Swedish University of Agricultural Sciences

Comparative analyses of Defense gene expression in Norway spruce sapwood

Sophie Rommel

Faculty of Natural Resource and Agricultural Sciences

Department of Forest Mycology and Plant Pathology

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Photograph: Carl Gunnar Fossdal

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Sophie Rommel

Supervisors: Docent Malin Elfstrand, Department of Forest Mycology and Plant Pathology
Dr. Jonàs Oliva, Department of Forest Mycology and Plant Pathology
Miguel Nemesio Gorriz, Department of Forest Mycology and Plant Pathology

Examinator: Docent Åke Olson, Department of Forest Mycology and Plant Pathology

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Sveriges lantbruksuniversitet
Swedish university of Agricultural Sciences
Faculty of Natural Resources and Agricultural Sciences
Department of Forest Mycology and Plant Pathology

1 Abstract

The fungus Heterobasidion annosum (Fr.) Bref. sensu lato is the most serious pathogen of Norway spruce [Picea abies (L.) Karst.] in the Northern hemisphere. It is the cause of extensive root and butt rot and leading to extensive yield losses, due to high storm breakage rate and reduction of timber quality. Efforts in studying the defense response of P. abies to this necrotrophic fungus have revealed the formation of the reaction zone in both roots and stems at the border of rot column and none-rotted sapwood due encounters with H. annosum s. l.. Also chemical and transcriptional responses, which were characterized as mainly unspecific responses in pathogen defense, were found upon H. annosum s. l. challenge. However, little is known about the association of those molecular defense mechanisms and the reaction zone formation. Thus this project analyzed the molecular response of P. abies towards H. annosum s. l. attack in tissue in proximate contact and distal from the reaction zone in two different materials, a controlled, long-term inoculation experiment and naturally infected trees. Gene expression of secondary metabolite and hormonal synthesis associated genes, as well as genes activated by jasmonic acid (JA) signal transduction were quantified using qPCR analysis. An up-regulation of defense responses, mainly the phenylpropanoid pathway and JA and ethylene (ET) synthesis at the inoculation site, was found when inoculation and wound treatment were compared. Brought in context with studies of the Norway spruce defense mechanism after bark inoculation, our data suggests that the defense mechanism is induced due to direct fungal contact and irrespective of the tissue type. However, a role of the reaction zone in triggering defense responses in the tree is hypothesized. The results indicate that carbon might be allocated from neighboring tissues to support defense response and phenolic enrichment in sapwood bordering the reaction zone.

2 Introduction

The basidiomycete Heterobasidion annosum (Fr.) Bref. sensu lato is one of the most widely spread fungal tree pathogens causing root and butt rot disease. Distribution of this fungus is documented from forests in north Russia, North America, as well as Europe, but also North Africa and Mesoamerica (reviewed by Korhonen and Stenlid, 1998). Interestingly, H. annosum s. l. is a species complex including five different species, which earliest divergence into two species clades was estimated to be between 75 to 85 million years ago (Dalman et al., 2010). Europe harbors three of the five species, namely Heterobasidion annosum sensu strict, belonging to the first clade and Heterobasidion parviporum and Heterobasidion abietinum, belonging to the second clade (Dalman et al., 2010; Niemelä and Korhonen, 1998). All three species mostly occur on conifers, but show different host preferences, even though Dalman and her colleagues (2010) could show that conifer radiation preceded fungal divergence. While H. annosum s. s. mainly infects pine, fir is infected by H. abietinum and spruce species get readily colonized by H. parviporum (Niemelä and Korhonen, 1998). Norway spruce [Picea abies (L.) Karst.], a highly ecological and economically important spruce species in Scandinavia, is highly susceptible H. annosum s. l. and infection leads to extensive losses in the forestry sector.

2.1 Heterobasidion annosum infection of Picea abies - Infection, spread and consequences

Many studies showed that H. annosum s. l. is capable to infect its hosts through wounds in stems and roots or by root-to root contact after infection of cuttings or stumps (reviewed by Stenlid and Redfern, 1998). In P. abies primary infection via stumps or roots in broken or wounded conditions were recorded in several studies (Dimitri, 1969; Schönhar, 1988, Swedjemark and Stenlid, 1993). In accordance to many infection studies basidiospores are considered to be the primary source of infection, although conidiospore infections, even though not found in nature, are also possible (reviewed by Redfern and Stenlid, 1998).

Spores are capable of infection when environmental conditions allow dispersal and reasonably long survival of the spores to germinate (reviewed by Woodward et al., 1998). The colonisation of P. abies was observed to occur preferably in heartwood (Korhonen and Stenlid, 1998), which H. annosum s. l. is capable to colonize due to its saprophytic ability. However, besides the spread of H. annosum s. l. through the heartwood, the basidiomycete also slowly expands into the sapwood of the tree.

Because of H. annosum s. l. prevalently growing in heartwood of Picea species butt rot mostly does not occur before tree ages between 25 to 40 years (Korhonen and Stenlid, 1998). Thus dying of infected individuals of P. abies directly due to H. annosum s. l. does not occur often (Bendz-Hellgren et al., 1998), but infection leads to a slower secondary growth of the trees (Bendz-Hellgren and Stenlid, 1995; Oliva et al., 2010). Besides decay of stems and secondary growth reduction, root rot enhances the risk of windthrown in these trees during storms (Oliva et al., 2008), demonstrating that H. annosum s. l. causes great damage to Norway spruce stands and the forest industry.

2.2 Defense mechanism in Picea abies

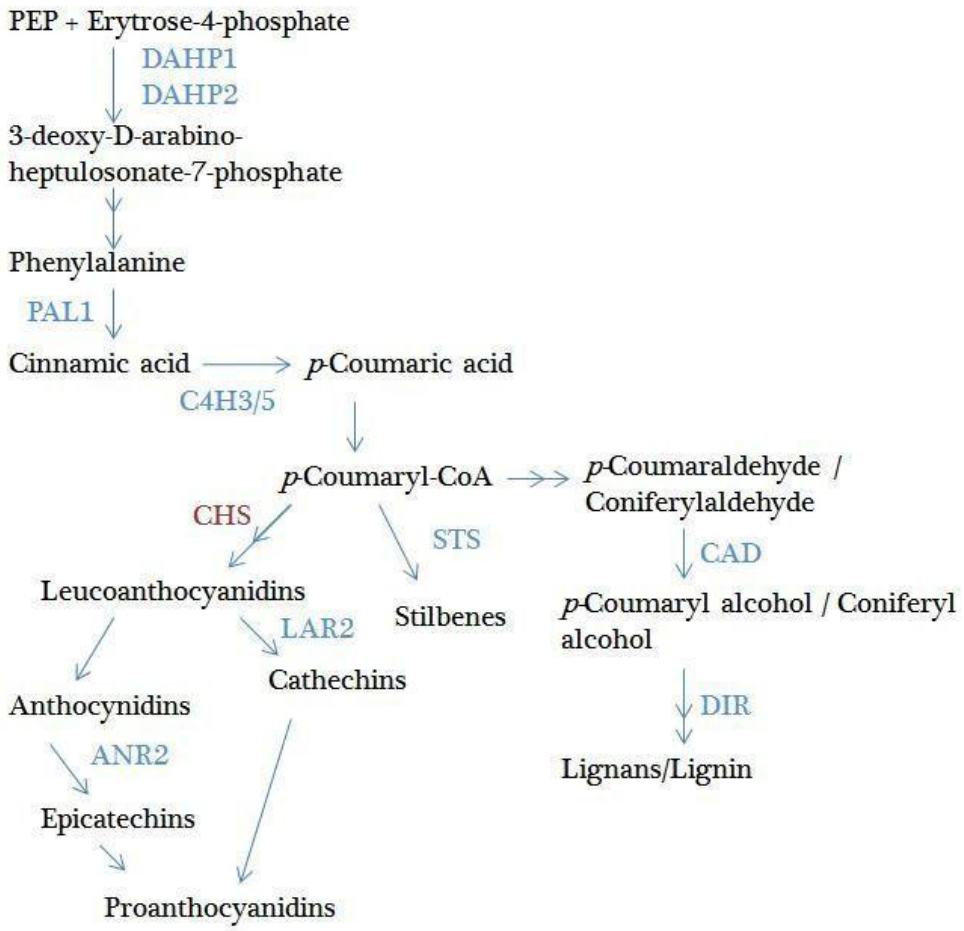
During its spread through the heartwood of infected trees, H. annosum s. l. will get in contact and interact with the sapwood, where then a reaction zone will be formed. The formation of a reaction zone

in spruce was first characterized by Shain (1971) as a non-pathogen-specific host response to compartmentalize and restrict fungal growth because of the presence and accumulation of phenolic compounds, mainly lignans, at the border to non-colonized sapwood (Shain, 1971; Shain and Hillis, 1971).

Both Oliva and colleagues (2010) as well as Bendz-Hellgren and Stenlid (1995) hypothesized that the observed diameter growth reduction in response to the fungus correlates with the reaction zone formation as it seems that carbohydrates are reallocated for production of antifungal compounds by starch degradation (Johansson and Stenlid, 1985; Shain and Hillis, 1971) instead of investment of secondary growth. This was especially underlined by the fact that secondary growth suppression showed a stronger correlation with reaction zone formation instead the status of rot within the stem (Oliva et al., 2010). Oliva et al. (2012) also found that smaller sapwood occurs, regardless of the amount of decay in rotten trees and suggested a qualitative response of the tree towards decay. They hypothesized, that sapwood reduction is the consequence of lower secondary growth because the reaction zone acts as a carbon sink and triggers allocation of photosynthates upon fungal attack, which might also include the initiation of defense responses in adjacent tissues.

Many studies thus have focused on studying secondary metabolism and transcriptional changes of genes encoding key enzymes in the secondary metabolite production upon challenging of spruce species with H. annosum s. l.. In many studies transcriptional regulation of the shikimate and phenylpropanoid pathway (see box 2.1) was often found to respond to the basidiomycete and therefore explored more closely. Arnerup et al. (2011) found that the shikimate pathway enzymes deoxy-D-arabinoheptulosonate 7-phosphate synthases (DAHPs) were transcriptionally regulated in H. parviporum inoculated bark of P. abies and interpreted the observed expression pattern, showing down-regulation of PaDAHP1 and 3 and up regulation PaDAHP2 a possible carbon shift towards secondary metabolite production. This might be possible to relate to the hypothesis of Oliva and his colleagues (2012). More evidence for up-regulation of secondary metabolite production could be provided by transcriptome 454-sequencing and quantitive PCR (qPCR) analysis of H. annosum inoculated Norway spruce showing a high up-regulations of various genes in the flavonoid pathway as well as activation of initial genes in the phenylpropanoid pathway like the phenylalanine ammonia lyase-like genes PaPAL1 and 2 and cinnamic acid 4-hydroxylase genes PaC4H3/5 and PaC4H2 (Danielsson et al., 2011). Besides flavonoids, lignan and stilbene synthesis could act as carbon sinks, because enrichment of both compounds in response to H. annosum s. l. was found and have been associated with the capacity to limit mycelial growth of H. annosum s. l. (Lindberg et al., 1992; Shain and Hillis, 1971).

Some studies now showed the influence of jasmonic acid (JA) on this secondary methabolite production (Arnerup et al., submitted; Yaqoob et al., 2012). Involvement of the JA pathway in the defense response against H. annosum s. l. is supported by the up-regulation of the lipoxygenase gene (LOX) (Arnerup et al., 2011), an upstream regulator of the JA production (Bell et al., 1995). Also the jasmonate ZIM-domain 1 gene (JAZ1), which characterizes a negative feedback loop in the JA signaling (Chini et al., 2007), response to inoculation with H. parviporum with increased expression (Arnerup et al., 2011). Direct influence upon PAL1 expression was also reported by Arnerup and her colleagues (submitted), further hinting an influence on the transcriptionally co-controlled phenylpropanoid production (reviewed by Rogers and Campbell, 2004). Also the influence of other hormone signaling was investigated by the two studies of Arnerup and colleagues (submitted; 2011), e. g. ethylene (ET), and suggested a synergistic cross-talk between the two pathways.



Box 2.1 Secondary metabolite synthesis as a defense response against Heterobasidion annosum sensu lato in Picea abies

The flow chart above depicts a simplified picture of parts of the shikimate pathway as well as the phenylpropanoid pathway. Enzymes which corresponding genes have been chosen for transcriptional investigation in this study are shown in blue next to the chemical transformation step they catalyze. Chalcone synthase (CHS) is added in red to underline its competitive role in the use of p-Coumaryl-CoA with STS.

The choice of which genes to study was made because of the association of those enzymes with the defense response against attack of necrotrophic pathogens and herbivores in previous studies. All of those genes are parts of larger gene families and the particular representatives were chosen, because they showed the most interesting and reliable expression patterns in previous studies.

Deoxy-D-arabinoheptulosonate 7-phosphate synthases (DAHP1 and DAHP2) participated in the synthesis of deoxy-D-arabinoheptulosonate 7-phosphate which later is converted to shikimate, thus they are enzymes of the shikimate pathway. Phenylalanine ammonia lyase-like 1 (PAL1) and cinnamic acid 4-hydroxylase 3/5 (C4H3/5) belong to gene families encoding enzymes of the general phenylpropanoid pathway and are important for the production of the precursors for lignin/lignan, flavonoids and stilbenes. Stilbene synthase (STS) is the initial enzyme in the stilbene synthesis forming resveratol which in the later pathway can be converted into different stilbenes than able to restrict fungal growth. Leucoanthocyanidin reductase (LAR2) and Anthocyanidin reductase (ANR2) lead to Catechin and Epicatechin synthesis, which are precursors for flavonoids that have antifungal properties. Cinnamyl alcohol dehydrogenase (CAD) and Dirigent-proteins (DIR) play important roles in lignifications and lignan production. While CAD catalyses the Monolignol formation DIR is needed for their polymerisation into lignins and lignans.

2.3 Many factors influence defense mechanism in Picea abies

Phenylpropanoid synthesis depicts an important line of defense in P. abies. Koutaniemi et al. (2007) and Emiliani and colleagues (2011) studied its expression in various different tissues, developmental stages, seasons and abiotic and biotic stress responses, showing that expression varies not only within the tissue upon the different treatments, but also among the tissues. Intriguingly, Koutaniemi and colleagues (2007) saw a similar response to stress in phloem and xylem, while expression varied strongly before stress was induced; however, they did not study tissue-specific spatial expression upon pathogen attack. In contrast, other studies presented differences in expression patterns between bark and sapwood of both Picea sitchensis and P. abies in various genes known to play a role in the defense response, including PaPAL1, PaPAL2, C4H3/5 and Cinnamyl alcohol dehydrogenase (CAD) (Deflorio et al., 2011; Yaqoob et al., 2012). Also, comparison of expression in sapwood and bark revealed significant differences for general defense related genes, such as Chitinase IV (PaCHIIV) (Deflorio et al., 2011).

The aim of this study was to associate transcriptional changes in the secondary metabolite pathways with reaction zone formation. Therefore transcriptional regulation of the secondary metabolite production associated with the defense response of P. abies towards H. annosum s. l., i. e. synthesis of phenylpropanoids, was investigated. Expression of genes related to the general phenylpropanoid pathway, as well as the flavonoid, stilbene and lignin/lignan synthesis pathway, was quantified using qPCR. Moreover, JA and ET signaling was studied, due to indications of its importance in P. abies defense mechanisms and its possible role as an upstream regulation of phenylproponaoid production. In contrast to other studies, material was inoculated with H. parviporum into the heartwood-sapwood boarder with duration of three months and gene expression was analyzed in sapwood adjacent to the induced reaction zone, the adjoining primary xylem and bark including phloem and cambium, to analyze radial changes in defense response. Further, natural infected trees were analyzed to determine the comparability of pathogen defense in P. abies induced after manual inoculation and long-term challenge with H. annosum s. l..

3 Material and Methods

3.1 Biological material and sampling

Sampling of naturally infected Norway spruce was done in mid-October. Trees were sampled at the base in two different positions labelled as inner and outer sapwood (Figure 3.1 A). Each position was sampled three to four times in different parts of the outer and inner sapwood. In total three infected and one healthy tree was sampled as a control. Samples were collected using a drill and then immediately transferred to liquid nitrogen and then stored at -80˚C until further use.

Inoculation of P. abies was performed by the research group of Carl Gunnar Fossdal at Skog og Landskap, Ås, Norway. In total three trees were inoculated with H. parviporum strain 87-257/1 at the heartwood-sapwood border or the beginning of the sapwood by drilling. The position was drilled two times into the tree and at the stopping point of the drill was treated either with H. parviporum or sapwood dust as a control (Figure 3.1 B). Samples were taken from as disks cut out of the trees at the drilling point position. Samples were frozen and stored at -70˚C until further use. Samples for RNA extraction were collected from i) the bark, cambium and phloem, ii) primary xylem (first three sapwood rings) and iii) sapwood above and around the position were the reaction zone has formed. In tree three the reaction zone was rather close to the primary xylem, in both inoculation and wounding treatment, however a sapwood sample was collected as well.

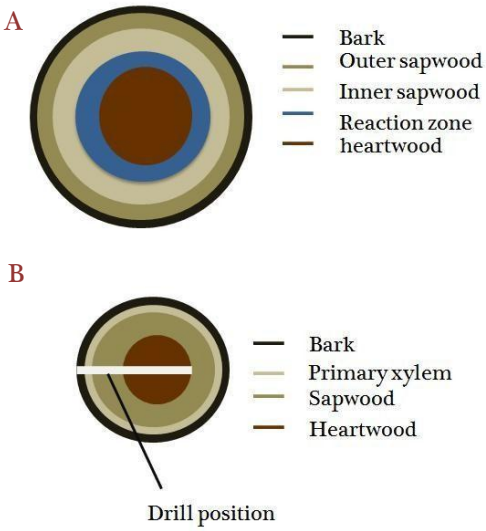


Figure 3.1 Experimental design.

Sampling of natural samples was done in inner and outer sapwood of healthy and infected trees. Inner and outer sapwood is exemplified in a drawing of an infected, horizontally cut tree (A); Sampling in material of the controlled inoculation experiment was done in sapwood, primary xylem and bark (including phloem and cambium) opposite the drill position, which was either inoculated with H. parviporum or dusted with sapwood dust (B). The different zones are color coded and legend can be viewed next to each picture.

3.1.1 Sample code

All samples that were collected and further used for RNA extraction and gene quantification are indicated in table 3.1 for the samples collected from naturally H. annosum infected trees and table 3.2 for samples that were inoculated with H. parviporum. The tables give the sample code of each sample and show further information on the tissue type and tree from which the samples originate. Further the tree status, i.e. if the sample is a control (healthy or wounded and treated with sapwood dust) or if it was treated with H. parviporum or naturally infected by H. annosum can be viewed in the table 3.1 and 3.2. In case of the controlled inoculation experiment position of treatment is indicated as well.

Table 3.1: Sample code for samples obtained from trees naturally infected with H. annosum, sample origin gives tissue and position in the tree where the sample was collected, tree number indicates from which of the four trees sampled the RNA sample was obtained and tree status indicates if the tree was infected with H. annosum or healthy.

Tree number	Tree status	Sample origin	Sample code
1	H. annosum infection	Inner Sapwood; tree base	S1I
1	H. annosum infection	Inner Sapwood; tree base	S2I
1	H. annosum infection	Inner Sapwood; tree base	S5I
1	H. annosum infection	Outer Sapwood; tree base	S1U
1	H. annosum infection	Outer Sapwood; tree base	S2U
2	H. annosum infection	Inner Sapwood; tree base	S12I
2	H. annosum infection	Inner Sapwood; tree base	S13I
2	H. annosum infection	Inner Sapwood; tree base	S14I
2	H. annosum infection	Outer Sapwood; tree base	S12U
2	H. annosum infection	Outer Sapwood; tree base	S14U
2	H. annosum infection	Outer Sapwood; tree base	S15U
3	healthy	Inner Sapwood; tree base	S25I
3	healthy	Inner Sapwood; tree base	S27I
3	healthy	Inner Sapwood; upper part of the tree	S28I
3	healthy	Outer Sapwood; tree base	S24U
3	healthy	Outer Sapwood; tree base	S26U
3	healthy	Outer Sapwood; upper part of the tree	S28U
4	H. annosum infection	Inner Sapwood; tree base	S33I
4	H. annosum infection	Inner Sapwood; tree base	S35I
4	H. annosum infection	Outer Sapwood; tree base	S35U

Legend: S – Sapwood; U – outer; I – inner

Table 3.2: Sample code of inoculated samples; Sample origin indicates the tissue from which the RNA was extracted, position point of inoculation or wounding indicates the point in the tree where drilling was stopped and either H. parviporum inoculum or sapwood dust was placed, Tree status gives information if the sample was treated with H. parviporum or sapwood dust, tree number shows which of the three trees was used.

Tree number	Tree status	Sample origin	Position point of inoculation or wounding	Sample number
31	H. parviporum	Bark, phloem, cambium	Beginning of sapwood	12B
31	H. parviporum	Primary xylem	Beginning of sapwood	12PX
31	H. parviporum	Sapwood	Beginning of sapwood	12S
31	Sapwood dust	Bark, phloem, cambium	Beginning of sapwood	15B
31	Sapwood dust	Primary xylem	Beginning of sapwood	15PX
31	Sapwood dust	Sapwood	Beginning of sapwood	15S
32	H. parviporum	Primary xylem	Beginning of sapwood	23PX
32	H. parviporum	Sapwood	Beginning of sapwood	23S
32	Sapwood dust	Primary xylem	Heartwood-Sapwood border	26PX
32	Sapwood dust	Sapwood	Sapwood	25S
33	H. parviporum	Bark, phloem, cambium	Sapwood	32B
33	H. parviporum	Primary xylem	Sapwood	32PX
33	H. parviporum	Sapwood	Sapwood	32S
33	Sapwood dust	Bark, phloem, cambium	Sapwood	36B
33	Sapwood dust	Primary xylem	Sapwood	36PX
33	Sapwood dust	Sapwood	Sapwood	36S

Legend: B – bark; PX – primary xylem; S – sapwood

3.2 RNA extraction

RNA extraction was performed using a modified protocol based on the Plant RNA extraction method described by Chang et al. (1993). Samples were ground using liquid nitrogen and 2 ml to 3 ml of RNase free extraction buffer (heated for 30 minutes at 65°C in before use; 2 M Sodium chloride (NaCl), 50 mM hexadecyl-trimethyl-ammonium bromide (CTAB), 0.69µM Polyvinylpyrrolidone (PVP), 25 mM

ethylenediaminetetraacetic acid (EDTA, pH 8), 0.1M Tris-HCl (pH 8), diethylpyrocarbonate (DEPC)-water (H_2O), 2% β-Mercaptoethanol (added directly before use)) were added. Samples in extraction buffer were incubated at 65°C for 15 minutes. Then the samples were extracted twice with an equal volume of chloroform : isoamyl alcohol (24 :1) and centrifuged at 7000 rpm for 20 minutes at room temperature. The aqueous phase was precipitated at 4°C over night using ¼ of 8M lithium chloride (Sigma Aldrich) and then centrifuged at 4°C for 7000 rpm and 40 minutes. The supernatant was carefully poured out. The dried pellet was dissolved in 100µl of Nuclease free H_2O (Fermentas) and transferred to new tube where it was further precipitated with 2 volumes of cold absolute alcohol and 1/10 of the volume of 3M sodium acetate (NaAc; pH 7) (Sigma Aldrich). RNA was precipitated at -20°C for at least 1 hour. After precipitation the samples were centrifuged at 4°C and 9000 rpm for 20 minutes and then the supernatant was poured out carefully. The pellet was washed with cold 70% ethanol. After washing the supernatant was carefully discarded and the pellet was dried for several minutes and then dissolved in 17 µl of DEPC-H_2O.

3.3 DNase treatment

Extracted samples were treated with DNase1 (Sigma-Aldrich, USA) according to the manual description with the exception that no duplicate reaction was performed. After the DNase treatment the concentration of the RNA was measured either using a NanoDrop (Spectrophotometer ND 1000, Saven Werner) or the Bioanalyzer (Agilent 2100 Bioanalyzer, Caliper Life Science Inc.). Preparation of concentration measurement with the Bioanalyzer was performed with to the Agilent RNA 6000 Nano kit (Dalco Chromtech AB, Märsta, Sweden) according to the Agilent RNA 6000 Nano assay protocol (Edition April 2007, Dalco Chromtech AB, Märsta, Sweden).

3.4 messenger RNA purification of naturally infected samples

messenger RNA (mRNA) purification was done after DNase treatment, using the Dynabeads® mRNA-purification kit (Invitrogen, Oslo, Norway) according to the protocol modified by Jenny Arnerup. It was aimed to use 630 to 1000 ng of RNA of all samples for mRNA purification and samples were diluted to 24 µl in 10 mM RNase free Tris-HCl (Invitrogen, Oslo, Norway). Exception was made for samples with insufficient amount of RNA extracted but samples were processed as described above. Further, in case of S12U, S14U, S24U and S28Uthe whole sample (20 µl), irrespective of the amount of total RNA was used for mRNA purification and the volumes were adjusted accordingly. Equal volumes of sample and binding buffer (Invitrogen, Oslo, Norway) were heated to 65°C for 2 minutes and then cooled on ice. Dynabeads Oligo (dT)25 were equilibrated and resuspended in binding buffer. Samples were added to the beads and RNA was annealed by incubating the samples at room temperature for 3 to 5 minutes while vortexing carefully. Beads were washed twice with washing buffer B (Invitrogen, Oslo, Norway). mRNA was eluted in 10 mM RNase free Tris-HCl while incubating at 65°C for 2 minutes followed by immediate transfer to a new RNase free tube.

3.5 complementary DNA synthesis

complementary DNA (cDNA) was created using the iScript™ cDNA Synthesis Kit (BIO-RAD, Sundbyberg, Sweden). The reaction was performed according to the Reaction Set Up in the iScript™ cDNA Synthesis Kit manual using 14 µl of purified mRNA obtained from the natural infection samples and 1 µl Nuclease free H_2O (Fermentas) in each reaction, with exception for the samples S12U, S14U, S24U and S28U, where 5 µl of purified mRNA and 5 µl of Nuclease free H_2O (Fermentas)

was used in each in reaction, because of the different treatment in the mRNA purification protocol. All samples were diluted in a 1 : 1.06 ratio of sample to Nuclease free H_2O (Fermentas).

In case of the RNA obtained from the controlled inoculated samples cDNA was synthesised using the iScript™ cDNA Synthesis Kit (Bio-Rad, Hercules, CA, USA) according to the manual using 675 to 1000 ng total RNA in each reaction. After a test run with Phosphoglucomutase (PG mutase) and PaPAL1 samples were diluted to a 1 : 1 ration of sample to Nuclease free H_2O (Fermentas).

3.6 quantitative Polymerase-chain-reaction

3.6.1 Primer design

Primers for the Norway spruce gene Defensin 1 (SPI1) were designed based on the mRNA sequence of SPI1 found in the NCBI BLASTN database (Accession number AF548021.1). For primer design the software Vector NTI advance® (life technologies, Stockholm, Sweden) was used. Dirigent protein/ Dirigent protein-like gene pDIR2/32 was selected based on EST expression values of The Gene Index Project (Computational Biology and Fuctional Genomics Laboratory, Harvard) and expression data obtained from Ralph et al. (2007) and primers were designed based on the sequence obtained at the The Gene Index Project website (Computational Biology and Fuctional Genomics Laboratory, Harvard; Accession number TC128949 for pDIR2/32) using Primer BLAST, searching for an amplicon length between 50 and 250 bp. All primers were ordered at TAG Copenhagen. For Primer sequence, amplicon length and annealing temperature see table 9.1.

3.6.2 Preparation of standard curves

Standard curves were created for the genes SPI1, PaCHIIV and pDIR2/32 by first amplifying the gene fragment from cDNA of 2 week old seedlings of Norway spruce. Genes were amplified in a 50 µl PCR reaction using 1x Dream Taq green buffer (with 20 mM magnesium chloride; stock solution concentration 10x; Fermentas), Nuclease free H_2O, 0.2 mM dNTPs (stock concentration 2 mM), 0.1µM of each primer (stock solution concentration 10 µM; ATG Copenhagen), 1 µl template and 6.25U Dream Taq Polymerase (stock concentration 5U/µl; Fermentas). PCR reaction included 5 minutes initial denaturation at 95°C, 35 cycles of 95°C denaturation for 10 seconds, annealing for 10 s (for annealing temperature see table 9.1) and elongation at 72°C for 1 minute, the reaction ended with final elongation step at 72°C for 3 minutes.

The PCR product was purified using the GeneJet™ PCR-purification kit (Fermentas, Thermo Scientific) according to the GeneJet™ PCR-purification kit manual, including the optional step of adding half the volume of Isopropanol to binding buffer and PCR reaction. Elution was made in 50 µl elution buffer. The standard curves for SPI1 and pDIR2/32 were directly created using the purified PCR product, measuring the DNA concentration with the NanoDrop (Spectrophotometer ND 1000, Saven Werner), and apply a dilution series from 10^7 to 10^2 DNA copies/ µl based on the concentration measurement. The copy number was calculated using DNA copy number calculation (Finnzymes, Thermo Scientific) for custom DNA fragment.

Purified PCR-product of PaCHIIV was ligated using the TOPOTA Cloning pCR® 2.1 kit (Invitrogen), by ligating 4.5µl template with 0.5 µl TOPO® 2.1 – vector and 1 µl salt solution. The ligation reaction was incubated for 30 minutes at room temperature.

Transformation was made using ready competent Escherichia coli TOP10 cells (Invitrogen). 2.5 µl ligation product was added µl of competent E. coli and incubated on ice for 15 minutes, so that the cDNA will be adjacent to the bacterial membranes. Bacteria will be heat-shocked at 42°C for 30 to 45 seconds in a water bath and then placed briefly on ice. Then transformed cells will be equilibrated to room temperature before 100 µl of SOC medium (Invitrogen) was added and the cells will be grown for 1 hour at 37°C on a shaker and then plated on LB plates (pH 7; Sambrook and Russel, 2001) with ampicillin and x-gal and incubated at 37°C over night in a heating oven. White colonies will be picked and streaked out on LB plates with ampicillin (final concentration 100 µg/ml) and x-gal and further incubated over night at 37°C in a heating oven to gain sufficient material of transformed cells.

To screen the white E. coli colonies for the presence of the insert some E. coli cells were scratched of the plate and solved in 100 µl Nuclease free water, heated for 10 minutes at 90°C in a heating block to break the cells and centrifuged 1 minute at 13000 rpm. 5 µl of the supernatant were used as a template in the 10 µl PCR-reaction using 1x Dream Taq green buffer (with 20 mM magnesium chloride; stock solution concentration 10x; Fermentas), Nuclease free H_2O (Fermentas), 0.2mM dNTPs (stock concentration 2 mM), 3µM M13 forward and 3µM M13 reverse primer (Stock solution concentration 100 µM; Invitrogen) (for the primer sequence see table 9.1) and 1.25U Dream Taq Polymerase (stock concentration 5u/µl; Fermentas). The reaction has an initial denaturation phase at 94°C for 5 minutes, 35 cycles of denaturation at 94°C for 30 seconds, annealing at 55°C for 30 seconds, elongation at 72°C for 30 seconds and a final elongation step for 7 minutes at 72°C.

An inoculum of those colonies that have the insert were picked and inoculated in 2 ml liquid LB medium and grown over night at 37°C on a rotary shaker. The plasmid was purified using the plasmid purification GeneJet™ Plasmid Miniprep kit (Fermentas, Thermo Scientific) according to the supplied manual.

The concentration of the isolated plasmid was measured using the Nanodrop (Spectrophotometer ND 1000, Saven Werner). Amount for a plasmid concentration of 10^7 was calculated using the DNA copy number calculation function (Finnzymes, Thermo Scientific). The standard curve was prepared using the dilutions from 10^7 to 10^2.

3.6.3 Quantification of gene expression in Picea abies

Quantification of gene expression was done using qPCR (iQ5™ Multicolor Real-Time PCR Detection System, BIO-RAD). For the qPCR-reactions the SsoFast™ EvaGreen® Supermix was used, adding 10 µl of Supermix, Nuclease free H_2O (Fermentas), 0.35 µM of the forward and 0.35 µM of the reverse primer (stock solution concentration 10 µM) and 1 µl template. For each gene that was tested a 96 well-plate (Bio-Rad) was used and closed with transparent film (Bio-Rad). Three technical replications of each sample were made. The efficiency of each run was estimated using a standard curve for the particular gene with a dilution series from 10^7 to 10^3. Three technical replicates of each dilution were prepared. For the samples from the natural infection experiment two housekeeping genes were used as reference genes: Elongation factor 1 α (ELF1α), and PG mutase, while three reference genes, ELF1α, PG mutase and Elongation factor 4 α (ELF4α) was used for quantification of the samples from the controlled inoculation experiment.

In the naturally infected samples expression of PAL1, DAHP1, DAHP2, JAZ, CAD and pDIR2/32 (for primer sequences see attachment table 9.1) were compared in i) infected trees to the healthy tree and ii) in inner and outer sapwood in both the infected and healthy trees. In the samples that were

inoculated with H. parviporum comparison of the expression of PAL1, DAHP1, DAHP2, C4H3/5, Stilbene synthase (STS), Leucoanthocyanidin reductase (LAR2), Anthocyanidin reductase (ANR2), CAD, pDIR2/32, SPI1, PaCHIIV, JAZ, LOX, 1-aminocyclopropane-1-carboxylic acid syntheses (ACS) and 1-aminocyclopropane-1-carboxylate oxidase (ACO) (for primer sequences see attachment table 9.1) was analyzed. Expression values of i) inoculation to wounding in bark, primary xylem and sapwood and ii) sapwood to primary xylem, sapwood to bark and primary xylem to bark was compared.

qPCR cycle included an initial phase of 2 minutes at 55˚C, followed by the initial denaturation phase at 95˚C for 10 minutes. Then 40 cycles of 95˚C for 15s, followed by 1 minute at 60˚C, in which the fluorescence detection by the machine was applied, exceptions were made for PaCHIIV (56.8˚C), CAD (58˚C), STS (65˚C) and pDIR2/32 (55˚C) . The melting curves were measured. Expression data was analyzed using the software REST 2009 (Technical University Munich, Germany and Qiagen).

4 Results

4.1 Secondary metabolism analyses

In this study transcriptional regulation of the metabolic pathway leading to the synthesis of secondary metabolites such as stilbenes, flavonoids and lignans, as well as the synthesis of lignin were analyzed by quantifying gene expression of key enzymes in the particular pathways.

4.1.1 3-deoxy-D-arabinoheptulosonate-7-phosphate synthase expression in Picea abies

PaDAHP1 and PaDAHP2 encode enzymes that can convert 3-deoxy-D-arabinoheptulosonat-7-phospahte into shikimate and are thus involved in the shikimate pathway in plant metabolism. In this project their expression was studied in both naturally H. annosum s. l. infected and manually H. parviporum inoculated P. abies.

Overall PaDAHP2 showed higher non-normalized Ct-values compared to PaDAHP1 in the controlled inoculation experiments. When expression was studied in naturally infected samples, Ct-values for PaDAHP1 were under the detection limit of the qPCR in all inner sapwood samples of both healthy and infected trees and most of the outer sapwood samples.

The data obtained from the controlled inoculation experiment revealed no significant changes in the PaDAHP1 and PaDAHP2 expression in bark, primary xylem and sapwood when inoculation is compared to wound treatment (Figure 4.1 A). It can be seen that while their expression showed lower relative expression values in bark, when H. parviporum treatment is compared to wounding, higher relative expression values were observed for gene expression in primary xylem and sapwood with relative expression values for PaDAHP1 of 1.197 and 1.439 and for PaDAHP2 of 1.3 and 2.562, respectively. Comparison of naturally infected trees to the healthy tree showed relative gene expression values lower than 1 independent of the tissue sampled, although only results for the outer sapwood were significant (data not shown).

Tissue comparison of either sapwood to primary xylem or sapwood to bark in both H. parviporum challenge and wounding demonstrated a relative expression value for PaDAHP1 gene expression significantly lower than 1. Relative expression values for PaDAHP2 in these comparisons were only significantly higher than 1 in the sapwood when challenged with H. parviporum (Figure 4.1 B; appendix figure 9.1). Tissue comparison in the naturally infected and healthy samples showed only insignificant changes in when inner sapwood is compared with outer sapwood for PaDAHP2 however the relative expression values were 1.345 and 0.766 for comparison in the naturally infected trees and the healthy trees, respectively (Figure 4.2).

4.1.2 Expression of genes in the general phenylpropanoid pathway

PaPAL1 and C4H3/5 expression was studied, as these two genes encode enzymes facilitating the conversion from phenylalanine to cinnamic acid and from cinnamic acid to p - Coumaric acid, respectively which are the precursors for flavonoids, stilbenes, lignin and lignans. While expression of both was measured in the samples of the controlled inoculation experiments, samples from the natural infection study were only analyzed for PaPAL1 expression.

When H. parviporum inoculation was compared with wound treatment, PaPAL1 and PaC4H3/5 showed relative expression values of 1.6 and 1.4, respectively, in the sapwood, while relative expression values of both genes remained below 1 in bark and primary xylem samples (Figure 4.1 A). While the expression values were significant for both genes in the comparison in bark and sapwood samples, only the PaPAL1 expression comparison showed significance in the primary xylem samples. Comparison of naturally infected trees to a healthy tree, showed relative expression values for PaPAL1 were below 1 irrespectively of the tissue type ($p < 0.001$ for the outer sapwood and $p < 0.05$ for the inner sapwood).

Tissue comparisons revealed significantly high relative expression values for PaPAL1 in both the controlled inoculation experiment when inoculated with H. parviporum and the natural infected samples. When expression in sapwood and bark was compared relative expression reached a value of 48.37, while inner sapwood versus outer sapwood showed a value of 26.2 (Figure 4.1 B and 4.2 B). The corresponding comparison for wounding treatment revealed a relative expression value 12.85 ($p < 0.001$) while it was below 1 when inner sapwood was compared to outer sapwood in healthy trees (Figure 4.1 B and 4.2). Relative expression values for PaC4H3/5 were similar to PaPAL1 in the sapwood – bark comparison (52.534 when inoculated and 19.009 when wounded) (Figure 4.1 B). Sapwood - primary xylem comparison showed similar results for both genes (Appendix figure 9.1). The comparison of bark to primary xylem showed no significant differences between the two tissues (Figure 4.1 B).

4.1.3 Flavonoid, Stilbene, Lignin and Lignan synthesis pathways

Expression of LAR2, ANR2, STS, CAD and pDIR2/32 was studied because they are representative genes involved in flavonoid, stilbene, lignan and lignin biosynthesis. While the flavonoid and stilbene pathway was only investigated in the controlled inoculation experiments, the lignan and lignin synthesis pathway was analyzed in both experiments.

LAR2 and ANR2 are involved in steps leading to the synthesis of proanthocyanidins. The expression of both of the corresponding genes was found to be below 1 ($p < 0.05$ and $p < 0.01$, respectively) in bark in samples inoculated with H. parviporum compared to wounding, while in sapwood at least ANR2 showed a significantly higher relative expression values above 1 ($p < 0.001$) (Figure 4.1 A). In a tissue comparison of sapwood to bark LAR2 had a relative expression value of 2.223 ($p < 0.05$) when challenged with H. parviporum, while upon wound treatment the relative expression value was 0.478 ($p < 0.05$) in sapwood compared to bark (Figure 4.1 B).

STS encodes an enzyme that converts p – Coumaryl-CoA and Malonyl-CoA into Resveratol, which is the first compound in the stilbene synthesis pathway. Expression of STS showed no significant changes, except for a relative expression value slightly lower than 1 in bark tissue when H. parviporum response is compared to the wounding response (Figure 4.1 A). However, in a tissue comparison relative expression values of STS were observed to be 0.275 and 0.166 in sapwood in H. parviporum and wounding treatment, respectively. Relative expression values observed in the primary xylem still were below 1 in comparison to bark in both treatments but the difference between the two tissues is not that strong compared to the difference obtained in the sapwood bark comparison. (Figure 4.1 B)

CAD and pDIR2/32 are two important enzymes participating in the lignin and lignan biosynthesis pathway. In figure 4.1 A it can be seen that CAD and pDIR2/32 showed no significant changes in their expression level when H. parviporum and wounding influence is compared in the different tissue types. Only in sapwood pDIR2/32 had a higher relative expression, with a relative expression value of

2.5 that was significant ($p < 0.001$). In the comparison of natural infection by H. annosum s. l. to a healthy tree relative expression of pDIR2/32 was lower than 1 in outer sapwood ($p < 0.05$) but no significant change was found in the corresponding comparison in the inner sapwood (data not shown). CAD expression was not measurable in the inner sapwood of the healthy tree; however, CAD expression showed no significant change for the outer sapwood samples when natural infected trees are compared to a healthy tree (data not shown).

In the controlled inoculation experiment sapwood showed a significantly higher relative expression of pDIR2/32 and CAD compared to bark (Figure 4.1 B) for both inoculation and wounding. The difference in relative expression of pDIR2/32, however, was 3.4 times higher when inoculated compared to wounding in the sapwood-bark tissue comparison. Genes encoding enzymes involved in stilbene, flavonoid and lignin/lignin biosynthesis showed significant changes in the primary xylem – bark comparison, with relative expression values below 1 in the primary xylem compared to the bark in inoculated samples. In wounding only STS and ANR2 relative expression values were significant in that comparison (Figure 4.1 B). Inner to outer sapwood comparison demonstrated the relative expression of CAD and pDIR2/32 was significant over 1 in the inner sapwood when compared to the outer sapwood in infected trees and in case of pDIR2/32 also in the comparison in the healthy tree (Figure 4.2).

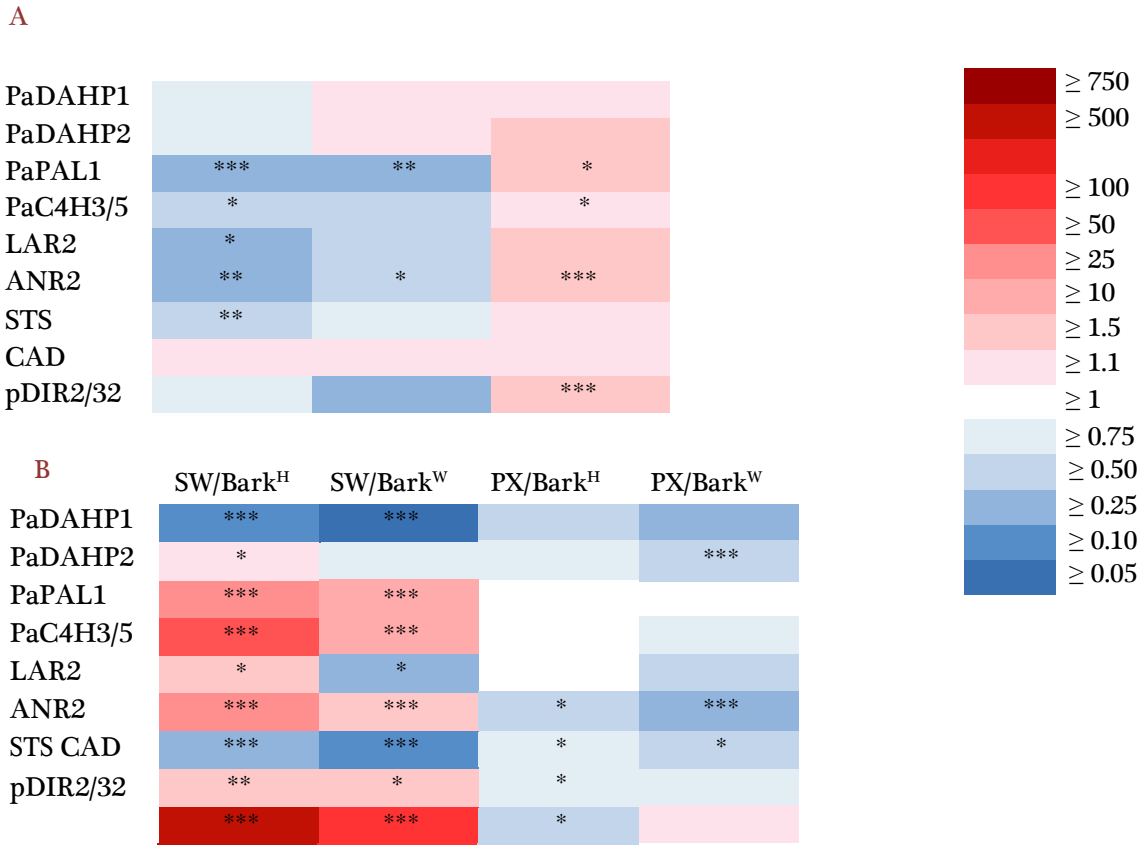


Figure 4.1 Heat map showing the relative expression values of genes acting in the phenylpropanoid pathway when comparing gene expression in samples inoculated with H. parviporum to wounding in bark, primary xylem (PX) and sapwood (SW) (A) and in tissue comparison of sapwood to bark or primary xylem to bark in samples inoculated with H. parviporum (•) or wounded (◦) (B); up-regulation is indicated in red color with higher up-regulation corresponding to a darker red and down-regulation indicated in blue with stronger down-regulation corresponding to a darker blue; *,** and *** indicate significance levels of $p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively, calculated by REST 2009

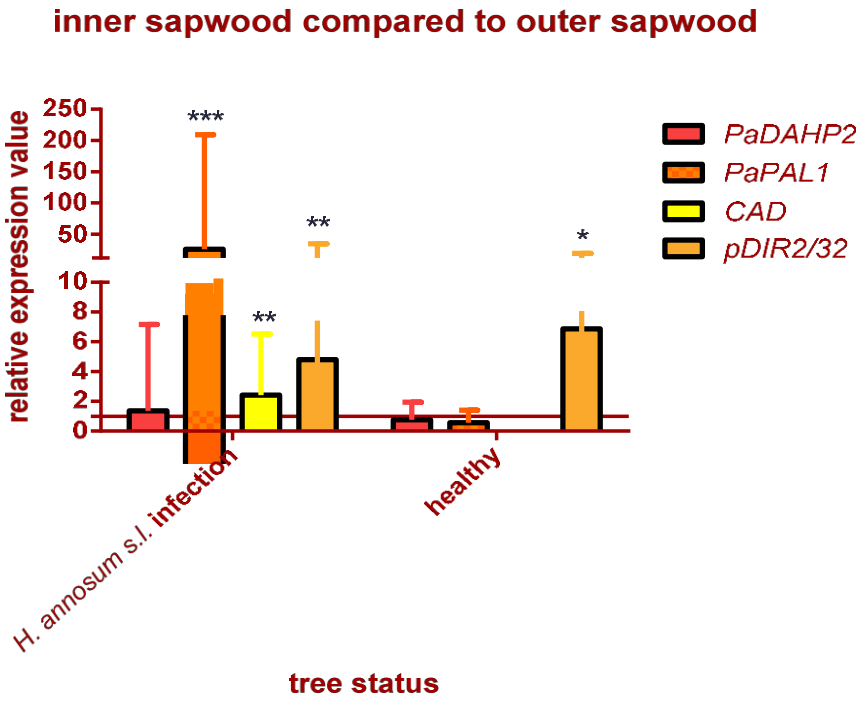


Figure 4.2 Relative expression values of PaDAHP2, PaPAL1, CAD and pDIR2/32 in inner sapwood compared to outer sapwood in either H. annosum s. l. infected trees or the healthy tree; Analysis of the relative expression value for CAD in the healthy tree could not be estimated, because CAD expression was not measurable in the inner sapwood of the healthy tree; relative expression values over 1 show an up-regulation of the particular gene; bars indicate the standard error calculated by REST 2009; *, ** and *** indicating p-values of $p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively

4.2 Hormonal pathway

Expression of genes included in hormonal pathways as well as expression of CHIIV and SPI1 was analyzed in the different tissues extracted from samples of the controlled inoculation experiments. Expression of JAZ was analyzed in both, samples of the controlled inoculation and samples that had been naturally infected with the fungus. However, in the natural infection the melting curves showed several peaks, as primers targeted more than one JAZ gene (data not shown). Primer concentrations were adjusted for expression analysis in the controlled inoculation experiment, resulting in one clear melting curve peak in the qPCR data analysis.

Relative expression values show that neither SPI1 nor CHIIV were significantly higher than 1 in any of the three investigated tissues (Figure 4.3 A). LOX and ACS expression showed only a significant change in sapwood in which its relative expression was estimated to be 7.371 and 28.053, respectively, when H. parviporum inoculation and wounding is compared. JAZ and ACO have significant relative expression values far below 1 in bark challenged with H. parviporum compared to wounding, showing relative expression values of 0.13 and 0.26, respectively, while in primary xylem JAZ was the only gene that showed significant relative expression when inoculation was compared to wounding.

Interestingly, differences between sapwood and bark are mostly only significant in a H. parviporum treatment, but not upon wounding. Only LOX, ACS and CHIIV gene expression was significantly changed in the wounded sample when expression in sapwood is compared to expression in bark. In case of LOX and ACS, the relative expression in sapwood was higher compared to bark and as clearly more pronounced upon H. parviporum inoculation than in wounding. Relative expression values of SPI1 were not significant in either tissue comparison. Also, in general no significant changes were observed when primary xylem was compared to bark. (Figure 4.3 B)

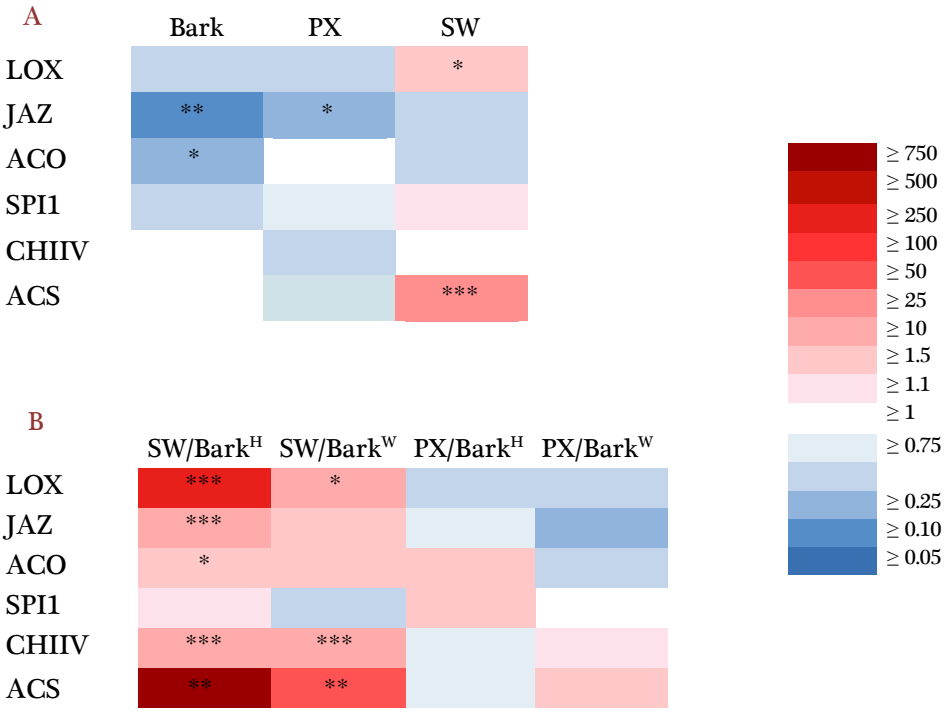


Figure 4.3 Heat map showing the relative expression values of genes involved in JA and ethylene (ET) synthesis as well as genes downstream of the JA signaling cascade; A comparison of gene expression in bark, primary xylem (PX) and sapwood of H. parviporum inoculation to wounding; B comparison of gene expression in sapwood to bark and primary xylem to bark in H. parviporum inoculation (^{H}) and wounding treatment (^{W}); red color indicates up-regulation of gene expression with darker red corresponding to a higher up-regulation and blue indicating down-regulation with darker blue showing a stronger down-regulation; *, ** and *** give significance levels of $p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively.

5 Discussion

5.1 Secondary metabolite production shows significant up-regulation at the site of treatment

The data obtained in this study revealed that the general phenylpropanoid pathway was significantly up-regulated in inoculated sapwood i.e. at the site of inoculation, whereas significantly down-regulated in bark in the corresponding position. Up-regulation of the phenylpropanoid pathway at the site of inoculation was also shown in other studies, although other tissues were inoculated (Arnerup et al., 2011 Danielsson et al., 2011; Deflorio et al., 2011; Koutaniemi et al., 2007; Yaqoob et al., 2012). Yaqoob et al (2012) analyzed the expression in sapwood after inoculation in bark and found a down-regulation of targeted genes distal to the inoculation in the radial plane. Taken together, this suggests a response independent of tissue type, but dependent on proximity to H. parviporum invasion.

5.2 The flavonoid and the lignin/lignan synthesis pathways shows signs of co-regulation with the general phenylpropanoid pathway

Flavonoid, lignin and lignan bisosynthesis pathways, which both lead to secondary metabolites important in plant-defense responses, seem to show the explicit up-regulation at the site of H. parviporum inoculation and the expression seems less important in more distal tissues compared to wounding, indicating a probable co-regulation of the flavonoid pathway with the general phenylpropanoid pathway. ANR2 and LAR2 which catalyse the formation of epicatechin and catechin, precursors for proanthocyanidins (Danielsson et al., 2011 and references there in), showed an up-regulation in inoculated material compared to after wounding alone (ANR2 2.065, $p = 2.065$; LAR2 1.67 and $p = 0.145$). This represents the expected co-regulation of the general phenylpropanoid and flavonoid pathway at the site of inoculation as found in our previous study on bark (Danielsson et al., 2011). However, in distal tissues (bark) down-regulation of both ANR2 and LAR2 was found after inoculation of sapwood compared to wounding of sapwood, underlining both the co-regulation of the general phenylpropanoid pathway and the flavonoid pathway as well as the decrease in response in radial direction.

We attempted to investigate the lignin/lignan synthesis pathway by analyzing the expression of CAD and a selected pDIR gene. Building of lignin and lignan requires a stereoselective coupling and oxidization of coniferyl alcohol to form lignans and lignin and p-coumaryl alcohol as a precursor for lignin (KEGG pathway map for the phenylpropanoid synthesis, 2012; Danielsson et al., 2011 and references there in; Davin et al., 1997). This catalytic step requires DIR proteins, which in conifers have been shown to be highly radiated (Davin et al., 1997; Ralph et al., 2007; Ralph et al., 2006). The one selected pDIR gene (pDIR2/32) showed a generally high expression in sapwood compared to other tissues, which is in accordance with high constitutive levels reported for pDIR2 and a significantly higher lignin content in sapwood compared to bark (Delflorio et al., 2011; Ralph et al., 2006). The significant up-regulation for pDIR2/32 found in inoculated sapwood compared to wounded sapwood, may indicate a role of pDIR2/32 in the Spruce defense and may influence the amount or structure of lignin or lignan in the cell walls of sapwood cells. However the analyzed CAD gene did not change significantly when fungal inoculation was compared to wounding raising the possibilities that either precursors of lignin and lignan are important and required in response to both treatments alternatively other members of the CAD gene family could be responsible for the production of the precursors for lignans in the reaction zone. Up-regulation of pDIR2/32 in defense response is in agreement with other

studies (Ralph et al., 2007), but its exact role in induced defense responses in Spruce is not yet well understood. However, an intriguing thought is a role of pDIR2/32 in the production and accumulation in lignans, due to their enrichment in the reaction zone as found by Shain and Hillis (1971).

5.3 Stilbene synthesis

The STS gene encodes the enzyme that catalyzes the first step in stilbene synthesis (Hammerbacher et al., 2011). The STS gene is up-regulated upon pathogen attack and its induction correlates with increased levels of stilbenes at the same time (Hammerbacher et al., 2011). Lindberg et al. (1992) as well as Woodward and Pearce (1988) demonstrated that stilbenes, especially Astringin, can have growth inhibiting effects on fungal pathogen. Results on the ability of stilbenes to hinder H. parviporum growth however have been contradictory (Lindberg et al. 1992; Woodward and Pearce, 1988).

This study provides no evidence for a role of stilbene production in induced defense response of P. abies against H. parviporum as the steady state mRNA levels of STS in sapwood were similar after wounding and inoculation. According to Hammerbacher and colleagues (2011) this would indicate constant stilbene production at the site of inoculation. Alternatively the fact that the the primers for the STS genes targets two STS genes could explain the lack of regulation as the different STS genes respond to different environmental and developmental signals (Hammerbacher et al., 2011). However the latter is less likely as we observed a significant down-regulation of STS genes in bark in an inoculation-wounding comparison as well as in the sapwood-bark-tissue comparison.

5.4 Possible carbon flow to the site of inoculation from neighboring tissues

In this project not only expression patterns of genes participating in the phenylpropanoid pathway, but also expression of PaDAHP1 and PaDAHP2 was measured. Both genes participate in the synthesis of a shikimate precursor, which can be a carbon input into the phenylpropanoid pathway (Danielsson et al., 2011 and references there in). In this study we found that PaDAHP2 is indicated to be the stronger expressed PaDAHP gene of the two analyzed ones (according to their Ct-values; data not shown). Its up-regulation upon H. parviporum inoculation in the comparison of sapwood to bark and primary xylem thus thought to have a significant influence onto the switch between primary and secondary metabolism and probably represents a higher carbon input in the secondary metabolism at the inoculation site.

However, our results depicted no significant regulation on either of the two PaDAHP genes in any of the three investigated tissues in a comparison of fungal challenge to wounding. This suggests that the carbon input into the shikimate pathway does not differ in response to wounding and H. parviporum. We propose that the distribution pattern of carbon among various secondary metabolite pathways is more important in the response to H. parviporum than a higher carbon allocation into the shikimate pathway. Up-regulation of the general phenylpropanoid and flavonoid pathway in inoculated sapwood compared to wounded sapwood and the down-regulation of those pathways in the corresponding bark comparison support this hypothesis.

Besides down-regulation of the phenylpropanoid and flavonoid pathway in bark, down-regulation of PAL1 and ANR2 indicate that both pathways may also be down-regulated in primary xylem when sapwood was inoculated with H. parviporum. The destination of the carbon allocated in the shikimate pathway in these two tissues however, remains unanswered so far. Interestingly, another study,

analyzing spatial expression of the general phenylpropanoid and lignin/lignan pathway, provided support in carbon production for xylem tissue by phloem tissue (Emiliani et al., 2011). Opening the attractive thought of carbon processed through the shikimate pathway being transported towards the inoculation site to support spatial defense responses. However, this hypothesis needs further proof, e. g. by conducting spatial and temporal chemical analysis using e. g. a liquid-chromatography coupled with mass-spectrometry approach to determine which type of carbon accumulates in which tissue.

5.5 Contradictory regulation of the lignin/lignan pathway in natural infections

In the tissue comparison of inner and outer sapwood PaPAL1 was found to be up-regulated in naturally infected trees, but showing no significant differences in the healthy tree. This supports that up-regulation of the general phenylpropanoid pathway depends upon challenge with H. annosum s. l. and not on the tissue type.

However, pDIR2/32 was significantly up-regulated in both comparisons. This not in accordance to the results obtained in the controlled inoculation experiment for pDIR2/32. Besides, that our knowledge about the function of pDIR proteins is still very little, the contradicting outcome might have resulted from sampling itself. Inner sapwood of the healthy tree was sampled closely to the heartwood, whilst the corresponding sample of the infected tree was taken next to the reaction zone, thus the positions do not correspond exactly to each other. Intriguingly, Nabaka et al. (2008), determined that onset of death of metabolically ray cells in the sapwood corresponds to their distance, meaning the number of annual rings away, from the cambium and showed a species specifity. Inner sapwood samples of the healthy tree yielded much less total RNA than the corresponding samples from the infected tree (data not shown), provoking the thought of the two different sapwood samples not being comparable in their amount of metabolically active ray cells. Since defense response projected in gene expression can vary strongly even within less or highly susceptible genotypes (Danielsson et al., 2011), the presence of different genotypes probably also influenced the outcome. Thus a bias in the comparison for the healthy tree is not excluded and it is suggested that the natural infections should be reanalyzed, using controls for inner and outer sapwood from healthy parts within the same tree, to obtain a less biased and more accurate result.

5.6 Hormonal defense response and expression of defense related genes

5.6.1 Jasmonic acid signaling is involved in defense mechanisms against Heterobasidion parviporum

The expression patterns in the three different tissues show an up-regulation of LOX in sapwood when inoculation is compared to wounding. As LOX is involved in JA synthesis in angiosperms (Bell et al., 1995), this suggests that JA mediated signaling plays a crucial role in the response to necrotrophic pathogens, not only in angiosperms (Marla and Singh, 2012), but also in defense response of gymnosperms. This observation is also consistent with the reported up-regulation of LOX by Arnerup and colleagues (2011), who studied LOX expression in seven-year old full-sibs of Norway spruce inoculated with H. parviporum in a greenhouse experiment. Interestingly, expression of JAZ did not significantly change in the inoculation – wounding comparison in sapwood, as it would have been expected due to its function as a negative feedback-loop (Chini et al., 2007). Given that the JAZ-COI complex together inhibits JA response and thus carefully triggers JA accumulation (Pieterse et al., 2009), this might indicate a switch in JA signaling with a different output when P. abies encounters H. parviporum compared to wounding. We see no difference in the response of LOX in either bark or primary xylem when H. parviporum inoculation in sapwood is compared to wounding in sapwood.

However, JAZ was significantly down-regulated, although no difference between the two treatments was expected. A stronger JA signaling in bark upon H. parviporum treatment in sapwood compared to wounding sapwood, however is unlikely, due to the LOX expression pattern. Thus down-regulation of JAZ might result from reduced JA accumulation in both tissues, thus the negative feed-back loop with which JAZ together with the F-box protein COI confers JA sensitivity of the plant (Chini et al., 2007, Paschold et al., 2008) does not need recovery, leading to down-regulation of JAZ expression.

We also tested the expression of SPI1 and CHIIV, which both have been shown to be responsive to the presence and accumulation of JA (Germain et al., 2012; Pervieux et al., 2004; Yaqoob et al., 2012). Interestingly, neither SPI1 nor CHIIV seem to be specifically expressed due to H. parviporum challenge when compared to wounding, because none of the two genes showed any transcriptional regulation in the H. parviporum inoculation – wounding comparisons in any tissue. Germain and colleagues (2012) showed that the SPI1 promoter also strongly response to wounding, which could explain the result for SPI1.They thus are suggested to be unspecific responses of the tree that occur equally in wounding and in defense response against H. parviporum. However, in contrast to SPI1 the CHIIV expression profile in a sapwood comparison to bark and primary xylem suggests a highly spatial response of the CHIIV gene in fungal and wounding treatment only in sapwood, but not in bark or primary xylem.

5.6.2 Ethylene synthesis corresponds with JA synthesis

ACS and ACO participate in ET synthesis and were found to be rate-limiting in this process (reviewed by Lelièvre et al., 1997). Participation of ET synthesis in response to H. parviporum was shown by Arnerup et al. (2011; submitted).

This study showed that expression of ACO was less or equally expressed in all tissues when H. parviporum inoculation was compared with wounding treatment. However, ACS showed a strong up-regulation in inoculated sapwood compared to wound treatment. Both genes showed up-regulation in sapwood compared to bark, indicating a higher ET synthesis at the site of inoculation compared to the bark. The reason of the less significant change of ACO can most likely be explained by two reasons: first this project suggests an equal response of ACO to wounding and fungal inoculation, an assumption confirmed by Arnerup et al. (2011) who also showed that wounding and H. parviporum treatment result in similar responses of ACO, second Lelièvre and colleagues (1997) reviewed that in fruit riping, a process which also requires ethylene, ACO accumulation predates ACS expression, suggesting that a similar procedure might take place in pathogen response as well. The data obtained in this study thus suggests an up-regulation of ET synthesis at the inoculation site which is in correspondence with Arnerup et al. (submitted). The fact that ET and JA synthesis both occur more pronounced upon inoculation with H. parviporum might indicate that, as in angiosperms ET and JA pathways act indeed synergistically in Norway spruce. Results of two studies already indicated this behavior of two pathways when H. parviporum threatens tree seedlings (Arnerup et al., 2011; Arnerup et al., submitted) and seems to keep up also in longer infections of older trees.

However, the hormonal network is very complex and to elucidate its responses and interactions within, a transcriptome analysis would probably lead to a more complete picture about hormonal regulation in gymnosperm defense, due to its higher throughput.

6 Conclusion

This study analyzed expression of genes associated with secondary metabolism and hormonal signaling. It can be shown that gene expression of genes involved in secondary metabolite production, namely genes covering the general phenylpropanoid pathway, flavonoid and lignin/lignan synthesis, as well as ET and JA synthesis are highly induced at the inoculation site. This provides the knowledge that the defense mechanism of P. abies is independent of which tissue type encounters the attack of H. parviporum. Thus, not only sapwood inoculations, but also inoculation in bark gives valuable and informative knowledge of the defense response of P. abies against H. parviporum.

Due to our results, we hypothesize that the reaction zone formation acts as the sink of flavonoids and probably lignans and thus triggers up-regulation of general the phenylpropanoid, flavonoid and maybe lignin/ lignan pathways in sapwood adjacent to it. A carbon transport from neighboring tissues towards the site of inoculation is also possible, but cannot be fully proved by our data.

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9 Appendix

Table 9.1: Primer sequences and annealing temperature used for the primers needed for creation of new standard curves

Gene	Forward Primer	Reverse Primer	Annealing temperature [°C]
ACO[7]	5' TCACTGAAAAACACTCAGTCATT 3'	5' TCCGATCTGGTTGCTTCTT 3'	60.0
ACS[7]	5' TCCAACGGCCAGTGTTGTCA 3'	5' ACCAGAGCTCGTCGAAGGT 3'	60.0
ANR2[6]	5' ATGGAGAACCCCTCTGCATAC 3'	5' TCAAATTGGGTTGTCACATTGT 3'	60.0
CAD[5]	5' AGGATTTGCCAGCAGTATGGT 3'	5' TGCTTCATTGGGCTGTAAACT 3'	58.0
CHIIV [1]	5' GCGAGGGCAAGGGATTCTAC 3'	5' GTGGTGCCAAATCCAGAAA 3'	56.8
ELF1 alpha[5]	5' TGGCAAGGAACTGGAGAAGGAA 3'	5' TAGTCCCTCACAGCAAAACGA 3'	60.0
ELF4 alpha[4]	5' AGTAAGCCCGTGAGGATTC 3'	5' AGTCAGCCAGTCAACCTTTC 3'	60.0
LAR2[6]	5' TTAGGATAGCTGAGGCGAACTT 3'	5' TGTATTTCAGGTCGGGATAGAGCT 3'	60.0
LOX[7]	5'ACCCTTGGTATAGCCCTCATA 3'	5' ATCGTCACTCCATTCTCTCGT 3'	60.0
JAZ[7]	5' TACGATGTGCCTGCTGAGAA 3'	5' TGTTCGATCTTACCGCTGGT 3'	60.0
M13[9]	5' GTAAAACGACGGCCAG 3'	5' CAGGAAACAGCTATGAC 3'	55.0
PG mutase[3]	5' AATGCAGTTGAAGCCATTCC 3'	5' CCAGTGCCGAAACTCTCTTTC 3'	60.0
PaC4H3/5[2]	5' CGCCCCGAGCGATTCT 3'	5' CCCCACACCGAAGGGTAGA 3'	60.0
PaDAHP1[7]	5' AGATCCCCAAAGGATGGTTC 3'	5' TGAAATCCAAGTTCCACTGAA 3'	60.0
PaDAHP2[7]	5' TGCAACAGGAGGTTATGCT 3'	5' ATGAATCCCAGTGCCTCAT 3'	60.0
PaPAL1[2]	5' GGCAGATCATTTGGGTGATC 3'	5' TAAAGTTCCATTTTCAACTATAGGACTAAT 3'	60.0
pDIR2/32	5' AGCTGTGCAGTTGTGCTTTG 3'	5' GCTTCTTCCAGCTATGCCCA 3'	55.0
STS[8]	5' GTGGCGAGCAGAACACAGACTTC 3'	5' CAGCGATGGTACCTCCATGAACG 3'	65.0
SPI1	5' ATGGCGGACAAGGGAGTTG 3'	5' GCAGTTGTTCCTACTCGCGC 3'	60.0

Primer sequences obtained from [1] - Yaqoob et al., 2011; [2] – Koutaniemi et al., 2007; [3] - Vestman et al., 2010, [4] - Palovarra and Hakman, 2008, [5] – Arnerup et al., 2011 [6] - Danielsson et al., 2011, [7] – Arnerup et al., 2011, [8] – Hammerbacher et al., 2011, [9] – supplied by Invitrogen

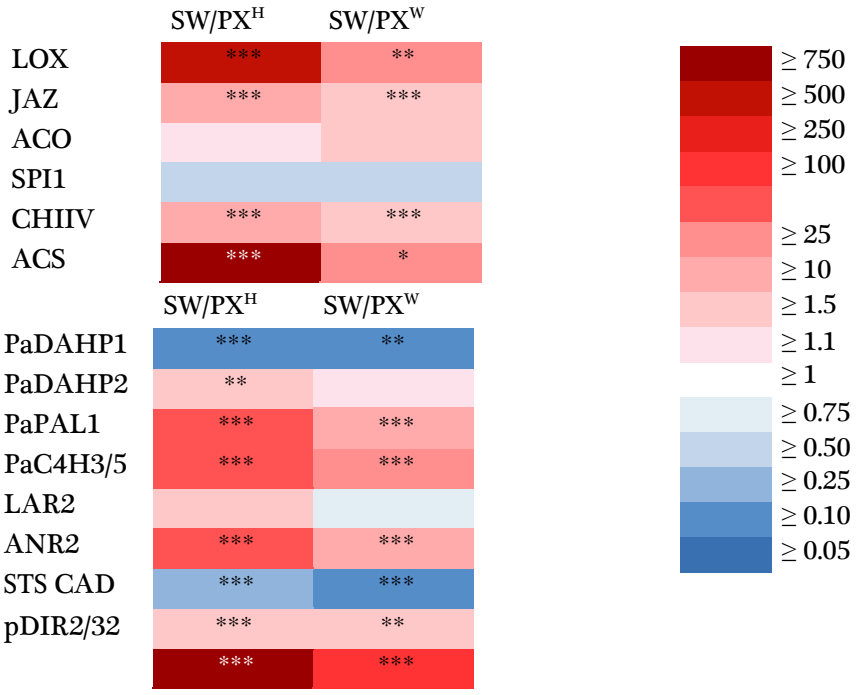

Figure 9.1 showing a heat map indicating relative expression values of various defense related genes when sapwood (SW) is compared to primary xylem (PX) in H. parviporum $^{(H)}$ and wounding $^{(W)}$ treatment