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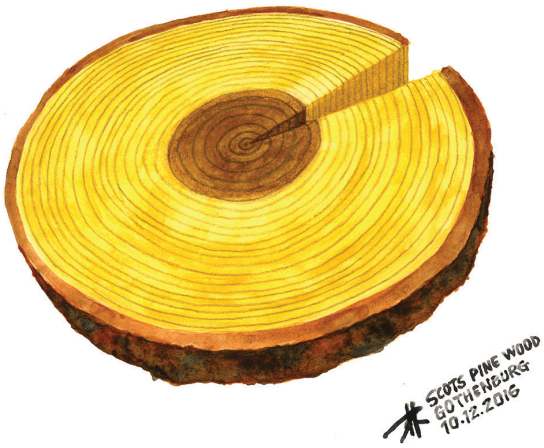
KEAN-JIN LIM

Scots Pine (*Pinus sylvestris* L.) Heartwood Formation and Wounding Stress: A View from the Transcriptome



DEPARTMENT OF AGRICULTURAL SCIENCES
FACULTY OF AGRICULTURE AND FORESTRY
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Scots pine (*Pinus sylvestris* L.) heartwood formation and wounding stress: A view from the transcriptome

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ACADEMIC DISSERTATION

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Cover: *Scots pine wood*, Kean-Jin Lim, watercolour. The pinewood disk was a gift from Martti Venäläinen during our sampling trip in Punkaharju.

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The glow of sunrise in Viikki was colourful and warm; a contrast to the cold, dark and lifeless winter. *Sunrise in winter*, Kean-Jin Lim, watercolour.

不经一番寒沏骨
焉得梅花扑鼻香

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List of original publications

This thesis is based on the following publications that are referred to in the text by their Roman numerals. The publications have been reprinted with permission from the publishers.

I **Lim KJ**, Paasela T, Harju A, Venäläinen M, Paulin L, Auvinen P, Kärkkäinen K, Teeri TH (2016) Developmental changes in Scots pine transcriptome during heartwood formation. Plant Physiology 172: 1403-1417.

II **Lim KJ**, Paasela T, Harju A, Venäläinen M, Paulin L, Auvinen P, Kärkkäinen K, Teeri TH (2017) Wounding response in Scots pine seedling stems – a transcriptomic view. Manuscript.

III Paasela T, **Lim KJ**, Pietiäinen M, Teeri TH (2017) The *O*-methyltransferase PMT2 mediates methylation of pinosylvin in Scots pine. New Phytologist 214: 1537-1550.

My contribution to the publications:

I I was involved in the experimental design, localised the transition zone, isolated total RNA from the sapwood and transition zone, prepared and constructed the sapwood and transition zone RNA-Seq libraries, and performed the RT-qPCR experiment. I performed bioinformatics analysis for RNA-Seq and RT-qPCR data. I was involved in data interpretation, wrote the manuscript and revised the manuscript together with my co-authors.

II I was involved in the experimental design, extracted total RNA from wood stems, prepared and constructed the wood stem RNA-Seq libraries. I performed bioinformatics analysis for RNA-Seq data. I was involved in data interpretation, wrote the manuscript and revised the manuscript together with my co-authors.

III I prepared and constructed the RNA-Seq libraries, carried out the RT-qPCR experiment and performed the bioinformatics analysis for RNA-Seq and RT-qPCR data. I revised the manuscript together with my co-authors.

Abbreviations

4CL	4-coumarate:CoA ligase
6PDH	6-phospogluconate dehydrogenase
AA	Amino acid
AAAT	Aromatic amino acid aminotransferase
AACT	Acetoacetyl-CoA thiolase
ACC	1-aminocyclopropane-1-carboxylate
ACO	ACC oxidase
ADT	Arogenate dehydratase
ALD	Aldolase
AUX	Auxin responsive
BFN	Bifunctional nuclease
C3H	4-coumaroyl shikimate/quinate 3'-hydroxylase
C4H	*trans*-cinnamate 4-monooxygenase
CAD	Cinnamyl-alcohol dehydrogenase
CCoAOMT	Caffeoyl-CoA *O*-methyltransferase
CCR	Cinnamoyl-CoA reductase
cDNA	Complementary deoxyribonucleic acid
CM	Chorismate mutase
CS	Chorismate synthase
CYP	Abietadienol/abietadienal oxidase (CYP720B)
CYP450	Cytochrome P450
CYP720B	Abietadienol/abietadienal oxidase
DAHP	3-deoxy-D-arabinoheptulosonate-7-phosphate
DAHPS	DAHP synthase
DHQ-SDH	Dehydroquinate dehydratase-shikimate dehydrogenase
DHQS	3-dehydroquinate synthase
DIR	Dirigent
DMAPP	Dimethylallyl diphosphate
DNA	Deoxyribonucleic acid
DP	Dirigent protein
DPR	Desiccation-related protein
DXP	1-deoxy-D-xylulose 5-phosphate
DXR	1-deoxy-D-xylylose 5-phosphate reductoisomerase
DXS	1-deoxy-D-xylulose 5-phosphate synthase
ENO	Enolase
EPSPS	5-enolpyruvylshikimate 3-phosphate synthase
EST	Expressed sequence tag
FDR	False discovery rate
FPP	Farnesyl diphosphate
FRK	Fructokinase
G3P	Glyceraldehyde 3-phosphate

G6PDH	Glucose 6-phosphate dehydrogenase
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GGPP	Geranylgeranyl diphosphate
GPP	Geranyl diphosphate
HCT	Hydroxycinnamoyl-CoA:shikimate/quinate hydroxycinnamoyltransferase
HDR	4-hydroxy-3-methylbut-2-enyl diphosphate reductase
HMG-CoA	Hydroxymethylglutaryl-CoA
HMGR	Hydroxymethylglutaryl-CoA reductase
HMGS	Hydroxymethylglutaryl-CoA synthase
HPI	Hexose phosphate isomerase
HPLC	High-performance liquid chromatography
HXK	Hexokinase
IAWA	International Association of Wood Anatomists
IPP	Isopentenyl diphosphate
IPPI	Isopentenyl diphosphate isomerase
IVT	Invertase
LAC	Laccase
LEA	Late embryogenesis abundant protein
LP3	Water deficit inducible
MEP	2-C-methyl-D-erythritol 4-phosphate
MVA	Mevalonic acid
MYB	Transcription factor family including the mammalian myeloblastosis oncogene
NAC	Transcription factor family including NAM, ATAF1/2 and CUC2
OMT	*O*-methyltransferase
PAL	Phenylalanine ammonia lyase
PAT	Prephenate aminotransferase
PCD	Programmed cell death
PCK	Phosphoenolpyruvate carboxykinase
PCR	Polymerase chain reaction
PDT	Prephenate dehydratase
PFK	Phosphofructokinase
PGAM	Phosphoglycerate mutase
PGK	Phosphoglycerate kinase
PGM	Phosphoglucomutase
PLR	Pinoresinol-lariciresinol reductase
PMT	Pinosylvin *O*-methyltransferase
PRX	Peroxidase
PS	Pinosylvin
PSME	Pinosylvin monomethyl ether
RNA	Ribonucleic acid
RNA-Seq	RNA sequencing
RPE	Ribulose 5-phosphate epimerase

RT-PCR	Reverse transcription polymerase chain reaction
RT-qPCR	Real time quantitative reverse transcription polymerase chain reaction
SIRD	Secoisolariciresinol dehydrogenase
SK	Shikimate kinase
STS	Stilbene synthase
SuSy	Sucrose synthase
TAL	Transladolase
TF	Transcription factor
TKL	Transketolase
TPI	Triose phosphate isomerase
TPS	Diterpene synthase
UDPG PPase	UDP glucose pyrophosphorylase
UPLC-MS/MS	Ultra performance liquid chromatography - tandem mass spectrometetry
UV	Ultraviolet
VND	Vascular-related NAC domain
XTH	Xyloglucan endotransglycosylase/hydrolase

Abstract

Scots pine (*Pinus sylvestris* L.) is an ecologically and economically important tree species in Nordic countries. In Finland, pinewood is extensively used and is popular in the woodworking industries because of its natural durability and timber characteristics. This quality trait is contributed by the pine heartwood extractives, and is mainly attributed to stilbenes. Pine stilbenes, pinosylvin and its monomethyl ether, are development and stress inducible metabolites. During heartwood formation, biosynthesis of pine stilbene takes place in a narrow zone between the heartwood and the sapwood, namely the transition zone. Pine stilbenes can also be induced in sapwood or needles in response to abiotic or biotic stress.

Despite the importance of pine heartwood, studies on the molecular development of heartwood formation from its transition zone are scarce. In addition, the timing and type of heartwood formation in Scots pine has not been satisfactorily described. Scots pine heartwood extractive content varies between individuals and is highly heritable, thus breeding for high extractives in pine is possible. Nevertheless, traditional forest tree breeding is time-consuming, particularly for a trait (heartwood quality) that can only be assessed in mature trees. A solution for early selection in forest tree breeding could be with a genomic approach, e.g. next-generation sequencing, coupled with bioinformatics analysis. In this work, the transcriptome changes during heartwood formation were studied in the transition zone compared to the sapwood of mature pine trees. In addition, the stress response transcriptome changes were studied by wounding the stems of pine seedlings.

Scots pine heartwood formation has been suggested to occur throughout the entire year. Here, the timing of Scots pine heartwood formation was investigated by studying the year-round expression profile of selected transcripts using quantitative RT-PCR. In fact, Scots pine heartwood formation did not occur throughout the year. Heartwood formation was initiated in spring, continued throughout the growth period and ceased in late autumn. The process was marked by programmed cell death. During heartwood formation, sucrose was metabolised for stilbene biosynthesis, indicating that pine stilbenes are biosynthesised *in situ* in the transition zone. Lipids were also probably utilised as carbon skeletons for stilbene biosynthesis and additionally as an energy source during heartwood formation.

The pine stilbene biosynthetic pathway is upregulated both during heartwood formation and in response to stress. Interestingly, distinct transcripts encoding phenylalanine ammonia lyase and 4-coumarate:CoA ligase, two enzymes acting at the beginning of the pathway, were induced during development and stress. We also showed that the previously characterised pinosylvin *O*-methyltransferase, PMT1, is probably not part of the stilbene pathway. A newly characterised *O*-methyltransferase, PMT2, turned out to be pinosylvin specific, and is strictly coexpressed with stilbene synthase.

Unexpectedly, the resin acid biosynthetic transcripts were not induced in concert with stilbene biosynthesis. The year-round expression study showed that the expression of resin acid biosynthetic transcripts were induced in early spring and ceased later in spring. Transcript levels were always stronger in sapwood than in the transition zone, indicating that resin acids are first synthesised in sapwood, then loaded into heartwood. Resin acid biosynthesis was not induced in response to wounding.

Single members of the MYB and NAC transcription factor families were upregulated in the transition zone compared to sapwood, and closely followed the expression of stilbene biosynthesis and its upstream pathways. However, other members of the MYB and NAC families were transiently induced in response to wounding. Similarly, distinct transcripts associated with cell wall modification, water deficit stress and plant defence were induced during development and stress.

This work demonstrated that only little similarity occurred in the transcriptome changes between heartwood formation and wounding response in pine. Despite stilbene synthase and PMT2 being commonly induced in both conditions, different sets of transcripts were induced, suggesting their physiological roles may be development and stress specific in Scots pine. The results presented in this study importantly contribute to the knowledge concerning conifer physiology and molecular mechanisms during developmental processes and stress challenges.

1. INTRODUCTION

Scots pine (*Pinus sylvestris* L.) is widely distributed from Western Europe to Eastern Siberia (Mason and Alía, 2000). In Finland, more than 60% of forestland is dominated by Scots pine (Peltola, 2014). It is one of the ecologically and economically important, and well studied boreal tree species (Krakau et al., 2013). Scots pine wood is extensively used in Finnish society because of its strength, straightness and visual appearance. Pinewood has been predominantly used as sawn timber for construction, making furniture, exterior wood cladding and interior decoration or wood panelling of buildings. In addition to these, a certain amount of pinewood is used for papermaking.

1.1 Composition of pinewood

The composition of pinewood can be divided into heartwood, sapwood and the transition zone (Figure 1); particularly the heartwood is valued for its properties such as dimensional stability and natural durability.

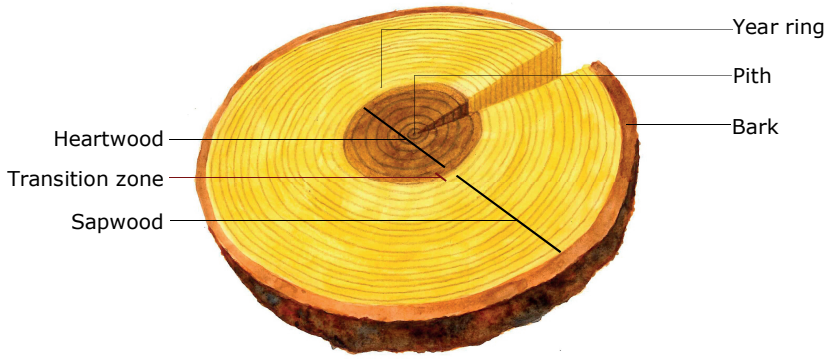

Figure 1. The composition of Scots pine wood. Sapwood is physiologically active and conducts water and minerals to the foliage. Heartwood, on the other hand, is dead, physiologically inactive and filled with heartwood extractives. Heartwood formation takes place in the transition zone, a narrow region between the sapwood and heartwood. It contains less living cells, is drier than sapwood and storage contents e.g. starch are consumed.

1.1.1 Heartwood

Heartwood is formed naturally in angiosperms and gymnosperms such as *Acacia*, *Catalpa*, *Juglans*, *Robinia, Cryptomeria*, *Larix*, *Picea* and *Pinus* tree species. However, not all tree species have heartwood. Heartwood is located in the centre of a growing tree, where moisture content is lowest and is often darker compared to sapwood (Rust, 1999; Beauchamp, 2011). Notably, heartwood is physiologically inactive and filled with extractives that contribute to its colouration and natural durability. The International Association of Wood Anatomists (IAWA) has defined heartwood as 'the inner layers of wood, which, in the growing tree, have ceased to contain living cells, and in which the reserve materials (e.g. starch) have been removed or converted into heartwood substances' (IAWA committee, 1964).

This notion, however, has been challenged by studies that define heartwood as the region where heartwood extractives have accumulated (Beekwilder et al., 2014; Celedon et al., 2016), pointing out that 'heartwood' can still be metabolically active. Celedon and colleagues (2016) regarded heartwood as the darkly coloured region due to the appearance of the extractives, and thus harvested the 'heartwood' samples on the basis of colour. They drilled the stem of tropical sandalwood (*Santalum album*) with a hand-driven drill, harvested the wood samples based on the colour of the shavings and defined sapwood as white or yellow, the transition zone as pink or red and heartwood as red or dark brown. It would be convenient to determine heartwood based on extractive colour. However, the metabolic activities associated with heartwood formation might not yet be complete despite the heartwood extractives having accumulated and extractive colour being visualisable (Taylor et al., 2002).

The cellular features in heartwood suggest that this region is void of living cells. Pekka Saranpää, who has studied cellular structures of Scots pine wood with an electron microscopy approach (Saranpää, 1988), observed that the cells of Scots pine heartwood are always void of cytoplasm in electron microscopic images (personal communication, 11th April 2016). Georg von Arx and colleagues (2015) recently published a study of ray parenchyma cells of Scots pine sapwood with an anatomical approach. He also confirms that the heartwood of Scots pine is vacant of living cells (personal communication, 25th April 2016). In addition, a study by Nakaba et al. (2008) showed that the survival rate of ray parenchyma cells drops from 100% to 0% within two annual rings in the latewood region of the Japanese red pine (*Pinus densiflora*) and pitch pine (*Pinus rigida*). Indeed, pine heartwood is in accordance to the heartwood definition of the IAWA committee.

Thus, if one would find 'heartwood' (defined based on colour) still metabolically active, this zone should be defined as the transition zone (see chapter below), perhaps, the 'inner transition zone'.

1.1.2 Sapwood

Sapwood (Figure 1), on the other hand, is defined as 'the portion of the wood that in the living tree contains living cells and reserve materials' (IAWA committee, 1964). It is physiologically active, has a higher moisture content compared to heartwood and conducts water between the roots and foliage (Rust, 1999; Taylor et al., 2002). Sapwood is less durable, as it does not contain heartwood extractives, but is rich in nutrients, thus being attractive to biotrophic pathogens.

1.1.3 Transition zone

Many tree species will eventually convert sapwood into heartwood as the tree matures. The process, heartwood formation, takes place in a narrow zone between the sapwood and the heartwood, namely the transition zone (Figure 1). Generally, the width of the transition zone varies from one to three annual rings (Hillis, 1987). The width of the transition zone in Scots pine, in particular, is approximately one to two annual rings (Gustafsson, 2001). Typically, the transition zone contains less living cells; reserve materials, such as starch and lipids are consumed, and it is drier than sapwood but not as dry as heartwood (Hillis, 1987; Bergström, 2003; Rust, 1999).

1.2 Heartwood formation

1.2.1 Types of heartwood formation

Up to date, two types of heartwood formation have been described. For type I or *Robinia* type heartwood formation, the heartwood extractives are biosynthesised *in situ* and accumulate in the transition zone, with no extractive precursors found in the ageing sapwood. In type II or *Juglans* type heartwood formation, the extractive precursors gradually accumulate in ageing sapwood and are transformed to heartwood extractives by hydrolysis, oxidation and polymerisation upon onset of heartwood formation in the transition zone (Magel, 2000). Heartwood formation of pine has been suggested to be of type II (Magel, 2000).

1.2.2 Timing of heartwood formation

The specific timing for Scots pine heartwood formation remains unclear. Studies have described the heartwood formation of various tree species takes place at different times of the year. Nelson (1978) showed that heartwood formation in the walnut tree (*Juglans nigra*) takes place during autumn, while Yang and colleagues (2004) suggested that the black locust (*Robinia pseudoacacia*) undergoes heartwood formation in late summer and autumn. Nakada and Fukatsu (2012) concluded that the deposition of heartwood extractives in the Japanese larch (*Larix kaempferi*) takes place in autumn and winter. Heartwood formation for pines has also been described as occurring at various times of the year. Shain and Hillis (1973) suggested that heartwood formation in the radiata pine (*Pinus radiata*) occurs during the winter dormancy period; on the other hand, Yang (1993) concluded that heartwood formation in jack pine (*Pinus banksiana*) is initiated in August. For Scots pine, interestingly, Bergström and colleagues (1999) suggested that there is no specific time of year for the heartwood formation. The timing of pine heartwood formation, particularly for Scots pine, has not been satisfactorily described.

1.3 Heartwood extractives

Heartwood extractives are secondary metabolites accumulating in the heartwood as the tree ages. Deposition of heartwood extractives marks the end of heartwood formation. Pine heartwood extractives are important elements that give unique colour characteristics to timber and resistance against wood degradation by microorganisms (Nakada and Fukatsu, 2012; Harju and Venäläinen, 2006; Leinonen et al., 2008). Scots pine heartwood extractives are comprised of the stilbenes pinosylvin (PS) and pinosylvin monomethyl ether (PSME), resin acids and free fatty acids (Saranpää and Nyberg, 1987). Heartwood phenolic extractive content is highly heritable (Partanen et al., 2011), although it is also influenced by the environment (Magel, 2000).

1.3.1 Stilbenes

Pine PS and PSME belong to the stilbenoid family. Unlike other common plant stilbenes, for example resveratrol in grapes or peanuts (Chong et al., 2009), pine stilbenes lack the hydroxyl group in one of the aromatic rings (Preisig-Müller et al., 1999). Accordingly, while 4-coumaric acid is the precursor for resveratrol biosynthesis, PS and PSME are derived from cinnamic acid.

The biosynthesis of pine stilbenes branches off from the general phenylpropanoid pathway (Figure 2). It begins with the conversion of phenylalanine, a primary metabolism substrate derived from the shikimate pathway, to cinnamic acid by the enzyme phenylalanine ammonia lyase (PAL). PAL catalyses the entry point reaction in the biosynthesis of stilbenes, flavonoids, lignins and other phenylpropanoid metabolites (Whetten and Sederoff, 1995; Butland et al., 1998). It can be induced developmentally, but also in response to abiotic and biotic challenges such as ultraviolet (UV) radiation (Teklemariam and Blake, 2004; Gehlert et al., 1990) or fungal attack (Grönberg et al., 2009). A handful of pine *PAL* genes have been identified from jack pine and loblolly pine, and their corresponding transcripts are expressed differently during development and stress (Bagal et al., 2012; Butland et al., 1998). *Pbpal1*, *2*, *4*, and 5, were found expressing in the developing xylem of a mature tree, as well as in a fungal-elicited cell suspension culture. *Pbpal3*, on the other hand, was only expressed in the fungal-elicited culture (Butland et al., 1998), suggesting that this *PAL* may only be induced in response to stress.

The next enzyme after PAL in the stilbene biosynthesis pathway is the enzyme that activates cinnamic acid to form cinnamoyl-CoA. Most of the plant 4-coumarate:CoA ligases (4CLs) do not use cinnamic acid as a substrate. Up to date, a pine 4CL that can utilise cinnamic acid for synthesising cinnamoyl-CoA has not been characterised. All pine 4CLs characterised so far are suggested to be involved in lignin biosynthesis (Zhang and Chiang, 1997).

Further downstream, cinnamoyl-CoA is converted to the stilbene pinosylvin through pine stilbene synthase (STS). Gehlert and colleagues (1990) showed that pine STS has six times higher preference for cinnamoyl-CoA as for 4-coumaroyl-CoA. Later, Schanz and colleagues (1992) also demonstrated that Scots pine STS preferentially utilised cinnamoyl-CoA as a substrate to produce pinosylvin. Thus, pine STS, which is encoded by a small gene family (Preisig-Müller et al., 1999), is categorised as cinnamoyl-CoA specific stilbene synthase (Kodan et al., 2002). Another enzyme in pine, chalcone synthase, also utilises cinnamoyl-CoA as a substrate to produce chalcone pinocembrin, a precursor for flavonoid biosynthesis (Fliegmann et al., 1992). Pinosylvin can be modified by methylation, and is converted to PSME by pinosylvin *O*-methyltransferase (PMT). A Scots pine PMT has been described and characterised by Chiron *et al.* (2000a).

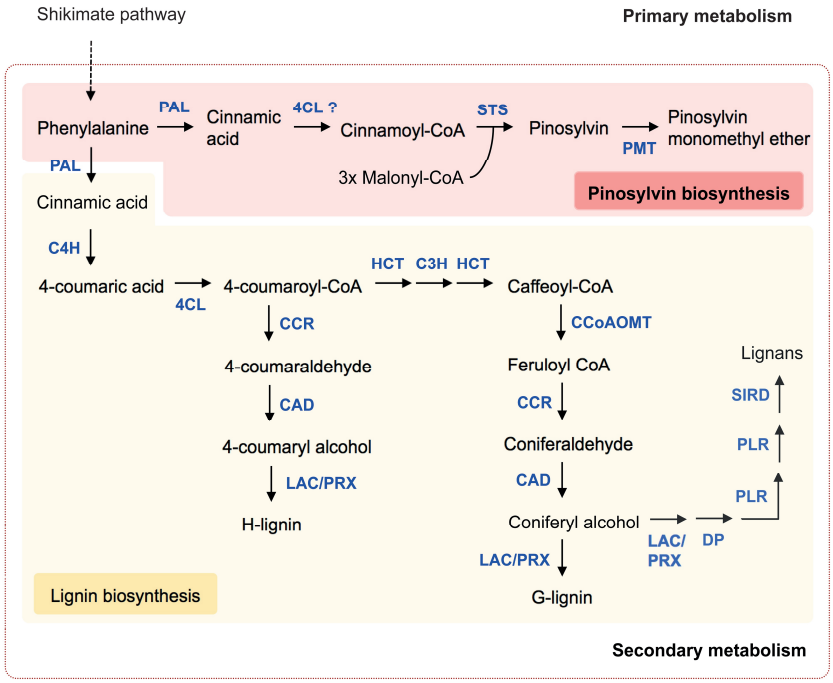

Figure 2. The pinosylvin and lignin biosynthetic pathways. The biosynthesis of pine stilbenes and lignin begins with the conversion of phenylalanine, a precursor derived from primary metabolism. The arrows represent enzymatic reactions. Abbreviations: PAL, phenylalanine ammonia lyase; 4CL, 4-coumarate:CoA ligase; STS, stilbene synthase; PMT, pinosylvin *O*-methyltransferase; C4H, *trans*-cinnamate 4-monooxygenase; HCT, hydroxycinnamoyl-CoA:shikimate/quinate hydroxycinnamoyltransferase; C3H, 4-coumaroyl shikimate/quinate 3'-hydroxylase; CCoAOMT, caffeoyl-CoA *O*-methyltransferase; CCR, cinnamoyl-CoA reductase; CAD, cinnamyl-alcohol dehydrogenase; LAC, laccase; PRX, peroxidase; DP, dirigent protein; PLR, pinoresinol-lariciresinol reductase; SIRD, secoisolariciresinol dehydrogenase.

1.3.2 Resin acids

Resin acids are major components of conifer oleoresin, and they account for 50% of extractives in Scots pine heartwood (Bergström, 2003). Oleoresins are comprised of a complex mixture of volatile monoterpenes and sesquiterpenes, along with non-volatile diterpenes (Zulak and Bohlmann, 2010). In general, the biosynthesis of terpenoids (Figure 3) involves the mevalonic acid (MVA) or methylerythritol phosphate (MEP) pathways that produce isopentenyl

diphosphate (IPP) and dimethylallyl diphosphate (DMAPP), which are then converted to monoterpenes, sesquiterpenes and diterpenes by prenyltransferases and terpene synthases (Zulak and Bohlmann, 2010). Sesquiterpenes are synthesised by the precursors from the MVA pathway; on the other hand, the MEP pathway is the primary route for the formation of mono- and diterpenes (Zulak and Bohlmann, 2010). Aided by cytochrome P450 enzymes, resin acids are converted from diterpenes in a multiple step reaction (Kolosova and Bohlmann, 2012).

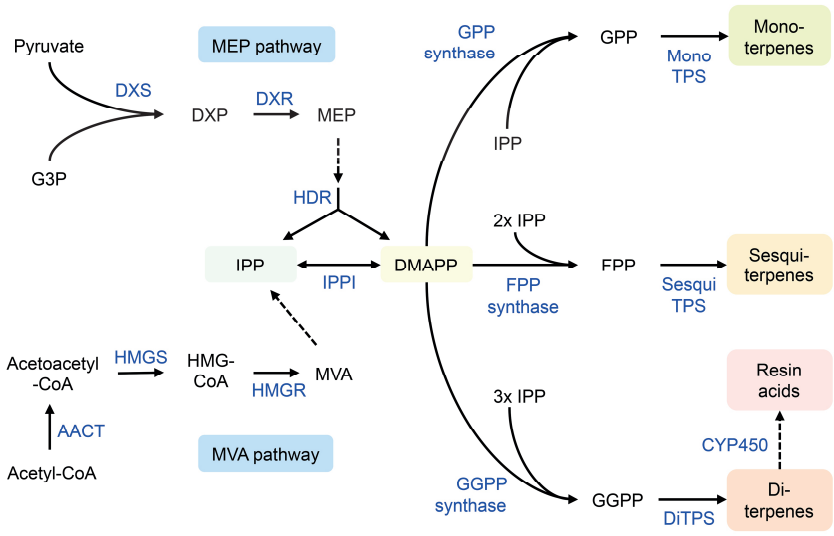

Figure 3. The pathway of mono-, sesqui- and diterpene biosynthesis. The arrows represent enzymatic reactions in the pathways. Abbreviations: MEP, 2-C-methyl-D-erythritol 4-phosphate; G3P, glyceraldehyde 3-phosphate; DXP, 1-deoxy-D-xylulose 5-phosphate; DXS, 1-deoxy-D-xylulose 5-phosphate synthase; DXR, 1-deoxy-D-xylylose 5-phosphate reductoisomerase; HDR, 4-hydroxy-3-methylbut-2-enyl diphosphate reductase; IPP, isopentenyl diphosphate; IPPI, isopentenyl diphosphate isomerase; DMAPP, dimethylallyl diphosphate; AACT, acetoacetyl-CoA thiolase; HMG-CoA, hydroxymethylglutaryl-CoA; HMGS, hydroxymethylglutaryl-CoA synthase; HMGR, hydroxymethylglutaryl-CoA reductase; MVA, mevalonic acid; GPP, geranyl diphosphate; FPP, farnesyl diphosphate; GGPP, geranylgeranyl diphosphate; TPS, terpene synthase; CYP450, cytochrome P450. Figure modified from Kolosova and Bohlmann (2012).

1.3.3 Free fatty acids

The concentration of free fatty acids is higher in pine heartwood than in the sapwood. It accounts for approximately 35% of extractives in Scots pine heartwood (Bergström, 2003). Studies showed that the amount of free fatty acids increased from the transition zone towards the heartwood, whereas the concentration of triglycerides was highest in the sapwood, began decreasing in the transition zone and was lowest in the heartwood (Saranpää and Nyberg, 1987; Bergström, 2003). This is in accordance with the observation of lipophilic droplets in the parenchyma cells of the transition zone during heartwood formation (Taylor et al., 2002; Kampe and Magel, 2013). The increasing free fatty acid content from the transition zone towards heartwood has been suggested to be due to the hydrolysis of triglycerides for secondary metabolite synthesis during heartwood formation (Saranpää and Nyberg, 1987; Bergström, 2003).

1.4 Heartwood extractives and defence

Trees are frequently challenged by herbivorous insects and pathogens during their lifetime, and have to deal with various environmental challenges from time to time (Eyles et al., 2010; Ahuja et al., 2012). Scots pine responds to these challenges with constitutive and induced defences. While constitutive defence forms an essential defence system in trees without need for elicitation, induced defence is triggered by a pathogen or herbivore attack (Goyal et al., 2012).

Pine secondary metabolites, such as pine stilbenes, play important roles in both constitutive and induced defences. Pine stilbene biosynthetic transcripts are induced during heartwood formation as part of a developmental process. Pinosylvin and its monomethyl ether are synthesised in the transition zone and deposited to the nearest cell walls, filling the parenchyma lumen (Spicer, 2005). A study by Harju and Venäläinen (2006) indicated that the concentration of PS and PSME in the heartwood correlates with its decay resistance against the decaying fungus *Coniophoira puteana*. *In vitro* assays have been used to describe PS and PSME as toxic to other wood-destroying fungi (Schultz et al., 1992; Venäläinen et al., 2004). Taken together, the accumulation of PS and PSME in heartwood plays a constitutive defence role in protecting wood against decaying fungi.

PS and PSME are also inducible in the sapwood and needles in response to various stressors. For example, Harju and colleagues (2009) reported that mechanically wounding stems of three-year-old Scots pine seedlings triggered

pine stilbene production in the xylem next to the wounded areas. Accumulation of PS and PSME was also reported in needles of six-week-old Scots pine seedlings upon ozone treatment (Rosemann et al., 1991). PS and PSME are also strongly induced in the phloem of seven-year-old pine saplings that were initially fumigated with ozone for two days, followed by fungal inoculation in a time-series experiment (Chiron et al., 2000b). In addition, pinosylvin biosynthetic transcripts are rapidly induced in needles of four-week-old pine seedlings when irradiated with UV-C (Gehlert et al., 1990).

PS and PSME are phytoalexins that play a role in induced defence in addition to constitutive defence. Pine PS is generally considered more toxic than PSME (Hillis and Inoue, 1968; Fries et al., 2000). Interestingly, a recent study by Villari and colleagues (2012) noted that pine PSME is significantly induced in response to inoculation of a fungal complex (*Ophiostoma brunneo-ciliatum* and *Hyalorhinocladiella macrospora*), although toxicity of PSME is generally more specific to some members of the brown-rot fungi (Hart, 1981). In addition, the study by Suga and colleagues (1993) suggested that PSME is a better substance than PS for killing pine wood nematodes. This evidence suggests that both PS and PSME play important roles in inducible defence.

Resin acids are the most abundant terpenoids produced in the pine family (Keeling and Bohlmann, 2006). Resin acids, along with other terpenoids, are produced and stored in resin ducts during development to serve as a constitutive defence in pine (Zulak and Bohlmann, 2010). The resin ducts are located within the phloem and xylem regions, and serve as a second layer of defence if the primary barrier, the heavily lignified bark, fails to protect trees against pathogenic invasion (Keeling and Bohlmann, 2006). The resins are released or 'cast out' from resin ducts once they are disrupted by herbivorous invasion. The 'cast out' resins can both push an invading insect out from the invaded site with a flow of resin or trap and kill the insect within the resin (Keeling and Bohlmann, 2006). The released resin also cleans the wound off microorganisms, as they are deterrent and toxic to microbial intruders (Keeling and Bohlmann, 2006; Zulak and Bohlmann, 2010). The volatile terpenoids will gradually evaporate from the wounded areas, while the non-volatile diterpenoids like resin acids will harden and eventually seal the wound from insects and pathogens (Kolosova and Bohlmann, 2012).

Besides constitutive defence, the biosynthesis of resin acids can be induced in response to stressors. Abiotic and biotic stressors, such as mechanical wounding or fungus infection, can trigger the formation of traumatic resin ducts to

increase resins production. The chemical composition of newly produced resin, also known as secondary resin, can be different from constitutively produced resin, and even more toxic to pathogens in some cases (Luchi et al., 2004; Kolosova and Bohlmann, 2012). Harju and colleagues observed that a high amount of resin acid accumulates in the xylem next to wounded areas three months after mechanical wounding on the stems of young Scots pine seedlings (Harju et al., 2009)

The amount of heartwood extractives has been correlated with resistance against wood-decaying microorganisms. Despite the fact that higher concentrations of resin acids can be found in Scots pine heartwood compared to sapwood (Harju et al., 2002), no direct evidence suggests that resin acids play a role in the decay resistance of Scots pine heartwood (Venäläinen et al., 2003). Nevertheless, resin acids concentration in decay-resistant pine heartwood is reported to be higher than in decay-susceptible heartwood (Harju et al., 2002). The role of resin acids in decay resistance is perhaps due to their hydrophobic properties in forming waterproofing layers within heartwood, which prevent further penetration and growth of fungi, rather than due to their general toxicity (Eberhardt et al., 1994).

The content of free fatty acids, one of the heartwood extractives, is higher in heartwood compared to sapwood. The role of free fatty acids in the decay resistance of heartwood has remained unclear (Harju and Venäläinen, 2006), but they may also contribute to waterproofing.

1.5 Other processes taking place during heartwood formation

1.5.1 Programmed cell death

Plant programmed cell death (PCD) takes place both during developmental processes and in response to abiotic and biotic challenges (Pennell and Lamb, 1997). The formation of xylem tracheary elements (Bollhöner et al., 2012) and leaf senescence (Pennell and Lamb, 1997) are examples of developmental PCD, while hypersensitive response upon recognition of pathogens and abiotic stressors, such as UV irradiation, heat or cold leading to cell death, are examples of stress-induced PCD (Olvera-Carrillo et al., 2015).

A recent genome-wide transcriptome study showed the transcripts involved in developmentally induced PCD to be largely distinct from those related to stress-induced PCD (Olvera-Carrillo et al., 2015). One of the distinctive features of PCD is the degradation of cell nuclei by endonucleases (Pennell and Lamb, 1997). A study by Olvera-Carrillo and colleagues (2015) indicated that endonucleases, for example BIFUNCTIONAL NUCLEASE 1 (BFN1), are grouped in a cluster that belongs to developmentally induced PCD. BFN1 is an endonuclease expressed during leaf senescence and developmental cell death (Farage-Barhom et al., 2008). Degradation of ray cell nuclei has been observed in the transition zone (Spicer, 2005; Nakada and Fukatsu, 2012), suggesting that heartwood formation is a form of developmentally induced PCD. Heartwood formation can also be seen as tissue senescence within the transition zone, where ray parenchyma cells die and all physiological activities eventually cease (Spicer, 2005; Kampe and Magel, 2013).

1.5.2 Cell wall modification

Plant cell walls are made of a complex matrix of polysaccharides and polyphenols including cellulose, hemicelluloses, pectin and lignin. In addition, plant cell walls contain structural glycoproteins and various enzymes (Chong, 2014; Keegstra, 2010). Plant cells are mainly encrusted within the primary cell wall during cell division and differentiation. They undergo wall loosening and expansion involving a set of protein and carbohydrate modifying enzymes such as expansins and xyloglucan endotransglycosylases/hydrolases (XTHs) (Van Sandt et al., 2007). When cells mature, secondary cell wall formation involving wall thickening and lignification takes place to confer mechanical support against turgor pressure and stressors. In addition, lignification also increases the level of defences against pathogenic invasions (Yoon et al., 2015; Sasidharan et al., 2011).

Cell wall modification also takes place when plants are under stress. For example, a microarray analysis of maize roots showed upregulation of cell wall modifying enzymes in the root tips that experienced water-deficit stress (Spollen et al., 2008). When plants are under drought stress, downregulation of expansins and XTHs is observed, which results in slower shoot growth. Root growth, on the other hand, is stimulated with upregulation of expansins and XTHs to search for water-rich areas (Sasidharan et al., 2011). A transcriptomic study of aphid infestation on celery showed that expansins and XTHs are the major upregulated transcripts in response to infestation (Divol et al., 2005). Harju and colleagues (2009) observed that lignans in Scots pine seedlings

accumulated in the region next to the wounding site three months after mechanical wounding. Lignans are derived from lignin monomers (Figure 2) and have been described as having a role in plant defence against pathogens (Naoumkina et al., 2010; Dixon et al., 2002). Notably, in Scots pine, lignans cannot be detected in normal stem wood, only in knot wood close to branches (Holmbom et al., 2003).

1.6 Transcriptional control

Transcription factors (TFs) are DNA binding regulatory proteins. They activate expression of their target transcripts by binding to specific promoter regions that trigger cascades of downstream metabolic reactions (Franco-Zorrilla et al., 2014). TFs play essential roles both in developmental processes and in response to abiotic and biotic stressors. For example MYB TFs, members of one of the largest TF families in plants, are key regulators of plant secondary metabolism in many cases (Dubos et al., 2010; Liu et al., 2015). PtMYB1 (Patzlaff et al., 2003b), PtMYB4 (Patzlaff et al., 2003a) and PtMYB8 (Bomal et al., 2008) from loblolly pine have been suggested to regulate lignin biosynthetic transcripts. The studies indicated that these TFs induce the lignification of xylem cells that leads to secondary wall formation in conifers. Study of Höll and colleagues (2013) showed that MYB14 and MYB15 TFs play a role in regulating the grapevine stilbene biosynthetic pathway by activating the promoter of the *STS* genes. The expression of MYB14 and MYB15 is correlated with stilbene biosynthesis in the skins and seeds of developing grape berries (Höll et al., 2013). NAC TFs, members of another large TF family in plants, have also been reported to mediate development- and stress-induced response. For example, the arabidopsis (*Arabidopsis thaliana*) VASCULAR-RELATED NAC-DOMAIN (VND) 1 to 7 were identified as the main regulators of xylem secondary wall formation (Kubo et al., 2005). The authors reasoned that VND6 and VND7 play an important role in xylem vessel formation, as these TFs effectively induced transdifferentiation of various cell types into xylem vessel elements (Kubo et al., 2005). In addition to secondary wall biosynthesis, VND6 and VND7 also act as master switches to regulate cell death-related transcripts (e.g. BFN1) during tracheary element development (Bollhöner et al., 2012).

MYB and NAC TFs are also expressed in response to various stressors besides their developmental roles. For example, in response to wounding and elicitors, MYB2 from tobacco binds to the *PAL* gene promoter in order to activate phenylpropanoid biosynthesis (Sugimoto et al., 2000). Höll and colleagues (2013)

demonstrated that MYB14 and MYB15 TFs are regulators of stilbene biosynthesis in response to abiotic and biotic stressors in grapevine. They observed that the expression level of these TFs correlates with that of STS encoding transcripts upon UV-C irradiation and downy mildew (*Plasmopara viticola*) infection of leaf tissues (Höll et al., 2013). A recent study by Pascual and colleagues (2015) demonstrated that NAC TFs of maritime pine (*Pinus pinaster*), *PpNAC2* and *PpNAC3*, are strongly induced in response to methyl jasmonate treatment and wounding. These stressors induced pine NAC TFs that is homologous to arabidopsis ATAF1 and ATAF2, the NAC TFs induced in response to drought stress (Pascual et al., 2015).

2. AIMS OF THE STUDY

Scots pine is an economically and ecologically important forest tree species in Finland because of its heartwood quality traits that are derived from pine heartwood extractives (mainly stilbenes). The phenolic heartwood extractives strongly correlate with natural decay resistance in pine, as trees with highest phenolic content are most decay resistant. The extractive content varies between individual trees and the phenolic fraction is highly heritable. Breeding for high extractive content in Scots pine heartwood should therefore be efficient. However, phenotyping pine heartwood can only be done from mature trees, approximately 20 to 30 years after planting. Pine stilbenes, pinosylvin and its monomethyl ether, are biosynthesised in the transition zone during the developmental process of heartwood formation. They are also produced in response to abiotic and biotic stressors in sapwood or needles. Genetic studies indicate reasonable heritability between the heartwood stilbene content of mature trees and stress-induced stilbene accumulation in seedlings, indicating that stress stilbenes could function as a proxy for selecting the desired heartwood trait.

The aim of this study was to characterise the transcriptome changes of Scots pine during heartwood formation (development) and in response to wounding (stress), using a next-generation sequencing and bioinformatics approach. By following the pine stilbene biosynthetic pathway during development and as stress response, this study aimed to achieve the following objectives

1. To investigate the transcriptome changes during heartwood formation of Scots pine.

2. To study co-expressed metabolic and regulatory activities during heartwood formation at the molecular level.

3. To compare similarities and differences in transcriptome changes between heartwood formation and wounding response.

3. MATERIALS AND METHODS

Materials and methods used in this thesis are summarised in Table 1. Details have been described in the original publications **I** to **III**.

Table 1 Materials and methods used in this study.

Material or method	Publication
Scots pine (*Pinus sylvestris* L.) trees	**I**
Scots pine (*Pinus sylvestris* L.) seedlings	**II, III**
Localisation of the transition zone	**I**
RNA extraction and first strand cDNA synthesis	**I, II, III**
Semiquantitative RT-PCR	**I, II, III**
Ribosomal RNA depletion	**I, II, III**
SOLiD whole transcriptome RNA-Seq library preparation	**I, II, III**
Paired-end sequencing with the SOLiD sequencing platform *	**I, II, III**
Mapping of RNA-Seq reads to *Pinus* EST collection	**I, II, III**
Handling of mapped data	**I, II, III**
Differential expression analysis	**I, II**
Gene Ontology Enrichment analysis	**I, II**
Trinity *de novo* assembly and mapping of RNA-Seq reads	**I, II**
Real time quantitative RT-PCR	**I, II, III**
Pearson correlation analysis	**I**
Bayesian hierarchical clustering analysis	**II**
Gene set analysis	**II**
Isolation of enzyme coding cDNA molecules *	**III**
Cloning of PMT2 encoding genes *	**III**
Protein expression, purification and enzymatic assays *	**III**
HPLC and UPLC-MS/MS analyses *	**III**

* Methods were conducted by the co-author.

4. RESULTS AND DISCUSSION

4.1 Scots pine transcriptomics

Gene expression changes during development and stress response in the stems of Scots pine were studied with a transcriptomic approach. For studying heartwood formation (development), approximately 120 increment cores were harvested from four 46-year-old trees at breast height during summer. The sapwood and transition zones were sampled from increment cores of each tree, respectively, and total RNA was extracted for RNA sequencing. As a result, a total of 12–17 million paired-end reads were obtained from the sapwood and transition zone RNA samples.

For studying the wounding response of Scots pine (stress response), a time-course experiment of mechanical wounding of four half-sib families of five-year-old Scots pine seedling stems were conducted during early summer. Three seedlings from each of the four families were wounded and one unwounded seedling was used as a control. Unwounded (0H) and wounded stems were sampled three hours (3H), one day (1D), and four days (4D) after wounding. The bark and phloem of the stems was removed prior to sample preparation and RNA sequencing. At this age seedlings do not contain any heartwood. A total of 37–126 million paired-end reads were obtained from each time point of the wounding experiment.

All transcriptome libraries were mapped against the *Pinus* expressed sequence tag (EST) collection version 9.0 (The Gene Index Databases, 2014). Average mapping rate was 65% (Table S2 in paper **I**) for sapwood and transition zone libraries, and 88% for wounded and unwounded stem libraries (Table S1 in paper **II**). Differential expression analysis was carried out using *edgeR*, an R package. The analysis showed that a total of 1673 transcripts were statistically significantly (false discovery rate, FDR < 0.05) differentially expressed in the transition zone compared to sapwood (Tables S3 and S4 in paper **I**) and 4595 transcripts in the stem (xylem) at 3H, 1D or 4D after wounding, compared to the control (Table S2 in paper **II**).

For comparison, an annotated Trinity (Haas et al., 2013) assembly of sapwood and transition zone was generated from their respective RNA-Seq reads. Then, each of the RNA-Seq libraries was mapped against the annotated assembly. Similar assembling and mapping processes were carried out for wounded and unwounded stem libraries. The average mapping rate of sapwood and transition

zone libraries, and wounded and unwounded libraries to the Trinity assemblies was 70% (Table S2 in paper **I**) and 77% (Table S1 in paper **II**), respectively. Differential expression analysis was performed with *edgeR* after mapping. Although, in some cases, the fold changes of differentially expressed transcripts of the RNA-Seq libraries mapped against Trinity assemblies were higher, the obtained expression data were very similar to those where the *Pinus* EST collection was used as a reference.

Differential expression results from *Pinus* EST collection are presented in this work. The advantage of using *Pinus* EST is that the expression level of transcripts that are practically not expressed in the studied samples can be followed. Besides, the *Pinus* EST assemblies are longer. As discussed in paper **I**, the *Pinus* EST collection is a combined collection of ESTs from different pine species. High sequence similarity between different pine species allowed assembly of the ESTs into tentative consensus (TC) contigs as if the sequences originated from a single species (Quackenbush et al., 1999).

Interesting transcripts that were clearly upregulated (or downregulated) in the transition zone and characteristic of heartwood formation were selected based on the transition zone transcriptomic results. In a year-round expression study using real time quantitative reverse transcription polymerase chain reaction (RT-qPCR) the aim was to investigate the timing of heartwood formation. Transcripts associated to resin acid biosynthesis (not expressed during heartwood formation) were also selected for the study to understand when the pathway initiates. Transcripts encoding action and histone were used as a reference for RT-qPCR. A list of the selected transcripts is in Table S8 of paper **I**.

Four increment cores of two 35–36-year-old Scots pine trees were harvested monthly from March 2011 to March 2012. The January 2012 increment cores were lost due to severe fragmentation of the samples upon removal from the increment borer. The total RNA of sapwood and transition was first isolated. Then, first strand cDNA of the sapwood and transition zone was synthesised for the expression profile study with RT-qPCR. The expression levels of the tested transcripts in each tested tree varied much during the autumn and winter months. Nonetheless, they shared expression patterns during the late spring and summer months, suggesting the trees may have had different responses to local challenges or experienced tree-specific environmental effects. Each tree apparently responded similarly when it comes to the developmental process of heartwood formation.

4.2 Localisation of the transition zone

Heartwood formation takes place in the transition zone, a narrow zone located between heartwood and sapwood. It is essential to correctly localise the transition zone within the increment core before sample preparation and RNA sequencing (**I**). In the field, a pencil was used to mark the dry (heartwood) and wet (sapwood) zones of the increment cores before transfer to the laboratory in dry ice (Figure 4). The frozen increment cores were photographed under UV illumination in the laboratory to visualise the fluorescing Scots pine stilbenes (Harju et al., 2009).

Figure 4. Boundary between the dry (heartwood) and wet (sapwood) of an increment core is marked with a pencil in the field. Photo: Teemu Teeri.

Fluorescing stilbenes can be visualised in the entire dry (heartwood) zone, except for the outermost zone, approximately one annual ring in width, near to the pencil mark (Figure 5A). To determine the transition zone in molecular terms, paired annual rings of increment cores were sectioned from zones 1 to 8 around the pencil mark (Figure 5A) and total RNA was extracted. A good RNA yield (based on the A_{260}) was obtained from zones 5 to 8 and less from zone 4.

Practically no RNA could be obtained from zones 1 to 3, which was expected because the inner part of the dry zone (heartwood) did not contain any living cells (Figure 5B). Semiquantitative RT-PCR for the pine STS encoding transcript was performed for each sample. The results (Figure 5C) showed that the strongest *STS* signal was observed from the outermost dry zone, zone 4, in all sample series. Thus, this zone was designated (yellow triangle, Figure 5A) as the transition zone (**I**). Our definition is in accordance with the description by Bergström (2003).

Identifying the location of the transition zone was one of the challenges for the pine heartwood formation study. Determination of the transition zone with the help of both pencil mark and fluorescent stilbenes under UV illumination allowed us to discard samples where the fluorescing stilbenes were present in the sapwood (wet zone), indicating a response to the stress challenge.

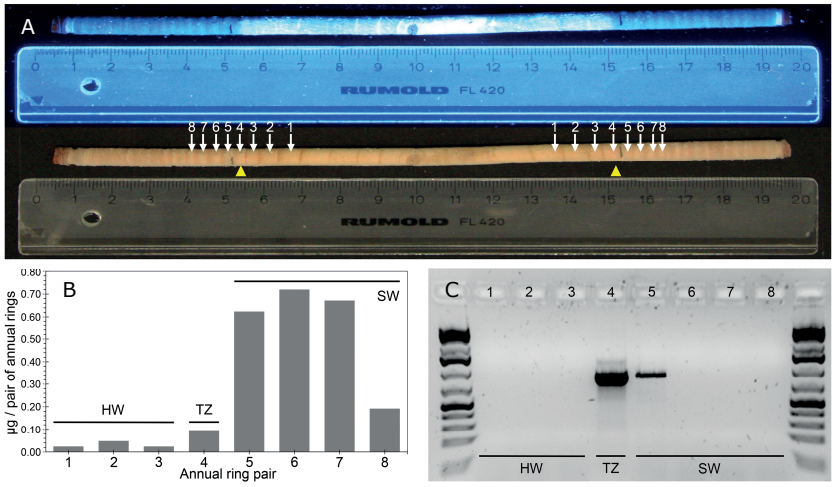

Figure 5. Localisation of the transition zone between sapwood and heartwood. **A)** Pine stilbenes give strong fluorescence under UV illumination (above) allowing a direct visualisation of heartwood extractives in the dry (heartwood) zone. Pairs of annual rings of zones 1 to 8 (below, under ambient light) were sampled for RNA extraction. **B)** The RNA yield (based on A_{260}) of zones 5 to 8 was comparable, less RNA was obtained from zone 4, and almost no RNA could be obtained from zone 1 to 3. **C)** Semiquantitative RT-PCR for pine *STS* was carried out for each zone. Strongest *STS* expression was observed in the outermost dry zone (zone 4), which was designated as the transition zone (yellow triangle). Abbreviations: HW, heartwood; TZ, transition zone; SW, sapwood. Figure modified from Lim et al. (2016), copyright American Society of Plant Biologists.

4.3 The timing of heartwood formation is marked by programmed cell death

Various studies have described pine heartwood formation happening at different times of the year. For Scots pine, Bergstöm (1999) concluded no specific time of the year for heartwood formation. However, the year-round expression study indicated that a certain time of the year was relevant for Scots pine heartwood formation. First, stilbene biosynthetic transcripts were shown to always be more strongly expressed in the transition zone than in the sapwood (**I**). These transcripts shared an expression pattern in late spring, the summer months, as well as a varied (between trees) expression pattern in late autumn and in winter. Its upstream pathways (e.g. the shikimate pathway) were also reacting in concert with stilbene biosynthesis, as was the expression of the two TFs (MYB and NAC) (Figure 3 in paper **I**).

The year-round expression study showed that the transition zone was undergoing PCD during the growth period (from May to October). An endonuclease that associates with plant PCD, BFN, was expressed in the transition zone in synchrony with the pine stilbene pathway during summer. However, the expression did not follow the pine stilbene pathway peaks in the late autumn and winter. Notably, the expression of BFN was observed only in the transition zone, never in the sapwood (Figure 4 in paper **I**). These observations point out that heartwood formation is initiated in spring, continues throughout the growth period and ceases in autumn, marked by PCD. The stilbene pathway was upregulated in late autumn and winter independent of PCD, i.e. in our interpretation, independent of heartwood formation.

4.4 Scots pine heartwood formation is of type I

Pine heartwood formation has been assumed to be of type II, or *Juglans* type, in which heartwood extractive precursors gradually accumulate in ageing sapwood and transform in the transition zone (Magel, 2000). This opinion, however, was contradicted by our heartwood formation transcriptomic data (**I**). We observed that, during heartwood formation, the transcripts encoding pine stilbene biosynthetic enzymes were strongly upregulated in the transition zone. Concomitantly, the transcript encoding enzymes for its upstream pathways, shikimate and primary metabolic pathways, were upregulated in the transition zone compared to the sapwood during heartwood formation (Figure 6). Transcripts for sucrose synthase were also upregulated, indicating that sucrose

is broken down as a carbon source and channelled for downstream metabolic activity, e.g. stilbene biosynthesis. The activation of primary and secondary metabolism pathways as well as stilbene biosynthesis within the transition zone indicate that heartwood formation in Scots pine is of type I (**I**), where heartwood extractives are biosynthesised *in situ* and accumulated in the transition zone.

4.4.1 Possible other carbon sources for stilbene biosynthesis

Though the primary and secondary metabolism pathways were activated within the transition zone during heartwood formation, the transcripts corresponding to sugar metabolism, glycolysis and oxidative pentose phosphate and shikimate pathway were, however, not upregulated in response to mechanical wounding of pine stems (**II**). They were either being constitutively expressed (therefore not needing induction) or were not expressed at all through the time points (Figure 7). The stilbene biosynthetic pathway, however, was strongly induced in both conditions. This observation raised the question of whether the carbon source used for stilbene biosynthesis in response to wounding would be different from that used for heartwood formation. Interestingly, the transcripts encoding phosphoenolpyruvate carboxykinase (PCK) turned out to be upregulated in the wounding transcriptomes (**II**). This enzyme converts oxaloacetate, the derivatives of triglycerides from plant fat storage tissues (glyoxysomes), to phosphoenolpyruvate, a precursor for the shikimate pathway (Bowsher et al., 2008). Therefore, breaking down of lipid storage into fatty acids could serve as the carbon source for stilbene biosynthesis in response to stress in addition to functioning as an energy source. PCK upregulation was also observed during heartwood formation (**I**). Considering a high amount of free fatty acids is found in the transition zone and heartwood, but not in the sapwood (Saranpää and Höll, 1989; Bergström, 2003), they could also be used as an energy and carbon source for stilbene biosynthesis.

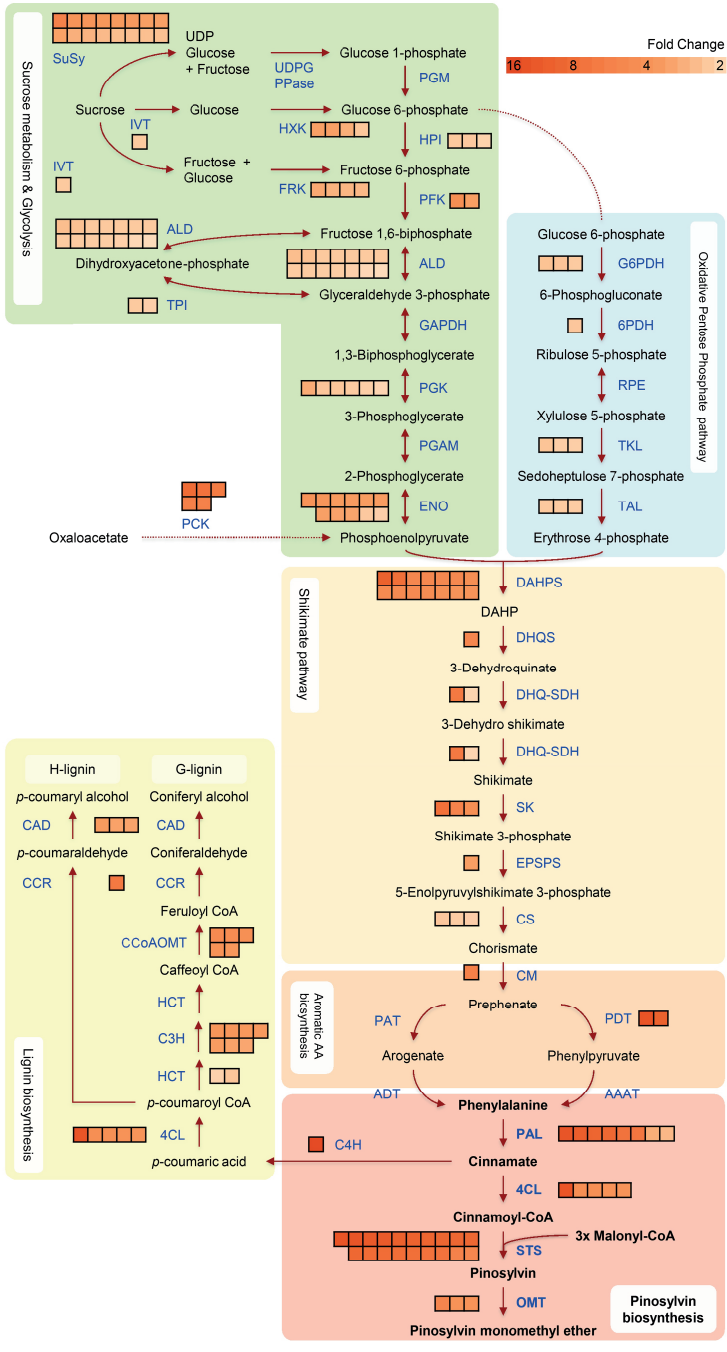
Fold Change
16
8
4
2
Sucrose metabolism & Glycolysis
SuSy
UDP
Glucose
+ Fructose
UDPG
PPase
Glucose 1-phosphate
PGM
Sucrose
Glucose
Glucose 6-phosphate
IVT
HXK
HPI
IVT
Fructose +
Glucose
Fructose 6-phosphate
FRK
PFK
ALD
Fructose 1,6-biphosphate
Dihydroxyacetone-phosphate
ALD
TPI
Glyceraldehyde 3-phosphate
GAPDH
1,3-Biphosphoglycerate
PGK
3-Phosphoglycerate
PGAM
2-Phosphoglycerate
ENO
PCK
Oxaloacetate
Phosphoenolpyruvate
Glucose 6-phosphate
G6PDH
6-Phosphogluconate
6PDH
Ribulose 5-phosphate
RPE
Xylulose 5-phosphate
TKL
Sedoheptulose 7-phosphate
TAL
Erythrose 4-phosphate
Oxidative Pentose Phosphate pathway
DAHPS
DAHP
DHQS
3-Dehydroquinate
DHQ-SDH
3-Dehydro shikimate
DHQ-SDH
Shikimate
SK
Shikimate 3-phosphate
EPSPS
5-Enolpyruvylshikimate 3-phosphate
CS
Chorismate
CM
Shikimate pathway
Prephenate
PAT
PDT
Arogenate
Phenylpyruvate
Aromatic AA biosynthesis
ADT
AAAT
Phenylalanine
PAL
C4H
Cinnamate
4CL
Cinnamoyl-CoA
3x Malonyl-CoA
STS
Pinosylvin
OMT
Pinosylvin monomethyl ether
Pinosylvin biosynthesis
H-lignin
G-lignin
p-coumaryl alcohol
Coniferyl alcohol
CAD
CAD
p-coumaraldehyde
Coniferaldehyde
CCR
CCR
Feruloyl CoA
CCoAOMT
Caffeoyl CoA
HCT
C3H
HCT
p-coumaroyl CoA
4CL
p-coumaric acid
Lignin biosynthesis

Figure 6. The primary and secondary metabolism pathways activated in the transition zone. The individual box next to the arrows is the transcript encoding enzymes that differentially expressed in the transition zone compared to sapwood during heartwood formation in summer. Arrows in the figure represent the enzymatic reactions. Abbreviations: SuSy, sucrose synthase; IVT, invertase; UDPG PPase, UDP glucose pyrophosphorylase; HXK, hexokinase; FRK, fructokinase; PGM, phosphoglucomutase; HPI, hexose phosphate isomerase; PFK, phosphofructokinase; ALD, aldolase; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; PGK, phosphoglycerate kinase; PGAM, phosphoglycerate mutase; ENO, enolase; TPI, triose phosphate isomerase; PCK, phosphoenolpyruvate carboxykinase; G6PDH, glucose 6-phosphate dehydrogenase; 6PDH, 6-phospogluconate dehydrogenase; RPE, ribulose 5-phosphate epimerase; TKL, transketolase; TAL, transladolase; DAHP, 3-Deoxy-D-arabinoheptulosonate-7-phosphate; DAHPS, DAHP synthase; DHQS, 3-dehydroquinate synthase; DHQ-SDH, dehydroquinate dehydratase-shikimate dehydrogenase; SK, shikimate kinase; EPSPS, 5-enolpyruvylshikimate 3-phosphate synthase; CS, chorismate synthase; CM, chorismate mutase; AA, amino acid; PAT, prephenate aminotransferase; ADT, arogenate dehydratase; PDT, prephenate dehydratase; AAAT, aromatic amino acid aminotransferase; PAL, phenylalanine ammonia lyase; 4CL, 4-coumarate:CoA ligase; STS, stilbene synthase; OMT, *O*-methyltransferase; C4H, *trans*-cinnamte 4-monooxygenase; HCT, hydroxycinnamoyl-CoA:shikimate/quinate hydroxycinnamoyltransferase; C3H, 4-coumaroyl shikimate/quinate 3'-hydroxylase; CCoAOMT, caffeoyl-CoA *O*-methyltransferase; CCR, cinnamoyl-CoA reductase; CAD, cinnamyl-alcohol dehydrogenase. Figure modified from Lim et al. (2016), copyright American Society of Plant Biologists.

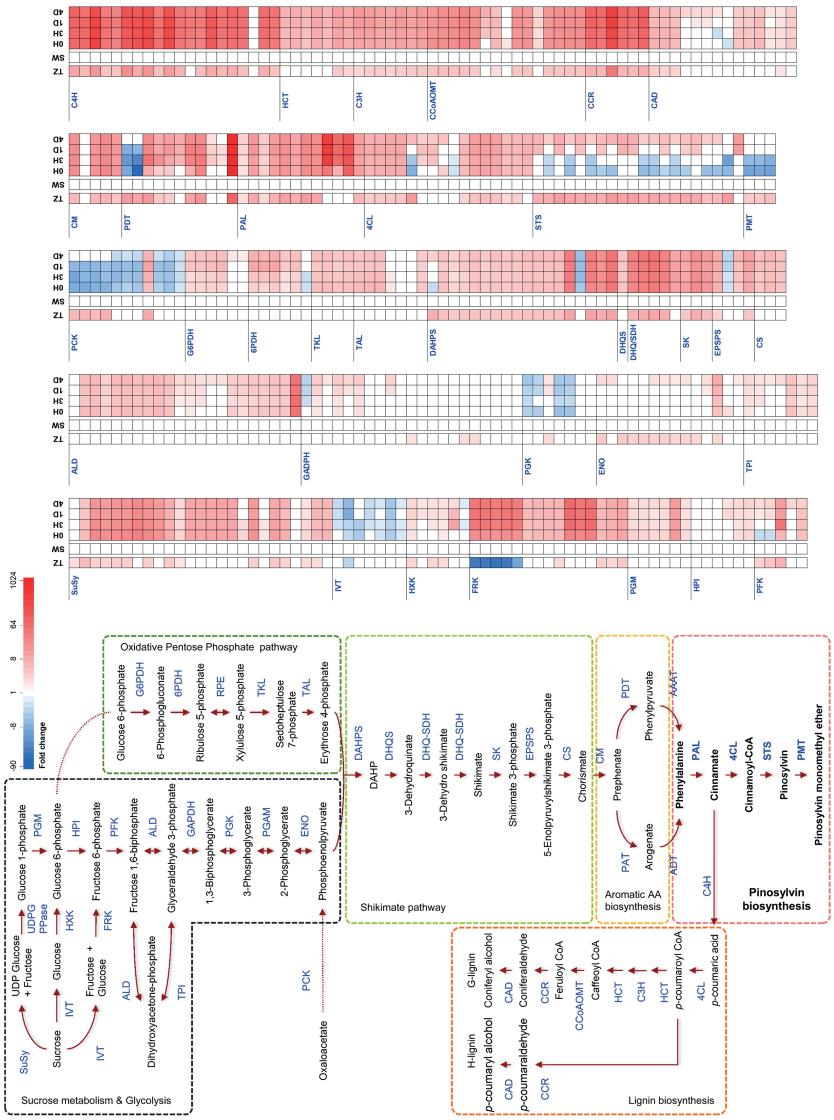

Figure 7. The expression profile of primary to secondary pathways during development and stress. Arrows in the figure represent the enzymatic reactions. The individual boxes represent fold change of transcripts encoding enzymes in the transition zone (TZ), sapwood (SW) of mature trees, in unwounded seedlings stems (0H), three hours (3H), one day (1D), and four days (4D) after wounding of seedling stems. All samples were compared to SW. See Figure 6 for the full abbreviations of the enzymes. Figure obtained from Lim et al. (2017), manuscript.

4.5 Biosynthesis of Scots pine heartwood extractives

Despite the difference in upregulation of primary pathways, stilbene biosynthesis was commonly upregulated during heartwood formation and in response to stress.

4.5.1 Distinct *PAL* and *4CL* genes might be specific for development and stress

The biosynthesis of pine stilbenes is initiated by converting phenylalanine to cinnamic acid by the enzyme PAL. Interestingly, the transcripts encoding PAL that were differentially expressed during heartwood formation (**I**) and wounding response (**II**) were not the same. Likewise, a different set of transcripts, which were annotated as 4CL in the *Pinus* EST collection, were found induced during development and wounding response. Cinnamoyl-CoA serves as the substrate for pine STS in the synthesis of the stilbene pinosylvin. However, the enzyme responsible for activation of cinnamic acid in pine is unknown, and one possibility is that it is a specific cinnamate-utilising 4CL. Cinnamate:CoA ligases have been described in some angiosperms (Klempien et al., 2012; Gaid et al., 2012) and sometimes they are acyltransferase not very close to 4CL. It is also important to note that both PAL and 4CL are also involved in other pathways of secondary metabolism, e.g. in the biosynthesis of lignin and lignans besides stilbenes.

4.5.2 Stilbene synthase and the correct *O*-methyltransferase for pine stilbene biosynthesis

Further downstream, stilbene pinosylvin is generated from cinnamoyl-CoA by pine STS. Though STS was both developmentally and stress-induced, two expression profiles of STS were observed in the wounding transcriptomic data, where STS was either constantly upregulated until 4D or upregulated and stabilised after 1D (Figure 2 in paper **II**, Figure 7). The same observation was also made for the Scots pine transcriptomic data from UV-C treated needles (Paasela et al., manuscript in preparation). From this observation, it could conceivably be hypothesised that at least two differently regulated *STS* genes occur in the Scots pine genome.

Pinosylvin is in part converted into pinosylvin monomethyl ether by PMT. A Scots pine PMT (here referred to as PMT1) was characterised by Chiron and

colleagues (2000a). Transcripts encoding PMT1, however, were not upregulated during heartwood formation (**I**) or in response to wounding (**II**). On the contrary, another *O*-methyltransferase was induced in both the developmental and stress contexts, and closely followed the expression of STS (**III**). For further investigation, the *O*-methyltransferase encoding transcript was isolated, heterologously expressed in *E.coli* and characterised (**III**). The enzymatic assay results demonstrated that this *O*-methyltransferase specifically utilises stilbenes as a substrate and forms PSME from PS. This is in contrast to the previously characterised PMT1, which acted as a multifunctional enzyme methylating several substrates *in vitro* (Table 1 in paper **III**). We conclude with great certainty that PMT1 is not the correct PMT that methylates PS to PSME, but instead it is the newly characterised *O*-methyltransferase, named PMT2 (**III**).

4.5.3 Biosynthesis of heartwood resin acids

Resin acids, one of the major components of pine heartwood extractives, are synthesised in resin duct epithelia during growth and also induced in response to herbivore and pathogen attacks (Keeling and Bohlmann, 2012). However, we did not expect that the transcripts associated with resin acid biosynthesis, e.g. diterpene synthase (TPS) and abietadienol/abietadienal oxidase (CYP720B), and its upstream pathways, were neither upregulated nor downregulated during heartwood formation during summer (**I**).

Further investigation was performed to follow their year-round expression via quantitative RT-PCR. Interestingly, the corresponding transcript level peaked in early spring, dropped in late spring or early summer and remained very low during the other seasons in both the transition zone and sapwood. Nevertheless, the expression level of these transcripts was always stronger in the sapwood. The result indicated that resin acid biosynthesis was in fact induced in early spring rather than during the summer, and that resin acids would be loaded to heartwood instead of being synthesised in the transition zone, indicating that from the resin acid perspective pine heartwood is of type II. Lorio (1986) proposed that pine prioritises the sink of photosynthates to growth during the growing season, and the resources are available for resin acid production only when growth ceases. The observation of resin acid biosynthesis in development transcriptomic data is in accordance with Lorio's concept.

In contrast to our expectations, CYP720B was rather downregulated when pine stem was wounded (**II**). Croteau and collegaues (1987) observed that the accumulation of resin acids in aseptically wounded two-year-old lodgepole pine

(*Pinus contorta*) seedlings stems was only 1.2 fold higher than in unwounded stems 12 days after treatment. However, the production of resin acids in the stems where aseptic wounding was followed by blue-stain fungus (*Ceratocystis clavigera*) infection was 2-fold higher than in the control. Harju and colleagues (2009) also observed that resin acids accumulated next to the wounding sites in pine seedling stems three months after mechanical wounding. Resin acids biosynthesis could be induced later than four days after wounding.

4.6 Different sets of MYB and NAC transcription factors are induced during heartwood formation and stress response

Plant transcription factors, for example MYB and NAC TFs, play important roles in both developmental and abiotic or biotic responses. Both MYB and NAC transcription factors have been suggested to regulate secondary cell wall biosynthesis, secondary metabolism and developmentally induced PCD (Nakano et al., 2015; Duval et al., 2014; Patzlaff et al., 2003a; Dubos et al., 2010; Bollhöner et al., 2012), as well as responses to stressors such as pathogen attacks and wounding (Pascual et al., 2015; Mellway et al., 2009).

In this study, single transcripts encoding MYB ($MYB_{TC188897}$) and NAC ($NAC_{TC164798}$) transcription factors were differentially expressed in the transition zone compared to sapwood during heartwood formation (**I**). The year-round expression study showed that the expression of these transcription factors strongly correlated with the expression of the transcripts associated with stilbene biosynthesis and its upstream pathways (Figure 8), both during heartwood formation and independent of this process during winter. This indicates them as putative regulators of pine stilbene biosynthesis in the transition zone (Figure 8).

Interestingly, the same transcripts were not expressed in response to wounding (**II**), instead different MYB and NAC encoding transcripts were differentially expressed. The expression level of wounding-induced MYB and NAC encoding transcripts was transiently increased at three hours and one day, then decreased at four days after wounding. The results suggest that under development and stress conditions, different MYB and NAC encoding transcripts are involved in regulating metabolic processes in Scots pine.

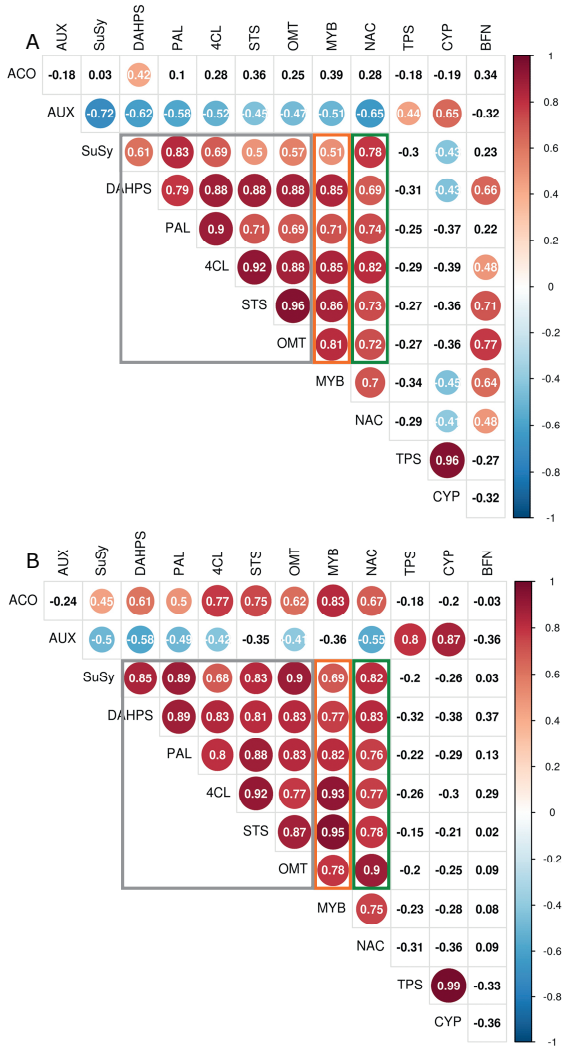

Figure 8. The correlation matrix between selected transcripts in the year-round expression study. The expression of MYB (orange box) and NAC (green box) strongly correlated with expression of stilbene biosynthesis and its upstream pathways transcripts (grey box) of **A)** tree C and **B)** tree D. Abbreviations: ACO, ACC oxidase; AUX, auxin responsive; BFN, bifunctional nuclease; MYB, transcription factor MYB-like; NAC, transcription factor NAC domain; SuSy, sucrose synthase; DAHPS, DAHP synthase; PAL, phenylalanine ammonia lyase; 4CL, 4-Coumarate:Coa ligase; STS, pinosylvin synthase; OMT, *O*-methyltransferase; TPS, diterpene synthase; CYP, abietadienol/abietadienal oxidase (CYP720B). Figure modified from Lim et al. (2016), copyright American Society of Plant Biologists.

4.7 Programmed cell death, cell wall modification, water deficit stress and other processes during development and stress response

4.7.1 Heartwood formation

Heartwood formation is described as a form of tissue senescence (Spicer, 2005). This can be observed from an upregulation of the endonuclease that is associated with plant PCD, BFN, in the transition zone (**I**). Upregulation of BFN is also in line with previous studies that report nuclear degradation of ray parenchyma cells in the transition zone, suggesting that PCD is indeed involved in heartwood formation (Taylor et al., 2002; Spicer, 2005; Nakada and Fukatsu, 2012). In addition, the transcripts encoding cell wall polysaccharide modifying and lignin biosynthetic enzymes (Table S5 in paper **I,** Figure 6) were upregulated in the transition zone during heartwood formation, indicating that lignification and cell wall modification of parenchyma cell walls was occurring in the transition zone, as also observed by Bergstöm (2003) and Yamamoto (1982). Towards the end of heartwood formation, storage compounds in the transition zone are consumed and depleted, parenchyma cell walls undergo modifications and the cells die.

Previous studies have described that water is withdrawn from the transition zone during heartwood formation (Nakada and Fukatsu, 2012; Bergström, 2003; Rust, 1999). Relating to this, we saw downregulation of aquaporins, which are related to plant cell-to-cell water transport and homeostasis (Bienert and Chaumont, 2011; Hachez et al., 2006) in the transition zone (**I**). On the other hand, the transcripts encoding desiccation-related proteins (DPRs), which have been suggested to be involved in plant tolerance to desiccation (Zha et al., 2013; Piatkowski et al., 1990), were upregulated in the transition zone (**I**). Upregulation of DPR encoding transcripts showed DPRs playing a role in protecting cells in the transition zone against water deficit while they carried out necessary physiological activities for heartwood formation.

In addition, a set of transcripts involved in plant defence, such as chitinase and pathogenesis-related proteins, were upregulated (**I**). Plant chitinases are known for hydrolysing the chitin of fungal cell walls, and are induced in response to fungal infection and developmental processes such as secondary cell wall lignification (Grover, 2012). Upregulation of these transcripts showed that they were developmentally inducible also during heartwood formation.

4.7.2 Comparison to stress response

The transcript encoding BFN was not induced in response to wounding stress (**II**), indicating that BFN is developmental PCD-related. In fact, no PCD-related transcripts were observed in the wounding response. Cell wall modification took place both during heartwood formation and in the wounding response. However, different transcripts were induced. The cell wall carbohydrate modifying enzymes, for example XTHs, were induced an average 17-fold, as early as three hours after wounding of the pine stem (**II**). Besides XTHs, a group of transcripts encoding cell wall modification enzymes, such as endo-beta-1,4-glucanase, expansin, glucan-1,3-beta-glucosidase, glucanase-like protein, mannan endo-1,4-beta-mannosidase, pectin methylesterase and pectin methylesterase-like proteins, were triggered as early as three hours or one day after wounding (**II**). These enzymes are involved in wall loosening, elongation and expansion during development and in response to abiotic or biotic stressors (Moreira and Filho, 2008; Schröder et al., 2009; Cosgrove, 2000; Sasidharan et al., 2011; Rose et al., 2002; Eklöf and Brumer, 2010). The upregulation of these transcripts suggest that cell wall remodelling takes place to improve stem integrity within wounded areas.

Lignan biosynthesis-related transcripts, such as dirigent (DIR) proteins, DIR-like proteins, pinoresinol-lariciresinol reductase (PLR), were also triggered one day after wounding. Lignans have been described to be involved in plant defence just as phytoalexins are (Naoumkina et al., 2010) and the study by Harju and colleagues (2009) also reported that lignans accumulated near the wounded xylem. The deposition of lignans in response to wounding is probably to enhance cell wall integrity and increases the level of difficulty for invasion attempts by pathogens.

Water deficit stress was observed in stress transcriptomics data (**II**). Once again, however, different corresponding transcripts were induced compared to what was developmentally induced. Transcripts encoding aquaporin, dehydrin, water deficit inducible (LP3) protein and early responsive to dehydration stress protein were induced three hours after wounding (**II**). The expression level then gradually decreased (Figure 2 in paper **II**). Furthermore, transcripts encoding the cold acclimation protein, dehydrin, and late embryogenesis abundant (LEA) proteins were triggered one day after wounding as a secondary response to wounding. The expression level of these transcripts then decreased during the following time points (Figure 2 in paper **II**). LP3 has been hypothesised of playing a role, similar to the DPR protein, in protecting the cell nucleus under

desiccation stress (Wang et al., 2002). In addition, dehydrin and LEA proteins are known to be associated with water deficit, drought and abiotic stress (Taji et al., 2002; Shinozaki and Yamaguchi-Shinozaki, 2006; Hanin et al., 2011). Mechanical wounding disturbs water conduction in the stem, causing water loss from the stem and subsequently leading to osmotic stress in the stem. Thus, upregulation of these transcripts may play a role in enhancing cell survival in wounded areas. Interestingly, DPR proteins were only upregulated in the transition zone during heartwood formation, but not in response to wounding, while *vice versa* was true for LP3 proteins, suggesting that Scots pine has two different sets of water deficit inducible transcripts that respond to development and stress.

The defence-associated transcripts, chitinase and pathogenesis-related proteins, that are upregulated during heartwood formation were also induced in response to wounding (**II**). A different set of transcripts associated with defence, such as antimicrobial peptides, defensin, elicitor responsive proteins, nematode resistance protein and pathogenesis-related proteins, were induced in response to wounding trauma (**II**). The expression of these transcripts was induced either three hours or one day after wounding, and was reduced at four days. This finding, albeit preliminary, suggests that defence mechanisms to all potential pathogens are triggered as an initial response to mechanical wounding trauma, while other physiological activities are concurrently ongoing.

5. CONCLUSIONS AND PROSPECTS

In this work, Scots pine transcriptome changes during development and stress were studied with a next-generation sequencing and bioinformatics approach. The molecular development of heartwood formation was investigated in the transition zone and sapwood of mature pine trees. In addition, stress response was studied in the stems of pine seedlings by wounding.

Up to date, several suggestions have been made regarding the timing of pine heartwood formation (Shain and Hillis, 1973; Yang, 1993). Bergström and colleagues (1999) suggested that the heartwood formation of Scots pine occurs year-round. This study demonstrated that actually there is indeed a specific time for Scots pine heartwood formation. The process takes place during the growth period and is marked by programmed cell death. We conclude that heartwood formation in Scots pine is of type I, supported by the transcriptomic data showing that pine stilbenes were synthesised in the transition zone and gained carbon skeletons from breaking down sugar *in situ*. In contrast to pine stilbenes, the expression of resin acid biosynthetic transcripts were induced in both sapwood and the transition zone, but were stronger in sapwood. The transcriptomic data also indicated that the biosynthesis of resin acids began earlier in the spring compared to stilbene biosynthesis and mainly took place in the sapwood. It can thus be concluded that resin acids were loaded into the heartwood instead of synthesised *in situ* in the transition zone. In fact, from the resin acids point of view, Scots pine heartwood formation is of type II.

Many interesting details were revealed when following the expression of transcripts associated with stilbene biosynthesis. For instance, distinct *PAL* and *4CL* genes appear to be induced in the developmental and stress-induced contexts. Likewise, different members of MYB and NAC transcription factors were expressed during heartwood formation and in response to wounding. The same observation applied to the induced transcripts associated with water deficit stress and cell wall modification. In addition, many defence-associated transcripts were induced in response to wounding, however, only a handful were shared among those induced under development. Taken together, Scots pine has little similarity in transcriptome changes between development and stress, except for the stilbene biosynthetic pathway and certain pathogenesis-related transcripts.

With this background, we remain uninformed of the genes that might connect the observed genetic correlation between heartwood stilbene content in adult

trees and wound-induced stilbene biosynthesis in seedlings – unless our results are pointing directly to the structural genes encoding STS and PMT2.

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