Molecular responses to *Heterobasidion annosum s.l.* in *Picea abies*

Miguel Nemesio Gorriz
Faculty of Forest Sciences
Department of Forest Mycology and Plant Pathology
Uppsala

Doctoral Thesis
Swedish University of Agricultural Sciences
Uppsala 2015

Acta Universitatis agriculturae Sueciae
2015:114

ISSN 1652-6880
ISBN (print version) 978-91-576-8426-4
ISBN (electronic version) 978-91-576-8427-1

Print: SLU Service, Uppsala 2015

Molecular responses to *Heterobasidion annosum s.l.* in *Picea abies*

Abstract

Norway spruce [*Picea abies* (L.) Karst.] is a main tree species in European forests and is important both ecologically and economically. The root rot fungus *Heterobasidion annosum sensu lato* (*s.l.*) is the main *P. abies* pathogen. Including resistance in breeding programs would help mitigating the impact of the pathogen but knowledge regarding defense mechanisms in *P. abies* needs a better understanding. The work within this thesis intended to expand the existing knowledge on *P. abies* resistance mechanisms focusing on hormone signaling, flavonoid biosynthesis and its transcriptional regulation. I found that jasmonic acid is the major hormone controlling defense signaling pathways in *P. abies.* Furthermore, we validated a candidate gene, *PaLAR3*, as a resistance marker for *H. annosum s.l.* in *P. abies*. *PaLAR3* encodes an enzyme responsible for the synthesis of (+) catechin, which showed a fungistatic effect on *H. parviporum*. Analysis of genetic diversity revealed two allelic lineages of *PaLAR3* that showed significant differences in fungal resistance and (+) catechin content that were explained by dissimilarities in inducibility. We studied the role of PaNAC03, a transcription factor that is associated with *H. annosum s.l.* infection. PaNAC03 not only showed repression of multiple genes including *PaLAR3,* but bound only to the promoter of one of the *PaLAR3* allelic lineages explaining at least partly the differences in allelic expression that were observed. Finally, we identified a full repertoire of members of a MYB/bHLH/WDR transcription factor complex in Norway spruce, which showed differences in protein interactions and expression patterns, and also in ability to control the expression of genes in the flavonoid biosynthetic pathway including *PaLAR3*.

Keywords: Picea abies, *Heterobasidion*, defense, phytohormones, flavonoid, catechin, LAR, MYB, NAC, promoter

Author's address: Miguel Nemesio-Gorriz, SLU, Department of Forest Mycology and Plant Pathology, P.O. Box 7026, SE-750 07 Uppsala, Sweden.
E-mail: miguel.nemesiogorriz@slu.se

Dedication

To my grandparents Amparín, Benito, Enrique and María.

Contents

List of Publications

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:

I Arnerup J., **Nemesio-Gorriz M.**, Lundén K., Asiebgu F., Stenlid J. and Elfstrand M. (2013). The primary module in Norway spruce defense signaling against *H. annosum s.l.* seems to be jasmonate-mediated signaling without antagonism of salicylate-mediated signaling. *Planta*, 237/4, pp. 1037-1045.

II **Nemesio-Gorriz M.**, Hammerbacher A., Ihrmark K., Källman T., Olson Å., Lascoux M., Stenlid J., Gershenzon J. and Elfstrand M. (2015). Different alleles of a gene encoding leucoanthocyanidin reductase (LAR3) influence resistance against the fungus *Heterobasidion parviporum* in *Picea abies* (submitted).

III **Nemesio-Gorriz M.**, Blair P.B., Dalman K., Hammerbacher A., Arnerup J., Stenlid J., Muhktar S.M. and Elfstrand M. Identification of Norway spruce WDR and bHLH proteins forming complexes with MYB transcription factors that regulate gene expression in the flavonoid pathway (manuscript).

IV Dalman K., Wind J., **Nemesio-Gorriz M.**, Hammerbacher A., Lundén K., Ezcurra I. and Elfstrand M. Overexpression of *PaNAC03*, a stress induced NAC gene family transcription factor in Norway spruce leads to reduced flavonol biosynthesis (manuscript).

Additional publications that are not part of this thesis

Lundén, K., Danielsson M., Brandström Durling M., Ihrmark, K. **Nemesio-Gorriz M.**, Stenlid J., Asiegbu F.O. and Elfstrand M. (2015). Transcriptional Responses Associated with Virulence and Defense in the Interaction between *Heterobasidion annosum s.s.* and Norway Spruce. PloS ONE, 10, e0131182.

Oliva J., Rommel S., Fossdal C.G., Hietala A.M., **Nemesio-Gorriz M.**, Solheim H. and M. Elfstrand (2015). Transcriptional responses of Norway spruce (*Picea abies*) inner sapwood against *Heterobasidion parviporum*. Tree Physiology, 35**,** pp. 1007-1015.

The contribution of Miguel Nemesio-Gorriz to the papers included in this thesis was as follows:

I He carried out the *in vitro* seedling treatments, extracted RNA and tested candidate genes with qPCR, performed data analysis on expression data and wrote the corresponding parts of the manuscript.

II He planned the experimental design, performed the phenotyping of the plants, extracted RNA and measured allelic expression levels with qPCR, performed the sequence assembly and phylogenetic analyses, analyzed the genetic differences between allelic forms, compared the genetic diversity with *Picea glauca* and wrote the manuscript with comments and suggestions from co-authors.

III He identified and isolated the candidate genes, put them into destination vectors, performed phylogenetic analyses of the candidates, measured expression levels in different tissues with qPCR and analyzed expression data, took part in the transformation of the Norway spruce transgenic cell lines, extracted RNA from them to test expression of candidate genes and wrote the manuscript in cooperation with the co-authors.

IV He participated in designing the experiments. He performed the biochemical analyses of the *PaNAC03* transformant lines and contributed to the transcriptomic data analyses. He sequenced the promoter region of *PaLAR3A* and *PaLAR3B*. He wrote the corresponding parts of the manuscript.

Abbreviations

AIP	2-amino-indan-2-phosphonic acid
ANR	Anthocyanin reductase
bHLH	Basic helix-loop-helix protein structural motif that characterizes a family of transcription factors
BLAST	Best linear alignment search tool
cDNA	Complementary deoxyribonucleic acid
CHS	Chalcone synthase
DIECA	diethyldithiocarbamic acid
dpi	Days post inoculation
EBG	Early biosynthetic gene
ET	Ethylene
FGS	Fungal growth in sapwood
GF	Growth factor
GUS	Beta-glucuronidase reporter gene
JA	Jasmonic acid
LAR	Leucoanthocyanidin reductase
LBG	Late biosynthetic gene
MAMP	Microbe-associated molecular patterns
MeJA	Methyl jasmonate
MeSA	Methyl salicylate
mRNA	Messenger ribonucleic acid
MYB	Transcription factor family characterized by the common amino acid motif "MYB"
NAC	Protein family composed by the transcription factor types NAM, ATAF and CUC
PA	Proanthocyanidin
PAMP	Pathogen-associated molecular patterns
PCD	Programmed cell death
PCR	Polymerase chain reaction
PP	Polyphenolic parenchyma
PR	Pathogenesis-related
qPCR	Quantitative polymerase chain reaction
QTL	Quantitative trait locus
ROS	Reactive oxygen species
RZ	Reaction zone

s.l.	*Sensu lato* = in the broad sense
s.s.	*Sensu stricto* = in the strict sense
SA	Salicylic acid
SNP	Single-nucleotide polymorphism
STS	Silver-tiosulfate
TF	Transcription factor
WDR	Transcription factor family characterized by the retetition of amino acid blocks that end with a WD motif
WRKY	Transcription factor family characterized by the common amino acid motif "WRKY"
YEP	Yeast extract peptone
EST	Expressed sequence tag
eQTL	expression QTL

1 Introduction

Forests are the dominant terrestrial ecosystem covering about a 30% of the world's land area and accumulating 80% of the total plant biomass on Earth (http://www.skogsstyrelsen.se). Forests host biodiversity and provide human societies resources and leisure. Most of the land area in Sweden is covered by forest (57%). Forests amount to about 3% of Sweden's Gross Domestic Product and provide most of the employment in sparsely populated areas. Forest products accounted for 12% of the total Swedish exports and 18% of the country's energy came from forest-based biofuels already in 2008 (http://www.nordicforestry.org/facts/Sweden.asp). Conifer forests are the dominant forest type in the country (82%), primarily represented by Scots pine (*Pinus sylvestris*) and Norway spruce [*Picea abies* (L.) Karst.]. Both conifers have long rotation periods (65-110 years) and during their lifetime they interact with other plants, animals and particularly fungi. Fungi play a crucial role in forest ecosystems by establishing symbiotic mycorrhizal interactions and decomposing dead matter (Waksman, 1931). Some fungi, like *Heterobasidion annosum sensu lato* (*s.l.*), establish pathogenic interactions and attack trees causing damage and economical losses (Woodward, 1998).

1.1 The Heterobasidion-conifer pathosystem

Among the forest pathogens, species of the *H. annosum s.l.* have become economically the most devastating forest pathogen in Sweden. This is partly due to the fungus being a facultative necrotroph, which means that *H. annosum s.l.* not only can live as a necrotroph killing host tissue for feeding, but also as a saprotroph feeding on dead wood by breaking down lignin and cellulose (Olson *et al.*, 2012). Forest management during the 20th century, with intensification of all-year-round harvesting, has favored *H. annosum s.l.* proliferation due to the capacity of this pathogen to colonize freshly cut stumps

and spread through the roots to neighboring living trees (Figure 1) (Woodward, 1998). In Sweden, 15-20 % of the Norway spruce trees, ready for harvesting, are infected by *H. annosum s.l.* (Thor *et al.*, 2005; Thor & Stenlid, 1998). *H. annosum s.l.* attack causes root and stem rot, increasing the probability of windsnap and decreasing tree growth and forest value. The estimated losses due to this pathogen are in the order of €790 million annually for the European forest industry (Woodward, 1998).

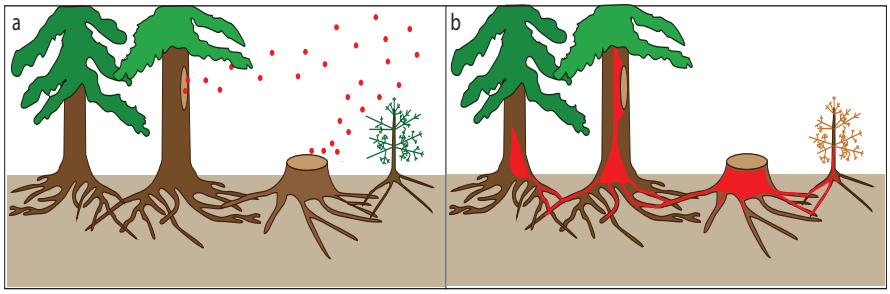

Figure 1. a) Colonization of a fresh stump and wound by *H. annosum s.l.* spores. b) Spread of the pathogen to neighboring trees.

Even though, since 1992, Swedish forest management has implemented stump treatment to prevent *H. annosum s.l.* establishment on fresh thinning stumps, *H. annosum s.l.* remains as a major concern for forest owners (Thor et al 2005). The stump treatment does not eradicate the already established rot in the root systems and the option of stump removal is not viable (Vasaitis *et al.*, 2008). In light of this, a more resistant plant material would be able to counteract the spread of the fungus.

1.1.1 The Heterobasidion annosum sensu lato species complex

Originally considered one single taxa, the *H. annosum s.l.* species complex is now known to be composed by five species that infect at least 200 different species in 31 genera plant taxa, the majority of which are conifers (Korhonen & Stenlid, 1998). The species within the complex show different host preference being Norway spruce the main host of *H. parviporum,* and Scots pine and silver fir the main hosts of *H. annosum sensu stricto* (*s.s.*) and *H. abietinum,* respectively (Niemelä & Korhonen, 1998). The two American species, *H. irregulare* and *H. occidentale,* also show host preference (Garbelotto *et al.*, 1996). All five species can be classified in two clades. While *H. annosum s.s.* and *H. irregulare* belong to one pine-infecting clade, *H. occidentale*, *H. parviporum,* and *H. abietinum* form a pine-non-infecting clade (Figure 2) (Dalman *et al.*, 2010).

One of the main aspects considered in the *H. annosum* genome project (Olson *et al.*, 2012) was the pathogenicity mechanisms that allow the pathogen to infect its hosts. Among them, fomannosin biosynthesis, carbohydrate degradation, membrane transporters and oxidative stress creation were the most remarkable. Fomannosin is a toxin that causes drooping, water soaking and browning of needles on loblolly pine needles (Kuhlman, 1969). Carbohydrate degradation helps degrading host cell wall and coverts it into nutrients that allow further growth of the pathogen. Transmembrane transportation allows the pathogen not only to secrete small molecules, toxins, and peptides, but also to take up nutrients. Finally, production of reactive oxygen species (ROS) contributes to the host-mediated oxidative stress and facilitates infection. Knowing how *H. annosum s.l.* attacks its host, is essential to understand the host´s defense reactions.

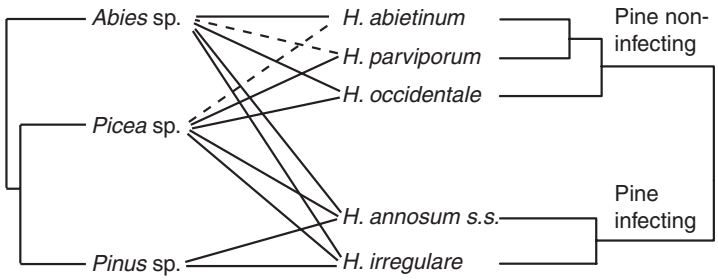

Figure 2. Illustration of incongruence between the phylogeny of *Heterobasidion annosum* species complex and the host genus. Lines indicate preferred hosts and dashed lines rare hosts (Dalman *et al.,* 2010).

1.1.2 Importance of conifers and Norway spruce

Conifers are one of the 12 divisions of the plant kingdom and include eight families, 68 genera, and 630 living species. They are the dominant plants over large areas, most notably the boreal forests of the northern hemisphere, which are the main type of forest in Sweden. Norway spruce is one of the approximately 35 species of the genus *Picea* (Farjon, 1990). It grows in boreal and montane conifer-dominated forests and is native to the European Alps, the Balkans, the Carpathians and its range extends north to Scandinavia and merging with Siberian spruce (*Picea obovata*) in northern Russia. Norway spruce is one of the most widely planted spruce species, both in and outside of its native range including North America (www.fs.fed.us/database/feis/), and is one of the most economically important coniferous species in Europe.

In Sweden, Norway spruce represents 40% of the standing volume and its wood is used for producing timber, paper, pulp and firewood (http://www.skogsstyrelsen.se). Around half of planted Norway spruce plants

originate from seed orchards that come from breeding programs, which have focused primarily on increasing wood production while maintaining or increasing wood quality and adaptation to climate. Even though *H. annosum s.l.* causes large economic losses, resistance to *H. annosum s.l.* has not yet been included as a trait for selection in forest breeding programs. Thus far, changes in forest management have been the main action taken to mitigate the effect of the pathogen in the forests, while the host's natural ability to produce trees with a higher level of resistance has not yet been exploited. This is not due to a lack of knowledge on resistance, but to the absence of tools that allow knowledge to be implemented into breeding strategies. Development of reliable resistance markers that facilitate early selection of trees with increased resistance is one of the ways in which resistance to *H. annosum s.l.* could be included in breeding programs.

1.1.3 Conifer genomes and resistance

The Norway spruce genome was published in 2013 (Nystedt *et al.*, 2013), being the first conifer genome ever published. The publication was followed by the white spruce genome (Birol *et al.*, 2013) and the loblolly pine genome (Neale *et al.*, 2014). The main difference between the three conifer genomes and most other plant genomes, is that they are much larger (Figure 3) despite having a similar number of genes. Conifers have one of the largest genomes known in diploid plants (Figure 3), which is mainly due to the accumulation of a diverse set of long-terminal repeat transposable elements. The two other remarkable characteristics are the presence of large introns (up to >10.000 bp) in some of the genes and a high amount of both small and very long non-coding mRNAs.

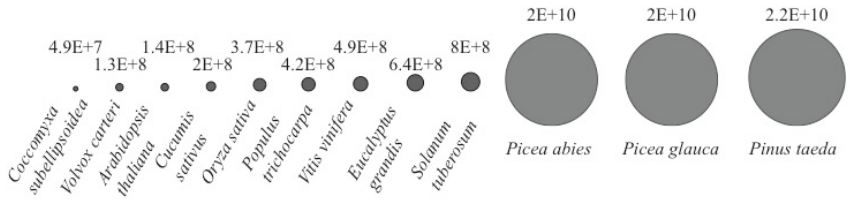

Figure 3. Genome size in different plant species. Circle areas are equivalent to the genome size in base pairs.

Resistance aspects were included in the loblolly pine genome article (Neale *et al.*, 2014), which mentioned the existence of several gene families containing multiple proteins with R-protein domains that points at the capacity of conifers to recognize potential pathogens. Another example is the updated

version of the white spruce genome, which was centered in terpenoid and phenolic defense metabolism (Warren *et al.*, 2015). Terpenoids and phenolics are specialized metabolites that have been broadly studied in conifers due to their involvement in defense against pests and pathogens (Hammerbacher *et al.*, 2014; Danielsson *et al.*, 2011; Zulak & Bohlmann, 2010; Phillips & Croteau, 1999; Brignolas *et al.*, 1995; Woodward & Pearce, 1988).

1.2 Defense mechanisms in conifers

Even though conifers face numerous challenges, they count with defense systems that allow them to respond and defend themselves. These defense systems can be divided into constitutive and induced defense mechanisms that allow conifers to defend themselves againts biotic threats (Figure 4). Defense responses in conifers are similar between different tissues (Oliva *et al.*, 2015) and have been suggested to be organ-specific (Adomas & Asiegbu, 2006). Also, these responses seem to grow in intensity depending on the stressor being mechanical wounding, a saprotroph like *Phlebiopsis gigantea,* or a necrotroph like *H. annosum s.l.,* (Arnerup *et al.*, 2011).

1.2.1 Constitutive and induced defense in conifers

Conifers have bark, a natural barrier that protects them against abiotic agents but also against insect, herbivore and pathogen attacks. Bark contains terpenoids, suberins and phenolics that are repelant or toxic to insects and fungal pathogens acting as a mechanical and chemical defense barrier (Franceschi *et al.*, 2005). Conifer needles that are not protected by the bark have a cuticle that also act as a natural barrier.

Resin is a viscous and odoriferous liquid that is part of the constitutive defense of conifers and it accumulates in conifers in different structures depending on the species (Fahn, 1988). Resin contributes to conifer defense by chemically disguising the host, adding toxins and altering the levels of pheromone precursors, which are attractants for predators or hormone mimics to disrupt insect development (Phillips & Croteau, 1999).

In the secondary phloem, polyphenolic parenchyma (PP) cells accumulate phenolics in their vacuoles and form the main constitutive defense structure (Franceschi *et al.*, 1998). PP cells can also contain calcium oxalate crystals (Hudgins *et al.*, 2003) and every year a row of this type of cells differenciates from the cambium to form a new layer. Even though most cells die as sapwood gets older, PP cells remain alive in the sapwood. 70-year old PP cells were found alive in a 100 year-old Norway spruce tree (Krekling *et al.*, 2000) pointing at the important role that these cells may play in defense in sapwood.

Another cell type that is relevant in constitutive defense are the stone cells. They are highly lignified cells that are located in the inner tissues and in the bark, and have been related to resistance to fungi and insects (Franceschi *et al.*, 2005; Wainhouse & Ashburner, 1996; Wainhouse *et al.*, 1990).

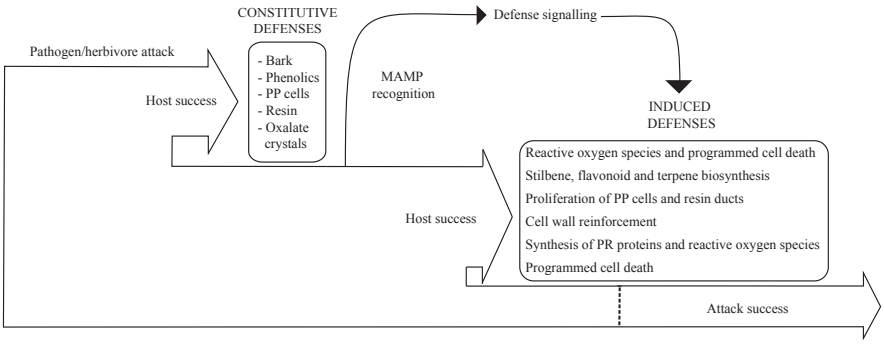

Figure 4. Schema of the constitutive and induced defense responses in conifers against an attack.

When primary barriers are breached, conifers dispose of mechanisms that activate inducible defenses. This recognition happens via specific detection of microbe-associated molecular patterns (MAMPs) like chitin or chitin fragments from the fungal cell wall (Salzer *et al.*, 1997). After the detection, studies suggest that a signal cascade is activated in conifers where hormone signaling plays a major role and this response is similar for either insect (Miller *et al.*, 2005) or fungal attack (Arnerup *et al.*, 2011). One of the effects of the activation of defense signaling pathways, is a transcriptional reprogramming followed by an induction of the phenylpropanoid biosynthetic pathway (Warren *et al.*, 2015), which leads to the production of specialized metabolites (SMs) of the stilbene and flavonoid families (Arnerup *et al.*, 2011). Structural changes related to the biosynthesis of specialized metabolites also occur , e.g., reinforcement of the cell wall (Franceschi *et al.*, 2000) and a profliferation of PP cells and traumatic resin (Nagy *et al.*, 2005; Nagy *et al.*, 2000). On the other hand, there is an induction of pathogen-related (PR) proteins (Jøhnk *et al.*, 2005; Liu & Ekramoddoullah, 2004; Nagy *et al.*, 2004; Asiegbu *et al.*, 2003; Ekramoddoullah & Hunt, 2002), defense-related genes (Yaqoob *et al.*, 2012; Arnerup *et al.*, 2011; Adomas *et al.*, 2007; Ralph *et al.*, 2006), and reactive oxygen species (ROS) (Ralph *et al.*, 2006; Fossdal *et al.*, 2001), which can trigger a hypersensitive response leading to programmed cell death (PCD) (Jones, 2001).

1.2.2 Phytohormone signaling in defense

The three phytohormones that are primarily involved in plant defense are salicylic acid (SA), regulating defense response against biotrophs, and jasmonic acid (JA) and ethylene (ET), which regulate defense response against necrotrophs and herbivores (Kunkel & Brooks, 2002). Even though ET is a very multifaceted hormone that is connected with defense, it is also involved in growth and development, and senescence (Chang & Bleecker, 2004). The two signaling pathways, SA and JA/ET, are partially antagonistic in angiosperms (Glazebrook, 2005). There are other hormones that are better known for their roles in stress tolerance and development (abscisic acid, brassinosteroids, cytokinins, gibberellins, strigolactones and auxins) that can also play a role in defense together with SA, JA and ET or independently (Robert-Seilaniantz *et al.*, 2011). Plants rely on complex phytohormone signaling through crosstalk between different phytohormones in order to activate defense responses against pathogens, while pathogens have evolved different strategies to manipulate host defense signaling for their benefit (Kazan & Lyons, 2014). For this reason, interactions vary greatly among pathosystems and it is difficult to draw general conclussions about how phytohormones regulate plant defense.

Genes involved in the biosynthesis or response to the different phytohormones have been well-studied in plants. Measuring their expression levels gives information on when particular phytohormones are being synthesized or activated. Among these, PR1 is a salicylic acid-responsive protein that has been long known (Ohshima *et al.*, 1990), *LURP1* is a gene that is induced directly by salicylic acid and it encodes a protein that acts as *PR1* regulon (Knoth & Eulgem, 2008). LOX genes encode enzymes that are involved in the biosynthesis of JA from lipid acids (Creelman & Mullet, 1997). While JAZ genes are induced by JA and repress its biosynthesis as part of a feed-back loop (Chini *et al.*, 2007), *MYC* genes encode transcription factors (TFs) that are responsive to JA (Boter *et al.*, 2004). *ACS* and *ACO* genes are involved in ethylene biosynthesis (Wang *et al.*, 2002) and ERF1 is an ethylene-responsive transcription factor (Lorenzo *et al.*, 2003).

In conifers, SA has been shown to induce PR proteins in *Pinus elliottii* seedlings (Davis *et al.*, 2002) and SA has been shown to accumulate in Norway spruce seedlings after inoculation with *H. annosum s.l.* (Likar & Regvar, 2008). However, JA has been pointed out as the main hormone controlling defense signaling in conifers (Franceschi *et al.*, 2002) and is often used as a treatment to induce defense reponses (Hudgins *et al.*, 2004). However, little is known about crosstalk in hormone signaling in conifers apart from the synergistic activity of JA and ET (Hudgins & Franceschi, 2004). One

of the objectives of this thesis was to elucidate if there is any crosstalk between SA and JA/ET in Norway spruce.

1.2.3 Norway spruce defense against *Heterobasision annosum s.l.*

H. annosum s.l. infection in forests happens via root contact of living Norway spruce plants through infected roots from neighbouring trees or stumps. After infection, the fungus will typically be growing saprotrophically in the heartwood of the tree, which lacks living cells, avoiding host induced defense responses. When *H. annosum s.l.* reaches the sapwood, cell wall degrading activity and MAMPs activate defense responses. The fungus will then switch from saprotrophic to necrotrophic growth to be able to deal with host defense responses and to access the carbon of the living tissues and reach the outside of the tree to form fruit bodies. Most trees develop a reaction zone (RZ), which is a non-specific response in the inner part of the sapwood that is in contact with the pathogen. In Norway spruce, the RZ has a high pH (8.0) and high levels of phenols (Shain & Hillis, 1971) in order to compartimentalize the pathogen and block its growth (Figure 5a). There is, however, a metabolic cost for RZ formation. Infected trees that have developed a RZ are more efficient in controlling the fungal growth but show a lower yearly growth increment than infected trees that have not (Oliva *et al.*, 2010).

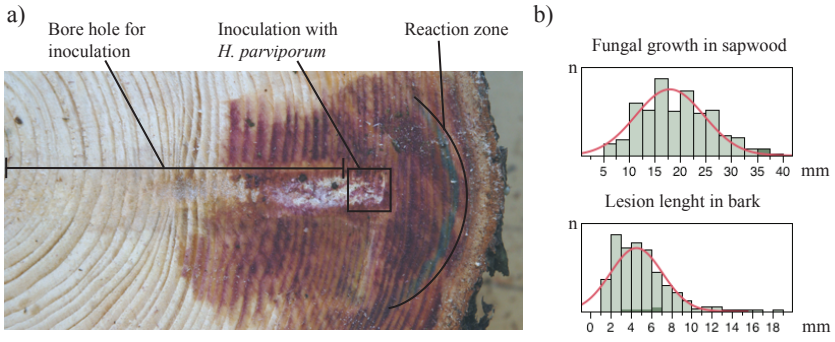

Figure 5. a) Picture by Dr. Carl-Gunnar Fossdal of a reaction zone in a Norway spruce stem inoculated with *H. parviporum* b) Distribution of FGS and LLB in a population of 717 individuals that were phenotyped during this thesis.

Susceptibility of Norway spruce to *H. annosum s.l.* growth is a quantitative trait (Arnerup *et al.*, 2010; Swedjemark *et al.*, 1997), meaning that it is a measurable phenotype that depends on the cumulative actions of multiple genes. Host resistance can be phenotyped in different ways depending on the type of plant material that is being used, but the most common are to measure

fungal growth in sapwood (FGS) or lesion length in bark (LLB) after inoculation with the fungus (Figure 5b). Finally, in adult trees the severity of stem rot can be estimated by measuring differences in conductance in the stem caused by the rot. Alternatively, by felling trees, the condition of the stems regarding the rot can be observed.

The interaction of Norway spruce and *H. annosum s.l* appears to lead to a non-specific defense response to the pathogen (Arnerup *et al.*, 2011). Differences in resistance can be seen among individuals (Arnerup *et al.*, 2010; Swedjemark & Karlsson, 2004; Swedjemark *et al.*, 1997). Differences in transcriptional regulation of the defense response and chemical profile determine the resistance level of an individual (Arnerup *et al.*, 2011; Danielsson *et al.*, 2011). These differences have a genetic background that can be determined by conducting genetic association studies (Lind *et al.*, 2014), but results of these studies need to be validated to confirm the effect in resistance of particular genetic components. On the other hand, epigenetic factors might also play a role in resistance as it has been shown for biotic stress in other conifers (Vivas *et al.*, 2013).

1.3 Specialized metabolites and conifer resistance

1.3.1 General overview

Specialized metabolites (SMs) are one of the main components of conifer defense. Insect and pathogen attacks induce transcriptional reprogramming in the specialized metabolism of conifers, especially in the flavonoid, terpenoid and stilbenoid biosynthetic pathways causing an increase in these compounds.
Terpenes are the main SMs in the conifer resin. In Norway spruce, as in other conifers, resin is composed 95% by mono- and diterpenes, which are volatile, in aproximately equal poportions and a smaller ammount of non-volatile sesquiterpenes (Martin *et al.*, 2002). Due to the volatility of monoterpenes and sequiterpenes, their individual effect is complicated to study. Terpenes are still important in defense against insects (Bohlmann, 2012; Zulak & Bohlmann, 2010) and also against fungi and bacteria (Himejima *et al.*, 1992). Terpenes' antimicrobial activity is partly based on their capacity to cause perturbation in the lipid fraction of the plasma membrane (Trombetta *et al.*, 2005).

Stilbenes inhibit fungal growth by interfering with microtubule assembly (Adrian *et al.*, 1997; Woods *et al.*, 1995), disrupting plasma membranes and uncoupling electron transport in fungal spores and germ tubes (Adrian & Jeandet, 2012; Pont & Pezet, 1990). A study in Austrian pine showed specific stilbenes that were negatively correlated with disease susceptibility (Wallis *et al.*, 2008). However, some pathogens have evolved mechanisms to detoxify

stilbenes that are toxic to them (Hammerbacher *et al.*, 2013; Woodward & Pearce, 1988).

Flavonoids represent a large family of SMs in plants. Even though they fulfill a wide range of functions in plants, including coloration, symbiosis signaling, physiological regulation and UV filtration, they also have antimicrobial (Cushnie & Lamb, 2005) and insecticide properties (Salunke *et al.*, 2005; Upasani *et al.*, 2003). In the particular case of conifers, different flavonoids from Norway spruce seem to have an antimicrobial effect on *E. polonica* (Hammerbacher *et al.*, 2014; Lieutier *et al.*, 2003; Brignolas *et al.*, 1998; Brignolas *et al.*, 1995), *H. annosum s.l.* (Danielsson *et al.*, 2011) and *Cylindrocarpon destructans* (Tomova *et al.*, 2005). Furthermore, Wang *et al.* (2015b) used a flavonoid extracted from conifers and showed its insecticide properties against the Colorado potato beetle, which points at their potential role in defense against other conifers pests. Moreover, flavonoids and, in particular, (+) catechin constituted one of the main differences between Norway spruce trees with different levels of resistance (Danielsson *et al.*, 2011). Finally, flavonoids are also the precursors of the proanthocyanidins, which are oligomerized flavonoids that play an important role in defense.

1.3.2 Biosysnthesis of specialized metabolites

SMs are synthesized in plants via several biosynthetic pathways in different cell compartments in pant cells. While mono- and diterpenoid biosynthesis takes place in the chloroplast, sesquiterpenes, stilbenes and flavonoids are synthesized in the cytoplasm. Figure 6 provides an overview of these pathways showing the most relevant enzymatic steps and compounds.

The study of the genes that encode the enzymes in these biosynthetic pathways is not an easy task. Conifers have, in many cases, gene families that encode isoenzymes that catalyze the reaction of the same metabolic step in different environments. This has been observed for the leucoanthocyanidin reductase (LAR) gene family in Norway spruce, where four genes were identified (Hammerbacher *et al.*, 2014). Spruce RNA libraries (Warren *et al.*, 2015; Nystedt *et al.*, 2013; Rigault *et al.*, 2011; Wang *et al.*, 2000) and specially the open access to conifer genome databases (www.congenie.org) facilitate the identification of candidate genes.

The flavonoid biosynthetic pathway is common to a wide range of plant lineages (Tohge *et al.*, 2013) and the genes encoding enzymes within the pathway are divided in two groups, early biosynthetic genes (EBGs) and late biosynthetic genes (LBGs) (Petroni & Tonelli, 2011). EBGs are *CHS, CHI, F3H, F3'H/F3'5'H/F5'H* and *FLS*. They lead to the synthesis of common precursors (tetrahydroxychalcone, naringenin, flavanones, dihydroflavonols

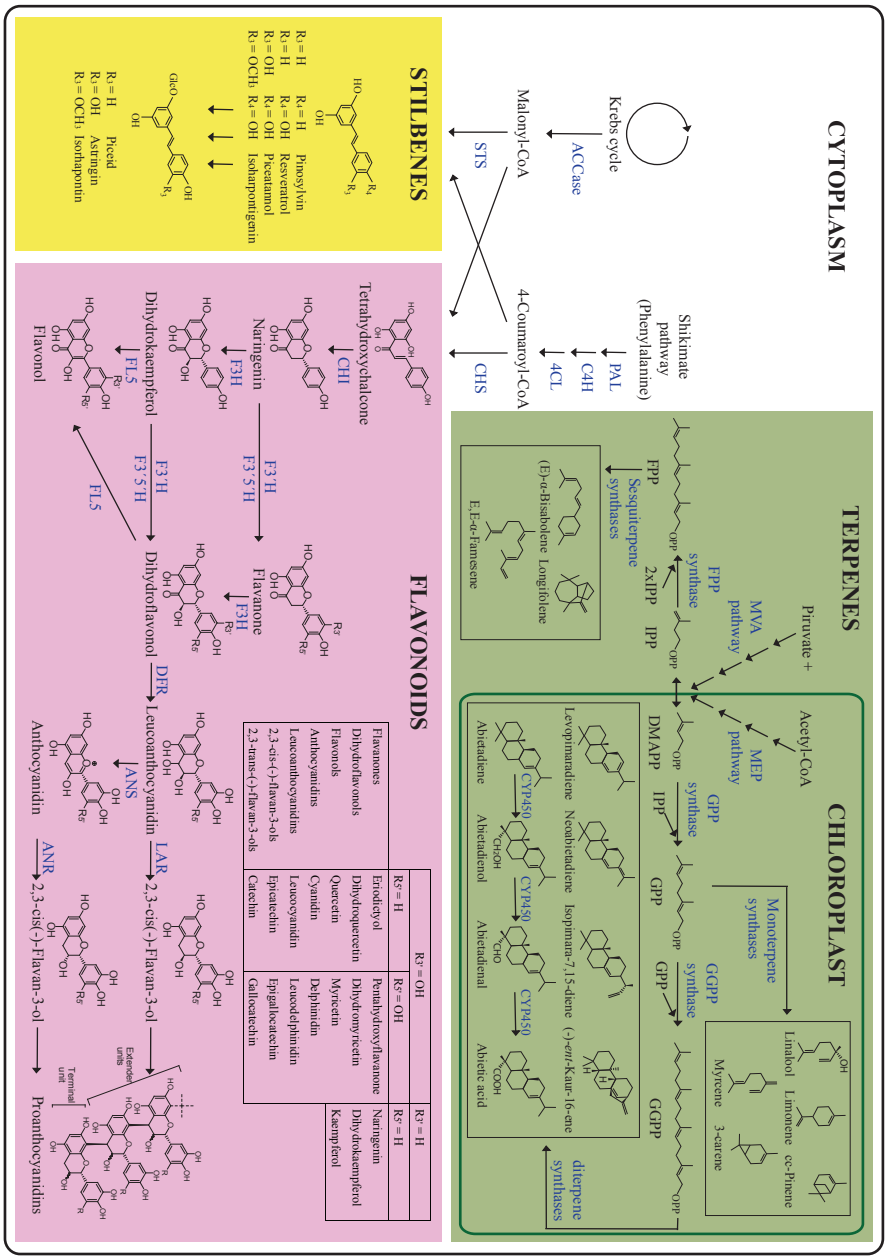

Figure 6. Terpene (green), stilbene (yellow) and flavonoid (pink) biosynthetic pathways in conifers. Information extracted from (Zulak & Bohlmann, 2010) and contribution from Dr. Almuth Hammerbacher.

and flavonols), while the late biosynthetic genes (LBGs), *DFR, ANS, ANR* and *LAR* are located downstream in the biosynthetic pathway and lead to the synthesis of flavonoids belonging to specific families (anthocyanidins, leucoanthocyanidins and flavan-3-ols) (Figure 6). One focus of this thesis is the specific role of flavonoids and their transcriptional regulation in defense against *H. annosum s.l.* in Norway spruce. The work in this thesis covers the role of a LBG, *PALAR3*, in defense against the pathogen *H. parviporum* in Norway spruce.

1.3.3 Transcriptional regulation of flavonoid biosynthesis

Biosynthesis of SMs has a high metabolic cost for the plant (Gershenzon, 1994). Because of this, it is essential for the plant to activate the biosynthesis of these compounds only when and where it is needed. In a large family of SMs, like the flavonoids, where specific subgroups of compounds fulfill different functions in plants, a finely tuned and complex regulation system is required. This regulation is controlled by transcription factors (TFs), which are proteins that are able to bind to specific DNA sequences in the promoter regions of the genes that they regulate, thereby controlling the rate of transcription of genetic information from DNA to messenger RNA.

Transcriptional regulation of flavonoid biosynthesis in angiosperms is controlled by TFs belonging to different gene families, among them WRKYs, NACs, bHLHs, MYBs, HD-ZIPs and WDRs (Xu *et al.*, 2015; Ré *et al.*, 2012), which act either as activators or repressors of their target genes. The division of the genes in the flavonoid biosynthetic pathway in EBGs and LBGs is not only based on the type of compounds that are produced by the enzymes encoded by them, but also on the type of regulation that each type of genes have. While EBG expression is controlled by individual R2-R3-MYB TFs, LBG expression is regulated by MYB-bHLH-WDR (MBW) TF complex (Petroni & Tonelli, 2011). All these TFs can be activated in response to environmental signals like light, temperature or stress (Petrussa *et al.*, 2013). Many of the TFs controlling flavonoid biosynthesis have been identified and characterized due to the fact that plant mutants that are defective for them often show changes in pigmentation due to the lack of flavonoids (Lepiniec *et al.*, 2006).

In conifers, Duval *et al.* (2014) presented a large number of TFs from different families including MYBs, NACs and WRKYs and predicted their effect on the regulation of several biosynthetic pathways in *P. glauca*. Lundén *et al.* (2015) reported a bHLH with homology to TT8, an *A. thaliana* TF that controls flavonoid biosynthesis (Nesi *et al.*, 2000). MYB TFs have been relatively well studied regarding their regulatory role on the biosynthesis of

different families of specialized metabolites in conifers. Xue *et al.* (2003) identified ten black spruce R2R3-MYBs. One of them, *MBF1*, induced pigment accumulation during transient overexpression in maize cell lines and showed transactivation of the anthocyanidin-related *Bz2* promoter in spruce and larch cell lines. Bedon *et al.* (2007) further identified 18 R2R3-MYBs in white spruce and loblolly pine and determined their tissue specificity hypothesizing about the role of some of them in lignin biosynthesis. This role was confirmed by Bomal *et al.* (2008). White spruce lines over expressing two of the pine R2R3-MYB TFs showed increased lignin accumulation in cell walls and induction of genes in the shikimate and monolignol biosynthetic pathways. In a different study, another of the R2R3-MYB TFs, PgMYB14, was shown to induce terpene and flavonoid accumulation in transgenic lines overexpressing this TF (Bedon et al. 2010). Like in *Arabidopsis,* there appears to be a high degree of complexity associated with the R2R3-MYB component of the transcription complex in conifers, resulting in specific transcriptional responses. Bomal *et al.* (2014) analyzed the expression of miss-regulated genes in the transgenic lines studied by Bomal *et al.* (2008) and Bedon *et al.* (2010). They identified a total of 70 genes that were up- or downregulated depending on which R2R3-MYB TF was being overexpressed, pointing at the opposite action of closely related R2R3-MYB TFs in conifers.

In *A. thaliana*, MYBs regulate LGBs after forming a MBW ternary complex with bHLH and WDR proteins. The modularity of the MBW complex allows different bHLHs to interact with several MYBs and one single WDR, thereby determining the activation of different genes in the flavonoid pathway in different environments and allowing a fine-tuned control of the expression of the target genes (Xu *et al.*, 2015). The knowledge on MYB TFs contrasts with the lack of information regarding bHLH and WD40 members of the MBW complex in conifers. Both Xue *et al.* (2003) and Bedon *et al.* (2010) identified bHLH-binding motifs in the R2R3-MYB TFs that they isolated and hypothesized about the relevance of this protein interaction for the completion of their regulatory roles. The bHLH in Norway spruce with similarity to TT8 reported by Lundén *et al.* (2015) is the only published reference of a bHLH in conifers and no WDR genes have been identified so far. The work in this thesis tries to cover this gap in knowledge by identifying and characterizing the members of a MBW complex in Norway spruce.

Another of the largest TF family in plants, NAC [for NAM (no apical meristem), ATAF (Arabidopsis *transcription* activation *factor)*, CUC (cup-shaped cotyledon)], are key regulators in developmental processes in plants, but they also have been shown to control stress response (Olsen *et al.*, 2005) and flavonoid biosynthesis (Morishita *et al.*, 2009). NACs are induced in

response to abiotic (Puranik *et al.*, 2012; Jensen *et al.*, 2010; Wu *et al.*, 2009) and biotic (Wang *et al.*, 2009; Wu *et al.*, 2009; Delessert *et al.*, 2005) stress, acting as activators or repressors of their target genes depending on the motifs that are present in their C terminus (Hao *et al.*, 2010). A part of this thesis covers the role of a stress-induced NAC gene, *PaNAC03*, controlling flavonoid biosynthesis.

2 Objectives

The overall aim of this thesis was to gain knowledge about the molecular defense responses to *H. annosum s.l.* in Norway spruce. The work had the following specific objectives:

- To elucidate the role of the phytohormones jasmonic acid and salicylic acid in the regulation of the Norway spruce defense response to *H. annosum s.l.* The hypothesis that jasmonic acid is the main hormone regulating defense response signaling in Norway spruce was tested in this study. (Paper I)
- To validate *PaLAR3* as a resistance marker for Norway spruce against *H. parviporum* and to understand the mechanisms behind the effect of *PaLAR3*. In this study, we hypothesized that *PaLAR3* associates with variation in resistance against *H. parviporum* in Norway spruce and that this variation in resistance is related to the presence of genetic variation in *PaLAR3* (Paper II)
- To investigate the role of specific transcription factors controlling specialized metabolism in Norway spruce defense (Paper III and IV). The specific aims were:
  - To identify and characterize members of a MYB-bHLH-WDR transcription factor complex in Norway spruce. Here we raised the hypothesis that Norway spruce has a full repertoire of members of the MYB-bHLH-WDR transcription factor complex. (Paper III)
  - To study the effects of the over expression of MYB and NAC transcription factors on downstream genes (Papers III and IV) Here we hypothesized that i) members of the MYB-bHLH-WDR transcription factor complex can regulate flavonoid biosynthesis (Paper III) and ii) the NAC transcription factor *Pa*NAC03 is a transcriptional repressor of *PaLAR3* (Paper IV).

3 Material and Methods

3.1 Plant and fungal material

For the experiments in Paper I, four-year-old spruce plants from a full-sib family of Norway spruce [*Picea abies* (L.) Karst.] propagated at The Forestry Research Institute of Sweden (Ekebo, Sweden) were used. Four rooted-cuttings from ten genotypes were planted in pots and kept in the greenhouse during the experiment. Norway spruce seedlings from the progeny Rörby FP-65 (Skogforsk) were used for seedling treatments.

In Paper II, 773 trees from 102 half-sib families from four populations in Sweden, Finland and Russia, were used for genotyping. The plants were grown at the Lugnet plant nursery in Håbo (Sweden). For re-genotyping, phenotyping, chemical analysis, protein activity study and qPCR, 42 of these trees were selected based of their genotype for the GQ03204_B13.1 locus.

Paper III, integrated work carried on with embryogenic cells of the same Norway spruce embryogenic cell line, 95:61:21 (Högberg *et al.*, 1998). Wild type cells and a transformant overexpressing *GUS*, which were used as control, and transformants overexpressing *PaMYB29*, *PaMYB32*, *PaMYB33* and *PaMYB35* were used for qPCR and chemical analyses. For the stress panel, wild type 95:61:21 cells were used and for the tissue expression panel, Norway spruce clones S21K0420041, S21K0420136, S21K0420259 and S21K0420949, S21K0421131, were used.

In Paper IV, eight Norway spruce genotypes: S21K7822405, S21K7825237, S21K7827398 and S21K7828590 (less susceptible genotypes) and S21K7823178, S21K7823340, S21K7825278, S21K7828397 (highly susceptible genotypes) previously classified for their susceptibility to natural infection by *Heterobasidion* spp. (Karlsson & Swedjemark, 2006), were selected for biotic and abiotic stress induction. Wild type cell lines 95:61:21, cell lines overexpressing GUS (controls) and 95:61:21 overexpressing

PaNAC-1 for qPCR, RNA-seq and chemical analysis. Wild type 95:61:21 was used for the stress panel. Finally, the 42 plants from Paper III were used for sequencing the *PaLAR3* promoter region.

Rb175, a well-defined strain of *H. parviporum*, was used in the experiments of Paper I, II and III. Sä 16-4 (Stenlid & Karlsson, 1991), a *H. annosum s.s.* strain, was used for the biotic stress induction in Paper IV and the Rotstop S strain of *Phlebiopsis gigantea* (InteragroSkog) was also used for inoculation in Paper I.

3.2 Plant inoculations

For all inoculations, fungal strains were grown on Hagem's medium (Stenlid, 1985) and inoculum was prepared as described by Lind *et al.* (2007).

For the inoculation experiments in Papers I and II, inoculations were done by inserting the inoculum wood plugs aseptically in wounds made using a cork borer with a diameter of 0.5 cm in the bark of one-year-old twigs. Inoculations were covered with Parafilm and daytime temperatures varied between 15 and 25°C until the twigs were harvested.

In the experiment of Paper I, samples for RNA extraction were harvested at 3 and 7 days post-inoculation (dpi). Negative controls, i.e. bark not previously wounded, were collected at the time of inoculation. On each branch, three wounds/inoculations were made and later pooled as one sample. At the time of harvest, bark surrounding the wounds and inoculation sites was cut into three sections: (A) 0–0.5 cm around the wound, (B) 0.5–1.5 cm from the wound and (C) 1.5–2.5 cm from the wound. Two ramets of each genotype treated in the same way were harvested at each time point and pooled as one sample.

In the experiment of Paper II, every plant was inoculated seven times on separate twigs. At 21dpi the inoculated twigs were harvested for phenotyping of fungal sapwood growth (FGS). Needles were removed from the twigs and five-centimeter sections upward and downward from the inoculation point were cut. Each of these sections was cut into ten five-millimeter pieces and they were placed on a Petri plate on moist filter paper together with the plug that was used for the inoculation and the inoculation point. Plates were then left under moist conditions at 21°C and in darkness for one week. After that, a stereomicroscope was used to determine the presence or absence of *H. parviporum* conidia on each one of the five-millimeter plugs under 50x magnification. For each twig, the sum of the FGS upward and downwards from the inoculation point was annotated. Plates where no conidia could be observed on the inoculation point or on the inoculation plug were treated as inoculation failures and were discarded.

For the biotic stress induction in paper IV, three ramets per clone and two roots per ramet were used. On one root, a colonized woody plug was attached to an artificial wound on the root surface with Parafilm®; the other root was wounded only and sealed with parafilm. Samples for RNA extraction were harvested at the start of the experiment (0 days post inoculation) and at 5 and 15 days post inoculation (dpi), and preserved in RNAlater (Ambion) for subsequent RNA extraction.

3.3 Hormone and stress treatments

In paper I, 2-week-old Norway spruce seedlings were transferred under axenic conditions to Petri plates with filter paper (five seedlings/plate) moistened with fertilized liquid media (Ingestad & Kähr, 1985). The plates were treated with 2 ml of homogenized *H. parviporum* (Rb175) liquid culture. Thereafter, a final concentration of 750 µM diethyldithiocarbamic acid (DIECA), which inhibits JA synthesis (Farmer *et al.*, 1994), 25 µM 2-amino-indan-2-phosphonic acid (AIP), a highly specific inhibitor of PAL activity (Zon & Amrhein, 1992) or 2.5 µM silver thiosulfate (STS), which blocks the ethylene signaling pathway (Veen, 1983), were added. For treatments with MeJA or MeSA, plates with five Norway spruce seedlings (each on filter paper treated with 2 ml Hagems media), placed in 1 l glass containers to which 75 µl of 10 % of either MeJA or MeSA was added and allowed to evaporate. The inhibitors were applied as above. Treatments were repeated every 24 h for 72 h. Mock plates were treated with 2 ml Hagems medium. Every treatment was performed in triplicate. At 72 h, seedlings were immediately frozen in liquid nitrogen and stored at -80 °C until use.

In Papers III and IV, Norway spruce cells (line 95:61:21) that were grown on LP agar (von Arnold & Eriksson, 1981) without plant growth regulators were treated with several types of abiotic stress for 48 hours The cold treatment consisted on keeping the cells at 4°C for 48 hours. The rest of the treatments were held at 21°C and included the addition of different agents to the medium. The salt medium consisted on placing cells on LP medium with 100mM of NaCl. The abscisic acid treatment was based on the addition of 8 µg/ml of abscisic acid to the medium. Finally, to study the effect of jasmonic acid and salicylic acid, unsealed plates with cells on them were placed in a sealed jar and 25 µL of either 10% methyl salicylate or 10% methyl jasmonate were placed in a cotton ball inside of the jars next to the plates in the beginning of the treatment and after 24 hours. At harvest, cells were collected and put in liquid nitrogen until further use.

3.4 Molecular methods

3.4.1 RNA extraction and cDNA synthesis

Total RNA extraction was done essentially according to the protocol by Chang *et al.* (1993). Samples were DNase treated with DNase1 (Sigma Aldrich, USA) according to the manufacturer's instructions and RNA concentration was determined with the NanoDrop (Spectrophotometer ND 1000, Saven Werner). 300 ng of total RNA was reverse transcribed to cDNA with the iScript™ cDNA Synthesis Kit (BIO-RAD) according to the manufacturer's instructions.

3.4.2 Quantitative-PCR

For preparation of standards for the qPCR reactions in Papers I, II, III and IV, PCR reactions consisting of 1x Dream-Taq green buffer, 0.25µM of each of the qPCR primers, 0.2mM dNTPs, 6.25U Dream-Taq Polymerase (Fermentas) and 1µl of *P. abies* cDNA. Initial denaturation was at 95°C for 5 min, followed by 35 cycles of: 15 s at 95°C, 20 s at 60°C and 120 s at 72°C and a final elongation step of 3 min at 72°C. PCR products were cloned into TOPO® vectors (Invitrogenh) following the manufacturer's instructions. Plasmids were purified using The PlasmidPrep minikit® (Fermentas) and dilution series were then prepared from 10^8 to 10^3 copies/µl. Three repetitions per standard, sample and negative control were run.

Quantitative PCR reactions were performed with the SsoFast™ EvaGreen® Supermix (BIO-RAD) according to the instructions in the manual, using 0.3µM of each primer. The qPCR were carried out in an iQ5™ Multicolor Real-Time PCR Detection System thermo cycler (Bio-Rad) using a program with a 30 seconds initial denaturation step at 95°C, followed by 40 cycles of 5 seconds denaturation at 95°C and 10 seconds at 60°C. Melt curve analyses were used to validate the amplicon.

3.4.3 Primer design

For the Norway spruce genes studied in this thesis, Primers were designed using the Norway spruce genome database (www.congenie.org) or GenBank sequences (http://www.ncbi.nlm.nih.gov/genbank/) as a reference and Primer3 (http://biotools.umassmed.edu/bioapps/primer3_www.cgi) to predict and design PCR primers. Primer quality and properties were checked at www.bioinformatics.org/sms2/pcr_primer_stats before primers were synthesized at TAG Copenhagen. Primer sequences are found in the correspondent papers that are part of this thesis.

3.4.4 Sequencing

For the sequencing of *PaLAR3* of Papers II and III, 50µl PCRs with 1x Dream-Taq green buffer, 0.2µM of each of the primers, 0.2mM dNTPs, 1.25U Dream-Taq Polymerase (Fermentas), a final concentration of MgCl2 of 3.25mM, and 0.5-5ng/µl reaction volume of genomic DNA, was run. Initial denaturation was at 95°C for 5 min, followed by 35 cycles of: 30 s at 95°C, 30 s at 57°C and 2 min at 72°C and a final elongation step of 7 min at 72°C. PCR products were cloned into TOPO® vectors (Invitrogen) following the manufacturer's instructions. Colony PCR was run on selected colonies with M13 primers and the PCR products were sent to Macrogen (Amsterdam, The Netherlands) for Sanger sequencing.

For the sequencing of candidate genes in Paper III, PCR reactions consisting of 1x Dream-Taq green buffer, 0.25µM of each of the primers with AttB borders, 0.2mM dNTPs, 6.25U Dream-Taq Polymerase (Fermentas) and 1µl of Norway spruce cDNA. Initial denaturation was at 95°C for 5 min, followed by 35 cycles of: 15 s at 95°C, 20 s at 58-60°C and 2 min at 72°C and a final elongation step of 3 min at 72°C. PCR products were cloned into Gateway pDONR/Zeo entry vectors that would be later used for Vector construction. Colony PCR with specific primers was run on colonies to verify the presence of the insert in the plasmid. Selected colonies were grown overnight in liquid LB medium with 50 mg/liter zeocin at 37°C and plasmids were purified using The PlasmidPrep Miniprep®. 2 µl of purified plasmid were sent to Macrogen for sequencing of the insert.

3.4.5 Vector construction

For papers III and IV, PCR reactions consisting of 1x Dream-Taq green buffer, 0.25µM of each of the primers with AttB borders, 0.2mM dNTPs, 6.25U Dream-Taq Polymerase (Fermentas) and 1µl of Norway spruce cDNA. Initial denaturation was at 95°C for 5 min, followed by 35 cycles of: 15 s at 95°C, 20 s at 58-60°C and 2 min at 72°C and a final elongation step of 3 min at 72°C. PCR products were cloned into the Gateway pDONR/Zeo entry vector and later into destination vectors following the manufacturers instructions. The destination vector pMDC32 (Curtis & Grossniklaus, 2003) was used for transformation of Norway spruce embryogenic cells and the pDest-AD-CYH2 and pDest-DB destination vectors were used for for the yeast-two hybrid protein interaction experiment.

3.5 Norway spruce transformation

The pMDC32 destination vectors carrying the constructs were transformed into the *Agrobacterium tumefaciens* C58C1 strain carrying additional virulence plasmid pTOK47. Transformed bacteria were then grown overnight in YEP (yeast extract peptone) medium supplemented with 100 mg/liter rifampicin, 5 mg/liter tetracycline, 100 mg/liter carbenicillin, and 50 mg/liter kanamycin. Cells were centrifuged at 4,500 *g* for 5 min and resuspended in infiltration buffer (10 mM $MgCl_2$, 10 mM MES, pH 5.5, and 150 µM acetosyringone). The OD_{600} of the cultures was adjusted to 10 with the infiltration buffer. *A. tumefaciens* was transformed with the constructs and incubated at room temperature for 1 h. One ml of the *A. tumefaciens* mixture was added to 5 ml of a cell pellet of 7-d-old suspension culture grown in the presence of GF. The volume of the mixture was adjusted to 10 ml with Plant Growth Regulators (PGR), and acetosyringone was added to a final concentration at 150 µM. After 4 h, the cells were plated on a filter paper placed on the top of solidified +PGR medium and incubated at room temperature in the darkness for 48 h. Then, filters were transferred on +PGR plates containing 400 mg/ml timentin and 250 mg/ml cefotaxime and incubated under the same conditions for 5 d. Subsequently, filter papers were transferred onto fresh +GF plates containing 20 µg/ml hygromycin, 400 µg/ml timentin, and 250 µg/ml cefotaxime and subcultured onto the same medium every week. The transgenic calli were picked from the plates after 3 wk. Lines were selected and verified by PCR and the expression levels of the transgenes were assessed by qPCR.

3.6 Yeast Two-Hybrid assay

DB and AD plasmids were individually transformed into haploid yeast (*S. cerevisiae*) strains Y8930 (MATα) and Y8800 (MATa) to create baits and preys, respectively, as described (Mukhtar *et al.*, 2011). Briefly, Y8930 and Y8800 strains were grown in liquid YEPD overnight. A 0.1 OD culture was prepared the following morning. Once the OD reached 0.4-0.6, the cells were harvested and prepared for transformation. The baits and preys were selected on Difco™ yeast nitrogen base (YNB) with leucine dropout (-L) and tryptophan dropout (-T) selective media, respectively. The haploid bait and prey yeast strains were pairwise mated overnight in YEPD. The diploid yeast cells were selected onto YNB -LT selective liquid media, and subsequently spotted onto YNB -LTH as well as -LH containing cycloheximide (CHX) selective media. In addition, we also determined the strength of protein-protein interaction by supplementing -LTH and -LH with 3-Amino- 1, 2, 4-trizole (3AT), a competitive inhibitor of histidine biosynthesis. Yeast growth on -LTH

but not on -LH containing CHX media were scored as positive interactions. Yeast growth found on both –LTH and –LH containing CHX were due to *de* novo autoactivation and hence removed from the data set.

3.7 Phylogenetic analyses

For the sequencing of *PaLAR3* in Paper III and IV, sequence assembly was done with Seqman (DNAstar). Sequences were then imported into MEGA6 (Tamura *et al.*, 2013) and aligned by ClustalW algorithm with gap opening penalty 15, gap extension penalty 6.66, IUB DNA weight matrix and transition weight 0.5. A Maximum Likelihood tree was created with a bootstrap phylogeny test of 1000 replications and pairwise deletion of missing data. Genetic distances between haplotypes were imported into HapNet (http://www.pawfal.org) to create a *Minimum Spanning Network using default software settings*. The network was edited in Illustrator (Adobe) to incorporate allele sizes and genotype provenances.

For the construction of Phylogenetic trees in Paper III, sequences were also imported into MEGA6 and aligned by ClustalW algorithm with gap opening penalty 15, gap extension penalty 6.66, IUB DNA weight matrix and transition weight 0.5. A Maximum Likelihood tree was created with a bootstrap phylogeny test of 1000 replications and pairwise deletion of missing data.

3.8 Catechin effect on *H. annosum s.l.* growth

H. parviporum Rb175 strain was pre-grown for 10 days at 25°C and darkness in solid Hagems medium. A 50mg/mL stock solution of (+) catechin (Sigma-Aldrich) in water with 10% ethanol was prepared. Petri plates (Ø = 9cm) with Hagems solid medium with no (+) catechin, physiological, and induced concentration of (+) catechin in *P. abies* bark, were prepared (Hammerbacher *et al.*, 2014). Five plates for each concentration were used in total.

A piece of agar (Ø = 8mm) with inoculum was placed in the center of each plate. Two perpendicular lines intersecting in the center of the plate were drawn on the bottom of each plate forming four axes around the inoculation point. Plates were left at 25°C and darkness. Every 24 hours a line was drawn in each of the four axes in the point where hyphal growth had reached. The average of the measurements of the four axes was taken as the colony radius. Colony area was calculated with the formula $a=\pi r^2$ minus the area of the initial inoculum ($a=\pi*4^2$).

4 Results and discussion

4.1 Jasmonic acid is the major hormone controlling defense signaling in Norway spruce

Determining the roles of specific hormones in the defense signaling pathways was the first aim of this thesis. The interaction between JA/ET-mediated signaling and SA-mediated signaling is complicated and the prioritization between the modules varies among interactions and plant taxa (Thaler *et al.*, 2012; Sato *et al.*, 2010). The presumed SA-dependent genes *PR1* and *LURP1* were significantly upregulated after exogenous treatment of the Norway spruce seedlings with MeJA or MeSA (Figure 7a). Two possible explanations for this observation were considered: (1) MeJA treatment directly induces transcription of *PR1* and *LURP1*, or (2) MeJA treatment induces an accumulation of SA in the seedlings, as reported by Kozlowski *et al.* (1999), which activates SA-dependent gene expression. Our data show that the induction of *PR1* is clearly dependent on the accumulation of JA after *H. parviporum* infection. Even though *PR1* was induced after application of MeSA, the addition of the JA synthesis inhibitor DIECA effectively eliminated the induction of the gene after treatment with *H. parviporum* (Figure 7c).

The effect of the addition of the PAL inhibitor AIP is more difficult to interpret as it clearly reduced, but did not eliminate, the induction of *PR1* after *H. parviporum* infection (Figure 7b). AIP acts as reversible competitive inhibitor; thus, it is possible that a certain PAL activity that could convert phenylalanine to cinnamic acid remains after the treatment with AIP (Appert *et al.*, 2003), and that this residual PAL activity produces sufficient cinnamic acid for some pathogen-induced SA production. Most likely, however, the JA-mediated induction of *PR1* in Norway spruce is not dependent on PAL-mediated production of cinnamic acid. It has been reported that the *Sp-AMP* gene of Scots pine (*Pinus sylvestris* L.), active in response to *H. annosum s.l.,*

is also induced in response to treatment with SA and ACC (1-aminocyclopropane-1-carboxylic- acid, an ethylene precursor) but not to MeJA treatment (Sooriyaarachchi *et al.*, 2011). These observations indicate that the signaling network in conifers in response to *H. annosum s.l.* may be dependent on synergistic, and possibly unique junctures, between JA/ET-mediated and SA-mediated signaling. Conifer genomes offer now new opportunities for an improved understanding of the role of defense hormone signaling in the response to *H. annosum s.l.* through the possibility of comparative analyses of the promoter regions of *PR1, LURP1, ERF1, LOX, JAZ* and other defense-regulated genes.

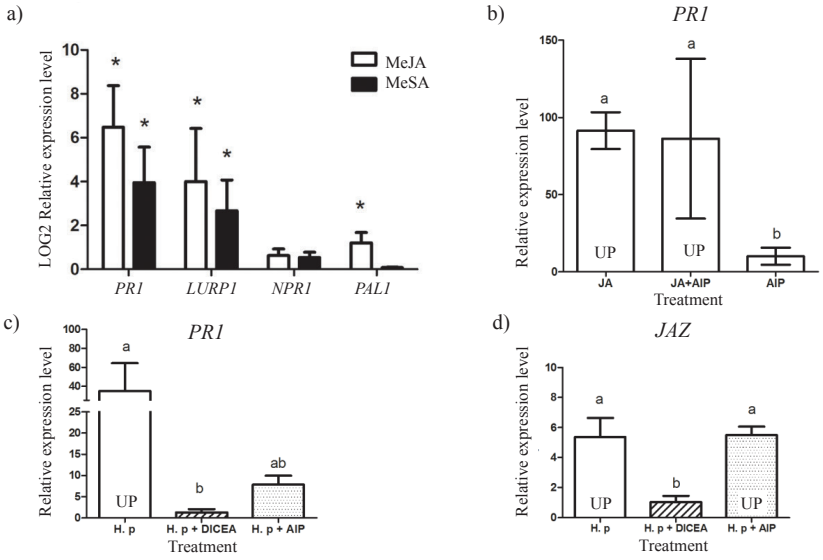

Figure 7 a) Relative expression of Norway spruce genes with similarity to *PR1*, *LURP1, NPR1*, and *PAL1* after 48 h of treatment with MeJA or MeSA. Expression were normalised to the constitutive expressed genes phosphoglucomutase, *elF4A* and ELF1α and the expression was calculated using REST 2006. * Indicate a significant upregulation compared to untreated seedlings (P<0.05). b) Relative expression of a Norway spruce gene with similarity to *PR1* after three days of treatment with MeJA, MeJA + AIP, or AIP alone. c). d) *PR1* and *JAZ*, three days after *H. parviporum* inoculation (H.p, open bars), or inoculation with *H. parviporum* in combination with 750 µM DIECA (H.p +DIECA, hashed bars), or with 25µM AIP (H.p +AIP, dotted bars). Bars denoted UP represent expression levels that are significantly (P< 0.05) higher than the mock treated control calculated with REST 2006. Letters indicate differences in expression levels between treatments calculated with REST 2006.

4.2 Regulation of hormone defense signaling depends on the type of stress, biotic agent and distance from the treatment point

All the genes related to hormone signaling that were tested in our study were induced proximal to the inoculation site at 3 dpi of the 4-year-old plants (Table 1). The induction of gene expression in the *H. parviporum*-inoculated samples was significantly stronger compared to wounding. The response to the saprotrophic fungus *P. gigantea* was, in principle, intermediate between the two. The pattern between treatments remains the same at 7 dpi (Table 1). The stronger reaction after fungal inoculation, compared to wounding alone, is in accordance with previous studies, where anatomically changes such as induction of TD and PP cells were shown to be stronger and faster in *H. annosum s.s.* inoculation than the control treatment (Krekling *et al.*, 2004). *P. gigantea* is a saprotrophic fungus living on dead wood and freshly cut stumps. Although the initial reaction to *P. gigantea* is reported to be similar to that to *H. annosum* in Scots pine, the size of the necrosis formed by *H. annosum* still increases after three weeks post-inoculation compared to wounding, while the necrosis caused by *P. gigantea* remains constant (Sun *et al.*, 2011). Hence, in our system, it is possible that *P. gigantea* has the ability to sustain itself by living on dead or damaged cells in the proximity of the wound, and thus elicits a stronger reaction than wounding.

The genes in this study showed similar patterns of activation at the site of wounding or inoculation with fungi with different trophic strategies. When interpreting these data, it is important to remember that the inoculation methods used to infect Norway spruce plants with wood degrading fungi are likely to induce wound responses in the tissue proximal to the inoculation site.

The induced defense responses are also very similar between wounding and inoculation in this and other studies (Yaqoob *et al.*, 2012; Arnerup *et al.*, 2011; Deflorio *et al.*, 2011). It has been shown that the transcriptional responses differ in intensity between bark and sapwood after inoculation (Oliva *et al.*, 2015; Yaqoob *et al.*, 2012; Deflorio *et al.*, 2011). In our study, fungal inoculation increased the transcript levels of *LOX, PR1, LURP1* and *ACS* distal to the wound, while wounding did not affect the gene expression levels distal to the wound. The level of gene induction of the analysed genes decreased with the increasing distance from the wound. This observation is in accordance with the report by (Yaqoob *et al.*, 2012), whereby the induction of peroxidase and chitinase genes was also lower distal to the wound or inoculation site, and is also in accordance with the work presented in Oliva *et al.* (2015). The suggestion by Katagiri and Tsuda (2010) that plant defense responses are

Table 1. *Log2 of the fold-change in expression levels in the treated material in comparison to untreated bark*

	W3_A		W3_B		W3_C		H3_A		H3_B		H3_C		P3_A		P3_C	
Gene	log2 ± se		log2 ± se		log2 ± se		log2 ± se		log2 ± se		log2 ± se		log2 ± se		log2 ± se	
LOX	2,4 ± 1,2	*	2,8 ± 1,2	*	-0,6 ± 0,1		**6,1 ± 2,5**	*	3,6 ± 1,8	*	0,5 ± 0,2		5,5 ± 2,0	*	2,8 ± 2,4	
ACS	3,3 ± 2,4	*	-0,7 ± 0,1		0,0 ± 0,0		**8,9 ± 4,4**	**	5,1 ± 2,6		**4,0 ± 1,6**	*	6,1 ± 1,6	*	2,7 ± 0,8	
ERF1	2,2 ± 0,9	*			2,1 ± 0,8	*	3,7 ± 0,8	*			**3,4 ± 1,0**	*	2,9 ± 1,0	*	3,4 ± 1,3	
PR1	7,7 ± 3,3	***	3,1 ± 2,0	*	1,6 ± 2,0		**11,3 ± 5,1**	*	6,9 ± 2,7	*	**6,1 ± 3,6**	*	10,7 ± 3,0	*	6,3 ± 5,6	
LURP	4,3 ± 2,5	*	-3,3 ± 0,0	*	-3,2 ± 0,0	***	**10,8 ± 3,3**	***	3,6 ± 1,7	*	**1,4 ± 0,6**		7,9 ± 2,7	*	0,3 ± 0,3	
NPR1	1,7 ± 0,9	*			1,1 ± 0,5		2,2 ± 0,9	*			1,8 ± 0,8	*	1,4 ± 0,6	*	2,2 ± 0,9	
JAZ	5,7 ± 3,4	*					**8,0 ± 4,0**	*								
MYC	1,7 ± 0,9	*					1,9 ± 0,8	*								
	W7_A		W7_B		W7_C		H7_A		H7_B		H7_C		P7_A		P7_C	
Gene	log2 ± se		log2 ± se		log2 ± se		log2 ± se		log2 ± se		log2 ± se		log2 ± se		log2 ± se	
LOX	5,0 ± 2,8	*	2,5 ± 1,2	*	-0,3 ± 0,1		**8,5 ± 4,2**	***	6,9 ± 3,9	*	2,9 ± 4,4		7,4 ± 3,0	*	1,5 ± 0,7	
ACS	0,5 ± 0,3		-2,2 ± 0,1	*	-3,2 ± 0,0	***	**5,1 ± 4,4**	*	3,5 ± 2,5	*	**1,7 ± 1,8**		2,3 ± 1,9		-1,0 ± 0,2	
ERF1	3,1 ± 1,1	*			2,1 ± 1,3		4,6 ± 1,8	***			3,4 ± 3,1	***	3,8 ± 1,5	*	3,2 ± 1,5	
PR1	9,4 ± 3,6	*	3,8 ± 2,8	*	1,9 ± 1,7		**11,5 ± 4,3**	***	8,6 ± 3,4	*	**8,4 ± 8,3**	*	9,7 ± 3,8	*	6,3 ± 3,9	
LURP	5,2 ± 2,3	*	-4,0 ± 0,0	*	-6,4 ± 0,0	***	**8,5 ± 4,6**	*	3,3 ± 1,7	*	**1,3** ± 2,4		6,1 ± 3,2	*	-2,1 ± 0,1	
NPR1	1,6 ± 0,5	*			1,2 ± 0,7		1,9 ± 0,5	*			1,7 ± 1,5		1,9 ± 0,6	*	1,9 ± 0,7	
JAZ	6,6 ± 3,2	*					**8,9 ± 5,5**	*								
MYC	1,7 ± 0,6	*					1,7 ± 0,4	*								

W wounding treated samples

H *H. parviporum* treated samples

P *Plebiopsis gigantea* treated samples

A Samples harvested 0-0.5 cm around the wound

B Samples harvested 0.5-1.5 cm from the wound

C Samples harvested 1.5-2.5 cm from the wound

Samples harvested at 3 and 7 days post inoculation

* Asterisks indicate if the expression is significantly different from the unharmed control (* = p< 0.05, ** = p<0.01, *** = p<0.001).

Bold number indicat that *H. parviporum* treated samples were significantly different from wounding

chiefly determined by how the shared defense signaling network is used rather than by signaling machinery specific to each interaction type, offers a context in which the obvious similarities of the induced responses between wounding and inoculation with *H. annosum s.l.* can be interpreted. Upon the perception of MAMPs or of potential danger-associated molecular patterns (Boller & Felix, 2009) released by biotic (e.g. *H. annosum s.l.* infection) or abiotic stressors (e.g. wounding and drought), Norway spruce activates a non-specific structural defense such as TD and PP cell formation (Krekling *et al.*, 2004; Nagy *et al.*, 2004) and transcription of defense genes (Yaqoob *et al.*, 2012; Arnerup *et al.*, 2011; Deflorio *et al.*, 2011; Hietala *et al.*, 2004). According to the Katagiri and Tsuda (2010) theory, it is possible that the outcome of MAMPs detection from *H. annosum s.l.* or *P. gigantea* may be similar to the abiotic stress of wounding due to shared downstream cross-points in the signaling network. In the samples distal to the inoculation site, we found that genes presumably associated with the ET signaling pathway (*ACS* and *ERF1*) showed significantly higher induction levels after *H. parviporum* infection than after wounding, thus emphasizing the potential role of ET-mediated signaling distal to the inoculation site.

4.3 *PaLAR3* is represented in Norway spruce by two allelic lineages that affect resistance against *H. parviporum*

Genotyping of three SNPs in the locus that contained the *PaLAR3* gene was done in 773 individuals representing Norway spruce trees from Finland, Russia and Sweden. Genotype data showed that the three SNPs co-segregated in all individuals, forestalling the existence of two allelic forms of the gene, one major ($p = 0.78$) and one minor ($p = 0.22$). Both allelic forms showed similar frequencies for all provenances. *PaLAR3* is a dimorphic gene that presents two main haplotypes in Norway spruce. Re-sequencing of the gene in 28 individuals of the same population showed 27 SNPs and three indels that co-segregated in all individuals together with the original three SNPs delineating two allelic lineages (Figure 8). The major allele *PaLAR3A* is identical to the *PaLAR3* sequence previously reported by Hammerbacher *et al.* (2014). The minor allelic lineage, *PaLAR3B,* differs from the major allele by 33 co-segregating mutations of which one results in one amino acid substitution in the protein sequence replacing the asparagine at position 175 with a lysine.

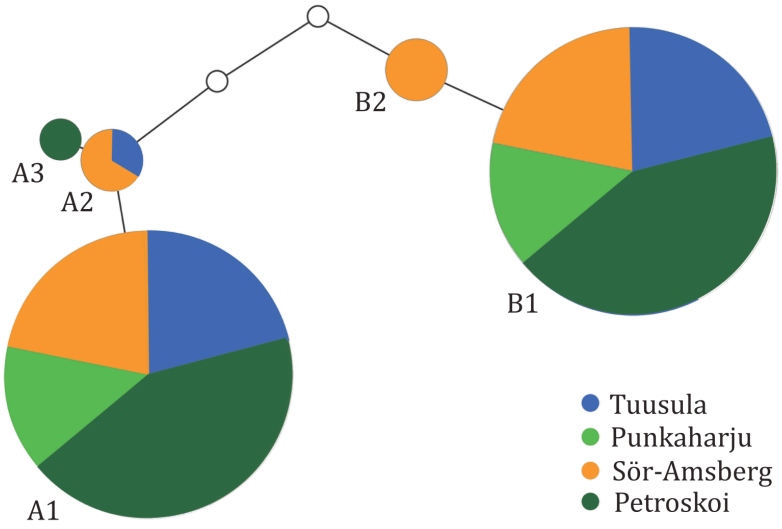

Figure 8. Haplotype network of *PaLAR3* sequences in *Picea abies* (n=36). Circle diameter is proportional to the number of sequences in a specific haplotype. Colors indicate the geographical origin of the recorded haplotypes and open circles indicate inferred missing haplotypes.

The presence of dimorphism in a gene has been previously reported in angiosperms (Aguade, 2001; Filatov & Charlesworth, 1999) and conifers (Gonzalez-Martinez *et al.*, 2006). Plants carrying at least one copy of *PaLAR3B* showed significantly reduced fungal growth in sapwood (FGS) after inoculation with *H. parviporum* than their half-siblings carrying only copies of *PaLAR3A*, pointing at dominance of *PaLAR3B* over *PaLAR3A*. An increased resistance against a fungal pathogen could be the result of a long-term balancing selection between two traits. Still, there must be some other beneficial trait for plants carrying *PaLAR3A* alleles given that *PaLAR3A* is the major allele in all the populations that we studied.

4.4 (+) Catechin has a fungistatic effect on *H. parviporum* and *PaLAR3* affects (+) catechin content in bark

The fungistatic effect of (+) catechin has already been tested on *E. polonica* (Hammerbacher *et al.*, 2014). However, in order to understand better the implication of *PaLAR3* in resistance in our study, we tested the fungistatic effect of (+) catechin on the *H. parviporum* strain that we used for inoculation (Rb175). Rb175 was inoculated on Hagems medium with no (+) catechin,

physiological levels and induced levels of (+) catechin in Norway spruce bark (Hammerbacher *et al.*, 2014). By measuring the radial growth and estimating the colony area we found that (+) catechin has a fungistatic effect on *H. parviporum*. Our data showed that the growth inhibition is much stronger with induced (+) catechin concentration than with physiological concentration (Hammerbacher *et al.*, 2014) (Figure 9a).

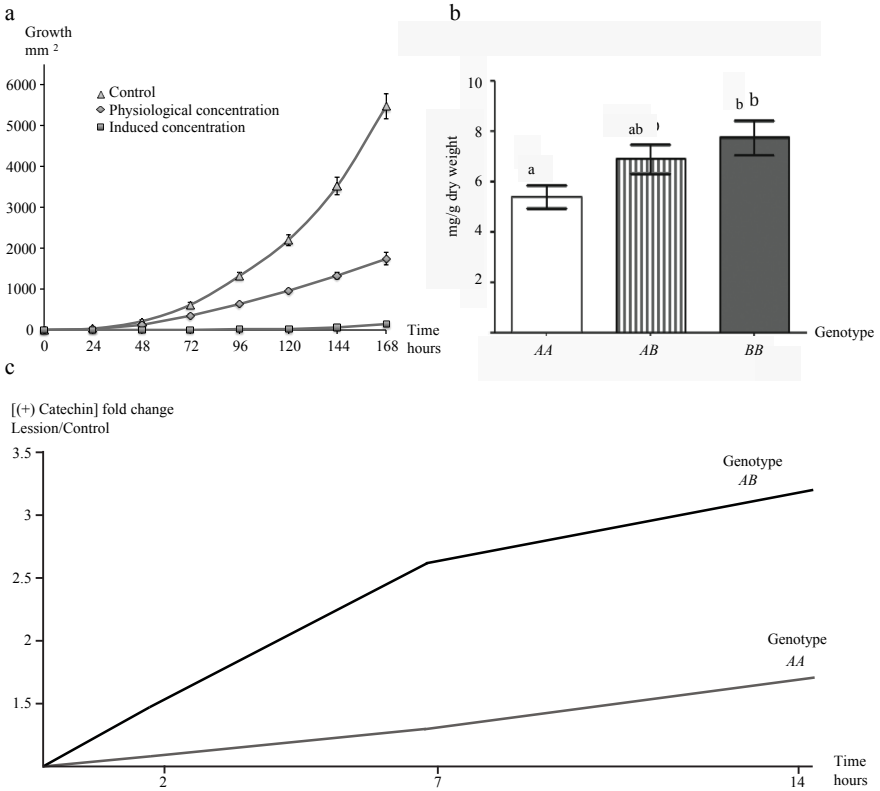

Figure 9. a) *H. parviporum* Rb175 growth at different (+) catechin concentrations. b) (+) Catechin content in bark in plants with different *PaLAR3* phenotype. c) Ratio between the average (+) catechin content in bark inoculated with *E. polonica* and untreated bark in one *PaLAR3A* homozygote and a *PaLAR3* heterozygote.

Our data also showed that *PaLAR3B* homozygotes have significantly higher levels of (+) catechin than *PaLARA* homozygotes (Figure 9b). The fungistatic effect of (+) catechin and the differences that we observe between genotypes may give a hint on how the two alleles could affect FGS. Transgenic poplar lines overexpressing a *LAR* gene (*PtrLAR3*) were shown to have higher (+) catechin and proanthocyanidin levels and enhanced fungal resistance (Yuan *et al.*, 2012), which indicates that (+) catechin levels play a role on fungal

resistance and also that levels depend on expression of LAR genes. It also has been shown that Norway spruce cell lines overexpressing *PaLAR3* have higher (+) catechin levels (Hammerbacher, 2011). Finally, after inoculation of two Norway spruce plants with different *PaLAR3* genotype with blue-stain pathogen *E. polonica*, we observed different patterns for (+) catechin accumulation around the inoculation point. While the AB heterozygote showed a rapid increase in (+) catechin levels in bark, the AA homozygote showed a much slower increase in (+) catechin levels (Figure 9c). The experiment shows how *PaLAR3* genotype can determine the (+) catechin accumulation during time as it was seen previously for plants with different resistance levels by Danielsson *et al.* (2011).

4.5 The two *PaLAR3* alleles differ essentially in inducibility

Based on the available *Vv*LAR1 structure (Mauge *et al.*, 2010), it appears that the amino acid change that defines the difference between *Pa*LAR3A and *Pa*LAR3B is flanked by two of the putative members of the catalytic triad of the enzyme, namely the tyrosine at position 174 and the lysine at position 176. The substitution in *Pa*LAR3B leads to the change from a polar amino acid into a positively charged amino acid, potentially altering the specific activity of the protein as already demonstrated by site directed mutagenesis experiment manipulating the properties of amino acids at positions flanking members of the catalytic triad of subtilisin in a *Bacillus amyloliquefaciens* (Estell *et al.*, 1985). To compare the catalytic properties of *Pa*LAR3A and *Pa*LAR3B, both proteins were overexpressed in *N. benthamiana* and enzyme activity was determined on the native protein. No significant difference in activity or specificity for the two main substrates of the enzyme in Norway spruce, leucocyanidin (Figure 10a) and leucodelphinidin (Figure 10b), was found between *Pa*LAR3A and *Pa*LAR3B suggesting that the N175K amino acid substitution does not interfere with the catalytic properties of the enzyme and thus rejecting the hypothesis that variations in specific protein activity or specificity underlie the observed differences in FGS.

Specific allelic expression levels were measured in 14 *PaLAR3* heterozygotes showing that *PaLAR3B* transcript levels were, as an average, 6.9 times higher than *PaLAR3A* transcript levels (Figure 10c). These differences in expression levels could lead to higher protein levels in plants carrying *PaLAR3B* leading to higher (+) catechin levels and a consequent higher resistance.

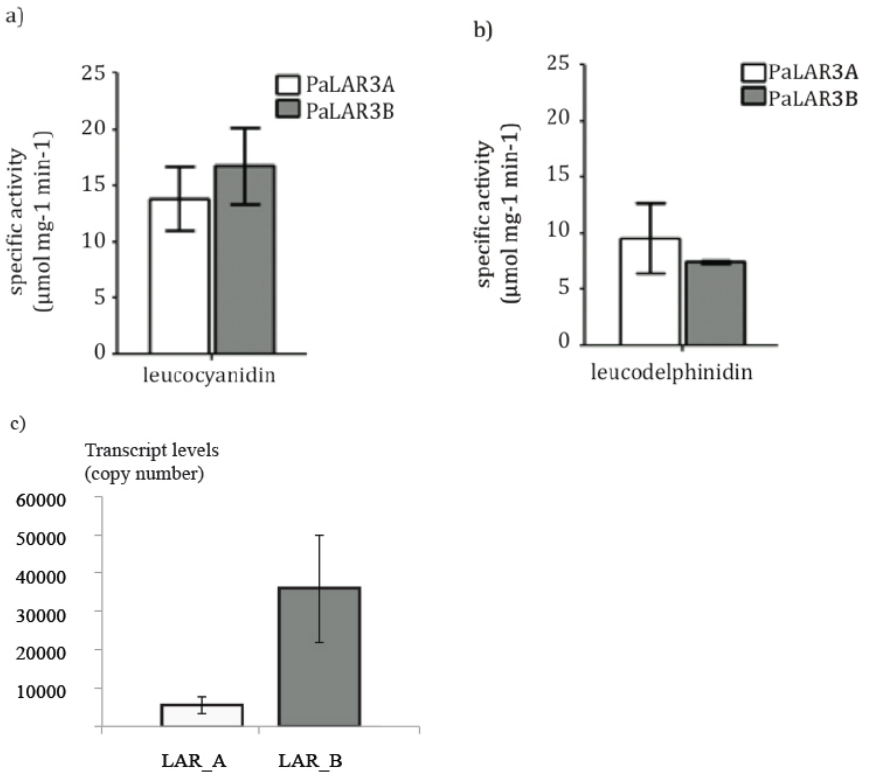

Figure 10. a) Specific activity of the two *Pa*LAR3 isoforms for leucocyanidin. b) Specific activity of the two PaLAR3 isoforms for leucodelphinidin. c) Transcript levels of the two *PaLAR3* alleles in 14 heterozygotes after challenge with *H. parviporum*.

4.6 The two *LAR3* allelic lineages might exist in other conifers

Our work confirms the relevance of *PaLAR3* in resistance against the basidiomycete *H. annosum s.l.* Furthermore, Porth *et al.* (2012) found, among other genes, the *PaLAR3* homolog to be linked to weevil resistance in an eQTL study in a *P. glauca* x *P. engelmanii* cross (interior spruce). The role of *LAR3* in resistance against very different pathogens and pests makes it especially interesting to know if the same allelic structure is also present in other *Picea* species.

We compared the SNP variation of *PaLAR3* with the SNP variation of the *LAR3* gene reported from white spruce EST sequences (Pavy *et al.*, 2013) to study the possibility that the allele lineages are conserved between the two species. We found eight SNPs that were shared between the two species, 24 that are white spruce-specific and 26 that are Norway spruce-specific. From the

eight SNPs that were shared between the two species, six differed between the *PaLAR3A* and *PaLAR3B* allelic lineages in Norway spruce while the other two SNPs belong to the *PaLAR3B*$_2$ subclass of the *PaLAR3B* allelic lineage (Figure 11).

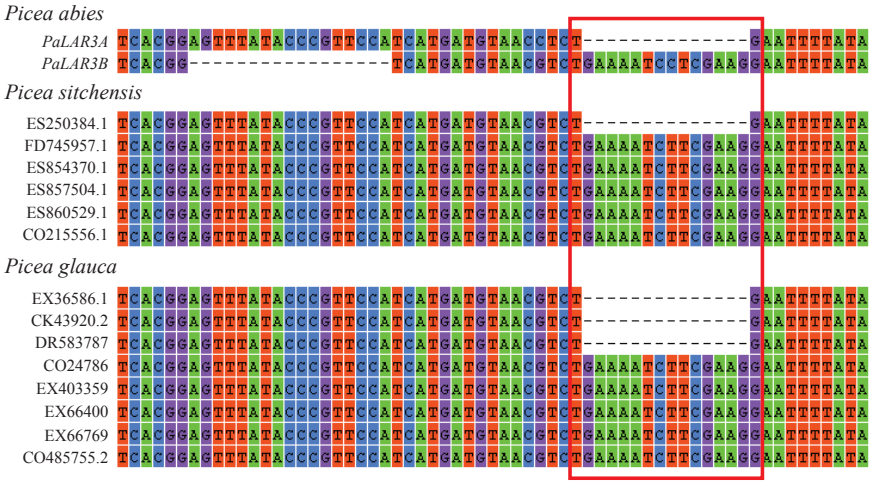

Figure 11. Comparison of the *PaLAR3* 3'UTR region in *P. abies, P. glauca and P. sitchensis*.

The 3'UTR region of *PaLAR3* shows two indels next to each other. We blasted the 3'UTR region of *PaLAR3* in GenBank to look for EST sequences of white spruce and Sitka spruce where variation in the indel area could be observed and we found that only one of the indels seems to be conserved between the species while the other one seems to be specific of Norway spruce (Figure 11).

Taken together, we found enough shared genetic diversity to suggest the possibility of a similar allelic structure in *PaLAR3* orthologs in other spruce species even though Norway spruce and white spruce are estimated to have diverged 13-20 million years ago (Nystedt *et al.*, 2013). Bouille and Bousquet (2005) reported vast numbers of trans-species shared polymorphisms in the genus *Picea,* indicative of an incomplete lineage sorting at speciation, highlighting the possibility the *PaLAR3* allele lineages predate the species-split. However, this cannot be confirmed unless *LAR3* is fully sequenced in other spruce species.

4.7 *PaNAC03* overexpression reduces expression of genes in the flavonoid pathway and flavonoids levels

Data analysis of the RNAseq in cell lines overexpressing *PaNAC03* showed a concomitant downregulation of three key genes in the flavonoid biosynthetic

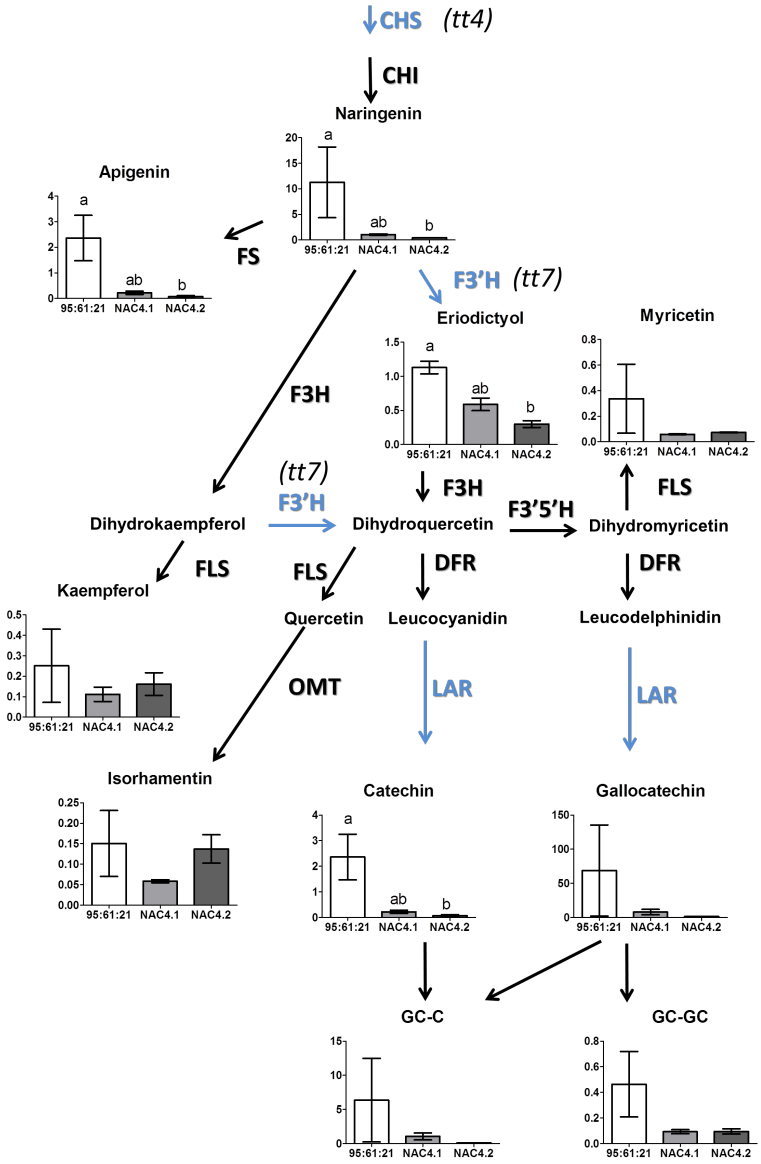

Figure 12. Flavonoid biosynthetic pathway with chemical levels for WT and two *PaNAC03* overexpressing lines. Next to the arrows in gray are the enzymes encoded by genes that are significantly downregulated in these lines.

pathway. We found that both naringenin and apigenin, which are products formed downstream of CHS but before steps catalyzed by either F3'H or *Pa*LAR3, were downregulated in the *PaNAC03* overexpression lines (as indicated in Figure 12). Eriodictyol, a catalytic product of F3'H was also reduced. The catalytic product of *Pa*LAR3, (+) catechin, was also significantly reduced in the over expression lines. Other metabolites, not directly associated with 3-flavanol production, accumulated to the same levels as in the wild type line showing that the downregulation of key members in the 3-flavonol pathway lead to a specific reduction in 3-flavonols (Figure 12). Although regulation of anthocyanin or proanthocyanin pathways by NAC TFs is not commonly reported in literature, the gene (*BL*) controlling the blood red flesh phenotype in Peach was recently shown to encode a NAC gene (Zhou *et al.*, 2015). Also, ANAC078 is a transcriptional activator of flavonoid biosynthesis genes and TFs controlling flavonoid biosynthesis under high-light conditions (Morishita *et al.*, 2009). The results by Morishita *et al.* (2009) could indicate that certain NAC domain proteins act as higher level switches in the flavonoid biosynthetic pathway similar to the role of VND6 and VND7 in secondary wall formation and lignin biosynthesis pathway (Yamaguchi & Demura, 2010). However, *PaNAC03* overexpression does not lead to misregulation of other TFs known to associate with flavonoid biosynthesis in conifers (Bedon *et al.*, 2010). Taken together, this suggests that *PaNAC03* could act as a negative regulator of 3-flavanol production in Norway spruce, possibly by acting directly on the misregulated flavonoid biosynthetic genes.

4.8 *Pa*NAC03 interacts differently with the promoter regions of the two *PaLAR3* alleles

Putting together two of our results, we found inducibility to differ between *PaLAR3A* and *PaLAR3B* and that overexpression of *PaNAC03* leads to downregulation of genes in the flavonoid biosynthetic pathway including *PaLAR3*. This lead us to hypothesize that *Pa*NAC03 could bind the promoter region of *PaLAR3* and suppress the transcription of the gene. We also hypothesized that this binding capacity might differ between the two allelic lineages of *PaLAR3,* being this the cause for the differences in inducibility that we observed between the two allele lineages. To test this possibility, we first sequenced and compared 1.5 kbp of the promoter regions of both allelic lineages and we identified differences in NAC-binding sites between alleles (Figure 13a). We also co-expressed *PaNAC03* with either the *PaLAR3A*- or *PaLAR3B* promoter in *N. bethamiana* leaves, hypothesizing that *Pa*NAC03

would reduce *PaLAR3A*- and *PaLAR3B* promoter activity. Interestingly, *Pa*NAC03 strongly activated the promoter of the *PaLAR3A* allelic lineage but did not affect the promoter of *PaLAR3B* activity (Figure 13b).

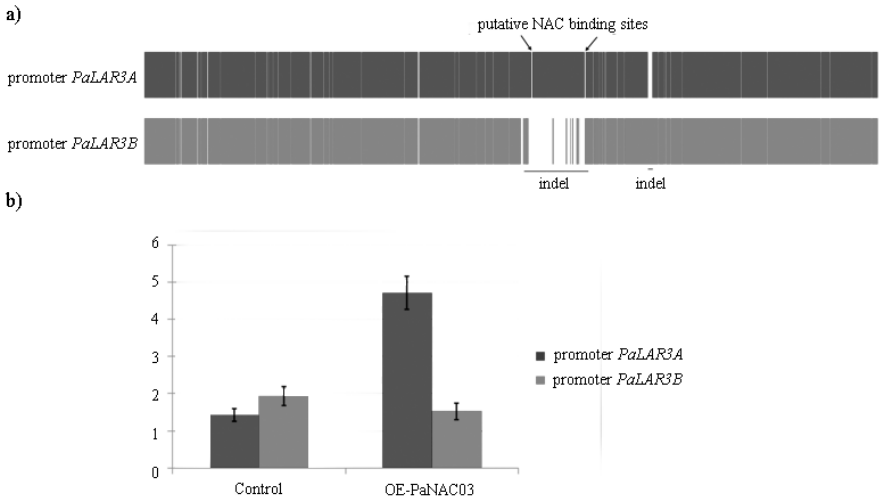

Figure 13. a) Comparison of the *PaLAR3A* and *PaLAR3B* promoter regions. b) Expression of *PaLAR3* in *N. benthamiana* after leave infiltration with 35S-*PaLAR3* and after leaf co-infiltration with 35S-*PaLAR3* and 35S-*PaNAC03*.

Our results confirmed the hypothesis that *Pa*NAC03 binds differently to the two promoters and that this is translated into differences in expression between the allele lineages. On one hand, however, our results showed a repression of *PaLAR3* in our transgenic cell lines overexpressing *Pa*NAC03 but, on the other hand, we also observed an activation of *PaLAR3* in *N. benthamiana*, suggesting that *Pa*NAC03 does not act as a negative regulator of 3-flavanol production, at least not through direct interaction with the target flavonoid biosynthetic genes. There is a possibility that the downregulation of the *CHS, F3'H* and *PaLAR3* genes in *PaNAC03* overexpressing lines is mediated by some other factor, possibly by the actions of the highly induced *GLABRA2* ortholog MA_122121g0010. An activation tagged mutant of *GLABRA2* has been shown to accumulate markedly lower levels of anthocyanins than WT *Arabidopsis* seedlings (Wang *et al.*, 2015a). Expression of the late biosynthesis genes *F3'H* and *ANS* were repressed in this mutant but not early biosynthesis genes such as *CHS* (Wang *et al.*, 2015a). Consequently, if MA_122121g0010 mediates the concerted downregulation of 3-flavanol biosynthesis, the mechanism by which MA_122121g0010 controls 3-flavanol production must differ from that of *GLABRA2*. Finally, it is possible that the reduced levels of

3-flavanols in the OE–lines is associated with the apparent interference of *Pa*NAC03 with the developmental program in Norway spruce embryogenic cultures. To test this, we would need to follow the expression of the target genes in different cell types and through the developmental stages, both in proliferating cultures and after initiation of embryo maturation.

4.9 Norway spruce has two functional *TT8* paralogs and one functional *TTG1* ortholog

The work presented in paper III gives a first insight into the subgroup IIIf of bHLH transcription factors (TFs) in a conifer. We isolated three putative genes encoding proteins that belong to the bHLH subgroup IIIf, *PabHLH-1*, *PabHLH-2* and *PabHLH-3,* from Norway spruce. The phylogenetic analysis suggested that the three bHLH proteins are paralogs, which are homologous to *TT*8, as the three bHLH candidates showed a shorter phylogenetic distance to *TT*8 than to the other *Arabidopsis* bHLH subgroup IIIf members (Paper III, Figure 1). Further analysis of the amino acid sequences of the three bHLH genes suggest that *PabHLH-1* and *PabHLH-2* encode functional bHLH proteins and that *PabHLH-3* might be a pseudogene. PabHLH-3 lacks large parts of protein domains that are essential for TT8 function (Feller *et al.*, 2011; Pattanaik *et al.*, 2008) (Paper III, Supplementary Figure 1). *Pa*bHLH-3 interaction with *Pa*WD40-1 is weaker than *Pa*bHLH-1 and *Pa*bHLH-2, and it did not interact with any of the subgroup 5 R2R3-MYB TFs included in the yeast two-hybrid assay. Alternatively, *Pa*bHLH-3 could have a regulatory role as a regulatory partner by forming heterodimers with other bHLH proteins as has been suggested for some bHLH proteins in *Arabidopsis* (Toledo-Ortiz *et al.*, 2003). Taken together, we conclude that the Norway spruce genome contains two functional *TT8* paralogs, *PabHLH-1* and *PabHLH-2*.

The level of sequence divergence between *Pa*bHLH-*1* and *PabHLH-2* and the presence of orthologous sequences in the white spruce and loblolly pine genomes (Paper III, Table 4), indicate that the split between *PabHLH-1* and *PabHLH-2* is not a recent duplication but that it predates the divergence between *Picea* and *Pinus*, which occurred approximately 90-100 Mya (Lu *et al.*, 2014). The case might not be the same for *PabHLH-3* since we could find an ortholog in white spruce but not in loblolly pine, suggesting either that the gene has been lost in loblolly pine or that *PabHLH-1* and *PabHLH-3* diverged in *Picea*. *PabHLH-1* and *PabHLH-2* show similar expression patterns in most tissues and in response to abiotic stress (Paper III; Table 1 and Figure 3), their protein interaction with subgroup 5 R2R3-MYB (Paper III, Figure 2) supports that there are differences between the two transcription factors. *Pa*bHLH-2

interacts with *Pa*MYB29, *Pa*MYB31 and *Pa*MYB33, while *Pa*bHLH-1 interacts only with *Pa*MYB33. Thus, the two paralogs appear to have diverged functionally since their separation.

In *Arabidopsis,* the WDR member of the MBW complex is represented by the protein single-copy ubiquitously expressed gene *TTG1,* influencing all traits associated with the MBW complex (Tominaga-Wada *et al.*, 2011). Consistent with these reports, we find a single potential ortholog of TTG1 in Norway spruce, *Pa*WD40-1, with a similarity of 65.6% and an identity of 85%. The predicted *Pa*WD40-1 protein sequence shows substantial similarity to TTG1 in the C-terminal region, which has been shown to be important for TTG1's interaction with TT8 (Matsui and Ohme-Takagi 2010). Consistent with the conservation of the predicted interaction domain, *Pa*WD40-1 interacts with all Norway spruce bHLH proteins included in the study. Finally, as expected of a *TTG1* ortholog, *PaWD40-1 is* expressed in all tissues and in response to various abiotic stress conditions. Taken together, our data suggests that that *PaWD40-1* is the *TTG1* ortholog in Norway spruce.

4.10 Norway spruce subgroup 5 R2R3-MYBs differ in function and expression patterns, and regulate genes in the flavonoid pathway

The R2R3-MYB transcription factors can act individually or as part of MBW complexes as regulators of the phenylpropanoid pathway (Xu *et al.*, 2014). In *Arabidopsis*, the R2R3-MYB transcription factor subgroup 5 is represented by the single member TT2 (Stracke *et al.*, 2001), which controls the regulation of the late flavonoids and proanthocyanidin biosynthesis (Nesi *et al.*, 2001). Together with the two MYB TFs with similarity to TT2 that have been reported from the genus *Picea* (Arnerup J., 2011; Xue *et al.*, 2003), we identified a total of six members of subgroup 5 of the R2R3-MYB transcription factor family (Stracke *et al.*, 2001) in Norway spruce. Our phylogenetic analyses suggested that the members of the *Picea* subgroup 5 could be further divided into the subgroups 5A, 5B and 5C based on sequence similarity (Paper III, Figure 4). The well-supported *Picea* subgroup 5A clustered together with TT2 and its orthologs in poplar, maize and grape (Mellway *et al.*, 2009; Terrier *et al.*, 2009; Nesi *et al.*, 2001; Pazares *et al.*, 1987), while *Pa*MYB35 clustered with to the grape transcription factor *Vv*MBPA1 (Bogs *et al.*, 2007) forming subgroup 5C. In contrast, the *Picea* subgroup 5B is formed by the two closely related MYB genes *PaMYB32* and *PaMYB33* and has no known homologs outside the gymnosperms, indicating that it represents a gymnosperm-specific subgroup of the R2R3-MYB transcription factor family. Examples of expanded

R2R3-MYB family transcription factor subgroups compared to *Arabidopsis* have already been reported from *Picea* and this expansion has been related to regulation of EBGs in conifers (Bomal *et al.*, 2014; Bedon *et al.*, 2010). Grotewold (2005) proposed a model to explain the functional divergence of recently duplicated regulatory genes and its relation to metabolic diversity in plants, which is based on functional differences. In our study, we observe these differences in the contrasting expression pattern of the MYB genes that we studied. The gene pair *PaMYB32* and *PaMYB33* (Subgroup 5B) is the most noteworthy example of distinct separation of tissue- or stress-dependent expression patterns between very closely related TFs (Paper III, Figure 5). All the studied MYB genes show individual expression patterns, which are consistent with the proposed modularity of the MBW complex where variation in inducibility or the tissue specificity among R2R3-MYB TFs would determine the regulation of individual traits (Xue *et al.*, 2014; Gonzalez, 2009; Baudry *et al.*, 2004; Dias *et al.*, 2003) in combination with particular bHLH proteins (Ramsay & Glover, 2005).

In the model plant *Lotus japonicus* there are three members of this subgroup capable of restoring function in *tt2* mutants (Yoshida *et al.*, 2008). When co-expressed with *LjTT8* and *LjTTG1,* the three *LjTT2*s show different activation of LBG in the flavonoid biosynthesis pathway (Yoshida *et al.*, 2010) and this variation in activation strength is associated with substitutions in the amino acid sequences of the different *LjTT2*s. To test the effect of our R2R3-MYB TFs in the regulation of genes in the flavonoid pathway, we generated transgenic lines overexpressing *PaMYB29*, *PaMYB32*, *PaMYB33* and *PaMYB35*. Overexpression of these TFs upregulated the expression of the LBGs *LAR3* and *ANR3* compared to wild type lines. Additionally, *PaMYB32* appeared to regulate *LAR4*. Overexpression of the close *PaMYB32* paralog, *PaMYB33,* and *PaMYB35,* activated more strongly the expression of the LGBs *ANR3, LAR3* and *LAR4* and also activated the EBG *PAL1,* and the LBGs *ANR2* and *ANR5* (Table 2). Together with the previously mentioned contrasting transcriptional responses to hormones and abiotic stress, this observation suggests that the gene duplication that gave rise *PaMYB32* and *PaMYB33* has been followed by a sub-functionalization of the paralogs, as predicted from Grotewold (2005)'s model and similar to that observed for R2R3-MYB TFs in several angiosperms (Chai *et al.*, 2014; Zhao & Bartley, 2014; Dias *et al.*, 2003). The more generalized LBG upregulation seen in *PaMYB33*- and *PaMYB35* overexpression lines compared with the expression pattern of *PaMYB33* and *PaMYB35,* which showed a higher degree of tissue specificity and more restricted responses to abiotic stress compared to *PaMYB29* and *PaMYB32,* suggests that the more generally expressed and induced TFs

(*Pa*MYB29 and *Pa*MYB32) might fulfil general functions in the plant. The more specific TFs (*Pa*MYB33 and *Pa*MYB35) may perform their regulatory role in particular organs or distinct cell types as suggested for *Arabidopsis*' subgroup 7 R2R3-MYB transcription factor family members (Stracke *et al.*, 2007) or for paralogous sequences in poplar (Chai *et al.*, 2014), and to some extent among conifer subgroup 4 R2R3-MYB family TFs (Bedon *et al.*, 2010; Bedon *et al.*, 2007).

Table 2. *Effect of the overexpression of four Norway spruce MYB genes in spruce cell lines compared to untransformed cells. Numbers indicate average fold-change in expression and standard deviation. Asterisk indicates significant regulation of the gene.*

	OE-*PaMYB29*	OE-*PaMYB32*	OE-*PaMYB33*	OE-*PaMYB35*
PAL1	0.2 ± 7.6	1.4 ± 1.2	7.1 ± 2.6*	5.9 ± 1.6*
ANR2	0.7 ± 5.2	4.9 ± 4.3	6.3 ± 1.4*	12.3 ± 1.4*
ANR3	8.7 ± 2.0*	5.7 ± 1.6*	26.7 ± 1.5*	11.5 ± 2.7*
ANR5	1.0 ± 2.5	1.4 ± 2.6	3.0 ± 2.1*	3.2 ± 2.7*
LAR3	3.5 ± 1.9*	7.7 ± 2.7*	13.2 ± 1.8*	5.4 ± 2.5*
LAR4	1.0 ± 12.3	4.1 ± 2.7*	2.6 ± 1.2*	2.9 ± 1.6*

5 Concluding remarks and future prospects

The work in this thesis has the focus of increasing the knowledge on the genetic control of defense responses in Norway spruce, which have been studied using mainly the necrotrophic pathogen *H. annosum s.l.*, but also the blue-stain pathogen *E. polonica*, the saprotroph *P. gigantea* and different stressors including hormones and mechanical wounding. This thesis highlights the role of hormones and in particular JA in defense response signaling in Norway spruce. These defense responses are similar in different plant tissues; and in response to mechanical wounding; and inoculation with saprotrophs and necrotrophs, but they vary in intensity (Oliva *et al.*, 2015; Yaqoob *et al.*, 2012). For this reason it is not only important to know what elements are involved in resistance but also how these elements are regulated. The work in this thesis reveals the involvement of the *PaLAR3* gene and (+) catechin in resistance against *H. parviporum* and also studies the regulation of genes in the flavonoid pathway, including *PaLAR3*, by different types of transcription factors.

In Paper I, it was shown that even though SA and ET are phytohormones active during defense response, JA is the major phytohormone that regulates defense response in Norway spruce. JA induced *PR1*, a gene that is responsive to SA, in the absence of SA. In contrast, in the absence of JA, SA could not induce *JAZ1*, a gene that is responsive to JA. Knowing the major role of JA in defense response signaling in Norway spruce, supports practices like seed treatments with the phytohormone in order to increase resistance to pests and pathogens as it has been already observed in conifers (Zas *et al.*, 2014). On the other hand, the ET biosynthetic gene *ACS* was induced distal to the treatment point. Due to its volatility, ET has the potential to be involved in defense signaling between tissues. By using ethylene inhibitors like STS, it could be tested if defense responses still happen distal from the treatment point as we observed in our study or if they no longer exist.

The work in Paper II validated *PaLAR3* as a gene contributing to resistance in Norway spruce. The novel allele *PaLAR3B* has a dominant effect over the already known allele *PaLAR3A* conferring higher levels of resistance to *H. parviporum* and higher (+) catechin levels to plants carrying it. As it has been observed in *E. polonica* (Hammerbacher *et al.*, 2014), we see that (+) catechin has a fungistatic effect on *H. parviporum*. This effect partly explains the differences in resistance that we observe between plants with different *PaLAR3* genotypes. Both alleles encode enzymes with similar catalytic properties, but plants carrying *PaLAR3B* show higher (+) catechin levels in bark and expression levels, suggesting that the two alleles differ essentially in inducibility. Papers III and IV answer the questions raised by this work regarding the differential inducibility of the two alleles. On the other hand, our work provides a molecular marker that is linked to resistance to one of the main Norway spruce pathogens, which could be implemented in the forest breeding programs by within-family selection for individuals carrying the *PaLAR3B* allele.

In Paper IV, we present *PaNAC03*, a gene induced by *H. annosum s.l.* that encodes a NAC TF. The overexpression of *PaNAC03* in Norway spruce cell lines leads to a decrease in the flavonoid contents and down-regulation of specific genes in the phenylpropanoid pathway including *PaLAR3*. On the other hand, we observe an interaction of *Pa*NAC03 with the *PaLAR3A* promoter that leads to the activation of the gene. No interaction was observed with the *PaLAR3B* promoter. If the mechanism through which *PaLAR3* is repressed in the overexpressing lines depends on the interaction of the gene with *Pa*NAC03, then *PaLAR3B* would not be subjected to this regulation explaining the differences in inducibility that we observe in Paper II. The cell line in which we overexpressed *Pa*NAC03 is homozygotic for *PaLAR3A*. By overexpressing *Pa*NAC03 in a cell line homozygotic for *PaLAR3B,* this could be confirmed. Our results indicate that *Pa*NAC03 is an activator of *PaLAR3* but that there are other factors in Norway spruce that lead to the down-regulation of *PaLAR3* and the other genes in the phenylpropanoid pathway during the overexpression of *PaNAC03*. One way to identify these other factors would be to use the *PaLAR3* promoters as a bait to identify TFs binding them during the overexpression of *PaNAC03*. These promoter-TF complexes could be isolated, using for instance, Yeast one-hybrid (Y1H), followed by colony isolation to determine what proteins are interacting with the *PaLAR3* promoters. The results of sequencing would give a list of transcription factors that regulate the expression of *PaLAR3* that would help explaining how defense response is regulated in Norway spruce.

Finally, Paper III identifies a full repertoire of members of the MBW complex in Norway spruce. This plant species and other *Pinaceae* seem to have a single WDR member of the complex as it happens in *Arabidopsis*. In contrast, we find two bHLH paralogs corresponding to a single bHLH member in *Arabidopsis* and six MYB homologs corresponding to a single MYB member in *Arabidopsis.* While functional differences between bHLHs seem to be based on protein interaction with the MYB TFs that were studied, the MYBs differ greatly in tissue specificity and stress inducibility. At least four of these MYBs regulate the expression of genes in the flavonoid pathway including *PaLAR3*. However, there are differences between the genes targeted by the four different MYBs. Taken together, the presence of multiple bHLH and MYB members in the MYB-bHLH-WDR complex that show differences in function and expression patterns suggests a higher complexity in the regulation of the flavonoid pathway in Norway spruce that does not exist in *Arabidopsis* and is consistent with the subfunctionalization model proposed by Grotewold (2005). Our work provides additional information regarding the regulation of the specialized metabolism by TFs that could be completed by testing the interaction of our bHLH TF family members with other spruce R2R3-MYB transcription factors. Additionally, comparing the interaction of different transcription factors with the promoter of their target genes would help to understand the regulation of these genes by transcription factors.

The work in this thesis contributes to the better understanding of the molecular responses in Norway spruce to *H. annosum s.l.* by covering different aspects including hormone signaling, validation of molecular resistance markers, and the study of the transcriptional control of the biosynthesis of specialized metabolites. All this work complements the existing knowledge and provides ground for further research in the area, but most importantly this work provides a important step, through the validation of the first molecular marker for resistance to *H. annosum* s.l., towards the inclusion of pathogen resistance in the Swedish Norway spruce breeding programs.

References

Adomas, A. & Asiegbu, F.O. (2006). Analysis of organ-specific responses of *Pinus sylvestris* to shoot (*Gremmeniella abietina*) and root (*Heterobasidion annosum*) pathogens. *Physiological and Molecular Plant Pathology,* 69(4), pp. 140-152.

Adomas, A., Heller, G., Li, G.S., Olson, A., Chu, T.M., Osborne, J., Craig, D., Van Zyl, L., Wolfinger, R., Sederoff, R., Dean, R.A., Stenlid, J., Finlay, R. & Asiegbu, F.O. (2007). Trranscript profiling of a conifer pathosystem: response of *Pinus sylvestri*s root tissues to pathogen (*Heterobasidion annosum*) invasion. *Tree Physiology,* 27(10), pp. 1441-1458.

Adrian, M. & Jeandet, P. (2012). Effects of resveratrol on the ultrastructure of *Botrytis cinerea* conidia and biological significance in plant/pathogen interactions. *Fitoterapia,* 83(8), pp. 1345-1350.

Adrian, M., Jeandet, P., Veneau, J., Weston, L.A. & Bessis, R. (1997). Biological activity of resveratrol, a stilbenic compound from grapevines, against *Botrytis cinerea*, the causal agent for gray mold. *Journal of Chemical Ecology,* 23(7), pp. 1689-1702.

Aguade, M. (2001). Nucleotide sequence variation at two genes of the phenylpropanoid pathway, the *FAH1* and *F3H* genes, in *Arabidopsis thaliana*. *Mol Biol Evol,* 18(1), pp. 1-9.

Appert, C., Zon, J. & Amrhein, N. (2003). Kinetic analysis of the inhibition of phenylalanine ammonia-lyase by 2-aminoindan-2-phosphonic acid and other phenylalanine analogues. *Phytochemistry,* 62(3), pp. 415-422.

Arnerup, J., Lind, M., Olson, A., Stenlid, J. & Elfstrand, M. (2011). The pathogenic white-rot fungus *Heterobasidion parviporum* triggers non-specific defence responses in the bark of Norway spruce. *Tree Physiology,* 31(11), pp. 1262-1272.

Arnerup, J., Swedjemark, G., Elfstrand, M., Karlsson, B. & Stenlid, J. (2010). Variation in growth of *Heterobasidion parviporum* in a full-sib family of *Picea abies*. *Scandinavian Journal of Forest Research,* 25(2), pp. 106-110.

Arnerup J. (2011). *Induced defence responses in Picea abies triggered by Heterobasidion annosum s.l.* Diss. Uppsala: Swedish University of Agricultural Sciences.

Asiegbu, F.O., Choi, W.B., Li, G.S., Nahalkova, J. & Dean, R.A. (2003). Isolation of a novel antimicrobial peptide gene (Sp-AMP) homologue from *Pinus sylvestris* (Scots pine) following infection with the root rot fungus *Heterobasidion annosum*. *Fems Microbiology Letters,* 228(1), pp. 27-31.

Baudry, A., Heim, M.A., Dubreucq, B., Caboche, M., Weisshaar, B. & Lepiniec, L. (2004). TT2, TT8, and TTG1 synergistically specify the expression of *BANYULS* and proanthocyanidin biosynthesis in *Arabidopsis thaliana*. *The Plant Journal,* 39(3), pp. 366-80.

Bedon, F., Bomal, C., Caron, S., Levasseur, C., Boyle, B., Mansfield, S.D., Schmidt, A., Gershenzon, J., Grima-Pettenati, J., Seguin, A. & MacKay, J. (2010). Subgroup 4 R2R3-MYBs in conifer trees: gene family expansion and contribution to the isoprenoid- and flavonoid-oriented responses. *Journal of Experimental Botany,* 61(14), pp. 3847-3864.

Bedon, F., Grima-Pettenati, J. & Mackay, J. (2007). Conifer R2R3-MYB transcription factors: sequence analyses and gene expression in wood-forming tissues of white spruce (*Picea glauca*). *BMC Plant Biology,* 7.

Birol, I., Raymond, A., Jackman, S.D., Pleasance, S., Coope, R., Taylor, G.A., Saint Yuen, M.M., Keeling, C.I., Brand, D., Vandervalk, B.P., Kirk, H., Pandoh, P., Moore, R.A., Zhao, Y.J., Mungall, A.J., Jaquish, B., Yanchuk, A., Ritland, C., Boyle, B., Bousquet, J., Ritland, K., MacKay, J., Bohlmann, J. & Jones, S.J.M. (2013). Assembling the 20 Gb white spruce (*Picea glauca)* genome from whole-genome shotgun sequencing data. *Bioinformatics,* 29(12), pp. 1492-1497.

Bogs, J., Jaffe, F.W., Takos, A.M., Walker, A.R. & Robinson, S.P. (2007). The grapevine transcription factor VvMYBPA1 regulates proanthocyanidin synthesis during fruit development. *Plant Physiology,* 143(3), pp. 1347-1361.

Bohlmann, J. (2012). Pine terpenoid defences in the mountain pine beetle epidemic and in other conifer pest interactions: specialized enemies are eating holes into a diverse, dynamic and durable defence system. *Tree Physiology,* 32(8), pp. 943-945.

Boller, T. & Felix, G. (2009). A renaissance of elicitors: perception of microbe-associated molecular patterns and danger signals by pattern-recognition receptors. *Annual Review of Plant Biology,* 60, pp. 379-406.

Bomal, C., Bedon, F., Caron, S., Mansfield, S.D., Levasseur, C., Cooke, J.E.K., Blais, S., Tremblay, L., Morency, M.J., Pavy, N., Grima-Pettenati, J., Seguin, A. & MacKay, J. (2008). Involvement of *Pinus taeda* MYB1 and MYB8 in phenylpropanoid metabolism and secondary cell wall biogenesis: a comparative in planta analysis. *Journal of Experimental Botany,* 59(14), pp. 3925-3939.

Bomal, C., Duval, I., Giguere, I., Fortin, E., Caron, S., Stewart, D., Boyle, B., Seguin, A. & MacKay, J.J. (2014). Opposite action of R2R3-MYBs from different subgroups on key genes of the shikimate and monolignol pathways in spruce. *Journal of Experimental Botany,* 65(2), pp. 495-508.

Boter, M., Ruíz-Rivero, O., Abdeen, A. & Prat, S. (2004). Conserved MYC transcription factors play a key role in jasmonate signaling both in tomato and *Arabidopsis*. *Genes & Development,* 18(13), pp. 1577-91.

Bouille, M. & Bousquet, J. (2005). Trans-species shared polymorphisms at orthologous nuclear gene loci among distant species in the conifer *Picea* (*Pinaceae*): Implications for the long-term maintenance of genetic diversity in trees. *American Journal of Botany,* 92(1), pp. 63-73.

Brignolas, F., Lacroix, B., Lieutier, F., Sauvard, D., Drouet, A., Claudot, A.C., Yart, A., Berryman, A.A. & Christiansen, E. (1995). Induced responses in phenolic metabolism in two

Norway spruce clones after wounding and inoculations with *Ophiostoma polonicum*, a bark beetle-associated fungus. *Plant Physiology,* 109(3), pp. 821-827.

Brignolas, F., Lieutier, F., Sauvard, D., Christiansen, E. & Berryman, A.A. (1998). Phenolic predictors for Norway spruce resistance to the bark beetle *Ips typographus* (*Coleoptera* : *Scolytidae*) and an associated fungus, *Ceratocystis polonica*. *Canadian journal of forest research,* 28(5), pp. 720-728.

Chai, G.H., Wang, Z.G., Tang, X.F., Yu, L., Qi, G., Wang, D.A., Yan, X.F., Kong, Y.Z. & Zhou, G.K. (2014). *R2R3-MYB* gene pairs in *Populus*: evolution and contribution to secondary wall formation and flowering time. *Journal of Experimental Botany,* 65(15), pp. 4255-4269.

Chang, C. & Bleecker, A.B. (2004). Ethylene biology. More than a gas. *Plant Physiology,* 136(2), pp. 2895-2899.

Chang, S., Puryear, J. & Cairney, J. (1993). A simple and efficient method for isolating RNA from pine trees. *Plant molecular biology reporter,* 11(2), pp. 113-116.

Chini, A., Fonseca, S., Fernandez, G., Adie, B., Chico, J.M., Lorenzo, O., Garcia-Casado, G., Lopez-Vidriero, I., Lozano, F.M., Ponce, M.R., Micol, J.L. & Solano, R. (2007). The JAZ family of repressors is the missing link in jasmonate signalling. *Nature,* 448(7154), pp. 666-671.

Creelman, R.A. & Mullet, J.E. (1997). Biosynthesis and action of jasmonates in plants. *Annual Review of Plant Physiology,* 48, pp. 355-381.

Curtis, M.D. & Grossniklaus, U. (2003). A gateway cloning vector set for high-throughput functional analysis of genes *in planta*. *Plant Physiology,* 133(2), pp. 462-469.

Cushnie, T.P.T. & Lamb, A.J. (2005). Antimicrobial activity of flavonoids. *International Journal of Antimicrobial Agents,* 26(5), pp. 343-356.

Dalman, K., Olson, A. & Stenlid, J. (2010). Evolutionary history of the conifer root rot fungus *Heterobasidion annosum* sensu lato. *Molecular Ecology,* 19(22), pp. 4979-93.

Danielsson, M., Lundén, K., Elfstrand, M., Hu, J., Zhao, T., Arnerup, J., Ihrmark, K., Swedjemark, G., Borg-Karlson, A.K. & Stenlid, J. (2011). Chemical and transcriptional responses of Norway spruce genotypes with different susceptibility to *Heterobasidion spp.* infection. *BMC Plant Biology,* 11.

Davis, J.M., Wu, H., Cooke, J.E.K., Reed, J.M., Luce, K.S. & Michler, C.H. (2002). Pathogen challenge, salicylic acid, and jasmonic acid regulate expression of chitinase gene homologs in pine. *Molecular Plant-Microbe Interactions,* 15(4), pp. 380-387.

Deflorio, G., Horgan, G., Woodward, S. & Fossdal, C.G. (2011). Gene expression profiles, phenolics and lignin of Sitka spruce bark and sapwood before and after wounding and inoculation with *Heterobasidion annosum*. *Physiological and Molecular Plant Pathology,* 75(4), pp. 180-187.

Delessert, C., Kazan, K., Wilson, I.W., Van Der Straeten, D., Manners, J., Dennis, E.S. & Dolferus, R. (2005). The transcription factor ATAF2 represses the expression of pathogenesis-related genes in *Arabidopsis*. The *Plant Journal,* 43(5), pp. 745-57.

Dias, A.P., Braun, E.L., McMullen, M.D. & Grotewold, E. (2003). Recently duplicated maize *R2R3-MYB* genes provide evidence for distinct mechanisms of evolutionary divergence after duplication. *Plant Physiology,* 131(2), pp. 610-620.

Duval, I., Lachance, D., Giguere, I., Bomal, C., Morency, M.J., Pelletier, G., Boyle, B., MacKay, J.J. & Seguin, A. (2014). Large-scale screening of transcription factor-promoter interactions in spruce reveals a transcriptional network involved in vascular development. *Journal of Experimental Botany,* 65(9), pp. 2319-2333.

Ekramoddoullah, A.K.M. & Hunt, R.S. (2002). Challenges and opportunities in studies of host-pathogen interactions in forest tree species. *Canadian Journal of Plant Pathology,* 24(4), pp. 408-415.

Estell, D.A., Graycar, T.P. & Wells, J.A. (1985). Engineering an enzyme by site-directed mutagenesis to be resistant to chemical oxidation. *Journal of Biological Chemistry,* 260(11), pp. 6518-6521.

Fahn, A. (1988). Secretory tissues in vascular plants. *New Phytologist,* 108(3), pp. 229-257.

Farjon, A. (1990). *Pinaceae. Drawings and descriptions of the genera Abies, Cedrus, Pseudolarix, Keteleeria, Nothotsuga, Tsuga, Cathaya, Pseudotsuga, Larix and Picea.* Koeltz: Königstein: Koeltz Scientific Books.

Farmer, E.E., Caldelari, D., Pearce, G., Walkersimmons, K. & Ryan, C.A. (1994). Diethyldithiocarbamic acid inhibits the octadecanoid signaling pathway for the wound induction of proteinase-Inhibitors in tomato leaves. *Plant Physiology,* 106(1), pp. 337-342.

Feller, A., Machemer, K., Braun, E.L. & Grotewold, E. (2011). Evolutionary and comparative analysis of MYB and bHLH plant transcription factors. *The Plant Journal,* 66(1), pp. 94-116.

Filatov, D.A. & Charlesworth, D. (1999). DNA polymorphism, haplotype structure and balancing selection in the *Leavenworthia PgiC* locus. *Genetics,* 153(3), pp. 1423-1434.

Fossdal, C.G., Sharma, P. & Lönneborg, A. (2001). Isolation of the first putative peroxidase cDNA from a conifer and the local and systemic accumulation of related proteins upon pathogen infection. *Plant Molecular Biology,* 47(3), pp. 423-435.

Franceschi, V.R., Krekling, T., Berryman, A.A. & Christiansen, E. (1998). Specialized phloem parenchyma cells in Norway spruce (*Pinaceae*) bark are an important site of defense reactions. *American Journal of Botany,* 85(5), pp. 601-615.

Franceschi, V.R., Krekling, T. & Christiansen, E. (2002). Application of methyl jasmonate on *Picea abies* (*Pinaceae*) stems induces defense-related responses in phloem and xylem. *American Journal of Botany,* 89(4), pp. 578-586.

Franceschi, V.R., Krokene, P., Christiansen, E. & Krekling, T. (2005). Anatomical and chemical defenses of conifer bark against bark beetles and other pests. *New Phytologist,* 167(2), pp. 353-375.

Franceschi, V.R., Krokene, P., Krekling, T. & Christiansen, E. (2000). Phloem parenchyma cells are involved in local and distant defense responses to fungal inoculation or bark-beetle attack in Norway spruce (*Pinaceae*). *American Journal of Botany,* 87(3), pp. 314-326.

Garbelotto, M., Ratcliff, A., Bruns, T.D., Cobb, F.W. & Otrosina, W.J. (1996). Use of taxon-specific competitive-priming PCR to study host specificity, hybridization, and intergroup gene flow in intersterility groups of *Heterobasidion annosum*. *Phytopathology,* 86(5), pp. 543-551.

Gershenzon, J. (1994). Metabolic costs of terpenoid accumulation in higher plants. *Journal of Chemical Ecology,* 20(6), pp. 1281-1328.

Glazebrook, J. (2005). Contrasting mechanisms of defense against biotrophic and necrotrophic pathogens. *Annual Review of Phytopathology,* 43, pp. 205-227.

Gonzalez, A. (2009). Pigment loss in response to the environment: a new role for the WD/bHLH/MYB anthocyanin regulatory complex. *New Phytologist,* 182(1), pp. 1-3.

Gonzalez-Martinez, S.C., Ersoz, E., Brown, G.R., Wheeler, N.C. & Neale, D.B. (2006). DNA sequence variation and selection of tag single-nucleotide polymorphisms at candidate genes for drought-stress response in *Pinus taeda* L. *Genetics,* 172(3), pp. 1915-1926.

Grotewold, E. (2005). Plant metabolic diversity: a regulatory perspective. *Trends in Plant Science,* 10(2), pp. 57-62.

Hammerbacher, A. (2011). *Biosynthesis of polyphenols in Norway spruce as a defense strategy against attack by the bark beetle associated fungus Ceratocystis polonica* Diss. Jena, Germany: Max Planck Institute for Chemical Ecology.

Hammerbacher, A., Paetz, C., Wright, L.P., Fischer, T.C., Bohlmann, J., Davis, A.J., Fenning, T.M., Gershenzon, J. & Schmidt, A. (2014). Flavan-3-ols in Norway spruce: biosynthesis, accumulation, and function in response to attack by the bark beetle-associated fungus *Ceratocystis polonica*. *Plant Physiology,* 164(4), pp. 2107-2122.

Hammerbacher, A., Schmidt, A., Wadke, N., Wright, L.P., Schneider, B., Bohlmann, J., Brand, W.A., Fenning, T.M., Gershenzon, J. & Paetz, C. (2013). A common fungal associate of the spruce bark beetle metabolizes the stilbene defenses of Norway spruce. *Plant Physiology,* 162(3), pp. 1324-1336.

Hao, Y.-J., Song, Q.-X., Chen, H.-W., Zou, H.-F., Wei, W., Kang, X.-S., Ma, B., Zhang, W.-K., Zhang, J.-S. & Chen, S.-Y. (2010). Plant NAC-type transcription factor proteins contain a NARD domain for repression of transcriptional activation. *Planta,* 232(5), pp. 1033-43.

Hietala, A.M., Kvaalen, H., Schmidt, A., Johnk, N., Solheim, H. & Fossdal, C.G. (2004). Temporal and spatial profiles of chitinase expression by Norway spruce in response to bark colonization by *Heterobasidion annosum*. *Applied and Environmental Microbiology,* 70(7), pp. 3948-3953.

Himejima, M., Hobson, K.R., Otsuka, T., Wood, D.L. & Kubo, I. (1992). Antimicrobial terpenes from oleoresin of ponderosa pine tree *Pinus ponderosa:* A defense mechanism against microbial invasion. *Journal of Chemical Ecology,* 18(10), pp. 1809-1818.

Högberg, K.A., Ekberg, I., Norell, L. & von Arnold, S. (1998). Integration of somatic embryogenesis in a tree breeding programme: a case study with *Picea abies*. *Canadian journal of forest research,* 28(10), pp. 1536-1545.

Hudgins, J.W., Christiansen, E. & Franceschi, V.R. (2004). Induction of anatomically based defense responses in stems of diverse conifers by methyl jasmonate: a phylogenetic perspective. *Tree Physiology,* 24(3), pp. 251-264.

Hudgins, J.W. & Franceschi, V.R. (2004). Methyl jasmonate-induced ethylene production is responsible for conifer phloem defense responses and reprogramming of stem cambial zone for traumatic resin duct formation. *Plant Physiology,* 135(4), pp. 2134-2149.

Hudgins, J.W., Krekling, T. & Franceschi, V.R. (2003). Distribution of calcium oxalate crystals in the secondary phloem of conifers: a constitutive defense mechanism? *New Phytologist,* 159(3), pp. 677-690.

Ingestad, T. & Kähr, M. (1985). Nutrition and growth of coniferous seedlings at varied relative nitrogen addition rate. *Physiologia Plantarum,* 65(2), pp. 109-116.

Jensen, M.K., Kjaersgaard, T., Petersen, K. & Skriver, K. (2010). NAC genes: time-specific regulators of hormonal signaling in Arabidopsis. *Plant Signaling and Behavior,* 5(7), pp. 907-910.

Jøhnk, N., Hietala, A.M., Fossdal, C.G., Collinge, D.B. & Newman, M.-A. (2005). Defense-related genes expressed in Norway spruce roots after infection with the root rot pathogen *Ceratobasidium bicorne* (anamorph: *Rhizoctonia* sp.). *Tree Physiology,* 25(12), pp. 1533-43.

Jones, A.M. (2001). Programmed cell death in development and defense. *Plant Physiology,* 125(1), pp. 94-97.

Karlsson, B. & Swedjemark, G. (2006). Genotypic variation in natural infection frequency of *Heterobasidion spp*. in a *Picea abies* clone trial in southern Sweden. *Scandinavian Journal of Forest Research,* 21(2), pp. 108-114.

Katagiri, F. & Tsuda, K. (2010). Understanding the plant immune system. *Molecular Plant-Microbe Interactions,* 23(12), pp. 1531-1536.

Kazan, K. & Lyons, R. (2014). Intervention of phytohormone pathways by pathogen effectors. *Plant Cell,* 26(6), pp. 2285-2309.

Knoth, C. & Eulgem, T. (2008). The oomycete response gene *LURP1* is required for defense against *Hyaloperonospora parasitica* in *Arabidopsis thaliana*. *The Plant Journal,* 55(1), pp. 53-64.

Korhonen, K. & Stenlid, J. (1998). Biology of *Heterobasidion annosum*. In: Woodward, S., Stenlid, J., Karjalainen, R. & Hüttermann, A. (eds) *Heterobasidion Annosum: Biology, Ecology, Impact and Control* CAB International, pp. 43-70.

Kozlowski, G., Buchala, A. & Metraux, J.P. (1999). Methyl jasmonate protects Norway spruce [*Picea abies* (L.) Karst.] seedlings against *Pythium ultimum* Trow. *Physiological and Molecular Plant Pathology,* 55(1), pp. 53-58.

Krekling, T., Franceschi, V.R., Berryman, A.A. & Christiansen, E. (2000). The structure and development of polyphenolic parenchyma cells in Norway spruce (*Picea abies*) bark. *Flora,* 195(4), pp. 354-369.

Krekling, T., Franceschi, V.R., Krokene, P. & Solheim, H. (2004). Differential anatomical response of Norway spruce stem tissues to sterile and fungus infected inoculations. *Trees,* 18(1), pp. 1-9.

Kuhlman, E.G. (1969). Inoculation of loblolly pine seedlings with *Fomes annosus* in greenhouse. *Canadian Journal of Botany,* 47(12), pp. 2079-&.

Kunkel, B.N. & Brooks, D.M. (2002). Cross talk between signaling pathways in pathogen defense. *Current Opinion in Plant Biology,* 5(4), pp. 325-331.

Lepiniec, L., Debeaujon, I., Routaboul, J.M., Baudry, A., Pourcel, L., Nesi, N. & Caboche, M. (2006). Genetics and biochemistry of seed flavonoids. *Annual Review of Plant Biology,* 57, pp. 405-430.

Lieutier, F., Brignolas, F., Sauvard, D., Yart, A., Galet, C., Brunet, M. & van de Sype, H. (2003). Intra- and inter-provenance variability in phloem phenols of *Picea abies* and relationship to a bark beetle-associated fungus. *Tree Physiology,* 23(4), pp. 247-256.

Likar, M. & Regvar, M. (2008). Early defence reactions in Norway spruce seedlings inoculated with the mycorrhizal fungus *Pisolithus tinctorius* (Persoon) Coker & Couch and the pathogen *Heterobasidion annosum (Fr.) Bref. Trees,* 22(6), pp. 861-868.

Lind, M., Dalman, K., Stenlid, J., Karlsson, B. & Olson, A. (2007). Identification of quantitative trait loci affecting virulence in the basidiomycete *Heterobasidion annosum s.l. Current Genetics,* 52(1), pp. 35-44.

Lind, M., Källman, T., Chen, J., Ma, X.F., Bousquet, J., Morgante, M., Zaina, G., Karlsson, B., Elfstrand, M., Lascoux, M. & Stenlid, J. (2014). A *Picea abies* linkage map based on SNP markers identifies QTLs for four aspects of resistance to *Heterobasidio*n parviporum infection. *PloS ONE,* 9(7).

Liu, J.J. & Ekramoddoullah, A.K.M. (2004). Characterization, expression and evolution of two novel subfamilies of *Pinus monticola* cDNAs encoding pathogenesis-related (PR)-10 proteins. *Tree Physiology,* 24(12), pp. 1377-1385.

Lorenzo, O., Piqueras, R., Sanchez-Serrano, J.J. & Solano, R. (2003). ETHYLENE RESPONSE FACTOR 1 integrates signals from ethylene and jasmonate pathways in plant defense. *Plant Cell,* 15(1), pp. 165-178.

Lu, Y., Ran, J.H., Guo, D.M., Yang, Z.Y. & Wang, X.Q. (2014). Phylogeny and divergence times of gymnosperms inferred from single-copy nuclear genes. *PloS ONE,* 9(9).

Lundén, K., Danielsson, M., Durling, M.B., Ihrmark, K., Nemesio Gorriz, M., Stenlid, J., Asiegbu, F.O. & Elfstrand, M. (2015). Transcriptional Responses Associated with Virulence and Defence in the Interaction between Heterobasidion annosum s.s. and Norway Spruce. *PloS ONE,* 10(7), p. e0131182.

Martin, D., Tholl, D., Gershenzon, J. & Bohlmann, J. (2002). Methyl jasmonate induces traumatic resin ducts, terpenoid resin biosynthesis, and terpenoid accumulation in developing xylem of Norway spruce stems. *Plant Physiology,* 129(3), pp. 1003-1018.

Mauge, C., Granier, T., d'Estaintot, B.L., Gargouri, M., Manigand, C., Schmitter, J.M., Chaudiere, J. & Gallois, B. (2010). Crystal structure and catalytic mechanism of leucoanthocyanidin reductase from *Vitis vinifera*. *Journal of Molecular Biology,* 397(4), pp. 1079-1091.

Mellway, R.D., Tran, L.T., Prouse, M.B., Campbell, M.M. & Constabel, C.P. (2009). The wound-, pathogen-, and ultraviolet B-responsive *MYB134* gene encodes an R2R3-MYB transcription factor that regulates proanthocyanidin synthesis in poplar. *Plant Physiology,* 150(2), pp. 924-941.

Miller, B., Madilao, L.L., Ralph, S. & Bohlmann, J. (2005). Insect-induced conifer defense. White pine weevil and methyl jasmonate induce traumatic resinosis, de novo formed volatile emissions, and accumulation of terpenoid synthase and putative octadecanoid pathway transcripts in Sitka spruce. *Plant Physiology,* 137(1), pp. 369-382.

Morishita, T., Kojima, Y., Maruta, T., Nishizawa-Yokoi, A., Yabuta, Y. & Shigeoka, S. (2009). Arabidopsis NAC transcription factor, ANAC078, regulates flavonoid biosynthesis under high-light. *Plant and Cell Physiology,* 50(12), pp. 2210-22.

Mukhtar, M.S., Carvunis, A.R., Dreze, M., Epple, P., Steinbrenner, J., Moore, J., Tasan, M., Galli, M., Hao, T., Nishimura, M.T., Pevzner, S.J., Donovan, S.E., Ghamsari, L., Santhanam, B., Romero, V., Poulin, M.M., Gebreab, F., Gutierrez, B.J., Tam, S., Monachello, D., Boxem, M., Harbort, C.J., McDonald, N., Gai, L.T., Chen, H.M., He, Y.J., Vandenhaute, J., Roth, F.P., Hill, D.E., Ecker, J.R., Vidal, M., Beynon, J., Braun, P., Dangl, J.L. & Conso, E.U.E.

(2011). Independently evolved virulence effectors converge onto hubs in a plant immune system network. *Science,* 333(6042), pp. 596-601.

Nagy, N.E., Fossdal, C.G., Krokene, P., Krekling, T., Lonneborg, A. & Solheim, H. (2004). Induced responses to pathogen infection in Norway spruce phloem: changes in polyphenolic parenchyma cells, chalcone synthase transcript levels and peroxidase activity. *Tree Physiology,* 24(5), pp. 505-515.

Nagy, N.E., Franceschi, V.R., Kvaalen, H. & Solheim, H. (2005). Callus cultures and bark from Norway spruce clones show similar cellular features and relative resistance to fungal pathogens. *Trees,* 19(6), pp. 695-703.

Nagy, N.E., Franceschi, V.R., Solheim, H., Krekling, T. & Christiansen, E. (2000). Wound-induced traumatic resin duct development in stems of Norway spruce (*Pinaceae*): Anatomy and cytochemical traits. *American Journal of Botany,* 87(3), pp. 302-313.

Neale, D.B., Wegrzyn, J.L., Stevens, K.A., Zimin, A.V., Puiu, D., Crepeau, M.W., Cardeno, C., Koriabine, M., Holtz-Morris, A.E., Liechty, J.D., Martinez-Garcia, P.J., Vasquez-Gross, H.A., Lin, B.Y., Zieve, J.J., Dougherty, W.M., Fuentes-Soriano, S., Wu, L.S., Gilbert, D., Marcais, G., Roberts, M., Holt, C., Yandell, M., Davis, J.M., Smith, K.E., Dean, J.F.D., Lorenz, W.W., Whetten, R.W., Sederoff, R., Wheeler, N., McGuire, P.E., Main, D., Loopstra, C.A., Mockaitis, K., deJong, P.J., Yorke, J.A., Salzberg, S.L. & Langley, C.H. (2014). Decoding the massive genome of loblolly pine using haploid DNA and novel assembly strategies. *Genome Biology,* 15(3).

Nesi, N., Debeaujon, I., Jond, C., Pelletier, G., Caboche, M. & Lepiniec, L. (2000). The *TT8* gene encodes a basic helix-loop-helix domain protein required for expression of *DFR* and *BAN* genes in *Arabidopsis* siliques. *Plant Cell,* 12(10), pp. 1863-1878.

Nesi, N., Jond, C., Debeaujon, I., Caboche, M. & Lepiniec, L. (2001). The *Arabidopsis TT2* gene encodes an R2R3 MYB domain protein that acts as a key determinant for proanthocyanidin accumulation in developing seed. *Plant Cell,* 13(9), pp. 2099-2114.

Niemelä, T. & Korhonen, K. (1998). Taxonomy of the genus *Heterobasidion*. In: Woodward, S., Stenlid, J., Karjalainen, R. & Hüttermann, A. (eds) *Heterobasidion annosum. biology, ecology, impact and control* CAB International, pp. 27-33.

Nystedt, B., Street, N.R., Wetterbom, A., Zuccolo, A., Lin, Y.C., Scofield, D.G., Vezzi, F., Delhomme, N., Giacomello, S., Alexeyenko, A., Vicedomini, R., Sahlin, K., Sherwood, E., Elfstrand, M., Gramzow, L., Holmberg, K., Hallman, J., Keech, O., Klasson, L., Koriabine, M., Kucukoglu, M., Kaller, M., Luthman, J., Lysholm, F., Niittyla, T., Olson, A., Rilakovic, N., Ritland, C., Rossello, J.A., Sena, J., Svensson, T., Talavera-Lopez, C., Theissen, G., Tuominen, H., Vanneste, K., Wu, Z.Q., Zhang, B., Zerbe, P., Arvestad, L., Bhalerao, R., Bohlmann, J., Bousquet, J., Gil, R.G., Hvidsten, T.R., de Jong, P., MacKay, J., Morgante, M., Ritland, K., Sundberg, B., Thompson, S.L., Van de Peer, Y., Andersson, B., Nilsson, O., Ingvarsson, P.K., Lundeberg, J. & Jansson, S. (2013). The Norway spruce genome sequence and conifer genome evolution. *Nature,* 497(7451), pp. 579-584.

Ohshima, M., Itoh, H., Matsuoka, M., Murakami, T. & Ohashi, Y. (1990). Analysis of Stress-Induced or Salicyclic Acid-Induced Expression of the Pathogenesis-Related 1a-Protein Gene in Transgenic Tobacco. *Plant Cell,* 2(2), pp. 95-106.

Oliva, J., Rommel, S., Fossdal, C.G., Hietala, A.M., Nemesio-Gorriz, M., Solheim, H. & Elfstrand, M. (2015). Transcriptional responses of Norway spruce (*Picea abies)* inner sapwood against *Heterobasidion parviporum*. *Tree Physiology,* 35(9), pp. 1007-15.

Oliva, J., Thor, M. & Stenlid, J. (2010). Reaction zone and periodic increment decrease in *Picea abies* trees infected by *Heterobasidion annosum s.l. Forest Ecology and Management,* 260(5), pp. 692-698.

Olsen, A.N., Ernst, H.A., Leggio, L.L. & Skriver, K. (2005). NAC transcription factors: structurally distinct, functionally diverse. *Trends in Plant Science,* 10(2), pp. 79-87.

Olson, A., Aerts, A., Asiegbu, F., Belbahri, L., Bouzid, O., Broberg, A., Canbäck, B., Coutinho, P.M., Cullen, D., Dalman, K., Deflorio, G., van Diepen, L.T.A., Dunand, C., Duplessis, S., Durling, M., Gonthier, P., Grimwood, J., Fossdal, C.G., Hansson, D., Henrissat, B., Hietala, A., Himmelstrand, K., Hoffmeister, D., Högberg, N., James, T.Y., Karlsson, M., Kohler, A., Kues, U., Lee, Y.H., Lin, Y.C., Lind, M., Lindquist, E., Lombard, V., Lucas, S., Lundén, K., Morin, E., Murat, C., Park, J., Raffaello, T., Rouze, P., Salamov, A., Schmutz, J., Solheim, H., Ståhlberg, J., Velez, H., de Vries, R.P., Wiebenga, A., Woodward, S., Yakovlev, I., Garbelotto, M., Martin, F., Grigoriev, I.V. & Stenlid, J. (2012). Insight into trade-off between wood decay and parasitism from the genome of a fungal forest pathogen. *New Phytologist,* 194(4), pp. 1001-1013.

Pattanaik, S., Xie, C.H. & Yuan, L. (2008). The interaction domains of the plant Myc-like bHLH transcription factors can regulate the transactivation strength. *Planta,* 227(3), pp. 707-715.

Pavy, N., Gagnon, F., Rigault, P., Blais, S., Deschenes, A., Boyle, B., Pelgas, B., Deslauriers, M., Clement, S., Lavigne, P., Lamothe, M., Cooke, J.E.K., Jaramillo-Correa, J.P., Beaulieu, J., Isabel, N., Mackay, J. & Bousquet, J. (2013). Development of high-density SNP genotyping arrays for white spruce (*Picea glauca*) and transferability to subtropical and nordic congeners. *Molecular Ecology Resources,* 13(2), pp. 324-336.

Pazares, J., Ghosal, D., Wienand, U., Peterson, P.A. & Saedler, H. (1987). The regulatory *c1* locus of *Zea mays* encodes a protein with homology to MYB proto-oncogene products and with structural similarities to transcriptional activators. *The EMBO Journal,* 6(12), pp. 3553-3558.

Petroni, K. & Tonelli, C. (2011). Recent advances on the regulation of anthocyanin synthesis in reproductive organs. *Plant Science,* 181(3), pp. 219-229.

Petrussa, E., Braidot, E., Zancani, M., Peresson, C., Bertolini, A., Patui, S. & Vianello, A. (2013). Plant flavonoids—biosynthesis, transport and involvement in stress responses. *International Journal of Molecular Sciences,* 14(7), pp. 14950-14973.

Phillips, M.A. & Croteau, R.B. (1999). Resin-based defenses in conifers. *Trends in Plant Science,* 4(5), pp. 184-190.

Pont, V. & Pezet, R. (1990). Relation between the chemical structure and the biological activity of hydroxystilbenes against *Botrytis cinerea*. *Journal of Phytopathology,* 130(1), pp. 1-8.

Porth, I., White, R., Jaquish, B., Alfaro, R., Ritland, C. & Ritland, K. (2012). Genetical genomics identifies the genetic architecture for growth and weevil resistance in spruce. *PloS ONE,* 7(9).

Puranik, S., Sahu, P.P., Srivastava, P.S. & Prasad, M. (2012). NAC proteins: regulation and role in stress tolerance. *Trends in Plant Science,* 17(6), pp. 369-81.

Ralph, S.G., Yueh, H., Friedmann, M., Aeschliman, D., Zeznik, J.A., Nelson, C.C., Butterfield, Y.S.N., Kirkpatrick, R., Liu, J., Jones, S.J.M., Marra, M.A., Douglas, C.J., Ritland, K. & Bohlmann, J. (2006). Conifer defence against insects: microarray gene expression profiling of Sitka spruce (*Picea sitchensis*) induced by mechanical wounding or feeding by spruce budworms (*Choristoneura occidentalis*) or white pine weevils (*Pissodes strobi*) reveals large-scale changes of the host transcriptome. *Plant Cell and Environment,* 29(8), pp. 1545-1570.

Ramsay, N.A. & Glover, B.J. (2005). MYB-bHLH-WD40 protein complex and the evolution of cellular diversity. *Trends in Plant Science,* 10(2), pp. 63-70.

Ré, D.A., Raud, B., Chan, R.L., Baldwin, I.T. & Bonaventure, G. (2012). RNAi-mediated silencing of the HD-Zip gene *HD20* in *Nicotiana attenuata* affects benzyl acetone emission from corollas via ABA levels and the expression of metabolic genes. *BMC Plant Biology,* 12(1), p. 60.

Rigault, P., Boyle, B., Lepage, P., Cooke, J.E.K., Bousquet, J. & MacKay, J.J. (2011). A white spruce gene catalog for conifer genome analyses. *Plant Physiology,* 157(1), pp. 14-28.

Robert-Seilaniantz, A., Grant, M. & Jones, J.D.G. (2011). Hormone crosstalk in plant disease and defense: more than just jasmonate-salicylate antagonism. *Annual Review of Phytopathology,* 49, pp. 317-343.

Salunke, B.K., Kotkar, H.M., Mendki, P.S., Upasani, S.M. & Maheshwari, V.L. (2005). Efficacy of flavonoids in controlling *Callosobruchus chinensis* (L.) (*Coleoptera : Bruchidae)*, a post-harvest pest of grain legumes. *Crop Protection,* 24(10), pp. 888-893.

Salzer, P., Hubner, B., Sirrenberg, A. & Hager, A. (1997). Differential effect of purified spruce chitinases and [beta]-1, 3-glucanases on the activity of elicitors from ectomycorrhizal fungi. *Plant Physiology,* 114(3), pp. 957-968.

Sato, M., Tsuda, K., Wang, L., Coller, J., Watanabe, Y., Glazebrook, J. & Katagiri, F. (2010). Network modeling reveals prevalent negative regulatory relationships between signaling sectors in *Arabidopsis* immune signaling. *PloS Pathogens,* 6(7).

Shain, L. & Hillis, W.E. (1971). Phenolic extractives in Norway spruce and their effects on *Fomes annosus*. *Phytopathology,* 61(7), pp. 841-845.

Sooriyaarachchi, S., Jaber, E., Covarrubias, A.S., Ubhayasekera, W., Asiegbu, F.O. & Mowbray, S.L. (2011). Expression and beta-glucan binding properties of Scots pine (*Pinus sylvestris* L.) antimicrobial protein (Sp-AMP). *Plant Molecular Biology,* 77(1-2), pp. 33-45.

Stenlid, J. (1985). Population structure of *Heterobasidion annosum* as determined by somatic incompatibility, sexual incompatibility and isoenzyme patterns. *Canadian Journal of Botany,* 63(12), pp. 2268-2273.

Stenlid, J. & Karlsson, J.O. (1991). Partial intersterility in *Heterobasidion annosum*. *Mycological Research,* 95, pp. 1153-1159.

Stracke, R., Ishihara, H., Barsch, G.H.A., Mehrtens, F., Niehaus, K. & Weisshaar, B. (2007). Differential regulation of closely related R2R3-MYB transcription factors controls flavonol accumulation in different parts of the *Arabidopsis thaliana* seedling. *The Plant Journal,* 50(4), pp. 660-677.

Stracke, R., Werber, M. & Weisshaar, B. (2001). The R2R3-MYB gene family in *Arabidopsis thaliana*. *Current Opinion in Plant Biology,* 4(5), pp. 447-56.

Sun, H., Aidun, C.K. & Egertsdotter, U. (2011). Possible Effect From Shear Stress on Maturation of Somatic Embryos of Norway Spruce (*Picea abies*). *Biotechnology and Bioengineering,* 108(5), pp. 1089-1099.

Swedjemark, G. & Karlsson, B. (2004). Genotypic variation in susceptibility following artificial *Heterobasidion annosum* inoculation of *Picea abies* clones in a 17-year-old field test. *Scandinavian Journal of Forest Research,* 19(2), pp. 103-111.

Swedjemark, G., Stenlid, J. & Karlsson, B. (1997). Genetic variation among clones of *Picea abies* in resistance to growth of *Heterobasidion annosum*. *Silvae Genetica,* 46(6), pp. 369-374.

Tamura, K., Stecher, G., Peterson, D., Filipski, A. & Kumar, S. (2013). MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. *Molecular Biology and Evolution,* 30(12), pp. 2725-9.

Terrier, N., Torregrosa, L., Ageorges, A., Vialet, S., Verries, C., Cheynier, V. & Romieu, C. (2009). Ectopic expression of *VvMybPA2* promotes proanthocyanidin biosynthesis in grapevine and suggests additional targets in the pathway. *Plant Physiology,* 149(2), pp. 1028-1041.

Thaler, J.S., Humphrey, P.T. & Whiteman, N.K. (2012). Evolution of jasmonate and salicylate signal crosstalk. *Trends in Plant Science,* 17(5), pp. 260-270.

Thor, M., Ståhl, G. & Stenlid, J. (2005). Root rot incidence, correlation with environmental conditions and potential for predicting stand decay levels. *Scandinavian Journal of Forest Research,* 20, pp. 165-176.

Thor, M. & Stenlid, J. (1998). *Heterobasidion annosum* infection following mechanized first thinning and stump treatment in *Picea abies*. *Scandinavian Journal of Forest Research,* 20(2), pp. 154-164.

Tohge, T., Watanabe, M., Hoefgen, R. & Fernie, A.R. (2013). The evolution of phenylpropanoid metabolism in the green lineage. *Critical Reviews in Biochemistry and Molecular Biology,* 48(2), pp. 123-152.

Toledo-Ortiz, G., Huq, E. & Quail, P.H. (2003). The *Arabidopsis* basic/helix-loop-helix transcription factor family. *Plant Cell,* 15(8), pp. 1749-70.

Tominaga-Wada, R., Ishida, T. & Wada, T. (2011). New insights into the mechanism of development of *Arabidopsis* root hairs and trichomes. *International Review of Cell and Molecular Biology,* 286, pp. 67-106.

Tomova, L., Braun, S. & Fluckiger, W. (2005). The effect of nitrogen fertilization on fungistatic phenolic compounds in roots of beech (*Fagus sylvatica*) and Norway spruce (*Picea abies*). *Forest Pathology,* 35(4), pp. 262-276.

Trombetta, D., Castelli, F., Sarpietro, M.G., Venuti, V., Cristani, M., Daniele, C., Saija, A., Mazzanti, G. & Bisignano, G. (2005). Mechanisms of antibacterial action of three monoterpenes. *Antimicrobial Agents and Chemotherapy,* 49(6), pp. 2474-2478.

Upasani, S.M., Kotkar, H.M., Mendki, P.S. & Maheshwari, V.L. (2003). Partial characterization and insecticidal properties of *Ricinus communis* L foliage flavonoids. *Pest Management Science,* 59(12), pp. 1349-1354.

Vasaitis, R., Stenlid, J., Thomsen, I.M., Barklund, P. & Dahlberg, A. (2008). Stump removal to control root rot in forest stands. A literature study. *Silva Fennica,* 42(3), pp. 457-483.

Veen, H. (1983). Silver thiosulfate an experimental tool in plant science. *Scientia Horticulturae,* 20(3), pp. 211-224.

Vivas, M., Zas, R., Sampedro, L. & Solla, A. (2013). Environmental maternal effects mediate the resistance of Maritime pine to biotic stress. *PloS ONE,* 8(7).

von Arnold, S. & Eriksson, T. (1981). In vitro studies of adventitious shoot formation in *Pinus contorta*. *Canadian Journal of Botany,* 59 (5), pp. 870-874.

Wainhouse, D. & Ashburner, R. (1996). The influence of genetic and environmental factors on a quantitative defensive trait in spruce. *Functional Ecology*, pp. 137-143.

Wainhouse, D., Cross, D. & Howell, R. (1990). The role of lignin as a defence against the spruce bark beetle *Dendroctonus micans*: effect on larvae and adults. *Oecologia,* 85(2), pp. 257-265.

Waksman, S.A. (1931). *Principles of soil microbiology*. Baltimore: Williams & Wilkins.

Wallis, C., Eyles, A., Chorbadjian, R., Gardener, B.M., Hansen, R., Cipollini, D., Herms, D.A. & Bonello, P. (2008). Systemic induction of phloem secondary metabolism and its relationship to resistance to a canker pathogen in Austrian pine. *New Phytologist,* 177(3), pp. 767-778.

Wang, K.L.C., Li, H. & Ecker, J.R. (2002). Ethylene biosynthesis and signaling networks. *Plant Cell,* 14, pp. S131-S151.

Wang, S.X., Hunter, W. & Plant, A. (2000). Isolation and purification of functional total RNA from woody branches and needles of Sitka and white spruce. *Biotechniques,* 28(2), pp. 292-296.

Wang, X., Basnayake, B.M., Zhang, H., Li, G., Li, W., Virk, N., Mengiste, T. & Song, F. (2009). The Arabidopsis ATAF1, a NAC transcription factor, is a negative regulator of defense responses against necrotrophic fungal and bacterial pathogens. *Moecularl Plant Microbe Interactions,* 22(10), pp. 1227-38.

Wang, X., Wang, X., Hu, Q., Dai, X., Tian, H., Zheng, K., Wang, X., Mao, T., Chen, J.G. & Wang, S. (2015a). Characterization of an activation-tagged mutant uncovers a role of GLABRA2 in anthocyanin biosynthesis in *Arabidopsis*. The *Plant Journal,* 83(2), pp. 300-11.

Wang, Z., Zhao, Z., Cheng, X., Liu, S., Wei, Q. & Scott, I.M. (2015b). Conifer flavonoid compounds inhibit detoxification enzymes and synergize insecticides. *Pesticide Biochemistry and Physiology*.

Warren, R.L., Keeling, C.I., Yuen, M.M., Raymond, A., Taylor, G.A., Vandervalk, B.P., Mohamadi, H., Paulino, D., Chiu, R., Jackman, S.D., Robertson, G., Yang, C., Boyle, B., Hoffmann, M., Weigel, D., Nelson, D.R., Ritland, C., Isabel, N., Jaquish, B., Yanchuk, A., Bousquet, J., Jones, S.J., MacKay, J., Birol, I. & Bohlmann, J. (2015). Improved white spruce (*Picea glauca*) genome assemblies and annotation of large gene families of conifer terpenoid and phenolic defense metabolism. *The Plant Journal,* 83(2), pp. 189-212.

Woods, J.A., Hadfield, J.A., Pettit, G.R., Fox, B.W. & Mcgown, A.T. (1995). The interaction with tubulin of a series of stilbenes based on combretastatin A-4. *British Journal of Cancer,* 71(4), pp. 705-711.

Woodward, S. (1998). *Heterobasidion annosum : biology, ecology, impact, and control*. Wallingford, Oxon, UK ; New York: CAB International.

Woodward, S. & Pearce, R.B. (1988). The role of stilbenes in resistance of Sitka spruce (*Picea sitchensis* (Bong.) Carr.) to entry of fungal pathogens. *Physiological and Molecular Plant Pathology,* 33(1), pp. 127-149.

Wu, Y., Deng, Z., Lai, J., Zhang, Y., Yang, C., Yin, B., Zhao, Q., Zhang, L., Li, Y., Yang, C. & Xie, Q. (2009). Dual function of *Arabidopsis* ATAF1 in abiotic and biotic stress responses. *Cell Research,* 19(11), pp. 1279-1290.

Xu, W.J., Dubos, C. & Lepiniec, L. (2015). Transcriptional control of flavonoid biosynthesis by MYB-bHLH-WDR complexes. *Trends in Plant Science,* 20(3), pp. 176-185.

Xu, W.J., Grain, D., Bobet, S., Le Gourrierec, J., Thevenin, J., Kelemen, Z., Lepiniec, L. & Dubos, C. (2014). Complexity and robustness of the flavonoid transcriptional regulatory network revealed by comprehensive analyses of MYB-bHLH-WDR complexes and their targets in *Arabidopsis* seed. *New Phytologist,* 202(1), pp. 132-144.

Xue, B., Charest, P.J., Devantier, Y. & Rutledge, R.G. (2003). Characterization of a MYB-R2R3 gene from black spruce (*Picea mariana*) that shares functional conservation with maize C1. *Molecular Genetics and Genomics,* 270(1), pp. 78-86.

Xue, J., Schmidt, S.V., Sander, J., Draffehn, A., Krebs, W., Quester, I., De Nardo, D., Gohel, T.D., Emde, M., Schmidleithner, L., Ganesan, H., Nino-Castro, A., Mallmann, M.R., Labzin, L., Theis, H., Kraut, M., Beyer, M., Latz, E., Freeman, T.C., Ulas, T. & Schultze, J.L. (2014). Transcriptome-based network analysis reveals a spectrum model of human macrophage activation. *Immunity,* 40(2), pp. 274-88.

Yamaguchi, M. & Demura, T. (2010). Transcriptional regulation of secondary wall formation controlled by NAC domain proteins. *Plant Biotechnology,* 27(3), pp. 237-242.

Yaqoob, N., Yakovlev, I.A., Krokene, P., Kvaalen, H., Solheim, H. & Fossdal, C.G. (2012). Defence-related gene expression in bark and sapwood of Norway spruce in response to *Heterobasidion parviporum* and methyl jasmonate. *Physiological and Molecular Plant Pathology,* 77(1), pp. 10-16.

Yoshida, K., Iwasaka, R., Kaneko, T., Sato, S., Tabata, S. & Sakuta, M. (2008). Functional differentiation of *Lotus japonicus* TT2s, R2R3-MYB transcription factors comprising a multigene family. *Plant and Cell Physiology,* 49(2), pp. 157-169.

Yoshida, K., Kume, N., Nakaya, Y., Yamagami, A., Nakano, T. & Sakuta, M. (2010). Comparative analysis of the triplicate proathocyanidin regulators in *Lotus japonicus*. *Plant and Cell Physiology,* 51(6), pp. 912-922.

Yuan, L., Wang, L.J., Han, Z.J., Jiang, Y.Z., Zhao, L.L., Liu, H., Yang, L. & Luo, K.M. (2012). Molecular cloning and characterization of *PtrLAR3*, a gene encoding leucoanthocyanidin reductase from *Populus trichocarpa*, and its constitutive expression enhances fungal resistance in transgenic plants. *Journal of Experimental Botany,* 63(7), pp. 2513-2524.

Zas, R., Björklund, N., Nordlander, G., Cendán, C., Hellqvist, C. & Sampedro, L. (2014). Exploiting jasmonate-induced responses for field protection of conifer seedlings against a major forest pest, *Hylobius abietis*. *Forest Ecology and Management,* 313, pp. 212-223.

Zhao, K.M. & Bartley, L.E. (2014). Comparative genomic analysis of the R2R3 MYB secondary cell wall regulators of *Arabidopsis*, poplar, rice, maize, and switchgrass. *BMC Plant Biology,* 14.

Zhou, H., Lin-Wang, K., Wang, H., Gu, C., Dare, A.P., Espley, R.V., He, H., Allan, A.C. & Han, Y. (2015). Molecular genetics of blood-fleshed peach reveals activation of anthocyanin biosynthesis by NAC transcription factors. T*he Plant Journal,* 82(1), pp. 105-121.

Zon, J. & Amrhein, N. (1992). Inhibitors of phenylalanine ammonia−lyase: 2−aminoindan−2−phosphonic acid and related compounds. *European Journal of Organic Chemistry*(6), pp. 625-628.

Zulak, K.G. & Bohlmann, J. (2010). Terpenoid biosynthesis and specialized vascular cells of conifer defense. *Journal of Integrative Plant Biology,* 52(1), pp. 86-97.

Acknowledgements

On this path of becoming a PhD I have seen whole projects succeed and fail; I have lived moments of enthusiasm and moments of deception; I have gone through periods of moving around and periods of staying put; I have learnt stuff that I could use and stuff that I can only hope will be useful sometime in the future; I have enjoyed working with people and I have detested it, too. With so many ups and downs, some people might wonder what kind of force is needed to find a balance. Some would say that tenacity, hopefulness, necessity, patience or pure luck did it. In my case, I believe that the people I met along the way were the force that kept me moving forward on every stretch of the path.

My biggest thanks are owed to my main supervisor, Malin Elfstrand, for her great knowledge, for having tones of patience with me, for tempering me when I was too excited, too pissed, too disappointed, too skeptical, too wrong, too fast, too stuck or too "creative". I cannot recall a moment when I have felt that I could not count on her during these years. Thank you, Malin, for your guidance and support in the lab, in the field, preparing presentations and guiding me through the learning of scientific writing. Also, thank you for probably saving my life that time in the field when I almost met my demise incarnated as a viper and an ambulance, three paramedics, epinephrine, a helicopter with a pilot and a doctor, more doctors, nurses, cortisone, the antidote and some morphine, were needed to keep me in the land of the living. Together with Malin, I would like to thank my assistant supervisors, Jan Stenlid, who took me as close as to his home for a meeting with the group and as far as to a meeting and a fieldtrip in South Africa, and Åke Olson. Both of them contributed with their experience, advice and ideas to improve my work.

I am also very grateful to other researchers that contributed to improve my work, skills and personal growth. To Anki Rönnberg Wästljung and Sofia Berlin for introducing me to the world of research and making me want to be a PhD student; to Jenny Arnerup for leaving me a trace I could follow when I started; to Heriberto Vélëz for supervising me in the lab in the beginning and for helping me with the language correction in the end; to Jonàs Oliva for our sampling trip to Norway, which was the first of our many experiences together,

and for his advice whenever I asked for it; to Kerstin Dalman for being an excellent travel mate and coworker; to Sara von Arnold, Tina Olsson and Kanita Orozovic for their help with the challenging task of maintaining and transforming spruce cells; to Almuth Hammerbacher for bringing her perspective and the chemistry to my projects and for inviting me to work with her at the Max Planck Institute for Chemical Ecology. I would also like to thank the people at the Shahid Mukhtar lab at the University of Alabama. Thanks to Shahid Mukhtar for supervising me during my study visit at his lab and to Peter Blair, TC Howton, Ben Marsella and Yali Sun for being such great lab mates.

The environment at my institution, the department of Forest Mycology and Plant Pathology at SLU, has motivated a considerable part of my work. The people working here, the mykopats, provide an optimal broth for a student to become a scientist. For this reason I would like to thank all of them. Thanks to Rena, Maria and specially Katta for their help and advice in the lab. Thanks to Karin and Erica for their administrative support. Thanks to Miguel Ángel, Diem, Sara, Georgios, Tina, Yang and the rest of my fellow PhD students for creating an environment where we could share our experiences and sometimes hang out. Thanks, finally, to Audrius for being a fine office mate during my last year.

The possibility of travelling has been one most enriching factors during my PhD education. Thanks to the Organism Biology Research School and the SLU Travel Grant for providing me with travel funds for two study visits, two conferences and NOVA courses. At the same time, I have had the chance to be involved in different aspects of the non-scientific world at the university. Thanks to the people I worked with at the PhD student council, the faculty education board and the student union magazine for giving me a very different perspective of our university than the one I experienced while doing research.

My last bunch of gratitude goes firstly to my family, especially my parents, Jose Miguel and Tere; my siblings, Pablo and Teresa and also my grandma Maria. They have continuously sent me love, support and “real food” from home during all these years and they have made me feel home every time that I came back or that they came over. Secondly, I would like to thank all the special persons that I have shared Uppsala with. All their names compose my great second family in Sweden and their company has filled with experiences of all types these years, making even more worth this whole experience.