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TANJA PAASELA

The Stilbene Biosynthetic Pathway and Its Regulation in Scots Pine

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The stilbene biosynthetic pathway and its regulation in Scots pine

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The Doctoral School in Environmental, Food, and Biological Sciences (YEB)
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Lim K.-J., **Paasela T.**, Harju A., Venäläinen M., Paulin L., Auvinen P., Kärkkäinen K. and Teeri T.H. (2016). Developmental changes in Scots pine transcriptome during heartwood formation. Plant Physiology 172: 1403-1417 doi:10.1104/pp.16.01082.

II

Paasela T., Lim K.-J., Harju A., Venäläinen M., Paulin L., Auvinen P., Kärkkäinen K. and Teeri T.H. (2017). Scots pine transcriptional responses to UV-C treatment and inhibition of translation: Stilbene biosynthesis is a primary defense response. Manuscript.

III

Paasela T., Lim K.-J., Pietiäinen M. and Teeri T.H. (2017). The *O*-methyltransferase PMT2 mediates methylation of pinosylvin in Scots pine. New Phytologist 214: 1537–1550 doi:10.1111/nph.14480.

AUTHOR'S CONTRIBUTION

I

TP participated in planning of the experiments, conducted optimization of RT-qPCR analysis and did the RT-qPCR with K-JL. TP participated in interpretation of the results and writing of the manuscript.

II

TP did most of the experimental work, except the construction of the RNA-seq libraries and bioinformatics analysis. TP wrote the manuscript together with THT.

III

TP did all the experimental work, except the construction of the RNA-seq libraries and RT-qPCR analysis. TP wrote the manuscript together with THT.

ABBREVIATIONS

4CL	4-coumaroyl CoA ligase
ABA	abscisic acid
ACC	1-aminocyclopropane-1-carboxylic acid
AP2/ERF	apetala2/ethylene response factor
BFN	bifunctional nuclease
bHLH	basic helix-loop-helix
bZIP	basic leucine zipper
CHI	chalcone isomerase
CHS	chalcone synthase
CHX	cycloheximide
CML	compound middle lamella
CNL	cinnamate ligase
GRAS	Transcription factor family including GIBBERELLIC-ACID INSENSITIVE (GAI), REPRESSOR OF GAI (RGA) and SCARECROW (SCR)
GUS	β-glucuronidase
HW	heartwood
IAA	indole-3-acetic acid
MEP	2-C-methyl-D-erythritol 4-phosphate
MVA	mevalonic acid
MYA	million years ago
MYB	Transcription factor family including the mammalian MYELOBLASTOSIS ONCOGENE
NAC	Transcription factor family including NO APICAL MERISTEM (NAM), ARABIDOPSIS TRANSCRIPTION ACTIVATION FACTOR 1/2 [ATAF1/2] and CUP-SHAPED COTYLEDON 2 (CUC2)
NGS	next-generation sequencing
PAL	phenylalanine ammonia lyase
PCD	programmed cell death
PMT	pinosylvin *O*-methyltransferase
PS	pinosylvin
PSME	pinosylvin monomethyl ether
ROS	reactive oxygen species
STS	stilbene synthase
SW	sapwood
TF	transcription factor
TZ	transition zone
UTR	untranslated region
UV	ultraviolet
WRKY	transcription factor family containing a highly conserved WRKY (tryptophan/arginine/lysine/tyrosine) domain

ABSTRACT

Conifers dominate the boreal forests of the Northern Hemisphere, and especially members of the family Pinaceae have great economic and ecological significance. Part of their success is thought to arise from the vast array of secondary metabolites they produce. The products of secondary metabolism are essential for plants to survive in the ever-changing environment. In Scots pine (*Pinus sylvestris* L.), two groups of secondary metabolites, stilbenes and resin acids, are crucial for decay resistance of heartwood timber and for active defense responses against herbivores and fungal pathogens. Several studies have shown that stilbenes improve decay resistance of pine heartwood. Since there is wide variation in the concentration of stilbenes between individuals and the trait has high heritability, it may be possible to breed heartwood that is more decay-resistant. However, breeding for heartwood properties is slow, since the decay resistance characteristics can be estimated at the earliest from 30-year-old trees. Early selection methods utilizing genetic markers or chemical screening are needed, but we do not yet understand which genes control the biosynthesis of stilbenes and what the genetic differences are between individuals that explain the variation in the capacity to produce stilbenes. Importantly, there is genetic correlation between stress-induced stilbene biosynthesis in seedlings and the heartwood stilbene content in their adult mother trees.

Here, we examined the pine transcriptional responses under two conditions that were previously known to activate stilbene biosynthesis: heartwood formation in adult trees and ultraviolet (UV)-C treatment of needles in seedlings. We found that these two conditions had very little in common, except for the activation of stilbene pathway genes. For example, the regulators of the two responses seemed not to be shared. The activation of the stilbene pathway in response to UV-C treatment occurred a few hours after the onset of the treatment and was independent of translation. Stilbene biosynthesis seems to be an early defense response in Scots pine.

Heartwood formation, an important developmental process in the senescence of secondary xylem, is poorly understood. Based on transcriptomic analysis, stilbene biosynthesis occurs *in situ* in the transition zone between the sapwood and heartwood, but resin acids were synthesized primarily in the sapwood. Bifunctional nuclease, an enzyme involved in the process of developmentally programmed cell death (dPCD), is

a useful marker for heartwood formation and aided us in defining the timing of the process, from spring to late autumn. Expression of this marker, which is strictly confined to dPCD conditions, further clarified that heartwood formation truly is a process that is initiated by intrinsic programming instead of environmental cues.

The transcriptomic data revealed that the expression of the previously characterized pinosylvin *O*-methyltransferase gene, *PMT1*, was not induced under stilbene-forming conditions. A new PMT-encoding gene, *PMT2*, was identified by coexpression analysis. The gene showed an inducible expression pattern very similar to that of the stilbene synthase gene under all conditions studied. PMT2 furthermore methylated pinosylvin with high specificity, in contrast to PMT1, which accepted several substrates.

1 INTRODUCTION

Conifers dominated the flora for 160 million years (225-65 million years ago (MYA)) until the emergence and rapid expansion of the angiosperms, at the expense of gymnosperm abundance and diversity. The origin of the pine family Pinaceae is not known, but some evidence places it in the Late Triassic (235-208 MYA), and the genus *Pinus* arose in the Cretaceous 145 MYA (Donoghue & Doyle 2000; Miller 1977). Angiosperms dominate most terrestrial habitats with approximately 250 000 species, while the number of conifer species is only about 550 (Figure 1). Despite the rapid expansion of the angiosperms, conifers still dominate the boreal forests of the Northern Hemisphere, especially members of the family Pinaceae, many of which play significant roles in economics and the environment (Donoghue & Doyle 2000; Brodribb et al. 2012). The persistence of conifers among angiosperms is thought to arise from their adaptations to nutrient-poor soils and by their narrow tracheid structure, which is more tolerant of embolism caused by freezing, but a part of their success is explained by the vast array of secondary metabolites, such as phenolics and terpenoids, that confer resistance to many invading pests and pathogens (Phillips & Croteau 1999; Brodribb et al. 2012). Many of these secondary metabolites are produced actively, not only in response to harmful invaders, but also to developmental signals during the poorly understood process of heartwood (HW) formation.

The formation of HW, the innermost part of the stem with no living cells, is an important process trees use to control the amount of energy-consuming sapwood (SW) and to protect the dead plant material from microorganisms (Spicer 2005). In addition, it is a crucial determinant of the quality of the wood as a construction material in terms of durability and aesthetics, both of which are dependent on species-specific extractives. The HW of certain species is also a source of important chemicals for the fragrance industry and has been studied extensively for possible pharmaceutical applications (Kampe & Magel 2013; Jones et al. 2011).

Although important, there are several characteristics of conifers that hamper elucidation of the biosynthetic and regulatory pathways of these compounds. Their enormous genome size (20-40 Gb) has made sequencing of conifer genomes impossible until very recent years, when several next-generation sequencing (NGS) techniques have emerged, although it is still an extremely large task. The lack of mutant lines and efficient transformation techniques together with the slow growth of coniferous species makes metabolite profiling, transcriptomic analyses, and enzyme biochemistry crucially important for functional genomics.

NGS has made possible inexpensive and in-depth studies of plant responses to various environmental and developmental cues at the level of the whole transcriptome. Together with coexpression analyses, NGS has become a powerful tool for gene discovery in candidate gene approaches and in elucidation of biochemical pathways. Temporal and spatial localization of gene expression gives important clues that a given enzyme is involved in a pathway *in planta*, especially in species whose function cannot be confirmed by reverse genetics.

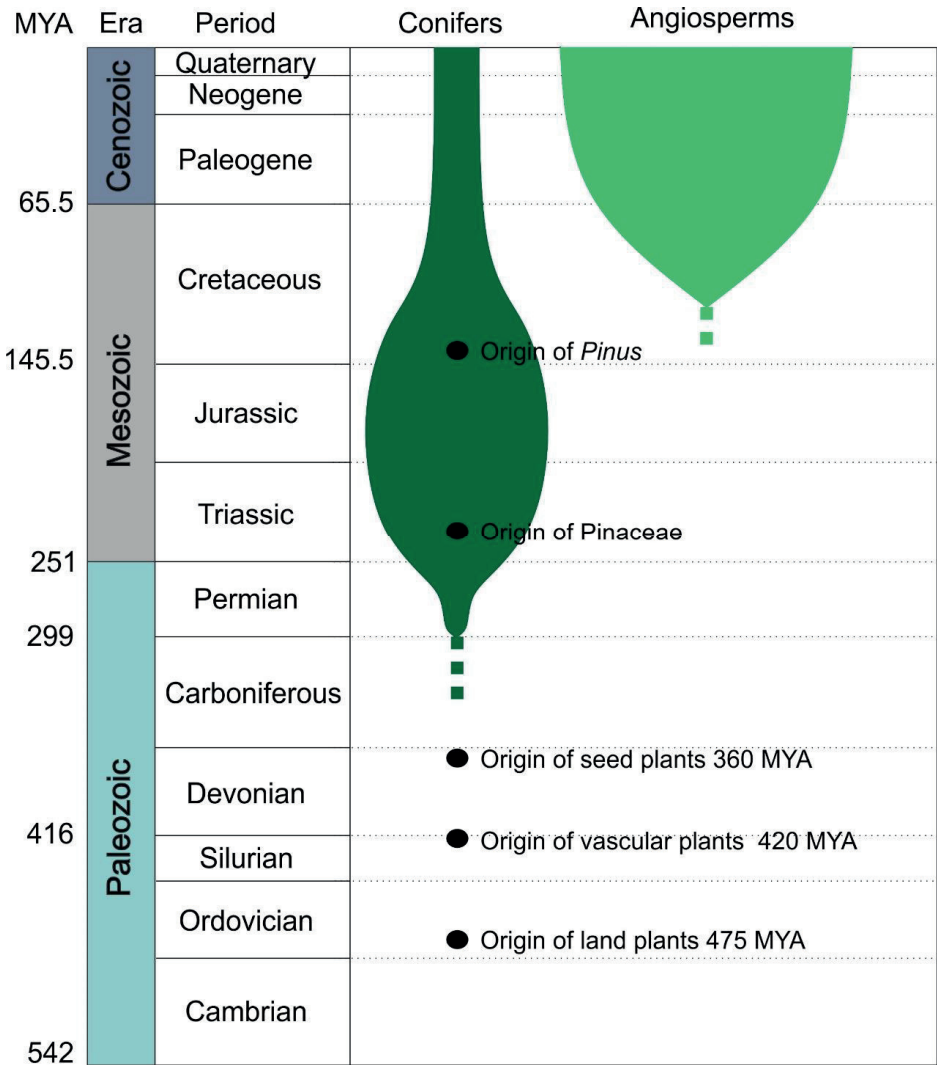

Figure 1. The major evolutionary events of conifers on the geological time scale. Redrawn based on Valentine (1978), Graham (1993), and Campbell & Reece (2005). MYA, million years ago.

1.1 Secondary metabolism, why is it so diverse?

Early interpretations saw secondary metabolites simply as (toxic) waste products of primary metabolism that the plant cell secreted or sequestered in vacuoles (Hartmann 2007). The current view, however, defines secondary metabolism as processes that are not needed for growth and development, but are often essential for plants to survive and interact with the environment. Secondary metabolites are needed for instance in defense against abiotic and biotic stresses, plant-plant communication, and for pollinator

attraction. The qualitative and quantitative secondary metabolite compositions in plants are diverse and vary within and between species (Kliebenstein & Osbourn 2012; Hartmann 2007). Terpenoids are excellent examples of diversity in secondary metabolites, with more than 70 000 different compounds identified (Vickers et al. 2014). Primary metabolism, in contrast, is common to all plants. It consists of processes that are needed to construct and maintain the cell and are essential for photosynthetic and respiratory metabolism, as well as for growth and development (Hartmann 2007; Kliebenstein & Osbourn 2012). Division into primary and secondary metabolism is not always clear. Some compounds have both primary and secondary functions.

The reason why an individual plant produces massive amounts of different metabolites, often seemingly inactive, has long intrigued scientists, not the least because investments in secondary metabolism divert resources away from growth and reproduction. Pathways for new secondary metabolites arise by gene duplication. The most common fate for a duplicated gene is that mutations silence the gene function; it becomes a pseudogene and is lost over time (pseudogenization) (Lynch & Conery 2000; Ober 2010). In subfunctionalization, the genes retain their original activity, but may lose specific expression domains, so that both copies are needed for the complete ancestral function (Lynch & Conery 2000; Ober 2010). A newly duplicated gene may, however, gain a novel function in the process called neofunctionalization through mutations in the coding region that alter the substrate specificity or in the regulatory areas that change the localization of the gene product, and eventually the enzyme encoded may become part of a new metabolic pathway. The genes encoding stilbene synthase (*STS*), enzymes at the focus of this thesis, have repeatedly emerged as neofunctionalized duplicates of chalcone synthase (*CHS*) genes, which, in contrast, is common to all plants (Tropf et al. 1994).

Vast arrays of different types of metabolites are needed for plants to defend themselves against multiple types of biotic and abiotic stressors, since as sessile organisms they cannot escape them. A driving force for the structural diversity of secondary metabolites is believed to be the promiscuous nature of secondary metabolism enzymes. Many enzymes can accept and modify different related structures, which aids their integration into new metabolic pathways (Moghe & Last 2015; Weng 2014). Furthermore, some enzymes are also highly promiscuous in their catalytic activity. For instance, the

sesquiterpene synthases (STPs) δ-selinene and γ-humulene synthases from grand fir (*Abies grandis*) produced *in vitro* 34 and 52 different structures from the same precursor, respectively (Steele et al. 1998). There are two often-cited hypotheses why chemical diversity is maintained in plants even in the absence of active roles for the compounds. One hypothesis is the so-called 'screening hypothesis', which is based on the idea that when a new metabolite arises through mutations, the likelihood of it being active is relatively low, and this shapes the evolution of secondary metabolite biochemistry (Jones et al. 1991; Firn & Jones 2003). The higher diversity of the various structures increases the chances for developing new highly active metabolites, and organisms that can generate and retain large numbers of chemicals would be favored. Plant-enemy coevolution, the so-called 'chemical arms race', is another hypothesis explaining the diversity of defensive compounds. In this, the chemicals produced by the plant furnish protection against invaders, and when the enemy gains resistance against one chemical, another compound is produced, and so on (Firn & Jones 2003; Speed et al. 2015). The theoretical model of plant-herbivore coevolution predicts that diversity of compounds increases plant fitness and decreases the fitness of the herbivore (Speed et al. 2015).

Although mechanisms of how new enzymatic functions appear are known, it is not well understood how whole metabolic pathways that consist of multiple steps are assembled from duplicated genes (Ober 2010; Kliebenstein & Osbourn 2012). Clustering is common in bacteria in which genes are organized in operons, but are only seldom detected in eukaryotes (Chu et al. 2011). However, some secondary metabolite pathways in plants are clustered physically in the genome. Clustering facilitates coinheritance of the gene combination. Losing one member of the cluster can lead to accumulation of toxic intermediates, which indicates that clustering may be a way to protect plants from these compounds. Clustering also aids coregulation of the pathway when the clustered genes are made accessible for transcription through chromatin modifications (Chu et al. 2011; Field & Osbourn 2008). In many cases, pathway-encoding genes may have scattered throughout the genome and still have ended up working as part of a common metabolic pathway. Adapting genes one by one to any pathway seems complicated, since it requires that intermediate steps along the process should be somehow beneficial to the plant. It is believed that these multistep pathways may have evolved by modification of genes that were already part of transcriptional or

biochemical modules. However, this alone does not explain the evolution of some well-known pathways, either. Multistep pathways have seemingly evolved by adapting and combining different modules in addition to recruitment of single enzymes. As an example, the glucosinolate pathway has evolved by the combination of modules for branched amino-acid biosynthesis, cyanogenic glucoside synthesis, sulfur assimilation, and glutathione utilization, and the backbone is further modified by independent recruitment of single enzymes (Kliebenstein & Osbourn 2012).

The evolution of the phenylpropanoid pathway is considered to be one of the key factors that facilitated the movement of plants to terrestrial habitats by providing a screen for ultraviolet (UV)-B radiation and eventually structural support, long-distance water transport, and protection from herbivores and pathogens (Weng & Chapple 2010; Weng 2014). The central phenylpropanoid pathway, common to all plants, is dependent on the shikimate pathway that generates the aromatic amino acid phenylalanine, the precursor for all phenylpropanoids. In the central position of the various pathways is 4-coumaroyl-coenzyme A (CoA), which is utilized in the biosynthesis of several groups of secondary metabolites, such as flavonoids, lignans, lignin, and sporopollenin (Figure 2; Vogt 2010). Chalcones, flavones, and flavonols appeared 500 MYA in the ancestors of bryophytes (mosses, liverworts and hornworts) after the appearance of the core phenylpropanoid and malonyl-CoA pathways (Koes et al. 1994; Stafford 1991). The key enzyme involved in biosynthesis of flavonoids is CHS, an enzyme shared by all land plants. Stilbenes are found sporadically in various plant families, such as Vitaceae, Pinaceae, Fabaceae, and Poaceae (Chong et al. 2009). There is seemingly no ancestral STS-encoding gene, but STSs have arisen independently in distinct species from CHS several times in evolution (Tropf et al. 1994). *STS* genes have evolved from *CHS* genes by neofunctionalization; in fact, only three amino-acid changes in CHS are sufficient to convert CHS to an enzyme with low STS activity (Tropf et al. 1994).

1.2 Extractives determine the durability of heartwood

One fundamental change that occurs when SW is converted to HW is the biosynthesis of extractives that impregnate the wood and function as a passive barrier against invading fungal pathogens. SW is that part of the wood that contains living cells, reserve materials, and is responsible for transporting water and minerals. HW, in contrast, is in

the center of the trunk and contains no living cells. In HW, reserve materials have been converted to HW extractives.

HW substances with fungitoxic properties in Scots pine (*Pinus sylvestris* L.) are composed mostly of the stilbenes pinosylvin (PS), its mono- and dimethylated derivatives, and resin acids of the pimaric and abietic acid type, with the latter predominating (Willför et al. 2003; Hovelstad et al. 2006). Pine stilbenes differ structurally from angiosperm stilbenes. PS lacks one hydroxyl group, compared with resveratrol, because the pine STS preferentially uses cinnamoyl-CoA as a substrate over 4-coumaroyl-CoA, which is used in angiosperms. Curiously, no resveratrol has been detected in pine, even though the STS can synthesize it. In contrast, products from both precursors can be found in flavonoids. Pine species can produce the unusual flavonoids pinocembrin and pinobanksin, which are derived from CHS activity against cinnamoyl-CoA instead of 4-coumaroyl-CoA as a substrate, in addition to typical 4-coumaroyl-CoA-derived flavonoids (Fliegmann et al. 1992; Pietarinen et al. 2006). Pinocembrin exists in low concentrations in HW and knots, but typical flavonoids have been determined only in needles (Willför et al. 2003). Lignans, optically active dimers of monolignols, are not present in stem HW, but are present in knot HW and are induced by mechanical wounding in SW (Willför et al. 2003; Harju et al. 2009). Both lignin and lignans are derived from 4-coumaroyl-CoA, also in pine (Suzuki & Umezawa 2007). The major phenylpropanoid pathways in Scots pine are shown in Figure 2. HW also contains different fatty acids and sterols, which in contrast may reduce the durability of the wood serving as nutrients (Fries et al. 2000).

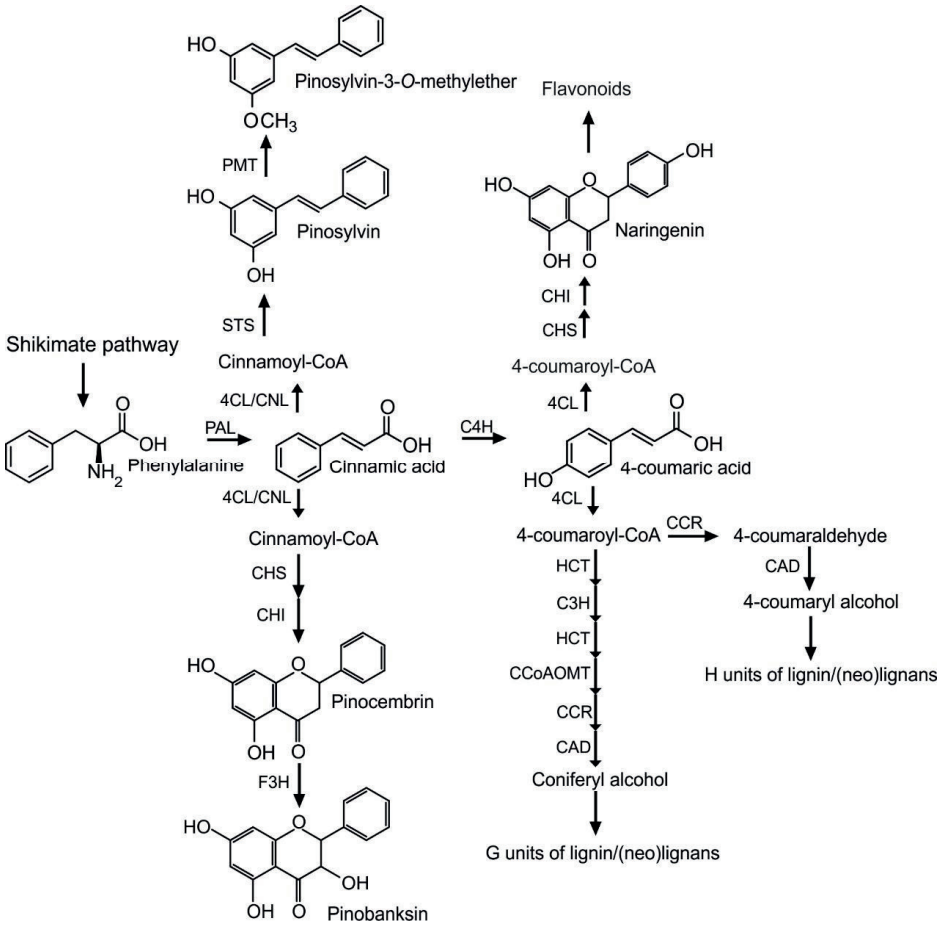

Figure 2. The phenylpropanoid pathway to flavonoids, lignin, and stilbenes in Scots pine. PMT, pinosylvin O-methyltransferase; CCoAOMT, caffeoyl-CoA O-methyltransferase; STS, stilbene synthase; 4CL, 4-coumaroyl-CoA ligase; PAL, phenylalanine ammonia lyase; CHI, chalcone isomerase; CHS, chalcone synthase; CCR, cinnamoyl-CoA reductase; HCT, hydroxycinnamoyl-CoA shikimate/quinate hydroxycinnamoyl transferase; CAD, cinnamoyl alcohol dehydrogenase; C3H, coumarate 3-hydroxylase; CNL, cinnamate ligase.

Several studies have shown the importance of extractives, mainly phenolic compounds and resin acids, for the durability of HW. The concentrations of the stilbenes PS and its monomethyl ether have strong negative correlation with the mass loss of HW caused by wood-destroying fungi (Harju & Venäläinen 2006; Leinonen et al. 2008). There is wide variation in the amount of stilbenes in the HW between individuals; the concentration of PS has especially high heritability (Harju & Venäläinen 2006; Partanen et al. 2011). Hence, it may be possible to breed for increasingly durable HW. The first annual rings of HW develop in Scots pine at ages of 15-20 years, while reliable

estimation of HW properties is possible only around the age of 30 years (Venäläinen 2002). To fulfill the aims of breeding, early selection of HW properties is essential. One interesting means to do this has arisen from the study of Harju et al. (2009), which showed a high offspring-parent heritability for PS. The induced stilbene production capacity in response to wounding of seedlings had a positive genetic correlation with the concentration of stilbenes in the HW of their mother trees. Since the developmentally regulated, constitutive stilbene biosynthesis seems to be correlated with the inducible biosynthesis, wounding of xylem was suggested as an early testing method for the selection of high producers for the breeding of more durable HW and, consequently, speeding up the breeding of HW properties (Harju et al. 2009). Furthermore, this suggests that breeding for more durable HW would simultaneously increase the active defense capacity of trees.

It has been proposed that HW extractives confer protection against fungi by various means. In addition to direct fungicidal activity, stilbenes are strong free-radical scavengers. The antioxidant properties of compounds are important, since many fungal pathogens use radicals to break down plant cell walls (Belt et al. 2017a; Schultz & Nicholas 2000). The stilbenes and resin acids also have water-repelling activity, which may have an important function in decreasing the water content in the HW and therefore hampering the metabolic functions of fungi (Eberhardt et al. 1994; Venäläinen et al. 2004). Stilbenes in pine HW consist of two major forms, PS and its monomethylated derivative, but minute amounts of its dimethylated derivative can also be found. More than half of the PS is methylated, but the effect of this modification in terms of activity against wood-destroying fungi has not been clearly shown. In general, methylation of hydroxyl groups affects the hydrophobic properties of the compound and changes its solubility (Lam et al. 2007). It has been suggested that PS is more toxic and a broad-spectrum fungicide. Pinosylvin monomethyl ether (PSME), in contrast, has more specific activity, while methylation increases the bioactivity of the stilbenes against brown-rot fungi (Schultz et al. 1990; Hart 1981). Synergistic effects of HW extractives are possible, but have not been studied.

1.3 Stilbenes as phytoalexins in active defense

Phytoalexins are defined as low-molecular-weight compounds that are synthesized and accumulate as a response to microorganisms or other elicitors at the site of the infection and are absent in uninduced tissues (Ebel 1986). Producing compounds only when needed is believed important for minimizing the costs of defense (Hartmann 2007). The first studies of active production of stilbenes in pine in response to stress were experiments showing strong induction of biosynthesis in response to UV-C stress in seedlings (Gehlert et al. 1990; Schoeppner & Kindl 1979; Preisig-Müller et al. 1999). Induced production of stilbenes as a response to wounding, fungal infection, and attack by wood wasps in the SW of adult trees, as well as in response to wounding of xylem in seedlings, has been described (Kemp & Burden 1986; Hillis & Inoue 1968; Jorgensen 1961; Kovalchuk et al. 2017; Harju et al. 2009). The wounded and infected area forms a 'reaction zone' where parenchyma cells lose their starch, die in advance of the fungal invasion, and the wood is impregnated with phytoalexins mimicking, in some aspects, the changes seen in HW formation (Kemp & Burden 1986; Shain & Hillis 1973). Pathogens also induce stilbene production in pine cell cultures and seedlings (Lange et al. 1994; Bonello et al. 1993; Kovalchuk et al. 2017), while ozone fumigation induced stilbene production, enzyme activity, and *STS* gene expression in pine seedlings (Zinser et al. 1998, 2000; Rosemann et al. 1991). Thus, many reactive oxygen species (ROS)-producing stress treatments induce stilbene biosynthesis both in needles and in the xylem. Studies undertaken before this thesis focused on following stilbene production or the kinetics of gene expression/enzymatic activity, and no large-scale transcriptomic analyses have been done.

1.4 Enzymatic steps in the stilbene biosynthesis pathway

1.4.1 Phenylalanine ammonia lyase

The pine stilbene pathway shares with the lignin and flavonoid pathways the first step in the core phenylpropanoid pathway, the conversion of phenylalanine to cinnamate by phenylalanine ammonia lyase (PAL). In jack pine (*Pinus banksiana*), PAL is encoded by 8-10 genes. The expressions of five genes (*pal1-pal5*) were studied in the xylem, needles, and cell cultures (Butland et al. 1998). *Pal1* was the only constitutively expressed gene in the cell cultures. All the others were expressed only after fungal

stimulus. All the genes, except *pal3*, were expressed in the developing xylem. This suggests that there is functional divergence between the PAL-encoding genes in jack pine. A gene identical to the constitutive jack pine's *pal1* was also isolated from the developing xylem of loblolly pine (*Pinus taeda* L.) and a similar gene from Scots pine megagametophytes (Butland et al. 1998; Dvornyk et al. 2002; Whetten & Sederoff 1992).

1.4.2 Activation of cinnamic acid

After the first step, the pine stilbene pathway diverges from the typical flavonoid and lignin pathways, since PS is formed by condensing cinnamoyl-CoA, instead of 4-coumaroyl-CoA, with malonyl-CoA. The enzyme responsible for this activity in pine has not been described, and 4CL (4-coumarate-CoA ligase) enzymes, in general, utilize cinnamic acid poorly as a substrate. There are, however, a few exceptions found in angiosperm species. Raspberry (*Rubus idaeus*) Ri4CL2 preferentially activates cinnamate over 4-coumarate, caffeate, ferulate, and sinapate (Kumar & Ellis 2003). Aaron's beard (*Hypericum calycinum*) and petunia (*Petunia hybrida*) have specific cinnamate-activating enzymes (CNLs) involved in the biosynthesis of benzoic acid and its derivatives. These enzymes have low sequence similarity with 4CLs, and they form a separate clade with other benzenoid-related CoA ligases (Klempien et al. 2012; Gaid et al. 2012).

Three 4CLs (4CL1, 4CL2, and 4CL3) have been characterized from loblolly pine. Two of the genes, *4CL1* and *4CL2*, had identical coding regions and were preferentially expressed in the xylem (Zhang & Chiang 1997; Chen et al. 2014; Voo et al. 1995). In different experimental systems, the enzymatic activities against different substrates showed varying results. In general, 4-coumaric acid, caffeic acid, and ferulic acid were favorable substrates, but utilization of cinnamic acid was either not detected or very low (Chen et al. 2014; Zhang & Chiang 1997). In other experiments, the activity could be measured, but the K_m for cinnamic acid was ten times higher than for other substrates (Voo et al. 1995). Downregulation of 4CL in Monterey pine (*Pinus radiata*), the enzyme corresponding to *4CL1* and *4CL2* in loblolly pine, reduced the lignin content, confirming the involvement of the enzyme in lignin biosynthesis (Wagner et al. 2009). *4CL3* from loblolly pine, in contrast, was expressed preferentially in the needles and roots, and no expression could be detected in the xylem (Chen et al. 2014).

Heterologous expression of the enzyme in poplar did not affect the amount of lignin. Instead, the amounts of 3-*O*-caffeoyl quinate and cinnamoyl-quinate increased, while the glucose esters of the hydroxycinnamates decreased. The accumulation of cinnamoyl-quinate suggested that the enzyme could utilize cinnamate to form cinnamoyl-CoA, which is then converted further to cinnamoyl-quinate, but the activity of the recombinant enzyme did not support this finding (Chen et al. 2014). Based on what is known about 4CL enzymes in pine, it is likely that the enzyme participating in stilbene biosynthesis in pine remains uncovered.

1.4.3 Stilbene synthase

STS is encoded by a small gene family in different *Pinus* species. Two genes have been described from eastern white pine (*Pinus strobus*) (Raiber et al. 1995), five genes (*PST1-PST5*) from Scots pine (Preisig-Müller et al. 1999) and three genes from Japanese red pine (*Pinus densiflora*) (Kodan et al. 2002). In Scots pine, different family members had very similar coding regions with an overall identity of over 95%, but the introns and, more importantly, the promoters varied substantially (Preisig-Müller et al. 1999). The promoter activities of P_{PST-1}, P_{PST-2}, and P_{PST-3} were tested in transgenic tobacco (*Nicotiana tabacum*) and in protoplasts, using the β-glucuronidase (GUS)-encoding gene *uidA* as a reporter. The various promoters were activated to different degrees in response to UV-C, fungal attack, and wounding, P_{PST-1} being the most active. They also showed differences in tissue specificities. P_{PST-1} and P_{PST-3} drove the reporter expression in the vascular bundles of tobacco, unlike P_{PST-2}.

Even with highly similar coding regions, the various STS enzymes showed different activities in *in vitro* experiments. In eastern white pine, a single amino-acid change dramatically affected the enzymatic activity and pH dependence (Raiber et al. 1995), while in Japanese red pine, a naturally occurring mutation in the enzyme-encoding gene led, through a frameshift, to a truncated enzyme that escaped feedback inhibition, had higher enzymatic activity, and produced more PS (Kodan et al. 2002).

1.4.4 Pinosylvin O-methyltransferase

A stress-inducible, S-adenosyl-l-methionine-dependent *O*-methyltransferase, PMT1, has been characterized from Scots pine. The gene expression of *PMT1* was induced in response to ozone in the needles and by fungal attack and wounding in the phloem (Chiron et al. 2000a, b). The enzymatic activity was characterized, using both purified

protein from the fungal-elicited cell cultures and recombinant protein produced in *Escherichia coli* (*E. coli*). The enzyme had broad substrate specificity. It methylated different stilbene aglycones, hydroxycinnamic acids, flavonoids, and caffeoyl-CoA, many of them with higher activity than PS. Chiron and colleagues suggested that this enzyme could have been responsible for PS methylation *in planta*, but in this thesis we have shown that PMT1 is not specific for the stilbene pathway in Scots pine, but that another *O*-methyltransferase has this function.

1.5 Regulation of the stilbene pathway

It is not known what factors control the stilbene pathway in pine. The pathway is transcriptionally regulated, but no specific transcription factors (TFs) have been identified so far. More is known about the stilbene pathway in grapevine (*Vitis vinifera*), which is activated by similar abiotic and biotic stress conditions as the pathway in pine. Two R2R3-type MYB family TFs, MYB14 and MYB15, have been experimentally proven to regulate *STS* genes in response to pathogen attack, UV-C stress, and during fruit ripening in grapevine (Höll et al., 2013). Other TFs from different families may also be involved, based on coexpression analysis (Wong et al. 2016). Furthermore, the stilbene pathway in grapevine is activated by inhibiting protein phosphatases and is regulated by the plant hormones ethylene and jasmonic acid (Belhadj et al. 2008; Tassoni et al. 2005). Analysis of the promoter of grapevine STS-encoding gene *Vst1* by a deletion series showed, additionally, that separate areas of the promoter are responsible for regulation of the gene expression in response to ozone fumigation, ethylene, and pathogen activities (Grimmig et al. 1997; Schubert et al. 1997).

1.6 Theories of heartwood formation

The tree trunk is roughly divided into bark, phloem, vascular cambium, and secondary xylem. In addition to mechanical support, the secondary xylem is responsible for transport and storage of water, storage of carbohydrates, and active defense against invading insects and pathogens (Spicer 2005). The secondary xylem and phloem are connected through radially oriented parenchyma cells. In most tree species, the amount of secondary xylem is controlled, and excess amounts of xylem are converted to HW by developmentally programmed SW senescence in a process called HW formation (Spicer 2005; Bergström 2003; Magel et al. 2001). Not all tree species, however, form HW, and these are called SW trees (Magel et al. 2001). During HW formation, SW goes

through chemical and anatomical changes in a narrow zone called the transition zone (TZ) that is located between the SW and HW (Bergström 2003).

The factors that trigger and regulate HW formation are unknown, although several hypotheses exist. The most crucial factor believed to control HW formation is the amount of SW needed to sustain foliar demands, the so-called 'pipe model theory'. The excess amounts of respiring SW are converted to HW to save resources. However, the experimental evidence to prove this is conflicting (Bergström et al. 2004; Beauchamp et al. 2013; Gartner et al. 2000; Taylor et al. 2002). The most accepted theory is that HW formation is an aging process, but in contrast to prior hypotheses in which ray parenchyma (RP) cells gradually lose their metabolic activity and die due to low oxygen content and high CO_2 levels, it is now understood as an active, controlled process of senescence (Spicer 2005; Spicer & Holbrook 2007).

Involvement of plant hormones controlling HW formation has been suggested. Since other aspects of wood formation are controlled by crosstalk of several hormones, it would not be surprising if secondary xylem senescence were under hormonal control as well. Vascular cambium activity is controlled by auxin, cytokinin, and ethylene, and xylem differentiation by auxin, cytokinin, brassinosteroids, and gibberellins (Ye & Zhong 2015). Localized and controlled conversion of SW into HW suggests that it is a highly regulated process, as are other aspects of wood formation. Very little, however, is known about the involvement of hormones in HW formation. Ethylene may play a role in HW formation. Ethylene treatment increased resin acid, PS, and its derivative production in the SW of Scots pine and Monterey pine (Nilsson et al. 2002; Shain & Hillis 1973), and ethylene production was higher in the TZ than in the outer SW during the dormant season in Monterey pine (Shain & Hillis 1973). Involvement of auxin has also been suggested, but there is no strong experimental evidence to support this (Shigo & Hillis 1973). One study, however, showed that the amount of indole-3-acetic acid (IAA) is lower or absent in the TZ than in the SW, and the amount of abscisic acid (ABA) is higher in the TZ than in the SW of Scots pine (Beauchamp 2011). The gibberellic acid GA_{24} was detected both in the SW and TZ together with ABA derivatives, but no differences in concentration between the TZ and SW were found.

The factors influencing HW formation may be a combination of many components. However, in general terms it is accepted that the initiation is genetically encoded, but can be influenced by the environment (Shigo & Hillis 1973; Taylor et al. 2002).

1.7 Chemical and cytological changes during heartwood formation

The biosynthesis of HW extractives is dependent on the activity of RP cells, which are the only living cells present in SW, in addition to the epithelial cells of the resin ducts, which are responsible for the biosynthesis of oleoresin. The RP are connected to each other and other cell types through the apoplast and the symplast, forming a continuum of cells from the xylem to the phloem (Spicer 2014). The chemical composition of wood and the cytological features of RP have been studied in Scots pine and other conifer species when transformation from SW to HW occurs (Bergström 2003; Nakaba et al. 2006, 2008).

In Scots pine, chemical and cytological changes occur within a single growth ring. In other pine species, extractive accumulation is made visible, using the polyphenol stain potassium permanganate in a wider area of two or three growth rings before the cell-death zone (Nakaba et al. 2008). Chemical changes are initiated earlier than structural changes (Bergström 2003). The decrease in moisture content and pit aspiration occurs early in the process of HW formation, but the exact timing in relation to extractive biosynthesis is unclear. In fully developed HW, the pits are encrusted with HW extractives.

Stilbenes are not present in unwounded SW, and their concentration abruptly increases in the TZ. Later, the concentration decreases somewhat when the HW ages and is therefore higher in the outer than inner HW. Stilbenes in Scots pine have been localized in the cell wall, middle lamella, and lumina of cells and may be bound to lignin (Belt et al. 2017b). Resin acids are already synthesized in the SW, but the amount gradually increases in the TZ, and the resin acid content in the HW is higher than in the SW. Unlike stilbenes, resin acids are not found in the cell wall, but only in the lumina of cells (Belt et al. 2017b). The amount of free fatty acids is higher in the TZ and HW than in the SW, but triglycerides show a contrasting trend (Zheng et al. 2014; Bergström 2003)

The middle ray cells without contact with ray tracheids (RTs) are responsible for extractive biosynthesis. They do not have lignified cell walls in the SW; only the compound middle lamella (CML) is present. Lignification begins in the middle of the TZ (Bergström 2003) and is completed in the HW, where the cell walls are completely lignified (Zheng et al. 2014). However, the structure of the fully lignified cell wall of rays is simpler, consisting only of the CML and the S_1 layer, while the S_2 and S_3 layers are missing (Zheng et al. 2014). The data by Zheng et al. (2014) suggest that lignification begins in the TZ and is completed in the HW. Lignification can progress even after programmed cell death (PCD), based on the presence of monolignols and oxidizing enzymes secreted by cells before their death and provided by neighboring cells (Pesquet et al. 2013). The biosynthesis of HW extractives and initiation of lignification is accompanied by the disappearance of storage carbohydrates. The concentration of triglycerides also decreases, and it is believed that both are used for the biosynthesis of extractives (Bergström 2003).

Cytological studies have shown that in the final stages of HW formation, the nuclei of the RP cells in conifers begin to disappear, and the cells die abruptly within a single growth ring, but the distance from the cambium varies between species, reflecting the fact that the initiation of HW formation in different species varies (Bergström 2003; Nakaba et al. 2006, 2008). The chemical and cytological changes are shown schematically in Figure 3.

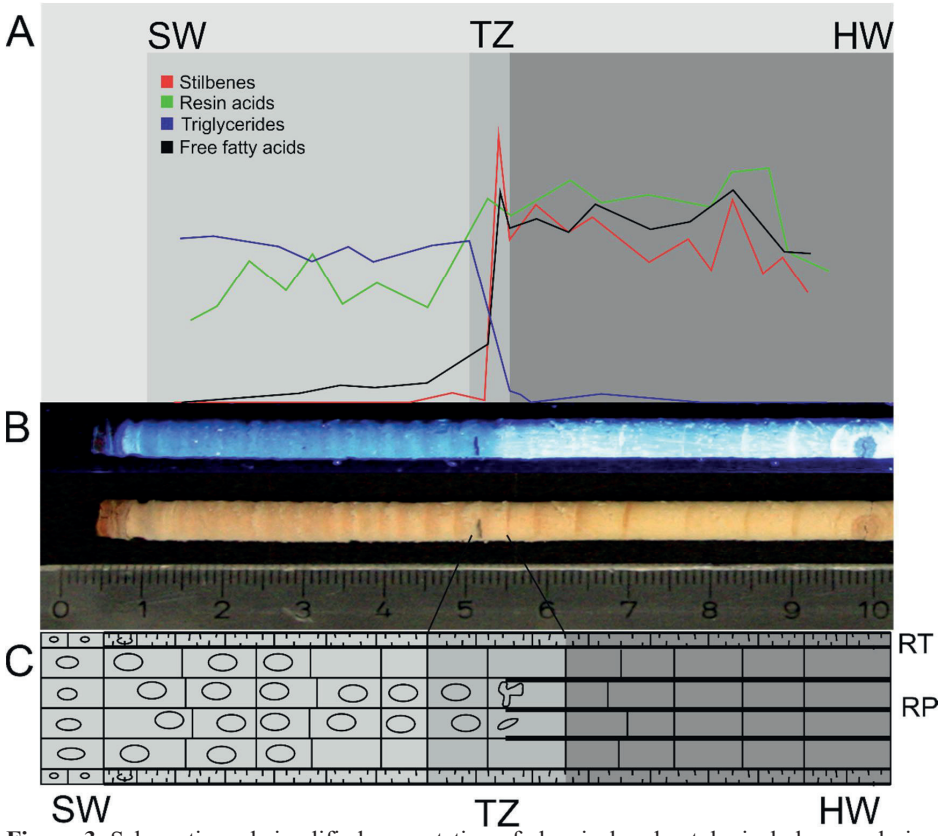

Figure 3. Schematic and simplified presentation of chemical and cytological changes during heartwood formation in pines. The figure is redrawn based on figures and data in Bergström (2003), Nakaba et al. (2008), and Zheng et al. (2014). **A:** Accumulation of heartwood extractives; Pinosylvins appear abruptly in the middle of the transition zone (TZ), resin acids are synthesized already in the sapwood (SW). The amount of free fatty acids is higher in the heartwood (HW) than in the SW, but the triglycerides show a contrasting trend (Bergström 2003; Zheng et al. 2014). **B:** Increment cores. Pinosylvins fluoresce under UV light. The border between the wet and dry zones is marked with a pencil. **C:** Nuclei begin to disappear in the TZ from the middle cells responsible for extractive biosynthesis, and the cell walls lignify (marked with thicker line). The ellipses represent normal nuclei and the other shapes degrading nuclei. The ray parenchyma (RP) cells in contact with the ray tracheids (RTs) do not synthesize extractives and lose their nuclei earlier. In the pine HW, no living cells are present (Bergström 2003; Nakaba et al. 2008).

2 AIMS OF THE STUDY

Previous studies have shown that stilbenes improve the decay resistance characteristics of HW. The concentrations of stilbenes have high heritability, and there is wide variation in these concentrations among trees, which suggests an opportunity to breed for more decay-resistant HW. Our ultimate aim is to understand what causes these genetic differences in stilbene biosynthesis capacity and to determine the means for early selection, utilizing genetic markers or chemical testing of seedlings.

The specific aims of this thesis were the following:

1) Practically nothing is known about the regulation of the stilbene pathway in pine, except that it is transcriptionally regulated. Our first aim was to characterize the transcriptomic responses to known stilbene-inducing conditions (wounding, UV-C, and HW formation) in pine. The data generated could then be used to answer general questions, such as what occurs at the transcriptional level during HW formation and how pines respond to UV-C treatment, as well as to more specific questions, such as which regulators are coexpressed with STS and may be important for controlling the pathway (I, II).

2) Utilize the transcriptomic data to determine the missing enzymes and possible common regulators of the pathway (II, III).

3 MATERIALS AND METHODS

The materials and methods used in this thesis are described in detail in the original publications I, II, and III, as indicated in Table 1.

Table 1 Methods used in publications I, II, and III. Methods that were conducted only by the coauthors are indicated with an asterisk (*).

Method	Publication
Total RNA extraction and first strand cDNA synthesis	**I, II, III**
Semiquantitative RT-PCR	**I, II**
Real-time quantitative (q)PCR	**I, III**
Chemical treatments of seedlings	**II**
Primer design	**I, II, III**
Isolation and cloning of enzyme-coding cDNA molecules	**III**
Protein expression, purification, western blot, and enzymatic assays	**III**
HPLC analyses	**III**
Sequence analysis and alignment	**III**
Phylogenetic analysis	**III**
UPLC-MS/MS analyses *	**III**
TLC analysis	**II**
Pearson correlation analysis	**I, III**
Mapping of RNA-seq reads to *Pinus* EST collection *	**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**
Bayesian hierarchical clustering analysis *	**II**

4 RESULTS AND DISCUSSION

4.1 Expression of secondary metabolite pathways during heartwood formation

One fundamental change that occurs when SW is converted to HW is the biosynthesis of extractives that accumulate in the HW. The process of extractive biosynthesis during HW formation is dependent on the activity of long-lived RP cells, in addition to the epithelial cells of the resin ducts.

The previously described chemical, cytological, and structural changes were strongly supported by our transcriptomics data and aided in the initial localization of the TZ. The transcriptional data were mapped against a pine expressed sequence tag (EST) collection that consisted of tentative consensus (TC) sequences. TC is a contig formed by assembling sequences from different pine species. Some of the TCs originated from single species and some were combinations of many, which means that different TCs do not represent different genes in Scots pine.

The TZ is described to have a lower moisture content than the SW, and this was utilized to localize the TZ from the increment core samples, together with stilbene fluorescence under UV light. The expression of STS was strongest in the outermost dry zone, and inwards to the HW from that region no RNA could be extracted. Some *STS* expression was seen on the SW side in the wet area. In general, *STS* expression was restricted to an area of one or two growth rings (I).

Extractives in different species form either *in situ* in the TZ (Type I), or the precursors accumulate in aging SW and are then transported to the TZ, where they are oxidized and/or hydrolyzed into extractives (type II) (Magel 2000). The extractives are biosynthesized from both local storage carbohydrates and triglycerides, in addition to transported carbohydrates from the outer SW and phloem (Magel 2000). The expression of sucrose synthase, enzyme involved in glycolysis, gluconeogenesis, and the oxidative pentose phosphate pathway was upregulated in the TZ, which supports the hypothesis that carbohydrates and triglycerides are utilized for building the backbones of HW extractives. In pine, extractives consist mostly of stilbenes (PS and PSME), resin acids, and free fatty acids. The increasing concentration of stilbenes in the TZ was supported by strong activation of the expression of both the stilbene pathway- and the shikimate

pathway-encoding genes in the TZ, compared with the SW. This confirms that, with respect to stilbene biosynthesis, HW formation is type I in Scots pine and is restricted to a relatively narrow area of one or two growth rings, as described in other studies (Bergström 2003).

An unexpected finding was the lack of induction of resin acid biosynthesis genes in the TZ, since resin acid concentrations increase in the TZ, as do other extractives, and are higher in the HW than in the SW (Bergström 2003; Venäläinen et al. 2003; Zheng et al. 2014). During our sampling time in June, the expression of resin acid biosynthesis genes was low in both the SW and TZ (I). In fact, the year-round experiment showed that the expression of these genes peaked much earlier, already in April, and were then downregulated. But even then, the genes expressed were five times higher in the SW than in the TZ. This suggests that resin acids are mostly produced in the SW and then loaded into the TZ from the outside SW. With respect to resin acid accumulation, Scots pine HW formation more resembles type II (I). However, the expression of resin acid biosynthesis genes was also induced slightly in the spring in the TZ, so it cannot be ruled out that some of the resin acids may have been synthesized in the TZ.

From the year-round expression profiles, we concluded that resin acid biosynthesis occurs during spring and stilbene biosynthesis in the summer and autumn. In the winter months, even when the temperature was well below zero, the transcripts of the stilbene pathway strongly accumulated. This is in some sense paradoxical, but the same phenomenon has been described in the needles of Douglas-fir (*Pseudotsuga menziesii*) and in the xylem of Norway spruce (*Picea abies*). In Douglas-fir, thousands of genes were expressed at the highest levels during winter dormancy (Cronn et al. 2017), while in Norway spruce the lignin pathway genes peaked in the winter when the temperature was lowest during the measuring time (Soile Jokipii-Lukkari, personal communication, July 17, 2017).

Pines are capable of synthesizing the unusual flavonoids pinocembrin and pinobanksin, in addition to the archetypical flavonoids, but flavonoids are not often described as major constituents of HW extractives in pine species. However, at least pinocembrin was present at low concentrations in the HW and knots of Scots pine (Willför et al. 2003). In the transcriptomics data, CHS was expressed at 2-fold higher levels in the TZ than in the SW and chalcone isomerase (CHI) was preferentially expressed in the TZ at

5-6-fold higher expression levels than in the SW. At the final stages of HW formation, lignification of the RP cells occurred in the TZ, and the transcription of the lignin biosynthesis pathway was concurrently activated (I).

4.2 Programmed cell death and timing of heartwood formation

Cytological studies have shown clearly that in the last phases of HW formation, the nuclei disappear and the parenchyma cells die. Bifunctional nuclease (BFN), an enzyme involved in degradation of DNA and RNA during the late stages of senescence and various PCD processes, was expressed exclusively in the TZ (I). PCD is divided into environmentally induced PCD (ePCD), which is triggered by external abiotic and biotic signals, and developmentally induced PCD (dPCD), which is induced by internal factors (Pérez-Amador et al. 2000; Farage-Barhom et al. 2008). *BFN* expression has only been detected in dPCD processes and is considered a marker for dPCD (Olvera-Carrillo et al. 2015). The activation of BFN expression strongly indicates that HW formation truly is a dPCD process that is regulated by internal factors, instead of being a stress response to drying, which has been suggested as an initiation factor for HW formation (Shigo & Hillis 1973). BFN expression was not induced in response to UV-C treatment, which is a trigger for ePCD (II). Following the BFN expression throughout the year showed that the occurrence of PCD is highest in the autumn and that BFN expression ceases after October, marking the end of HW formation (I).

4.3 Expression of secondary metabolite pathways in response to UV-C stress induction

Stilbene biosynthesis is strongly induced in pines and other species as a response to UV-C-induced stress. Whole-transcriptome-level responses have been studied in grapevine but not in any coniferous species. RNA sequencing (RNA-seq) data confirmed the activation of *STS*-encoding genes six hours after the onset of the UV-C treatment. Other enzymes involved specifically in the stilbene pathway of pine have not yet been characterized, but several *PAL*- and *4CL*-encoding TCs were also upregulated. The *O*-methyltransferase PMT1 (Chiron et al. 2000a), with PS-methylating activity, was not differentially expressed, but some other *O*-methyltransferases showed an inducible expression pattern (II).

Resin acid biosynthesis occurs through the chloroplastic 2-C-methyl-D-erythritol 4-phosphate (MEP) pathway, leading to mono- and diterpenes, and the cytosolic

mevalonic acid (MVA) pathway, leading to sesquiterpenes and triterpenes (Figure 4). The core MEP pathway genes together with specific mono- (MTS)- and diterpene synthase (DTS)- encoding genes were downregulated by UV-C in the 24-hour timeframe, as were many other genes involved in processes in chloroplasts (II). This may have occurred due to the damage to and malfunction of chloroplasts caused by a UV-C-induced ROS burst. On the other hand, rapid accumulation of the resin acids is not needed, since the first layer of defense is obtained by preformed compounds stored in the needles. One interesting finding was, however, that the gene encoding the sesquiterpene α-farnesene synthase, an enzyme functioning downstream of the cytoplasmic MVA pathway, was highly upregulated in response to UV-C treatment, together with genes encoding core MVA pathway enzymes. The importance of α-farnesene is not well defined, but it may have a signaling function in plant-insect interactions, which again may explain its rapid expression.

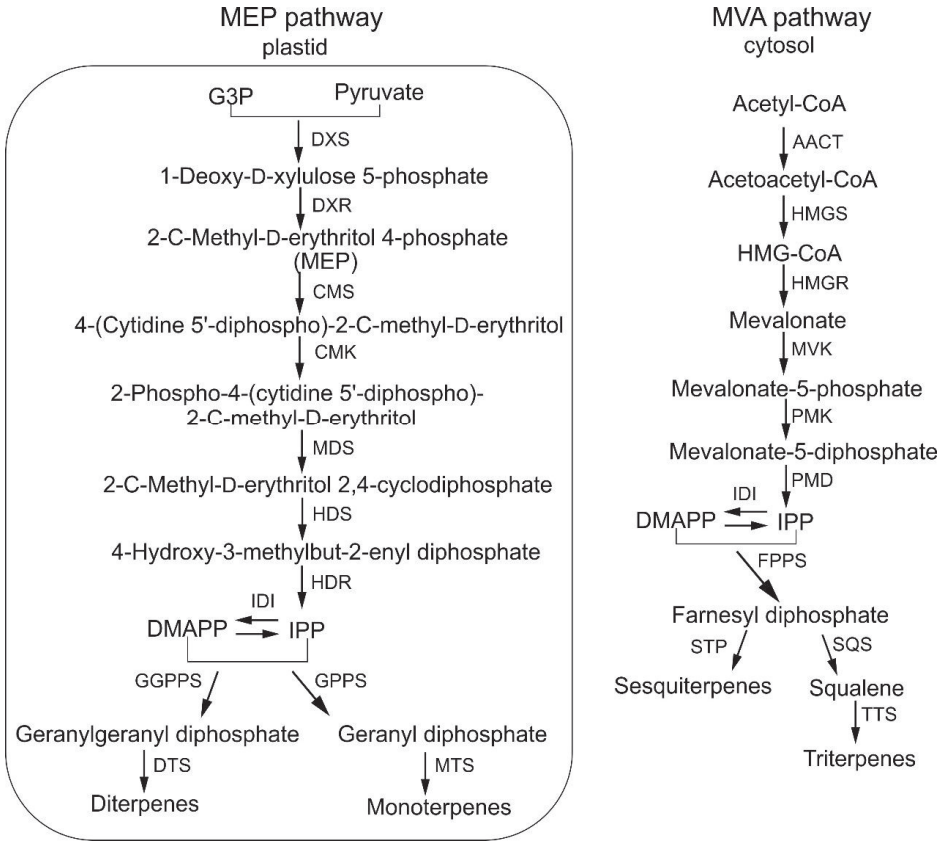

Figure 4. Biosynthesis of terpenoids through plastidic MEP (2-C-methyl-D-erythritol 4-phosphate) and cytosolic MVA (mevalonic acid) pathways. DXS, 1-deoxy-D-xylulose 5-phosphate synthase; DXR, 1-deoxy-D-xylulose 5-phosphate reductoisomerase; CMS, 4-diphosphocytidyl-2-C-methyl-D-erythritol synthase; CMK, 4-diphosphocytidyl-2-C-methyl-D-erythritol kinase; MDS, 2-C-methyl-D-erythritol 2,4-cyclodiphosphate synthase; HDS, (E)-1-hydroxy-2-methyl-but-2-enyl-4-diphosphate synthase; HDR, 1-hydroxy-2-methyl-2-(E)-butenyl 4-diphosphate reductase; IPP, isopentenyl pyrophosphate; DMAPP, dimethylallyl pyrophosphate; IDI, IPP/DMAPP isomerase; GGPPS, geranylgeranyl pyrophosphate synthase; GPPS, geranyl pyrophosphate synthase; DTS, diterpene synthase; MTS, monoterpene synthase; AACT, acetoacetyl-CoA thiolase; HMGS, 3-hydroxy-3-methylglutaryl-CoA synthase; HMGR, 3-hydroxy-3-methylglutaryl-CoA reductase; MVK, MVA kinase; PMK, phosphomevalonate kinase; PMD, MVA diphosphate decarboxylase; FPPS, farnesyl pyrophosphate synthase; STP, sesquiterpene synthase; SQS, squalene synthase; TTS, triterpene synthase. The figure is redrawn and simplified from Moses et al. (2013).

The flavonoid pathway genes were upregulated only mildly as a response to UV-C treatment. Only the branch leading to proanthocyanidins showed a slightly higher induction. In grapevine the induction of the stilbene pathway concurrently downregulates *CHS* and the flavonoid pathway genes (Vannozzi et al. 2012). At the biochemical level, in pine PS is an efficient inhibitor of CHS activity (Kodan et al.

2002). The biosynthesis of stilbenes is seemingly preferred at the expense of the flavonoid pathway. Suppression of the flavonoid pathway may be an important mechanism for sustaining sufficient substrate for stilbene biosynthesis, since the pathways utilize the same precursors. The genes involved in monolignol biosynthesis were also slightly upregulated, and it is known that lignin is produced as a response to ROS-generating conditions (Barros et al. 2015).

4.4 Transcriptional regulation of the stilbene pathway

There is wide genetic variation in the concentration of stilbenes in the HW of pine between individuals. One important aspect that may induce this variation is differences in regulation of the pathway. The regulators are still unknown and of the utmost importance to find. Based on *in silico* promoter analysis, the *STS* promoter has putative regulatory motives that can bind TFs from multiple families, such as the NAC, MYB, WRKY, basic leucine zipper (bZIP), basic helix-loop-helix (bHLH), apetala2/ethylene response factor (AP2/ERF), homeodomain, and GRAS families (II). The abundance of different sites binding the various families of TFs may reflect the fact that multiple abiotic and biotic stressors in addition to developmental signals activate the stilbene pathway.

It is not known if the same regulators are responsible for stilbene pathway activation in both environmental and developmental situations. Comparison of the TFs commonly expressed both in response to UV-C stress and in the TZ did not clearly reveal candidates involved in stilbene pathway regulation. Two possible candidates for stilbene pathway regulation in the TZ (coexpressed with *STS* in the TZ) (I) were not induced in response to UV-C treatment and TFs coexpressed with *STS* in UV-C-treated needles were not expressed in the TZ (II). It seems likely that a different set of regulators is induced in response to stress and HW development, and from both conditions several good candidates were chosen for future investigation.

Additional complications in the identification of possible regulator(s) utilizing coexpression analysis may arise from the result that stilbene pathway enzymes are, as we described in publication (II), primary response genes. This suggests that at least part of the regulation occurs through preexisting TFs (II). Pretreatment of plants with the inhibitors of translation cycloheximide (CHX) and anicomycin did not prevent activation of *STS* gene expression by UV-C (II). In fact, inhibiting translation without

UV-C treatment was sufficient to initiate transcription. This is a common phenomenon in regulation of plant hormone-responsive genes. Activating TFs are counteracted by labile repressors, and when these repressors cannot be replaced under CHX treatment, this leads to activation of transcription. The transcription of stilbene pathway genes may thus be regulated by both activating and repressive TFs.

4.5 Chemical genetics and hormonal regulation of the stilbene pathway

Determining the signaling pathways and mechanisms of gene regulation in Scots pine is hampered by the fact that transgenic approaches and mutant analysis are not currently feasible. However, chemical biology is increasingly valued as an approach to study the complex pathways in plants and to avoid the common problems of redundancy (Dejonghe & Russinova 2017). Chemical genetics could be used more in conifers to dissect the mechanisms of complex regulation of secondary metabolism pathways. The expression of *STS* is activated fast after UV-C induction and is not dependent on translation of transcriptional regulators (II). Protein phosphorylation is an important means of activating preexisting TFs in mammals. We tested how perturbation of the phosphorylation status of cells affects the activation of the stilbene pathway and found that inhibition of protein phosphatases chemically induces the transcription of *STS*, similar to what is seen in grapevine (II).

Hormonal regulation of the stilbene pathway, especially the involvement of ethylene during HW formation, has been discussed. Ethylene treatment of SW caused the induction of HW extractive production, and ethylene is formed in the TZ during dormancy (Nilsson et al. 2002; Shain & Hillis 1973). We observed the expression of the ethylene biosynthesis gene 1-aminocyclopropane-1-carboxylate oxidase upregulated more highly in the TZ than in the SW (I), but following the expression of the transcript did not support the involvement of ethylene in HW formation. When we treated pine seedlings with the ethylene precursor 1-aminocyclopropane-1-carboxylic acid (ACC), expression of *STS* was induced erratically (II). However, combination of ACC with jasmonic acid caused consistently strong activation of *STS*.

4.6 Characterization of stilbene-specific *O*-methyltransferase

The expression of the *STS*-encoding gene was consistently activated as a response to UV-C and wounding stresses and in the TZ during HW formation. To our surprise, the previously published gene *PMT1* (Chiron et al. 2000a), encoding the enzyme suggested

to catalyze the last step of the stilbene biosynthesis pathway, conversion of PS into PSME, showed no similar expression profiles. The gene was poorly expressed in the TZ, was not induced in response to wounding of xylem or UV-C treatment of the needles, and was constantly expressed primarily in the xylem (I, II, III).

Our RNA-seq data did not support the involvement of PMT1 in the methylation of PS, although the enzyme displayed such enzymatic activity *in vitro*. Correlation analysis of genes coexpressed with *STS* through all of our data uncovered a new *O*-methyltransferase-encoding gene, *PMT2*. This gene was strongly upregulated with STS under all of the experimental conditions examined. PMT2 was produced in *E. coli* as a histidine (His)-tagged recombinant protein and showed high specificity for methylating PS and structurally similar compounds (resveratrol and piceatannol) in *in vitro* enzymatic assays (III). PMT1, in contrast, showed broader substrate specificity, methylating many compounds such as flavonoids, stilbenes, and hydroxycinnamic acids and many of these more efficiently than PS. In addition, PMT2 had higher catalytic efficiency towards PS than did PMT1. PMT2 is encoded by three or four genes in the genome, based on intron sequences, and some of the gene copies were expressed preferentially as a response to UV-C treatment and some in the TZ (III).

4.7 Candidates for cinnamate-activating-CoA ligase

One important enzyme is still missing from the stilbene pathway, the enzyme that converts cinnamic acid to cinnamoyl-CoA. In some angiosperm species, this activity is attributed to cinnamate ligase (CNL) enzymes and in raspberry a 4CL enzyme variant is able to utilize cinnamic acid efficiently as a substrate. In general, cinnamic acid is a poor substrate for 4CL enzymes. The loblolly pine enzyme Pt4CL3 has been suggested to activate cinnamate *in vivo*, even when it did not use the substrate *in vitro*, based on its ability to form cinnamoyl-CoA-derived compounds when expressed heterologously in poplar (Chen et al. 2014). The TC in our collection corresponding to Pt4CL3 was not induced in response to UV-C, wounding, or CHX treatments, nor was it differentially expressed in the TZ, compared with the SW. Coexpression analysis with *STS* did not clearly reveal a candidate for this activity. Various 4CL enzymes were coexpressed in response to different treatments. However, one 4CL enzyme-encoding gene, similar to Pt4CL3, was induced in the UV-C and CHX treatments and was also present in the TZ, but was missing from the wounding libraries. When the expression of this gene was

followed throughout the year, it showed high correlation with the expression profile of STS and PMT2 (I, II). This enzyme may be a good candidate for cinnamate-activating-CoA ligase functioning in the stilbene pathway of pine. Nevertheless, additional candidates, similar to the CNL enzymes, should be searched for in the transcriptomic data.

4.8 Is there a division of labor between pine stilbene synthase pathway genes?

STS in Scots pine is encoded by at least five genes, but it is not known whether these genes function redundantly, have tissue specificity, or respond to different stimuli. The coding areas of the genes are very similar, but the promoters are different. In our RNA-seq data, the same set of TCs was induced in the TZ from adult trees and UV-C-treated needles. This suggests that the same genes are responsible for stilbene biosynthesis under different conditions. This was also the case in PMT2. However, the resolution of the mapping likely could not distinguish the extremely similar paralogs. In fact, more careful observation of the differences in the 3' untranslated regions (UTRs) and the expression of the different variants of the *PMT2* gene revealed some indication that there were preferences among the variants in their responses to different conditions (III). The resolution of this analysis was rather poor and should be verified by more rigorous experiments. Cloning of the complete *STS* and *PMT2* genes from all family members together with the 5' and 3' UTRs may furnish enough resolution to determine the divergence for different induction conditions.

Different phenylpropanoid pathway branches, however, do not apparently share common isoforms of the enzymes. In some angiosperm species, different 4CL isoforms functioned in branches leading to lignin and nonlignin metabolites, but also showed overlapping functions (Hu et al. 1998; Li et al. 2015). The PAL-encoding genes in pine showed differences in expression after fungal induction and in tissue specificities (Butland et al. 1998). The lignin, flavonoid, and stilbene pathway PAL and 4CL genes are likely not shared, which is also reflected in the very different expression profiles of different TCs. For instance, the strongest UV-C-responsive PAL TCs were not expressed in the TZ at all. The PAL- encoding TCs that were induced most strongly in the TZ showed a 2-fold induction in the UV-C samples and were very similar to the putative lignin pathway genes. The same applies to the 4CL-encoding TCs. Two TCs

were shared between the UV-C samples and the TZ, but most appeared either in the samples or in the TZ (I, II).

5 CONCLUSIONS AND FUTURE PROSPECTS

This thesis focused on the crucial secondary metabolism pathway of stilbenes in Scots pine. We studied whole-transcriptome-level responses to known stresses and the developmental cues inducing the pathway and shed new light on the poorly understood processes of HW formation. The most important individual finding was the identification of the true *O*-methyltransferase enzyme, PMT2, responsible for the last step of the stilbene pathway: methylation of PS to PSME. We were also able to show that a marker specific for developmentally induced PCD, BFN, was exclusively expressed in the TZ, which indicates that HW formation is truly induced by internal factors. With this marker, we were also able to pinpoint the timing of HW formation in Scots pine from spring until the end of late autumn. However, results from the year-round expression profiles are approximate because of the small number of replicates and high variation between the individuals.

Transcriptomic analysis revealed several possible candidates for stilbene pathway regulation in pine. The next step is the functional analysis of these candidates and dissection of the crucial binding areas for the regulators. In addition, we need to resolve whether all the *STS*-encoding genes in the pine genome are equally important for the synthesis of PS in the TZ, or whether there is a division of labor between the paralogs. Knowing the genes responsible for the biosynthesis and their regulatory areas would enable any polymorphism in these to be studied in high- and low- stilbene-producing individuals to correlate their differences in PS-producing capacity. Regulation of *STS* genes is interesting as such, because pine *STS* genes retain their stress inducibility when transformed into other plant species that do not normally synthesize stilbenes (Preisig-Müller et al. 1999; own unpublished data). This indicates that *STS* regulation occurs via ancient stress-response pathways that are shared between angiosperms and gymnosperms.

We could not demonstrate involvement of plant hormone signaling in the initiation of HW formation. More defined dissection of the intermediate area, combined with RNA-seq and hormonal analysis, may furnish answers to this and other intriguing questions,

such as what the mechanism is that restricts the TZ in the area of one or two growth rings, even though the continuum of cells reaches all the way to the phloem. Combining chemical, anatomical, transcriptomics and proteomics studies from same set of samples from sapwood via transition zone to heartwood would give more comprehensive view on HW formation.

In addition to molecular markers, a simple chemical screening method may be a way to hasten breeding of HW durability. This possibility arises from studies described previously, in which wounding-induced stilbene production correlated with the amount of stilbenes the plant was able to produce in its HW as an adult tree. However, wounding of the xylem is quite a destructive method. Another possibility may be to test whether UV-C-induced stilbene production in the needles could be utilized instead of estimating the stilbene production capacity.

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