCBI database (http://www.ncbi.nlm.nih.gov/). The GenBank accession numbers for the genes used in this study are *Pinus taeda* sequences *CCR*-AY064169, *CAD1*-AF549814, and *Pinus sylvestris* sequence for *PAL1*-AF353986. Primers for candidate genes were selected using the programs Primer3 (http://frodo.wi.mit.edu/), FastPCR (http://primerdigital.com/fastpcr.html) and checked by NetPrimer (http://www.premierbiosoft.com/netprimer/). For each gene, four to six primer pairs were tested and the ones with the most similar PCR effectivity with the endogenous control primers were selected for real-time PCR analysis. The primers were analysed using BLAST against the GenBank nucleotide and expressed sequence tag (EST) databases. The *CCR* gene primers were homologous to several unannotated *P. sylvestris* ESTs. The forward primer was 100% homologous to these sequences, while the reverse primer had one mismatch. However, this was in the penultimate 5'position, and so should not affect the PCR efficacy. The *CAD* gene primers did not find any significant homology among *P. sylvestris* sequences in the NCBI database. However, the primer sequences and expected product lengths were highly conserved in several other pine species, indicating that these gene sequences are conserved within *Pinus*, and that cross-species amplification using these primers should not be problematic, provided that the PCR efficacy of the primers is determined utilising the experimental material. The primers used for real-time PCR (5'- 3') sequences were: CADVF - ACAAGCAGCCCCTCT, CADVR - TCTTCCCGGGCTCTG, PALZF - CGGATCCATTAACTCACAAGC, PALZR - TCCCGTCCAAGACATACTCC, PtCCR1f - TCAGGCCTATGTCCATGTCA, PtCCR1r - CGAGTCAACCACATCACCAC. *GAPDH* gene primers for use as expression controls are described in ŠĶIPARS et al. (2011).

The *P. sylvestris* individuals analysed were from a pine plantation consisting of half-sib families established in 1982 in central Latvia (Vecumnieki, 56°41'N, 24°26'E). Distance between trees was 2 x 1 m and no thinning was done at this site. The analysed trees were open pollinated progenies from four populations growing in different regions of Latvia: Misa (Rigas region), Andrupene (Kraslavas region), Silene (Daugavpils region), Silene2 (Daugavpils region).

Wood density values were obtained using the Pilodyn instrument, which provides a non-

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destructive method for wood density measurement (HANSEN, 2000). KIEN et al. (2008) showed that wood density values obtained with destructive methods and by Pilodyn are comparable. Measurements were carried out at a height of 1 m from the base of the tree. The Pilodyn wood tester strikes a 40 mm long and 2.5 mm diameter pin into the tree trunk with a constant power of 6J. Wood density was determined by the depth of penetration of the pin. Wood density results are presented as the length in mm of the striker pin left outside of the tree trunk. Trees with higher wood density show a longer part of the pin left outside the tree trunk. For gene expression analysis 48 trees were selected (24 with higher wood density (HD) and 24 with lower wood density (LD)). The individuals with high wood density were from 4 half-sib families, and samples with low wood density were from 6 half-sib families.

For total lignin content determination, wood samples were obtained from all 48 trees at a height of approximately one metre in accordance with TAPPI T 257 "Sampling and Preparing Wood for Analysis", extracted with acetone according to TAPPI T 264 „Preparation of Wood for chemical Analysis". Klason lignin content was determined according to TAPPI 222om-98: the carbohydrates in wood were hydrolyzed and solubilized by 72% sulphuric acid, the acid-insoluble lignin was filtered off, dried and weighed. Lignin content analyses were carried out at the Latvian State Institute of Wood Chemistry.

Samples for RNA extraction were collected at two time points from each individual – in spring (April) during early wood (EW) formation and in autumn (October) during late wood (LW) formation (96 maples in total). 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. Samples at the two time points were collected at the same height, on opposite sides of the trunk. Biological replicates were not able to be collected due to the diameter of the trunks, and the fact that these individuals were part of a longer-term experiment, and so were required to be sampled non-destructively.

An adapted CTAB buffer based method was used for RNA extraction, (CHANG et al., 1993; PINA and PEREZ, 2011). RNA quantity was assessed fluorometrically using a Qubit fluorometer and Quant-iTreagents (Invitrogen) following the manufacturer's instructions. RNA extracts were treated with DNase I (Fermentas) and purified using phenol/chloroform extraction. DNA contamination of RNA was tested by standard PCR reactions, using intron-spanning primers (primer sequences (5'- 3'): 5SUSf: CCTGGTCTCTACCGTGTGGT; 5SUSr: GTAAGGCGATGCTGCTTTTC) and visualized by 1.2% agarose gel electrophoresis, stained with ethidium bromide. The expected amplicon size from cDNA was 119bp, and the amplicon size from genomic DNA was ~200bp.

For cDNA synthesis, 500 ng of total RNA in 50 µl reaction was reverse transcribed using Taqman reverse transcription reagents (Invitrogen) and Oligo(dT)18 primer (Fermentas) according to the manufacturer's instructions. cDNA for standard curves was obtained from 1 µg total RNA per reaction. cDNA quality was checked using a PCR reaction with intron-spanning primers as described previously. Gene expression analysis was performed by real-time PCR using a relative standard curve method and carried out on an Applied Biosystems StepOnePlus 96–well Real-time PCR system using a standard three step real-time PCR protocol using the Maxima SYBR Green PCR kit (Fermentas) in a total volume of 20 µl containing 2 µl of cDNA and 0.15 µM primers.

Each sample for each candidate gene was run in three technical replicates and the housekeeping control gene *GAPDH* was run on the same plate. Only samples with technical replicates with *Ct* values that were within ±0.3 cycles were analysed, otherwise reactions were repeated. After amplification, melting curve analysis was used 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 were analysed using the StepOne software v2.2, single factor 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 and low density) as well as according to sampling time points (early and late wood).

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Table 1. – PAL1, *CCR*, *CAD* gene expression during early wood (EW) and late wood (LW) formation in ten open-pollinated Scots pine families. Unshaded – tree families/individuals with higher wood density; shaded – tree families/individuals with lower wood density. Mean gene expression values given as % of *GAPDH* expression. nd – no data.

	Gene/Time point					
Family/Individual	PAL1/EW	PAL1/LW	CCR/EW	CCR/LW	CAD/EW	CAD/LW
Mis 34/1-I	15.235	1.421	8.591	4.523	46.484	46.090
Mis 34/1-II	34.837	0.382	61.501	10.032	135.529	3.994
Mis 34/1-III	54.927	nd	3.912	21.099	71.711	78.900
Mis 34/3-II	5.625	nd	5.291	49.703	153.480	104.100
Mis 34/5-VI	45.103	nd	3.117	9.814	58.232	400.251
Mis 34/9-VI	12.884	nd	16.546	nd	151.790	nd
S 25/5-III	2.709	61.278	5.094	nd	46.647	69.870
S 25/6-VI	3.774	57.350	2.796	3.821	32.023	5.219
S 25/7-IV	8.838	34.123	3.405	30.596	116.041	6.659
S 25/7-V	14.087	30.300	nd	4.928	69.633	5.599
S 25/8-IV	12.243	37.721	3.498	4.040	124.283	49.664
S 25/9- I	13.798	58.336	5.432	39.734	31.649	9.650
S 2 17/5-II	nd	59.667	nd	11.577	nd	2.992
S 2 17/10-VII	nd	34.792	1.892	3.713	nd	10.891
S 2 17/2-II	861.116	70.522	133.844	11.462	128.101	56.112
S 2 17/2-IV	402.152	39.892	48.326	1.123	84.188	6.508
S 2 17/3-VI	135.472	23.853	2.321	9.364	109.035	3.653
S 2 17/7-V	nd	62.945	7.098	0.810	192.256	12.832
S 2 17/9-IV	454.481	55.193	22.994	20.430	157.065	561.600
S 2 18/2-III	11.185	194.117	3.973	16.065	68.216	2.551
S 2 18/4-I	59.186	192.672	9.944	71.084	205.946	5.385
S 2 18/5-I	544.119	109.689	200.747	19.716	98.731	55.269
S 2 18/5-VIII	135.531	71.273	0.984	29.126	103.537	40.003
S 2 18/9-VIII	19.003	30.874	8.613	0.503	92.616	69.970
And 19/1-VII	7.422	51.198	7.706	10.268	110.510	103.050
And 19/4-VI	9.156	184.403	3.048	5.862	138.735	165.178
And 19/5-IV	55.550	2.389	4.878	8.898	89.651	26.863
Mis 60/5-III	5.625	54.95	190.317	20.694	145.685	49.211
Mis 60/6-VI	173.038	54.622	9.442	14.461	88.846	15.522
MIS 60/6-VII	12.884	52.090	6.103	5.950	178.004	8.110
MIS 60/7-VI	17.626	54.798	12.720	0.789	144.992	133.048
MIS 60/10-III	7.848	nd	7.918	nd	235.219	nd
MIS 60/1-IV	7.848	nd	3.988	nd	80.632	nd
MIS 60/5-VII	19.003	nd	30.668	nd	373.380	nd
S 2/10-IV	12.812	26.865	5.681	8.791	14.123	118.171
S 2/7-V	8.838	15.049	2.382	4.394	29.084	17.612
S 2/9 -VII	31.126	195.410	2.443	21.620	58.900	1.635
S 24/10-VIII	14.087	30.094	2.785	0.743	129.002	8.706
S 24/2-V	20.732	1.022	3.566	1.960	58.520	6.679
S 24/5-III	45.103	2.982	6.177	2.849	169.033	29.207
S 24/7-VII	nd	7.191	1.617	4.012	nd	65.427
S 24/8-II	6.748	39.786	6.152	1.097	61.863	49.310
S 8/1-VI	25.623	54.800	24.253	7.280	140.385	49.143
S 8/5-IV	31.126	69.366	1.154	13.683	16.868	75.096
S 8/8-III	35.235	54.831	108.634	5.262	387.435	49.110
S 9/1-III	20.270	23.546	8.004	0.933	255.318	nd
S 9/4-VIII	25.910	34.610	0.483	10.181	227.741	0.939
S 9/7-VIII	5.438	141.447	4.303	3.008	125.133	11.923

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Results

Expression levels of the three examined candidate genes varied widely between individuals, reflecting the genetic and environmental heterogeneity of these open-pollinated trees growing in natural conditions. Gene expression was compared for all samples between the EW and LW time points. The mean value of *PAL1* expression during early wood (EW) formation was 76.80% of *GAPDH* expression. The lowest individual expression was 2.70% and the highest was 861.10%. During late wood (LW) formation the average was 58.26% with the lowest expression in one tree 0.38% and the highest 195.40%. Expression of the CCR gene was lower than *PAL1* expression during EW formation, with an average of 21.90% with lowest expression 0.48% and highest 200.70% and during LW formation the average expression was 12.35% with lowest 0.50% and highest 71.00%. Mean values for *CAD* expression were 123.50% of *GAPDH* expression during EW and 60.70% during LW formation with lowest and highest expression levels 14.10% and 387.40% (EW); and 0.94% and 561.60% (LW) respectively (*Table 1*). For the PAL1 and CCR genes there were no statistically significant differences in gene expression levels between early and late wood formation. A statistically significant higher mean gene expression during early wood formation compared to late wood was detected for the *CAD* gene ($p < 0.01$).

Wood density values between the group of trees with low wood density (individuals from six families) and with high wood density (four families) varied significantly ($p = 1.06E\text{-}20$). Wood density as measured with the Pilodyn instrument varied from 22–30.5 mm. The average value for trees with lower density was 25.1 mm and for trees with higher density 29.5 mm. Lignin content ranged from 26.9% to 32.3% in the 48 analysed individuals. ANOVA indicated that the lignin content between groups with high and low wood density were not statistically significant. There was no correlation between lignin content and wood density when all individuals were analysed trees together. There was also no significant correlation between lignin content and wood density values for trees with lower wood density, but wood density values for trees with higher wood density were slightly negatively correlated with

Table 2. – Mean gene expression values given as % of *GAPDH* expression. Ratio of mean gene expression values between early wood (EW) and late wood (LW) formation and for trees with higher (HD) or lower (LD) wood density.

Gene/wood density	EW+LW	EW	LW	Expression ratio (EW/LW)
a. *CAD*				
HD+LD	-	123.03	60.27	2.0
HD	86.33	103.51	69.9	1.5
LD	98.67	141.7	49.2	3*
Expression ratio (HD/LD)	0.9	0.73	1.42	-
b. *CCR*				
HD+LD	-	22.05	12.23	1.8
HD	21.21	25.45	16.97	1.5
LD	13.49	18.93	7.27	2.6
Expression ratio (HD/LD)	1.6	1.34	2.33**	-
c. *PAL1*				
HD+LD	-	78.30	58.00	1.4
HD	99.33	135.54	61.32	2
LD	39.78	26.05	54.83	0.5
Expression ratio (HD/LD)	2.5	5.20**	1.12	-

*–$p < 0.01$, **–$p < 0.05$.

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Table 3. – Correlation of candidate gene expression, total lignin content and wood density during early and late wood formation, n = 26.

r crit=0,388	*CCR* LW	*PAL1* LW	*CAD* LW	*CCR* EW	*PAL1* EW	*CAD* EW	Density	Lignin content
CCR LW	1							
PAL1 LW	0.466*	1						
CAD LW	0.035	0.012	1					
CCR EW	0.045	0.047	0.035	1				
PAL1 EW	0.045	0.034	0.331	0.804*	1			
CAD EW	0.242	0.184	0.194	0.143	0.19	1		
Density	0.329	-0.054	0.051	0.374	0.397*	0.095	1	
Lignin content	-0.168	0.090	-0.232	0.096	0.184	-0.276	-0.050	1

lignin content (r = –0.36, p = 0.038). No significant correlations between candidate gene expression and lignin content were detected.

Samples were divided into high wood density (HD) and low wood density (LD) groups, and gene expression was compared between EW and LW time points within these groups. No statistically significant differences in *PAL1* and *CCR* gene expression were found comparing mean gene expression within these wood density groups between EW and LW formation. However, for the *CAD* gene, a significant difference in gene expression was found only for trees with lower wood density – during EW formation it was three times higher than during LW formation (*Table 2*). No significant difference in mean gene expression between time points was found for the high wood density group. Therefore, the previously observed significant difference in *CAD* gene expression for all analysed trees between EW and LW was due to the differences in *CAD* gene expression in the lower density group.

Further, samples were divided into early wood (EW) and late wood (LW) time points, and gene expression was compared between HD and LD groups. Comparing gene expression within wood density groups during EW and LW formation, significant differences were detected for the *PAL1* and *CCR* genes. During EW formation, the *PAL1* gene was five times more highly expressed in the HD group, compared to the LD group. During LW formation, there was no significant difference in *PAL1* expression between trees with HD and LD. During LW formation, the *CCR* gene was significantly overexpressed in trees from the HD group, compared to the LD group (*Table 2*).

As these candidate genes are involved in the same biosynthetic pathway, correlation between expression levels of the candidate genes was examined. A positive correlation was found for the *PAL1* and *CCR* genes during EW and LW formation. During EW formation, the correlation coefficient was almost two times higher (*Table 3*). A positive correlation during EW formation was also found between *PAL1* gene expression and wood density (*Table 3*). No cor-

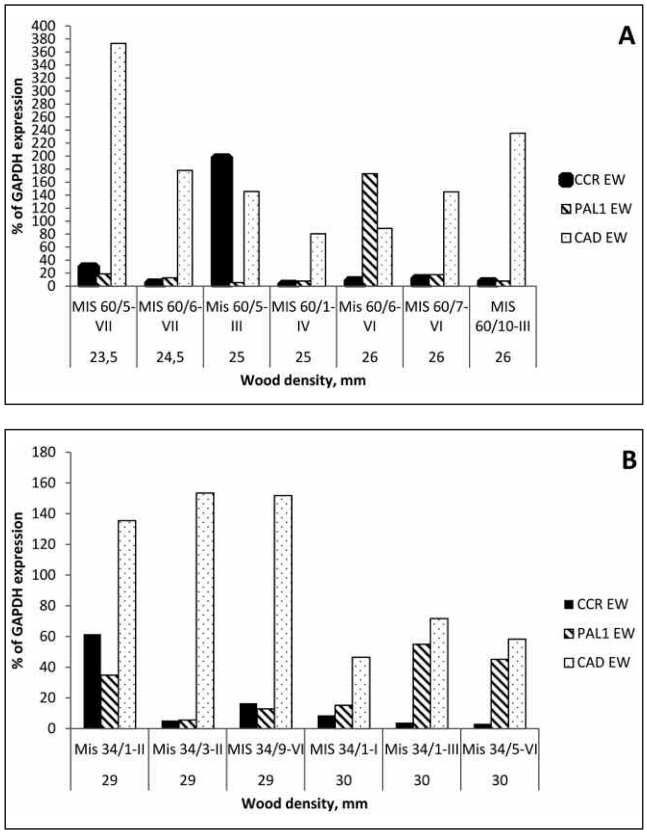

Figure 1. – *PAL1*, *CCR1* and *CAD1* gene expression during early (EW) formation in half-sib individuals from the Mis 60 (A) and Mis 34 (B) families.

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relation was found in expression of the *PAL1* gene between the EW and LW wood time points (*Table 3*). No correlation of gene expression with family structure was found. Gene expression within families varies greatly, for example, within the Mis 60 and Mis 34 families, one with lower and the other with higher wood density, gene expression varies by up to three times between samples belonging to the same family (*Figure 1*).

Discussion

The *PAL1*, *CCR* and *CAD* genes were selected for this study because of their previously described importance in wood formation in other species (Whetten and Sederoff, 1992; Whetten and Sederoff, 1995; Li et al., 2006; O'Malley et al., 1992). For the *PAL1* gene, a *P. sylvestris* gene sequence was available, while for the other two genes, sequences from *P. taeda* were utilised for primer design. To our knowledge, it has not been determined if these genes are a part of gene families in *P. sylvestris*, so it is possible that there are additional genes with similar functions that are expressed in different patterns than those examined in this study. These genes have been found to be part of gene families in other pine species (Butland et al., 1998; Bagal et al., 2012). However, the real-time PCR products did not reveal any additional peaks during melting curve analysis which would indicate the amplification of non-target sequences. The increasing availability of sequence data from *P. sylvestris* and other pine species will allow further elucidation of gene families involved in the phenylpropanoid biosynthetic pathway.

In this study, gene expression in a total of 48 individuals from 10 half-sib families growing in natural conditions was examined. A large amount of variation in gene expression was found, and there was no correlation of gene expression levels with family structure, which is not surprising given that the individuals sampled were growing in natural conditions and were from half-sib families, which contain a large amount of genetic variation even within families. Gene expression differences between individuals growing in natural conditions have not been widely studied in *P. sylvestris* previously. The expression of candidate genes related to xylem development has been studied in 400 unrelated loblolly pine clones, where significant variation was found for 106 of the 111 genes examined. The gene expression differences between high and low expressing clones ranged from 4.3 to 362 fold (average 20.8) (Palle et al., 2010). The results from this study also exhibit a large variation in gene expression between individuals, with the *PAL1* gene showing the largest expression differences between low and high expressing individuals, followed by *CCR* and then *CAD*. No correlation was found between expression of candidate genes in individuals between time points, indicating that there is not a systematic high or low gene expression level within individuals, or mutations within the gene or promoter which lead to a consistent relative level of expression. Similarly, no correlation of gene expression and family structure was found. Previous studies analysing expressed sequence tags (ESTs) for pine species such as *P. taeda* and *P. radiata* imply that genes involved in lignin biosynthesis can be differentially expressed during EW and LW formation as well as in juvenile and mature wood (Egersdotter et al., 2004; Yang and Loopstra, 2005; Kumar et al., 2009). Results from this study indicated that there was no statistically significant difference in *PAL1* and *CCR* gene expression between EW and LW formation (Li et al., 2009). However, our results showed a significantly higher expression of the *CAD* gene in EW compared to LW. Analysing this in more detail, it was found that for trees with lower wood density, expression of the *CAD* gene was three times higher during early wood formation, but for trees with higher density there was no statistically significant difference in *CAD* expression. *CAD* has been proposed as a lignin composition modulator (Mackay et al., 1997). Analysis of lignin biosynthesis related EST abundance found six *CAD* EST sequences during EW and three during LW formation (Li et al., 2009). One of the reasons for the lower density of early wood has been proposed to be the greater amount of lignin in early wood tracheids (Li et al., 2009; Bertaud and Holmbom, 2004).

Considering that the *CAD* gene *CAD1-n1* defective allele has been reported to have an important role in *P. taeda* breeding for improved wood properties (Whetten and Sederoff, 1995; Yu et al., 2005; Yu et al., 2006), these results may be able to be applied in Scots pine breeding as well. In contrast, in *P. radiata*, *CAD* was preferentially transcribed in high

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stiffness wood which has low microfibril angle and therefore higher density (Li et al., 2011b). In addition, *CAD* was preferentially expressed in latewood in *P. taeda* (Yang and Loopstra, 2005). It is possible that additional regulatory mechanisms also play an important role in monolignol transformation and deposition.

A more detailed comparison between trees with HD and trees with LD within EW and LW formation time points was made. *PAL1* expression was five times higher during EW formation in trees with higher wood density. Campbell and Sederoff (1996) suggested the involvement of *PAL1* in plant stress reactions and importance in normal tree development as well. As the trees used for these experiments were not growing in a controlled environment, it is possible that elevated *PAL1* levels in spring help both to protect tree from potential stress, while also providing enough substrate for monolignol biosynthesis. The *CCR* gene was more highly expressed in trees with higher wood density during late wood formation. *CCR* is reported to be a key regulator of monolignol biosynthesis as entry point enzyme (Whetten and Sederoff, 1995), and such genes coding for key regulatory enzymes may be good targets for manipulation of lignin content.

Correlation of gene expression was found between *PAL1* and *CCR* during both EW and LW formation. It is possible that elevated *PAL1* activity provides more substrate for *CCR*, and therefore the expression of the *CCR* gene increases. Correlation between expression levels of *PAL* and *CCR* was found in other studies as well (Palle et al., 2010). A positive correlation was also found between *PAL1* expression and wood density. In this study, wood density was measured using a Pilodyn instrument. Wood density measurements made using this instrument have been reported to correspond to values obtained by other methods (Kien et al., 2008), and while it has also been reported that the measurements are not as accurate (Raymond and MacDonald, 1998), the non-destructive sampling technique is advantageous. Wood density in pine trees is undoubtedly influenced by a number of genetic and environmental factors, and lignin content is only one factor in determining wood density. However, the genes involved in the phenylpropanoid biosynthesis pathway and their expression patterns are comparatively well characterised and studied in conifers. There are few reports of the relationships between phenylpropanoid gene expression and wood density, and in studies where gene expression was examined in the context of other wood properties or wood formation time points, the results are varied. Higher lignin content has been correlated with lower wood density in *Pinus pinaster* (Pot et al., 2002) and *Eucalyptus globulus* (Poke et al., 2006). We also found slight negative correlation between wood density and lignin content, but only in trees in the HD group, so our results partially support these previous reports.

In this study, expression of the *PAL1* and the *CCR* genes was increased in high density wood, which corresponds to previous reports, where expression of lignin biosynthesis genes was also found to be higher in latewood, which has higher density and lower lignin content (Egertsdotter et al., 2004). One explanation is that while the relative lignin content maybe lower in latewood, the cell walls are thicker, requiring increased lignin biosynthesis. In this study, the higher expression of *PAL1* and *CCR* in individuals with high wood density may indicate this, as well as reflecting the coordinated expression of these two genes. Studies of transgenic plants has shown that an increase in the expression of both *PAL* and *CCR* lead to an increase in lignin content (Vanholme et al., 2010). In contrast, reduced expression levels of *CAD* do not result in a change in total lignin content, but lead to differences in lignin composition (Mackay et al., 1997). In this study, a higher expression of *CAD* was found in individuals with lower wood density during early wood formation, and further determination of lignin composition will allow more detailed comparisons with wood density. There were no significant correlations detected between total lignin content and candidate gene expression levels in this experiment. Lignification processes, lignin content and composition can be highly variable and complex and alternative mechanisms may also exist when the main synthesis pathway is affected (Sederoff et al., 1999; Vanholme et al., 2010). Considering the fundamental role of lignification in plants and in trees in particular, moderate changes in gene expression may not have an effect on total lignin content due to redundancy of functional pathways and the complexity of lignin. One area of further investigation would be to determine the relationship of lignin composition with wood density values and gene expression in these individuals.

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Our results identified large natural gene expression variation within half-sib Scots pine families and that the expression of a particular candidate gene in an individual was not correlated between time points. Significant differences were found between mean gene expression levels of individuals grouped according to growing season and wood density. Gene expression differences play an important role in phenotypic variation within and between species (ALONSO-BLANCO et al., 2009), however relatively few studies have examined the variation of gene expression in natural populations. Correlations that can be established between individuals from different families with such large natural variation must be taken in consideration as important clues for understanding wood formation related molecular processes within trees. PALLE et al. (2010) found large differences in gene expression between *P. taeda* clones for lignin biosynthesis genes (11–27 fold differences) and found that there was greater variation between clones in gene expression for lignin biosynthesis genes than for genes involved in cellulose biosynthesis. Results from this study also found a large amount of variation between individuals, while also detecting significant differences in mean values of gene expression between groups of individuals with high and low wood density, and between early wood and late wood formation time points. This can give an important insight into gene expression patterns in trees growing in natural habitats and can contribute to our understanding of plant development and adaptation.

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Genetic and phenotypic correlations among volume, wood specific gravity and foliar traits in white spruce (*Picea glauca* (Moench) Voss)

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Abstract

White spruce is highly valued by the forest products industry in North America. Through tree improvement efforts, selected genotypes can exceed wild sources by 30% in volume. Negative correlations between growth and wood specific gravity have been established, but differences in leaf morphology between high and low performing genotypes are less well understood. We sampled five trees from each of 30 families at each of two locations in a 25-year old progeny test in Minnesota. One wood core was collected from each tree to sample wood specific gravity (WSG), along with a branch collected from the upper crown to examine foliar traits. We confirmed negative correlations between stem volume and WSG, but several families combined high wood volume with only small reductions in WSG. Leaf area ratio and specific leaf area were positively, genetically correlated with volume growth but not correlated with WSG. Increased growth rates of selected genotypes may be attributed, in part, to shifts in allocation to leaves and in leaf morphology that may optimize light interception.

Key words: white spruce, genetic correlations, wood specific gravity, specific leaf area, genotype by environment interaction, foliar nitrogen.

Introduction

White spruce is highly valued by the wood products industry in North America and is widely planted across Minnesota. Seed orchards, comprised of genotypes selected for superior growth are a common source used for tree planting on public and private land. Selection for fast growth is highly effective: differences between orchard-grown seedlings and local wild sources approach 30% in wood volume (PIKE et al., 2006; WENG et al., 2010). Neg-

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