Local and systemic defence responses in trees against pathogenic fungi: Differences revealed at the transcriptional level

Lokale og systemiske forsvarsresponser hos skogtrær mot patogen sopp: Transkripsjonsstudier avdekker forskjeller

Philosophiae Doctor (PhD) Thesis

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Ås 2011

Thesis number 2011: 43
ISSN 1503-1667
ISBN 978-82-575-1006-0

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Acknowledgements

Its matter of immense pleasure and satisfaction for me to thank my supervisor Professor Halvor Solheim for giving me opportunity to carry out research work that has led me to finish my doctoral thesis and providing excellent supervision.

I am also very much grateful to my co-supervisor Dr. Carl Gunnar Fossdal for opening the world of molecular biology on me and guiding me through this project. I learned from his discussions on the subject and life equally. His passion for molecular work was a strong inspiration for me.

I also would like to thank co-supervisor Professor Dzung Bao Diep at the department of chemistry and biotechnology (IKBM) at the Norwegian University of Life Sciences.

My thanks go to Paal Krokene for teaching me to transfer my lab findings into the construction of manuscripts and publications on Norway spruce work. It has always been pleasure to discuss lab issues and other scientific stuff with Igor Yakovlev. He has always been very generous in his help by saying “come to my office any time we shall discuss”.

I wish to thank also my colleagues Ari M. Hietala and Nina Elisabeth Nagy for their valuable comments and suggestions. Thanks to Adam Vivian-Smith and Siri Bjoner for their help in improving thesis. I am thankful to Inger Heldal and Mari Kjos for their technical assistance.

I appreciate the excellent supervision of Prof. Benedicte Riber Albrectson and Jan Karlsson at the Umeå Plant Science Centre, Department of Plant Physiology, Umeå University, Sweden for providing the *Populus* clones, *Melampsora* spores and for assisting with inoculations, microarray experiments and chemical analyses for manuscript II and III. They always have been very kind and quick to respond my enquiries. Thanks to Jan Karlsson for arranging my stay in Umeå during my visits. It has always been a matter of great pleasure to spend time with Iftikhar Ahmed and Basharat Ali in Umeå.

I am thankful to my friends especially Ibrahim mehmeti, Thair Mehmood, Rizwan Ahmed, Yousaf Bhatti, Shahid Nadeem, Tahir Mehmood Qureshi, M. Asif, Saqib, Abdul Samad Niazi, Asif khan, Burhan Ahmed, Mirza Faisal Mehmood, Khawaja Zeeshan and Mehmood Ayaz for their friendship, and help whenever needed. All time spend with you guys was lovely.

Sincere thanks to my old friends Waleed Anwar Bhatti, Arshad Nazir, Ishtiaq Awan, Afzal Nadeem, Irshad ullah for always being so helpful and sharing happiness and sorrows. I would like to thank all my previous teachers, colleagues, students and many others that I could not mention for helping me to learn science.

I thank to my uncles, aunts, brother in-laws, cousins, nephews and nieces.

I am thankful to my parents-in-law for their prayers, encouragement and support.

Whatever I am today is because of prayers of my mother. I have no words to thank my father for his love and support throughout my life. I am proud of you dad and thank you for high ambitions for me. Thanks to my grandmother for her prayers. I also thank my brothers Naeem Yaqub, Naveed Yaqub and sisters Asmat Jamila and Attiya Yasmin for their respect, care and support throughout my life.

Finally, but foremost I thank to my loving wife Samina Kauser Bhatti for her great understanding, sacrifices, continuous help and support during these years. Last but not least, my sons Muhammad Ismail Bhader and Muhammad Shafe Bhader, their sweet smiles always have been source of happiness for me. Ismail it was not easy to leave you at home when you asked me "Pappa kan du ikke være hjemme?"

All praises and thanks to The Almighty Allah (Al-'Aleem) and His prophet Muhammad (PBUH) who enabled me to contribute in this field of science.

Abbreviations

CC	Coiled Coil
CCR	*Cinnamoyl-CoA Reductase*
Cdc2	*Cyclin dependent kinase2*
cDNA	Complementary Deoxyribonucleic Acid
ChitiI	*Chitinase I*
CTs	Condensed Tannins
DNA	Deoxyribonucleic Acid
Dpi	Days post inoculation
ERD4	*Early Responsive toDehydration stress*
EST	Expressed Sequence Tags
GH3	*Gretechen Hagen 3 auxin responsive gene*
H. parviporum	*Heterobasidion parviporum*
HR	Hypersensitive Response
IR	Induced Resistance
LRR	Leucine rich repeat
MeJ	Methyl Jasmonate
Methyl-CpG	*Methyl-CpG-binding domain*
Mitoch-Cp	*Mitochondria carrier protein*
Mpk	*Mitogen-activated Protein Kinase*
NB	Nucleotide Binding
Omt	*O-Methyl transferase*
PaACO	*1-Aminocyclopropane-1-Carboxylate Oxidases*
PaACS	*1-Aminocyclopropane-1-Carboxylic acid Synthases*
PaC4H3/5	*Cinnamate-4-Hydroxylase*
PaChi4	*Chitinases class IV*
PaHCT1	*Hydroxycinnamoyl Transferase 1*
PAMPs	Pathogen Associated Molecular Patterns
PaPAL	*Phenylalanine Ammonia-Lyase*
PaPX	*Peroxidase*
PR proteins	Pathogenesis Related Proteins
qRT-PCR	Quantitative Reverse Transcription - Polymerase Chain Reaction
RNA	Ribonucleic acid
ROS	Reactive Oxygen Species
SAR	Systemic Acquired Resistance
Senesc	*Senescence-associated protein*
SwAsp	Swedish Aspen collection
TPs	Total Phenolics
Ubq	*Ubiquitin-like protein*
Wrky	*Wrky family transcrpion factor*

Abstract

Forest trees dominate the earth surface and has special place in the economy and ecology of the planet. Due to the long life span of woody trees they are challenged by the abiotic and biotic factors. Over the long history of existence trees have evolved defensive systems that still secure their longevity and dominance. Gymnosperms such as conifers are dominating the temperate forest together with the deciduous angiosperm trees and successfully defended their existence for millions of years. Norway spruce *(Picea abies)* is an important conifer species, and was selected to explore further its defensive system at the molecular level. Angiosperms are most diverse group of plants at present and contain many important tree species such as aspen (*Populus tremula*) and other *Populus* species. *Populus* having a sequenced genome guided us to aspen as our candidate angiosperm to study its defence responses at the transcriptome level. These most challenging pathogens for these species are the biotrophic and necrotrophic fungi. The resistance level within Norway spruce and *Populus* species show variation towards both biotrophic and necrotrophic fungi supporting that there is a genetic basis and variation in these genes for resistance.

The necrotrophic fungus *Heterobasidion parviporum* as able to colonize Norway spruce and is responsible for great economic losses. Up to twenty present of the spruce trees in Norway show decay caused by this fungus. In Norway spruce trees with high level of resistance to *H. parviporum* transcript marker genes such as Chitinase class IV and peroxidases are induced rapidly both locally and systemically at early stages after pathogen infection while in more susceptible plants the response is slower. There are also differences in how these are induced between bark and sapwood. The transcripts level of some genes such a *PaChi4* and *PaPX3* increased more in the sapwood while the genes like *PaPAL2 and PaHCT1* were more upregulated in the bark as a host response to infection. These results suggest that the systemic signalling of defence response may also occur through sapwood. In addition, the local defence responses to necrotroph infection, wounding and methyl jasmonate (MeJ) were found to show similarities.

The use of aspen offered a great opportunity to study different aspects including defence responses at the transcriptome level using microarrays. This study addressed the defence responses of a susceptible (23) and relatively resistant (72) SwAsp aspen clone after wounding, and inoculation with a necrotroph (*Ceratocystis* sp. NFLI 2004-466/501) and a

biotroph (*Melampsora magnusiana*) fungus by looking at the expression of a large part of the expressed genes in this species. Differences between the clones were evident at the transcripts level; the healthy un-inoculated clones showed 552 constitutive genes that were expressed differentially. Differential gene regulation in response to the pathogen attack was also evident between the two aspen clones after biotroph and necrotroph inoculation. In systemic leaves from the susceptible clone no differential regulation of genes found to biotroph while only 7genes in response to the necrotroph at 24 hour post infection. In contrast, 156 genes were differentially expressed in the more resistant clone to biotroph and 283 to necrotroph infection at the same time using microarrays. We also found that a larger portion of differentially regulated genes were upregulated in response to the biotroph while in contrast a majority of genes were downregulated in response to the necrotroph. qRT-PCR validation of selected genes supported that the systemic induction in leaves of clone 72 was higher after biotroph, necrotroph and wounding than in the susceptible clone 23.

The regulation of putative defence genes were also followed in the same two aspen clones to find the local and systemic defence response after necrotroph and wounding of the stem. An aggressive and newly discovered necrotroph *Ceratocystis* sp. from Norway was used to inoculate both clones. In general, clone23 showed as strong defence induction and was higher than in clone 72 at the early stages as a local response to infection. However, clone72 showed systemic response in leaves to the necrotroph and more so to wounding, while clone 23 showed little or no systemic inductions to wounding and necrotroph. The necrotroph was highly aggressive and the results suggest that it has the ability to suppress part of the host defence signalling seen towards wounding. These results also suggest that clone 72 has a fully functional local and systemic defence signalling, while clone 23 is deficient or delayed in its systemic response to wounding and necrotroph.

We also followed the defence induction markers, 21 NB-LRR Resistance gene-like homologues and five microRNA (miRNA) putatively targeting NB-LRRs in a relatively resistant Norway spruce clone. Ramets of the clone was wounded and inoculated with the necrotrophic fungus *Ceratocystis polonica*. The markers showed increase both locally and systemically indicative of a rapid and efficient host response and we also saw local and systemic changes in NB-LRR and miRNA transcript levels. However the transcriptional changes for the NB-LRRs and miRNAs followed were in general small, partly supporting the notion that Resistance-like genes are typically expressed at low and constitutive levels.

Comparing the host responses between these two tree species, the results suggest that the defence responses to pathogens and wounding in the gymnosperm Norway spruce and the angiosperm aspen show similarities despite their 300 million years of evolutionary separation. In both species we saw up regulation of defence related genes (such as Class IV chitinases) that are also upregulated in response to necroptrophic pathogens.

Sammendrag

Skogstrær er dominerende planter som har stor økonomisk og økologisk betydning. Fordi trær har et langt livsløp utsettes de for betydelige abiotiske og biotiske stressfaktorer. Trær har utviklet effektive systemer for å motstå forskjellige former for stress som gjør at de kan overleve og trives på samme sted over lange tidsrom. Gran (*Picea abies*) er det viktigste treslaget i Norge og ble valgt ut til videre molekylære studier av dens forsvarssystemer mot skadesopp. Løvtrærne er mer diverse enn bartrærne, men blant løvtrærne finnes mange viktige arter i de nordlige boreale skoger, blant annet osp (*Populus tremula*) og andre *Populus* arter. For *Populus* er genomet sekvensert, noe som ledet oss til å bruke osp i det molekylære arbeidet på transkriptom nivå. De mest utfordrende skadegjørere for trær er biotrofe og nekrotrofe sopper, men det er forskjeller i graden av resistens mot disse skadegjørerne innen treslagene noe som viser at det er en genetisk basis for dette og at det er variasjon i genene for motstandsdyktighet.

Granrotkjuka (*Heterobasidion parviporum*) er en nekrotrof sopp som effektivt angriper gran og gir store økonomiske tap. Over 20 % av grantrærne i Norge er angrepet av rotkjuke ved slutthogst. I grantrær med høy grad av resistens mot granrotkjuke ser man at gener slik som kitinaser (*PaChi4*) og peroksidaser (*PaPX3*) induseres raskt både lokalt og systemisk ved et soppangrep, mens mer mottagelige trær viser en forsinket respons. Det er også forskjeller mellom hvordan disse induseres i levende bark og yteved. Genuttrykket av *PaChi4* og *PaPX3* øker mest i yteved, mens gener som *PaPAL2* og *PaHCT1* oppreguleres mest i bark etter en infeksjon. Disse resultatene antyder at det også er systemiske forsvarsresponser i ved. I tillegg fant man at den lokale forsvarsresponsen mot det nekrotrofe patogenet viser likhet med responsen til skade og metyljasmonat (MeJ).

Bruk av osp har muliggjort studier av forsvarsresponser på transkriptom nivå ved bruk av mikromatriser. Studiene av osp ble utført på SwAsp kloner (23 og 72) med forskjeller i resistens mot den biotrofe soppen *Melampsora magnusiana*, men i tillegg ble responsen mot skade og en nylig oppdaget nekrotrof blåvedsopp *Ceratocystis sp.* (NFLI 2004-466/0501) studert. Det var store forskjeller molekylært mellom de to ospeklonene. I de friske klonene var 552 gener forskjellige, noe som reflekterer deres forskjellige genetiske bakgrunn. Forskjellen var også stor i hvordan disse reagerte på biotrof og nekrotrof sopp. Den mottagelige klonen (23) viste ingen systemisk induksjon av forsvarsresponser i blader 24 timer etter angrep med biotrof sopp, mens kun syv gener varierte i sitt uttrykk mot den

nekrotrofe soppen. I skarp kontrast til dette ble 156 gener regulert i den mer resistente ospeklonen (72) etter behandling med den biotrofe soppen, og hele 283 gener ble regulert etter inokulering med den nekrotrofe soppen. En større andel gener ble oppregulert i den sterke klonen etter angrep av den biotrofe soppen enn mot den nekrotrofe. Senere qRT-PCR validering på utvalgte gener støttet disse resultatene og viste at den systemiske responsen i blader var mye sterkere i den mer resistente klonen (72) enn i den mer mottagelige klonen (23).

Reguleringen av antatte forsvarsgener ble så fulgt i de to samme ospeklonene for å finne likheter og forskjeller mellom den lokale og systemiske responsen til den nekrotrofe soppen og til skade i bark på stammen. Den nekrotrofe *Ceratocystis* sp. ble brukt til å inokulere begge kloner. Klone 23 viste seg å gi lite systemisk rerspons, men like strek eller sterkere lokal respons mot den nekrotrofe soppen som klon 72. Den systemiske responsen mot skade var sterkere i klon 72 enn den var mot den nekrotrofe soppen. Nekrotrofen viste aggressiv vekst i begge klonene, og resultatene antyder at soppen har evnen til å undertrykke forsvarsresponsen i klon 72, mens det systemiske signalsystemet for forsvar mot skade og soppangrep ser ut til å være defekt eller tregt i klon 23.

Vi fulgte også transkripsjons nivået av utvalgte markører for indusert forsvar, 21 NB-LRR resistenslignende gener samt fem microRNA (miRNA) i en gran klone med relativt høyt resistens nivå etter inokulering med blåvedsopp (*Ceratocystis polonica*) og etter skade. Forsvarsmarkørene ble indusert lokalt og systemisk noe som tyder på at klonens forsvarssystemer ble effektivt slått på, vi så også endringer i NB-LRR og miRNA uttrykket lokalt og systemisk. Transkripsjonsforandringene for NB-LRR og miRNA var imidlertid generelt små, noe som delvis støtter det at resistensgener uttrykkes på et lavt og konstitutivt nivå.

Når man sammenligner de observerte forsvarsresponser mot nekrotrof sopp og skade mellom gran som er et bartre og osp som er et løvtre ser man likheter, selv om de har vært evolusjonært separert i mer enn 300 millioner år. For eksempel fant vi oppregulering av beslektede gener (som Klasse IV kitinaser) som oppreguleres raskt i respons mot nekrotrof sopp i begge treartene.

List of papers

This thesis is based on the following papers, which will be referred to in the text.

Paper I

Nadeem Yaqoob, Igor A. Yakovlev, Harald Kvaalen, Paal Krokene, Halvor Solheim and Carl Gunnar Fossdal. Defence-related gene expression in bark and sapwood of Norway spruce in response to *Heterobasidion parviporum* and methyl jasmonate

(Submitted)

Paper II

Nadeem Yaqoob, Benedicte Riber Albrectsen, Jan Karlsson, Igor A. Yakovlev, Halvor Solheim and Carl Gunnar Fossdal. Transcriptome differences between two SwAsp aspen clones and their systemic defence response to the biotroph *Melampsora manusiana*, a novel necrotrophic fungus *Ceratocystis* sp. and wounding

(Manuscript)

Paper III

Nadeem Yaqoob, Benedicte Riber Albrectsen, Jan Karlsson, Igor A. Yakovlev, Halvor Solheim and Carl Gunnar Fossdal. Rapid local and systemic host response in aspen (*Populus tremula*) to necrotrophic fungus and wounding

(Manuscript)

Paper IV

Igor A. Yakovlev, Nadeem Yaqoob, Paal Krokene, Halvor Solheim and Carl Gunnar Fossdal. Comparison of the local and systemic change in NB-LRR expression and microRNA targeting NB-LRRs in Norway spruce after *Ceratocystis polonica* infection and wounding

(Manuscript)

Introduction

Plant resistance to pathogens

Survival of a plant species in response to pathogen challenge depends on the components of its defence system. Plants have developed different strategies both constitutive and inducible to cope with an attacking pathogen. Pathogen success in causing disease is dependent on landing, attaching, and properly entering a host and furthermore remaining undetectable or having the capability to supress or overcome the host defence systems. Once pathogen is recognized, it triggers a number of defence related events within the plant body. These defensive measures may be manifested locally at the site of attack or systemically or a combination of the two.

Recognition of pathogen

Plants employ at least two essential strategies to detect the attacking pathogens. Pathogens characteristically have general external features such as flagellin, chitin and glycoproteins that can give rise to pathogen associated molecular patterns (PAMPs). These PAMPs elicit signal transduction from pattern-recognition receptors (PRRs) present on surface of the host cells and trigger the plant basal resistance and these have significant role in non-host type resistance or by directly recognizing a pathogen specific virulence factor that leads to an induced host response (Nürnberger & Kemmerling, 2009).

Some pathogens successfully overcome the plant basal immunity by releasing certain molecules known as effectors into the host cells to inactivate components of the defence apparatus and thereby be able multiply and cause disease. These effectors have been found to be mostly protein in nature and are translocated into the target host cell or into the surrounding apoplastic leaf spaces (de Wit *et al.*, 2009). Co-evolution of the host-pathogen system results in effector triggered immunity by which the plant has developed a system to recognize these pathogen effectors with intracellular receptors known as R proteins. These R proteins appear to ben pathogen specific. Resistant hosts are characteristically efficient in recognizing the pathogens externally through effective PAMPs or by endogenous by R proteins but must continue evolving mechanisms that allow for continuous and sometimes rapid adaption to keep up with the variation arising in new types of pathogen effectors.

Extracellular recognizing receptors

PRRs are categorised in two classes, i) transmembrane receptor kinases and ii) transmembrane receptor like proteins lacking any specific internal signalling domain (Zipfel, 2008). The best studied PAMP example is a bacterial flagellin that is recognised by Flagellin Sensing 2 a receptor kinase in *A. thalina* which activates a signalling complex. Another signalling complex is *Brassinosteroid insensitive 1-associated kinase 1* (*BAK1*) which is a leucine-rich repeat (LRR) class of proteins containing a PRRs receptor kinase (Chinchilla *et al.*, 2007; Heese *et al.*, 2007). This is an additional central regulator of PAMP triggered immunity against many pathogens, and effectors are known that can negatively affect its signalling (Shan *et al.*, 2008).

Plant pathogens overcome the plant innate immunity by introducing a suite of pathogen effectors into the host cells. One bacterial pathogen for example *Psuedomonas syringae* (*P. syringae*) encodes 20-30 effectors and injects them all directly into the host cell (Cunnac *et al.*, 2009). *P. syringae* effectors *AvrPto* and *AvrPtoB* both target FLS2-BAK1 complex to render it inoperable. *AvrPtoB* suppresses the host kinase in two ways; firstly this is done by an amino terminal interaction which interferes with the flagellin-mediated response, while at the same time the carboxyl terminal facilitates protein ubiquitination, thus marking this protein for degradation. In regards to eukaryotic effectors, much less data is available and their functions often remain uncharacterized compared to bacteria (Dodds & Rathjen, 2010). Both fungal pathogens and Oomycetes also deliver their effectors into host cells using unknown mechanisms (Kamoun, 2007; Panstruga & Dodds, 2009).

Intracellular effector recognising receptors

When plants successfully defend by recognizing PAMPs, pathogens may introduce effectors to overcome host. It is at this step plants recognize these effectors through intracellular effector recognising receptors (Chisholm *et al.,* 2006). These receptors are termed as resistance proteins (RPs) and possess nucleotide binding and leucine rich domains (NB-LRRs). PRs are specific for particular pathogens and therefore, genes encoding these proteins are represented by considerable numbers in plant genomes (Table 1). Furthermore there are at least 67 TIR-NBS-LRR genes in the conifer white pine (Liu & Ekramoddoullah, 2004). Plant NB-LRRs proteins consist of many types and diverse variants i) those containing an N-terminal TIR (Toll, interleukin-1 receptor, resistance protein) domain, ii) a more common class contain as N-terminal domain with coiled coil (CC) domain and there are others that do

not have conserved N-terminal region. To which extent a plant individual is able to defend itself is related to the kind of RPs it has. To resist new pathogens plant species needs to evolve new NB LRR gene(s) by accumulating changes and mutations within the repeat structure of the LRR domain (Tamura & Tachida, 2011). Selection occurs on this set of NB LRR proteins during pathogen challenge of plant populations containing this variation and those plants which efficiently recognise pathogen effectors, either directly through physical association or indirectly through an accessory protein, succeed in those pathogen challenged niches.

Table 1. Numbers of genes that encode domains similar to plant R proteins in *Populus trichocarpa*, *Vitis vinifera*, *Arabidopsis thaliana* and *Oryza sativa* based on identification by automated annotation, manual assessment of the genomic sequence, and by the prediction of protein domains (adapted from (Duplessis *et al.*, 2009) and references therein).

Predicted protein domains[a]	Poplar	Grapevine	Arabidopsis	Rice
Total NBS-LRR	317	459	149	480
Non-TIR-NBS-LRR	224	362	55	480
TIR-NBS-LRR	91	97	94	–
Total NBS type genes	85	76	29	55
Total[b]	402	535	178	535

[a]CC, coiled-coil domain; LRR, leucine-rich-repeat domain; NBS, nucleotide-binding site; TIR, Toll/interleukin-1-receptor,) [b]total number of genes for *Populus*, *Arabidopsis* and rice vary between 402, 178 and 535, respectively in (Kohler *et al.*, 2008) and 416, 174and 519, respectively in (Yang *et al.*, 2008) because the authors used different annotation procedures.

Induced plant defence responses

After recognizing a threat the plant launches a large number of integrated defence responses to ward off an invader. Generally, plant defence responses include the fortification of the cell wall through biosynthesis of lignin and by the deposition of callose. Hosts may also produce a diverse set of secondary metabolites such as phytoalexins to respond to attack. Furthermore pathogenesis related proteins (PRs) such as *Chitinases* and *Glucanases* are employed to specifically degrade and hydrolyse pathogen specific cell wall components to inhibit their growth (van Loon, 1985).

Induced defence responses such as the hyper sensitive response (HR) is employed for inducing localized cell death by programmed cell death, the generation of reactive oxygen species, and the activation of various protein kinase cascades. HR is thus very effective to restrict the pathogens with biotrophic life style (Glazebrook, 2005), which require living host tissues to thrive (Heath, 2000), however it is not so effectively able to inhibit necrotrophic

pathogens which extract nutrition from dead tissues by promoting death and killing of the host cells (Glazebrook, 2005). Plant defence responses to the necrotrophic invasion may be activated after toxin- or damage-related associated molecular patterns transported or diffuse from the pathogen or damaged tissue to responding living host cells (Dodds & Rathjen, 2010).

Signalling pathways

Once pathogen is detected by the plant, different cellular events are triggered that include early signalling responses by the influx of Ca^{++}, the subsequent activation of mitogen activated proteins kinase (*Mpk*) cascades and generation of the reactive oxygen species (ROS). These early signalling events are in fact interconnected to each other through certain proteins that lead to the activation of a complex set of defence related responses. These signalling *Mpks* cascades have gained attention because they transfer signals from the plant plasma membrane to cellular receptors which finally regulate the activity of transcription factors and other protein kinases. In *Arabidopsis Mpk3* and *Mpk6* play role in the activation of *Wrky*23 transcription factor after pathogen attacks (Asai *et al.*, 2002). Similarly *Mpk3* in *Populus* has been found to be highly upregulated after *Melampsora* infection (Nicole *et al.*, 2006). However there are other signalling pathways which work independently from mitogen activated proteins kinase cascades and in *Arabidopsis* there is one based on activation of the *Calcium dependent protein kinases* (*Cdpks*) (Boudsocq *et al.*, 2010).

Downstream responses

At the site of infection, local defence responses are initiated that often trigger systemic defence response in the distal parts of the plant. This protects the plant from subsequent pathogens infections and is termed as systemic acquired resistance (SAR) (Durrant & Dong, 2004). Two signalling pathways are known for SAR; salicylic acid (SA)-dependent that activates after biotroph and the jasmonic acid (JA)/ ethylene (ET)-dependent pathway which activates after nonbiotroph (necrotrophs, insects, and wound) infections. These pathway are also responsible for the regulation of the defence related genes expression in systemic plant tissues (Bari & Jones, 2009). SA signalling is believed to involved in SAR because mutants incapable of SA production were unable to develop SAR and do not show the normal activation of PR genes after pathogen infection (Durrant & Dong, 2004). The mechanistic action and translocation of signalling molecules which initiate SAR is not yet established, however it has been speculated that methyl-SA, JAs, glycerolipid-based factors and a lipid-transfer protein may play a role in distal transfer of SAR initiation (Vlot *et al.*, 2008). SAR

differs from induced systemic resistance (ISR) in many aspects. For instance ISR is i) induced from infection of beneficial soil-born microorganism e.g. rhizobacteria and mycorrhizal fungi (van Loon *et al.*, 1998; Pozo & Azcon-Aguilar, 2007), ii) milder but effective systemic resistance (Van Wees *et al.*, 2008), iii) and it is often regulated through JA/ET dependent signalling pathway which enhances defence through priming (Conrath *et al.*, 2006; Van Wees *et al.*, 2008).

Signalling pathways cross talk

A variety of plant pathogens from different groups have the ability to attack plant hosts and there are also abiotic stresses affecting the plants. SA-dependent and JA/ET-dependent signalling pathways work in close collaboration to provide the most efficient defence to the host from all types of biotic and abiotic stresses. Although host gene expression after biotrophic and non-biotrophic pathogens differ substantially, they are reported to still share a considerable number of core genes (Tsuda *et al.*, 2009; Dodds & Rathjen, 2010). It has been shown in Arabidopsis that SA and its derivative aspirin has antagonistic effect on JA signalling (Penninckx *et al.,* 1998) and JA and ET signalling has synergistic effect on each other (Katagiri, 2004). Low concentrations of exogenous JA and SA on *Arabidopsis* showed synergistic effect on the JA and SA responsive genes *PDF1.2* and *PR1* while this effect was antagonistic at higher concentrations (Mur *et al.*, 2006). Interaction in the SA-JA signalling pathways is also influenced by timing and the sequence of initiation in the SA and JA/ET responses (Koornneef *et al.*, 2008).

Host defence responses to biotrophs, necrotrophs and wounding

Fungal pathogens are the most destructive in trees and cause diseases in wide variety of the plants. The pathogenic interactions between fungi and plants are considered to have been established prior to 400 million years ago, host symptoms found in alga *Palaeo nitella* in thin sections of micro-plants in the Lower Devonian Rhynie chart had diseases symptoms with modern looking host responses already present (Taylor *et al.*, 1992). On basis of life style fungal pathogens are classified into biotrophic for those who feed on living host cells, necrotroph those who depend on killing their host cells and live on the organic compounds and hemibiotrophs those initially and transiently live as biotrophs but later during the colonization of the host switch to a necrotrophic lifestyle. There may also be saprophytic growth on dead substrate, as well as symbiotic interactions such as the mycorrhizae that do not normally harm the host but may be beneficial. In the arm races with the fungal pathogens,

plants have evolved different defence strategies and put them in force accordingly. Programmed cell death successfully stops an attacking biotroph but seems unable to restrict, and perhaps instead helps aid an invading necrotroph.

Pathogen-plant interactions are best studied where pathogen and host are sequenced and genomic tools are available. This thesis dealt with biotrophic fungi *Melampsora magnusiana* and necrotrophic fungi *H. parviporum*, *Ceratocysitis polonica and Ceratocystis sp.* It should also be noted that *H. parviporum* not only is a necrotroph but also able to thrive as a saprotroph on dead heartwood inside the stem of spruce.

Melampsora *leaf rust*

Populus plantations are severely affected by the *Melampsora* spp (Fig. 1). This pathogen causes huge economic losses by causing *Populus* rust disease (Pinon J, 2005). This pathogen is an obligate biotroph and affects the host through decreasing leaves ability for photosynthesis, an early defoliation and by increasing the susceptibility for other pests (Newcombe *et al.*, 1994; Gérard *et al.*, 2006). In compatible host-pathogen interactions this fungus proliferates after entering through the stomata, and completes its life cycle in 7 days by then releasing uridenia spores from golden pustules (Laurans & Pilate, 1999). The complete 101Mb genome sequence of *Melampsora larici-populina* (*Mlp*) was unrevealed recently and has provided a great opportunity to study molecular defence responses of both partners in the pathosystem in the already sequenced host *Populus (*Hacquard *et al.,* 2010*).* The related pathogen species *Puccinia graminis* has an 89Mb sized genome that causes rust disease in wheat and barley. Comparison of *P. graminus* and *Mlp* genome and transcriptomes was recently performed. Their genomes show large repertoire of effector-like small secreted proteins and a dramatic up-regulation of transcripts coding for small secreted proteins, secreted hydrolytic enzymes, and transporters *in planta* suggests that they play a role in host infection and nutrient acquisition (Duplessis *et al.*, 2011).

Figure 1. *Populus* plant infected with *Melampsora* leaf rust.

Heterobasidion annosum

Heterobasidion annosum (Fr.) *senso lato* (*H .annosum s.l.*) is a necrotroph and a saprotroph and cause root and butt rot of conifers (Fig 2). This pathogen is the most destructive pathogen of conifer forests in northern hemisphere. Economic losses caused by this pathogen annually are in millions of Euros (Woodward *et al.*, 1998). This pathogen causes root and butt rot, and consists of three intersterility groups in Europe, namely *H. parviporum, H. abietnum* and *H. annosum senso stricto*. Although *H. parviporum* causes root -rot mainly in Norway spruce (*Picea abies*), it also has been reported to kill Scots pine (*Pinus sylvestris*). On the other hand *H. annosum* is typically associated with mortality of pine trees (Asiegbu *et al.*, 2005), however conifers as well as broadleaves trees may be infected. *H. abietinum* infects mainly *A. bies* species and has not so far established in the Nordic countries. The *H. annosum* genome is comparatively compact at 33.6Mb and comparisons with other fungal pathogen genomes and its recent sequencing will help us to understand differences related to pathogenicity and wood degradation. Paper I is based on *H. parviporum* inoculations to Norway spruce.

Figure 2. (Left to right) Fruiting bodies of *Heterobasidion* on Norway spruce host trunk tissue (Photo: Skogoglandskap), decayed heart wood by *Heterobasidion* (Photo: Halvor Solheim) and colonization of *Ceratocystis* in the bark and sapwood of *Populus.*

Ceratocystis *spp.*

This particular necrotroph causes disease in woody trees and enters the host through wounded tissues. *Ceratocystis* disperses by association from insects, and bark beetles often act as efficient vectors. *Ceratocystis* species collectively populates a wide host range, including Norway spruce (Solheim, 1992) and *Populus* species (Fig 2) (Johnson *et al.*, 2005). In this work the well-known bark beetle vectored *Ceratocystis polonica* was inoculated to Norway spruce (paper IV) while the un-described but highly necrotrophic *Ceratocystis.* sp. (NFLI 2004-466/501) from Norway was used in inoculations on aspen (*Populus tremula*) (paper II & III) in order to study the molecular host defence responses.

Mechanical wounding

In nature trees are continuously challenged by abiotic and biotic stresses. Dead branches and mechanical injuries are important as physically damaged sites that pave an entry path for the pathogen. Often there is overlap in the observed defence responses between wounding and pathogen (Christiansen *et al.*, 1999; Cheong *et al.*, 2002), and this perhaps ensures that wounded sites are always rapidly guarded. Wound related defence mechanisms are activated at both the local tissue level, as well as at the systemic tissue level and may be mediated by JA through phloem tissue (Schilmiller & Howe, 2005). Wound based systemic defence response has already been studied at transcriptomic level in *Populus* trees (Christopher *et al.*, 2004; Smith *et al.*, 2004) and in common beech (*Fagus sylvatica*) (Schlink, 2011). Wounding

leads to the transcriptional changes in a suite of genes that reflect readjustments in metabolism, signal transduction and other cellular processes in the host. Microarray experiment in *Arabidopsis* and *Populus* models showed that differentially regulated transcripts after wounding were nearly 8% (Cheong *et al.*, 2002) and 11% of the total transcription (Christopher *et al.*, 2004) respectively. The differentially systemic expression of genes is reported slightly weaker in the downward pointing leaves than upward pointing leaves after wounding (Schlink, 2011). However local transcriptional response in *Populus* leaves was more rapid and strong than in systemic leaves (Babst *et al.*, 2009; Philippe *et al.*, 2010).

State of genome sequencing in gymnosperms and angiosperms

Gymnosperms and angiosperms were separated from 300 million years ago (mya). Unlike other model herbaceous plant species like *Arabidopsis*, maize, rice, wheat and tomato less attention has been given to the forest trees mainly because of their long generation times, significantly larger genomes, and the unavailability of mutants for reverse genetic studies. On the other hand forest trees makes more than 82% of the world's biomass but the number of researchers dealing with molecular and genomic studies of tree species are far less than those working on agricultural plant species. This situation was improved when US department of energy joint genome institute (JGI) approved the project of genome sequencing of first tree species black cottonwood (*Populus trichocarpa*). *Populus* being one of the first fully sequenced tree genomes has now become an established model system for woody perennials (Jansson & Douglas, 2007). Numbers of sequenced ESTs from different cDNA libraries (Sterky *et al*., 2004) and the fully sequenced genome (Tuskan *et al*., 2006) make it a tractable model, not only as a comparative plant model, but rapid growing *Populus* species are potential feedstock for future biofuel industries in the temperate areas of the world.

Gymnosperms have large genomes compared to angiosperms due to the presence of the presumed numerous noncoding regions and repetitive elements. Norway spruce has a large genome estimated to be 21-37 Gb and is the first gymnosperm tree which is to be sequenced this year in Sweden, while the sequencing of loblolly pine (*Pinus taeda)* is planned at JGI (Table 1). However genome size of angiosperms varies from smallest 64 Mb in *Genlisea* (Greilhuber *et al*., 2006) to an enormous 149 Gb in *Paris japonica* (Pellicer *et al*., 2010). *Populus* however is four times larger than *Arabidopsis* but still considerably smaller than any gymnosperm genome. It will be interesting to see the differences in molecular defence related

genes in the fully sequenced genomes and deep transcriptome studies of both Aspen and Norway spruce, but for now we have settled on transcriptome studies to look at the defence responses in these two species.

The work of this thesis uses two hosts, one angiosperm and one gymnosperm, to help make both comparisons and generalities in the investigated mechanisms of host responses to pathogenic fungi in trees. Aspen was used for transcriptomic studies and the obtained results are described in paper II and III while transcript work with Norway spruce is described in paper I and IV.

Table 2. Species, genome properties and genomic resources in seven genera of forest trees used in genomic research programmes ((Neale & Kremer, 2011) and references therein).

Genus	no. species	Genome size (2c)	chr. no.	Ref. sequence	no. Of genes	no. of ests	no. of UniGenes	Ref. genetic map	no. of snPs
Pinaceae									
Pinus	111	21.98–37.68 pg (~21–37 Gb)	2N = 24	*Pinus taeda* to begin in 2011	N/A	410,245	18,921	*Pinus taeda*	22,700
Picea	34	31.61–40.41 pg (~31–40 Gb)	2N = 24	*Picea abies* was begun in 2010	N/A	519,485	41,31	*Picea glauca*	10,432
Pseudotsugc	10	38.10 pg (~37 Gb) *(Pseudotsuga menziesii,*	2N = 24 (26 in *P. menziesii*)	N/A	N/A	18,142	N/A	*P. menziesii*	1,269
Salicaceae									
Populus	29	~485 Mb	2N = 38 (3N,4N exist)63	www.phytozome.net/poplar	44,976	421,592	24,617	*Populus trichocarpa*	2,550
Myrtaceae									
Eucalyptus	733	1.13 (~1.1 Gb) *(Eucalyptus globules)*	2N = 22	www.eucalyptusdb bi.up.ac.za/	N/A	36,981	N/A	*Eucalyptus grandis*	768
Fagaceae									
Quercus	531	1.17–2.00 pg (~1.14– 2 Gb)	2N = 24	N/A	N/A	145,827	29,303	*Quercus robur L.*	36,411
Castanea	12	1.57–1.67 pg (~1.53–1.63 Gb)	2N = 24	www.fagaceae.org	N/A	91,325	40,039	*Castanea dentata X Castanea mollissima*	12,000

Where no reference is indicated, data are taken from the National Center for Biotechnology Information (NCBI) website. N/A, not currently available.

State of defence in **Populus** *with emphasis on* **Melampsora-Populus** *interactions*

Black cottonwood became first woody perennial tree after completion of whole genome sequencing in 2006 (Tuskan *et al.*, 2006). It has relatively small genome (ca.485 Mb) than other economically important forest trees (Table 2). Almost fully annotated and improved second version of genome is available and third version is on its way. *Populus* is now an established model tree as it is fast growing, and relatively easy to manipulate and propagate (Jansson & Douglas, 2007). Aspen is found in Northern hemisphere and one of the important forest trees among 30-40 different species of the *Populus* genus. In aspen different level of resistance exist. Resistant aspen genotypes likely perceive and limit the pathogen spread much more effectively than susceptible ones (Bucciarelli *et al.*, 1998). Remarkable differences in induced resistance at the transcriptome (Babst *et al.*, 2009) and at the biochemical level (Osier & Lindroth, 2006) has also been shown. *Populus* provided a significant opportunity to understand defence mechanisms in woody angiosperms at transcriptome level.

Three major studies have been conducted recently in *Melampsora-Populus* pathosystem to uncover the defence responses at different -omics levels (Miranda *et al.*, 2007; Rinaldi *et al.*, 2007; Azaiez *et al.*, 2009). This transcriptomic data covered 1-9 days post inoculation (dpi) and also included the compatible and incompatible pathogens interactions. *Melampsora* haustoria formed within 12-24 hours post inoculations (hpi). At this stage neither disease symptoms, nor defence responses are observed in this compatible interaction (Laurans & Pilate, 1999; Rinaldi *et al.*, 2007). One report however shows that a few host PRs transcripts are upregulated before haustoria development (Miranda *et al.*, 2007). Incompatible hosts, on the other hand, trigger HR at 2dpi while the spread remains essentially unchecked in comparative compatible interactions (Laurans & Pilate, 1999). Compatibility in the *Melampsora* pathogen-host system can be monitored by fungal DNA measurements. In compatible reactions, *Melampsora* DNA within host tissues may increase to 600 times at 3dpi but in comparison no such increase was observable in incompatible interactions (Rinaldi *et al.*, 2007). Strong lignification of the host cell wall is evident histologically at 4dpi, and is another marker of an incompatible *Melampsora-Populus* interaction (Rinaldi *et al.*, 2007). Large sets of transcripts were found to be deregulated at 6dpi during strong quantitative resistance (Azaiez *et al.*, 2009). At this time point, most defence related transcriptional changes were noticed in signalling pathways and cell wall metabolism. At day 7 uridinea are released in compatible interactions thus completing the life cycle of the pathogen (Laurans & Pilate, 1999; Rinaldi *et al.*, 2007).

There are a limited numbers of transcriptome based studies in *Populus* addressing herbivory (Major & Constabel, 2006; Ralph *et al.*, 2006) and wounding and following *PopMV* virus infection (Smith *et al.*, 2004). In these cases the induced responses in hybrid poplar, in both local and systemic tissues, overlap extensively after both wounding damage and herbivore induced damage (Major & Constabel, 2006). Christoher and colleagues (Christopher *et al.*, 2004) initially profiled the gene expression resulting from systemically induced wound responses in hybrid poplar (*P. trichocarpa x P. deltoides*) and found that 11% of the studied ESTs were involved in pathogen defence. One report showed that Kunitz type trypsin inhibitors and *Chitinases* were among the most significantly up- regulated genes during the systemic activation in non-local *Populus* tissues after mechanical wounding (Christopher *et al.*, 2004).

State of defence in the conifers with emphasis on* H . parviporum-*Norway spruce interactions

Conifers such as pine, fir and spruce are successful gymnosperms that still dominate the largest temperate forest ecosystems of the world. These trees are not only important from timber and climate viewpoint but also as an important source to study resistance phenomena against different pathogens. Conifers must be considered to have effective defence strategies fungal pathogens, since they have likely faced these and other pests for the last 300mya. Their large size, long regeneration time, longevity in general, and large genome size has been hindrances making it difficult to cover all aspects of pathogen-conifer interactions. However, many studies were conducted in past to figure out the conifer defence to abiotic and biotic stresses at the anatomical, chemical, protein and transcript levels (Franceschi *et al.*, 2000).

Norway spruce an important conifer was introduced in Norway around 2000-2500 years ago and is now widely distributed in the country (Giesecke & Bennett, 2004). In nature different biotroph and necrotrophs are challenging this species. Most important is the pathogen *H. parviporum* a necrotrophic basidiomycete. *H. parviporum* colonize the Norway spruce through bark wounds caused by wind, animals, insects, it also infects healthy plants through root-to-root contact. Once fungus establishes itself in heartwood of Norway spruce, the colonization column can rise up to over 10meters (Woodward *et al.*, 1998). Annual vertical spread in heartwood is ca. 30cm; however lateral spread towards the sapwood is slower due to active host defence response called the reaction zone (Shain, 1971). A phenol containing reaction zone forms in the border area between the healthy sapwood and infected heartwood (Woodward *et al.*, 1998). There are special cells in the cortex known as polyphenolic parenchyma cells that contain phenolic compounds and paly a very important role in the tree defence (Franceschi *et al.*, 2000). These cells enlarged four times bigger than original cell size after wounding or pathogen attack (Krokene *et al.*, 2003). Phenolic compounds are released into the surrounding sieve cells and restricted spread through traumatic resin ducts (Nagy *et al.*, 2000). Beside wounding and pathogen attack certain elicitors such as ethylene and methyl jasmonate (MeJ) also initiate similar defence responses in Douglas fire and other conifers that leads to the activation of phenolics and terpenoids (Krokene *et al.*, 2003; Hudgins & Franceschi, 2004).

The defence responses are also evident in local and systemic tissues of Norway spruce. The transcripts level of peroxidases and *Chitinases* increase in local as well as in systemic tissues after wounding and *H. parviporum* treatments (Fossdal *et al.*, 2001; Hietala *et al.*, 2004; Nagy

et al., 2004). Hietala and co-workers (Hietala *et al.*, 2004) found that transcripts of *Chitinase IV* are higher in resistant than susceptible clones of Norway spruce. The induction of *ChitinaseIV*, *Phenyl-alanine ammonia lyase* (*PaPAL*) and *Glutathione-S-transferase* (*PaGST*) were recorded at two time points in resistant clones while only one peak was observed in case of susceptible clones (Hietala *et al.*, 2004; Karlsson *et al.*, 2007). Further, (Koutaniemi *et al.*, 2007) analysed the transcripts levels of some genes from lignin biosynthesis pathway in different tissues of Norway spruce sapling. However, no defence response between local as well as in systemic bark was studied that cover much larger area. Present work not only measured transcript level of defence related genes at the local site of infection but also nearly 3 and 5cm away from the infection site. Additionally, we also measured level of transcripts in corresponding sapwood after pathogen and MeJ treatment at 1, 3, 6 and 13dpi.

Comparison in angiosperm and gymnosperm tree defence

Considering the great evolutionary separation of gymnosperms and angiosperms (Magallon & Sanderson, 2005), they may have adopted different strategies to cope with abiotic and biotic factors (Agrawal, 2007), then again other components may have been conserved.

Necrotrophs seems to exploit defence system weaknesses in ta similar way both in gymnosperms and angiosperms. Molecular defence regulations are better studied in annuals and horticultural crops than in trees (Franceschi *et al.*, 2005). The availability of the *Populus* genome sequence and sequenced cDNA libraries in gymnosperm tree species make it possible to begin comparing the molecular defence responses. Furthermore resistance-like genes have been identified in tree species from both gymnosperms and in angiosperms. For example in poplar 1% (402) genes are from the NBS LRR class (Duplessis *et al.*, 2009) while in *loblolly pine* 67 such genes have been identified in the CC-NB-LRR subfamily (Liu & Ekramoddoullah, 2004) and a number of TIR-NB-LRRs have also been identified so far. Of the PR proteins Chitinases are very important as they degrade the cell wall of invading necrotrophic and biotrophic fungal pathogens and cause the release of oligimeric elicitors (PAMPs) that further induce the host defence response. These enzymes have been studied in Norway spruce and pine trees. It has been reported that chitinases in Norway spruce highly upregulated after necrotrophic fungal inoculations (Hietala *et al.*, 2004; Nagy *et al.*, 2005; Fossdal *et al.*, 2006). In *Populus* the *ChitinaseI* (*ChitiI*), for instance, is known to be differentially expressed after necrotrophic and biotrophic fungal invasion (Veluthakkal & Dasgupta, 2010). Also, in the present study *ChitiI* was also found to be upregulated after

wounding, biotrophic and necrotrophic fungal inoculations in aspen and *PaChi4* in Norway spruce. Peroxidases are important in defence responses in both gymnosperms and angiosperms, and they are up-regulated in response to in response to pathogen inoculation in spruce (Fossdal *et al.*, 2001; Fossdal *et al.*, 2007; Koutaniemi *et al.*, 2007). This seems to be the same case also in *Populus* because peroxidases genes were up-regulated upon *Melampsora* infection (Azaiez *et al.*, 2009). These examples of gene regulation in Norway spruce and *Populus* show similar trends after fungal infections. However, further work in studying the signalling pathways and transcripts regulation aided by a full genome sequence of both species is likely required to understand how the gene regulation differs and to what extent they show similarities in their defence responses, between these two evolutionary distant trees.

Objectives of this thesis

The gymnosperms and angiosperms together comprise the spermatophytes or seed plants and conifers are by far the largest group of gymnosperms. The gymnosperm trees include the largest and oldest living individual organisms on the planet, and angiosperm trees follow close behind. Their longevity suggests that these plants have evolved efficient constitutive and inducible defences toward necrotrophic and biotrophic pathogens, so it is possible that there are both differences and conserved properties in the defence strategies between and within these two major plant divisions.

Our lab is engaged with necrotroph-Norway spruce pathogen-host system and the focus has been on host defence mechanisms. Unfortunately the genome sequencing effort of Norway spruce has only just begun and was launched last year. Though this limits our analysis in that species, Norway spruce transcriptomic data allows us to initiate focussed studies. Broader studies in *Populus* could be achieved on the basis of the whole genome sequence (Tuskan *et al.*, 2006) and therefore, we initiated this project to study host defence mechanisms in *Populus*. The biotroph, necrotroph and wounding treatments were applied and transcriptional changes in whole genome were studied by microarray analysis. Long term goal of this study was to find out molecular components leading to resistance, giving insight and a better understanding into the biology of these pathogen-host systems as well as providing knowledge that can be used directly in resistance breeding. Therefore the specific objectives of this study are as follows.

Hypothesis 1: The systemic signalling of the defence response induces changes in transcription in both the secondary phloem (bark) and secondary xylem of Norway spruce.
Objective 1: Determination of spatiotemporal defence response in Norway spruce against necrotroph and MeJ separately in the bark and wood (paper I).

Hypothesis 2: The defence signalling induces systemic transcriptome changes to the biotroph infection and these differ from the response in necrotrophic interaction and wounding.
Objective 2: Determination of the defence transcriptome of aspen toward biotrophic and necrotrophic fungi (paper II).

Hypothesis 3: Differences in resistance are characterized by both by differences in the constitutive transcriptomic expression between healthy, yet genetically different plants and by their transcriptional responses to infection.
Objective 3: Examine differences in the molecular response of resistant *versus* susceptible trees toward a biotrophic fungus (paper II).

Hypothesis 4: The Local and systemic defence induction differ depending on type of pathogen and that the speed of systemic defence induction is more rapid in trees with higher level of resistance.
Objective 4: Comparison of the similarities and differences between aspen responses to necrotrophic and biotrophic pathogens (paper II).

Hypothesis 5: Highly necrotrophic pathogens are able to overcome or suppress the host defence response.
Objective 5: Comparison of the local and systemic induced defence response to necrotroph and mechanical wounding in aspen clones (paper III).

Hypothesis 6: NB-LRR proteins are involved in resistance to necrotrophic pathogens.
Objective 6: Comparison of the local and systemic change in NB-LRR expression and microRNAs targeting NB-LRR transcripts in Norway spruce after *C. polonica* and wounding (paper IV).

Summary of papers presented in this thesis

Paper I

Defence-related gene expression in bark and sapwood of Norway spruce in response to *Heterobasidion parviporum* and methyl jasmonate

Nadeem Yaqoob, Igor A. Yakovlev, Harald Kvaalen, Paal Krokene, Halvor Solheim, and Carl Gunnar Fossdal

Manuscript submitted to Plant Molecular Pathology and Physiology (2011)

Conifers dominate many temperate regions and Norway spruce (*Picea abies)* is one of the most economically and ecologically important tree species in the northern hemisphere. Forest trees are under continuous pressure from different pathogens. In Norway close to twenty percent of all the spruce trees show damages from the pathogenic fungus *Heterobasidion parviporum* upon harvest. *H. parviporum* is a necrotrophic basidiomycete that usually spread through root contact or wounds and it can also thrive inside the stem as a saprotroph. In response to this fungus Norway spruce typically elicits both local and systemic defence responses. Our research objectives in this paper were to characterize the local and systemic defence gene expression in bark and wood separately after pathogen inoculation and to compare this with the defence responses elicited by methyl jasmonate (MeJ). In order to study the spatio-temporal aspects due to the local and systemic host defence signalling, four time points (day 1, 3, 6 and 13 after infection/MeJ treatment) were selected and subsequent sections 8 cm up the stem from the site of treatment were analysed separately for gene expression changes using qRT-PCR.

The pathogen induced systemic up-regulation of *PaPX3, PaPX2, PaACO* and *PaChi4* in both bark and sapwood, whereas the local responses to MeJ were strongest in the bark. Genes involved in lignin biosynthesis (*PaPAL1, PaPAL2, PaC4H3/5* and *PaHCT1*) were up-regulated locally in the bark, and MeJ induced a stronger and more lasting response than the pathogen. These results demonstrate local and systemic host responses to pathogen infection

in both the bark and sapwood, and reveal similarities between the local responses to the necrotrophic pathogen and that to MeJ.

Paper II

Transcriptome differences between two SwAsp Aspen clones and their systemic defence response to the biotroph *Melampsora magnusiana*, a novel necrotrophic fungus and wounding

Nadeem Yaqoob, Benedicte Riber Albrectsen, Jan Karlsson, Igor A. Yakovlev, Halvor Solheim, and Carl Gunnar Fossdal

(Manuscript)

The recognition of attacking pathogens locally at site of attack and systemic signalling of the defence responses varies in different genotypes. There is also a constitutive difference in the expression of genes sets in healthy but genetically different individuals of a plant species, reflected in the basal expression of their genes, differences in growth, level of secondary metabolites and their ability to respond to external stimuli. Induced defence responses include physical, chemical, physiological and transcriptional changes in plants. Changes in the host defence gene expression locally at the site of infection and systemically at positions more distally located may also depend on the type of stress. Therefore; the response of the host plant may differ to mechanical wounding, biotrophic and necrotrophic fungal pathogens.

Genotypes within a plant species differ in their constitutive gene expression reflecting their basal level of resistance and the difference is also reflected in their ability to respond by inducing local and systemic defence gene expression upon exposure to biotic and abiotic stresses. To examine the constitutive and systemically induced defence responses to fungal infection by a biotroph and a highly pathogenic necrotroph, we used microarray for global transcription profiling of two *aspen* genotypes from the SwAsp collection (clone23 and 72). In healthy plants, difference in the constitutive gene expression was found between the two clones; among 552 significantly differentially expressed genes in clone 72, 250 were more highly expressed whereas 302 were less expressed constitutively as compared to clone 23. The clones also differed significantly in their transcriptional response to the biotrophic and necrotrophic pathogens at 24hours of treatment. Relative to healthy control plants, no differentially expression in response to biotroph treatment was seen in 23 whereas 166 genes were differentially regulated in clone 72. Similarly, in response to the necrotroph, only 7genes were differentially expressed in 23 while 118 genes changed their expression in clone 72. Principal Component analysis indicated a wide difference host response at the transcriptome

level between the two clones as well as in their systemic response to biotroph and necrotroph infection. Total phenolics and condensed tannins were also quantified from the leaf samples and revealed minor differences overall. Examination of a larger sample size including a time series by qRT-PCR confirm trends in gene expression observed by the array analysis and here wounded samples were also examined. Clone 23 showed no, little or no systemic response to all treatments, including wounding, suggesting a lack or delayed systemic defence signalling. Additionally, the higher level of transcripts induced systemically in response to wounding alone in clone 72 as compared to the necrotroph indicates that the pathogen suppress part of the host defence.

Paper III

Rapid local and systemic host response in Aspen (*Populus tremula*) to necrotrophic fungus and wounding

Nadeem Yaqoob, Benedicte Riber Albrectsen , Jan Karlsson, Igor A. Yakovlev, Halvor Solheim and Carl Gunnar Fossdal

(Manuscript)

Necrotrophic fungi are among the most damaging diseases of forest trees and are responsible for enormous losses to forestry worldwide. These pathogens enter living or dead plant parts through wounds or natural openings, kill the host tissues, produce enzymes that cause breakdown of membranes, cell contents and cell wall components, and absorb the resulting nutrients. Plant resistance against necrotrophs differ to that toward biotrophs that need to feed on living cells. Resistance to necrotrophs can be monogenic, but in trees it is usually complex, involves many mechanisms leading to resistance and may show similarities to the response to wounding. Effective defence against necrotrophic pathogens may involve both local and systemic defence signalling, production of secondary metabolites, lignification of cell walls and formation of pathogenesis-related proteins.

Two aspen SwAsp clones 72 and 23 were inoculated in the phloem with a newly discovered and highly aggressive necrotrophic fungus and wounded to examine the local at the local and systemic host response. The pathogen was able to colonize both clones but these were able to recover from wounding alone. The lesion length caused by the necrotroph was 3 to 4 times longer in secondary xylem than bark in the secondary phloem, suggesting that the bark defence is stronger than that of sapwood. Height growth was better in clone 72 indicating that it was the more vigorous. There were similar levels and changes in total phenol and tannins during the experiment for the two clones. The level of total phenols transiently dropped systemically in both clones, while a local and transient drop for the tannins was more prominent in clone 23. The local effect to inoculation and wounding in the bark was similar between the two clones in most transcripts examined. However, the local response to the necrotroph in clone 23 was in general stronger than clone 72.

The systemic effect in leaves was also examined 24 hours after treatments and revealed that clone 72 had a significantly more effective systemic signalling inducing increased transcript

levels. Clone 23 showed a general lack of systemic induction despite the clear local response. Furthermore, some transcripts increased to a higher level systemically in leaves of clone 72 in response to wounding than to the pathogen alone, suggesting that the pathogen has some ability to repress the induction and translocation of host systemic defence signalling.

Paper IV

Comparison of the local and systemic change in NB-LRR expression and microRNA targeting NB-LRRs in Norway spruce after *Ceratocystis polonica* and wounding

Igor A. Yakovlev, Nadeem Yaqoob, Paal Krokene, Halvor Solheim and Carl Gunnar Fossdal

(Manuscript)

NB-LRR resistance proteins are involved in recognizing pathogens and other exogenous signals. NB-LRRs may thus be the first step in inducing the defence response and are therefore of great interest to us. MicroRNAs (miRNA) are often involved in post-transcriptional regulating. We examined the expression of five Norway spruce miRNA putatively targeting putative NB-LRR related transcripts in secondary phloem (bark) of a resistance clone after wounding and inoculation with the necrotrophic blue stain fungus *Ceratocystis polonica*. We found local and systemic induction of the resistance marker genes *PaChi4, PaPAL* and *PaPX3* indicative of induced host defence response. There was also local and systemic change in the expression of 5 miRNAs and 21 NB-LRRs in bark between healthy and treated plants. However, only the putative NB-LRRs of PaLRR3 and PaLRR13 showed greater than two fold changes. The miRNAs showed a rapid local and systemic down regulation at day 1 that was followed by a later increase to and beyond the constitutive levels. Overall the changes in expression both locally at site of treatment and systemically away from the site of treatment were minor for most of the miRNAs and predicted NB-LRR homologue targets studied, suggesting that the expression of NB-LRR related genes are likely kept close to their constitutive levels and are possibly under tight transcriptional control in both stressed and healthy plants. The recognition of the invading pathogen may be specific or indirect and the regulation of the resulting local and systemic plant defence responses likely to be complex and multi layered.

Material and Methods

This work deals with the defence responses to pathogenic fungi and wounding in Norway spruce and aspen.

The Norway spruce clone (AL15886-B10), with high resistant was used for the work on Norway spruce defence mechanisms. Two year old spruce saplings generated by somatic embryogenesis were challenged with *H. parviporum*, *Ceratocystis polonica*, wounded and methyl jasmonate treated. The host gene expression of selected genes was followed at four time point (1, 3, 6, 13 days post inoculation). To study the local response in the stem a section of 1 cm long from site of treatment was collected. To study the systemic responses up the stem a second and third section were collected 3-5 cm and 6-8 cm above the treatment site.

In aspen the local response in the stem and the systemic response in leaves were studied following fungal infections and wounding. Systemic responses in aspen were studied after biotroph (*M. magnusiana*), necrotroph (*Ceratocystis* sp.) and wounding treatments. In paper II, constitutive transcriptome differences between clone 23 and 72 and their systemic defence response in leaves was studied at 24hpi. In manuscript III, we focused on the local and systemic defence responses after necrotroph and mechanical wounding of the stem. We looked the defence responses of host by looking into the gene expression. For this purpose we performed microarray experiments using aspen plants (paper II). Furthermore, we intensively used quantitative reverse transcriptase polymerase reaction (qRT-PCR) to follow the expression of the selected genes in host plants (Norway spruce, aspen). The level of induced host defence markers, NB-LRRS and miRNAs putatively targeting NB-LRRs was followed in *C. polonica* inoculated and wounded plants (paper IV).

qRT-PCR

In this genomic age, microarray and deep transcriptome sequencing are valuable tools, but qRT-PCR has emerged as the most reliable method of choice to detect and quantify transcript changes of limited number of candidates if large amount of samples are to be processed. The working principal of quantification is rather straight forward; target with higher copy number is detected at earlier cycles. The unit measured is critical threshold cycle (Ct) in the exponential phase of the real-time PCR. Typically an endogenous control is used to normalize the data and make comparison between samples more reliable. The detection is usually

obtained by incorporating fluorescent dyes that bind to double stranded DNA in the reaction mixture, but different setups including probes are also in common use. We used SYBR green in our studies. Obtained raw data for quantification purposes is usually normalized to an appropriate endogenous reference gene. The relative abundance of the transcripts in Norway spruce study was measured by using α-Tubulin as an endogenous reference gene. In paper I transcripts quantification was calculated using the ddCT method as X ddCT = α-Tubulin dCT-Target dCT. The ddCT method was also used for the determining the change in miRNAs levels in paper IV. In paper II, III and IV the quantity of target transcripts were calculated using absolute quantification as X Quantity = Target Quantity/endogenous transcript Quantity. The endogenous control was *Ubq* in paper II while the *Cdc2* was used in paper III. In our experimental setup with *aspen, Ubq* appeared more stable in leaf tissues than *Cdc2* (data not shown). On the other hand, *Cdc2* is expressed consistently in different type of tissues in *Populus* (Nicole *et al.*, 2006; Rinaldi *et al.*, 2007; Boyle *et al.*, 2010) and during this study also showed least variation in expression compared to other candidate endogenous genes.

Microarray

This technique was first described by Schena and associates in 1995 (Schena *et al.*, 1995). Microarray technology is an important tool to measure the relative expression levels of thousands of genes simultaneously. To understand the biological phenomena's including many transcriptional changes such as plant defence responses to biotic and abiotic stresses requires knowledge of genome-scale studies to identify candidate genes involved. Microarrays are typically the immobilization of DNA probes representing a large number of genes in the form of oligos or cDNA on a solid surface. The positions of probes are pre-defined on traditional arrays. Extracted RNA from the plants is reverse transcribed and labelled with fluorescent dyes. After hybridization to the array, the intensity of fluorescent signals is measured by scanning the spots on slides in a laser scanner. From images numerical data are generated and processed for measuring relative expression level. The microarrays experiments include in the present study were performed at The Umeå Plant Science Centre in Sweden. The RNA samples (target) were labelled with two fluorescent dyes, cyanine dye3 and 5. The microarray *Populus* POP2 that consists of 24,644 array elements and represents 16000 gene models were used (Sterky *et al.*, 2004).

Total phenols and condensed tannins

Secondary metabolites are important determinant of resistance in woody plants, among them phenolics are used as markers for resistance to pathogens. In poplar leaves the major defence related phenolics are the flavonoid-derived proanthocyanidins (CTs; condensed tannins)and the salicin-based phenolic glycosides (TPs; total phenols) (Mellway *et al.*, 2009). Tannins are biosynthesid on pathogen infection and mechanical wounding (Constabel *et al.*, 2000; Miranda *et al.*, 2007), both locally and systemically (Peters & Constabel, 2002). The changes in transcripts level is known to be regulated by interaction between several classes of transcription factors e.g. Myb and Wrky factors. Previously, in two aspen species, a correlation was found between their resistance level and phenolic profiles after shoot blight (*Vanturia tremulae*) during one growing season (Freiwald, 2008). We measured the TPs and CTs levels at constitutive and after pathogen and mechanical wounding in Clone23 and 72 from SwAsp aspen collection to demonstrate the trends in chemical defence responses. We used simple clorimeatric method for soluble phenols (TPs) and condensed tannins (CTs) in paper II & III.

Results and discussion

Though lot of new technologies are being employed in the scientific filed, still the knowledge about resistance mechanisms in plants is at its infancy. How these mechanisms of resistance varies in intra as well as extra-specifically in plants. A new era was opened eleven years ago when genome of small herbaceous plant *Arabidopsis thaliana* was unveiled. Although progress in plant genomes sequencing is much behind than animals; even then nearly 30 plant genomes are now available for analysis and comparisons (Piennisi, 2011). Among these sequenced genomes angiosperms that constitute major flora on earth are dominating. *Populus* is the first available genome from woody perennial published in 2006 (Tuskan *et al.*, 2006). To date, no genome is available from gymnosperms however genome of four conifers: the loblolly pine, sugar pine, Douglas fir and Norway spruce is in progress (Neale & Kremer, 2011; Piennisi, 2011). Comparative study of the available plant genomes indicate that plant evolution is surprisingly dynamic (Piennisi, 2011). This might be the basis for establishing significantly larger numbers of pathogenic interactions between fungi and plants.

In this thesis molecular defence responses of Norway spruce and European aspen were studied. Putative candidate defence related genes were identified in aspen after biotroph and necrotroph in local and systemic tissues. Similarly in Norway spruce expression of certain defence related genes were studied after necrotroph. It is interesting to see difference of gene regulation in these two species to defend themselves.

Molecular defence responses in Norway spruce and aspen after necrotrophic fungus and mechanical wounding

In order to study local and system defence responses we inoculated resistant clone of Norway spruce with necrotroph *H. parviporum* and collected bark and sapwood samples from inoculated site and from 3 to 5 cm up from the inoculation site. Addition to pathogen MeJ treatment was also applied in spruce study. In *Populus* two clones: more biotroph resistant 72 and susceptible 23 from SwAsp collection were inoculated with an aggressive necrotroph *Ceratocystis* sp. and collected bark sample from the site of infection as well as closest upper leaf from the inoculated site (ca. 5cm). In this study we used wounding treatment for comparison. Transcriptional response of few selected genes is discussed below.

Plant chitinases catalyse the hydrolysis of chitin an integral part of the fungal cell wall. On the basis of chitin-binding domain and carboxy-terminal extension domain plant chitinases are

grouped into seven classes (I to VII) (Hamel *et al.*, 1997). Chitinase class I, II, IV, VI and VII are placed in the pathogenesis related family 3 (PR3) while classification based on glycoside hydrolase (GH) places chitinas*e* class I, II and IV in GH family 19 (Neuhaus *et al.*, 1996). However class I and IV chitinase*s* are structurally more related to each other than class II (Graham & Sticklen, 1994). Chitinases are induced in woody trees such as Norway spruce, slash pine (*Pinus elliottii*) and *common beech* in response to wounding and pathogen attack ((Veluthakkal & Dasgupta, 2010) and reference therein). It has been demonstrated that resistant Norway spruce (Fossdal *et al.*, 2006) and poplar clones (Noël *et al.*, 2005) had higher level of chitinase transcripts. *Chitinase4* (*PaChi4*) from chitinase class IV in *P.abies* and *ChitiI* from *Populus* are closest homologue (NCBI blastp query coverage 98%, e-value 1e-80) and upregulated after wounding and necrotroph attack. In spruce we found higher transcripts level of *PaChi4* in local and systemic bark as well as in sapwood in two years old saplings. This rise in the transcript level was remained high till 6dpi and decreased at 13dpi (paper I, Fig. 2,). This is in accordance with previous studies on Norway spruce showing that in susceptible clone chitinase induction was delayed and weaker, particularly systemically in the more distal locations (Hietala *et al.*, 2004; Fossdal *et al.*, 2006). In *Populus* the highest transcript level of *ChitiI* was observed in the more biotroph resistant clone (72) at 1dpi that remain high at all three points of observations. *ChitiI* in susceptible clone (23) remained at much lower level than clone72 throughout (paper III, Fig. 1, 2, 3). Although, differences in resistance level of spruce and aspen and severity of pathogenicity of the necrotrophs is not comparable still we can have some idea how the host regulated their molecular defence responses locally at site of treatment and systemically at more distal locations. In these two pathosystems, aspen seems to respond at least as quickly as spruce but in Norway spruce (paper I), the increase in chitinase level was very much higher than in aspen. Later it returns closer to constitutive levels by the end of the experiment in spruce. The early increase followed later by a return close to constitutive levels is the hallmark of a successful host defence response, while in susceptible plants the levels tend to rise with time indicative of an ongoing colonization of the tissues. It shows that the necrotroph after the initial strong host defence response is defeated by the spruce clone and the infection is halted and the transcript levels return close to normal as the plants recovered completely from the inoculations, showing very short lesions that did not expand.

Trends of Chitinase regulation in systemic tissues of resistant Norway spruce and *Populus* show similarities. The major difference being that *ChitiI* was higher in systemic than local

tissues in *Populus* at 1dpi while in spruce it was typically lower systemically in response to stress (Fig 2 in paper I; Fig 1, 4 in paper III, Fig. 1 paper IV). (Philippe *et al.*, 2010) found that the systemic induction of *ChitiI* was less in local leaves of *Populus* after wounding and herbivore attack at 1dpi. The clone 72 showed higher level of *ChitiI* in systemic than local tissues, while clone 23 had *ChitiI* upregulation in local but less systemically. This is similar to what has previously been observed In Norway spruce, the transcript level of *PaChi4* was more rapidly unregulated systemically in resistant clone than susceptible clone after wounding and necrotroph *H. parviporum* by 3dpi (Hietala *et al.*, 2004). However, it is possible that we have missed part of (at least the local) increase to infection in our system as in tissue cultures such increase occurred for *PaChi4* already within 6hpi in the more resistant Norway spruce clone (Hietala *et al.*, 2004; Karlsson *et al.*, 2007). Douglas-fir *(Pseudotsuga menziesii)* showed increased *Chitinase* IV level first at 12hpi after attack by the necrotroph fungus *Phellinus sulphurascens* (Islam *et al.*, 2010).

Biosynthesis and alteration in cell walls are likely important in the plant defence in both angiosperm and gymnosperm plants. *CCR* is considered one of the crucial gene that regulate the carbon flux towards lignin (Piquemal *et al.*, 1998). It catalyses the conversion of feruloyl-CoA to coniferaldehyde and is considered the first enzyme in the monolignol-specific branch of the phenylpropanoid pathway. Lower level of *CCR* leads to lower lignin levels in tobacco (Chabannes *et al.*, 2001). In *Arabidopsis CCR* gene expression is related to increase in carbon flux into the monolignol pathway (Hano *et al.*, 2006). Down regulation of the *CCR* gene may result in the 50% reduction of lignin contents in *Populus* cell wall (Leplé *et al.*, 2007).Transgenic Norway spruce expressing antisense *CCR* gene showed 8% decrease in lignin contents (Wadenback *et al.*, 2008). The expression of *CCR* in Norway spruce was upregulated significantly in the xylem after *H. parviporum* infection at 14dpi (Koutaniemi *et al.*, 2007). In Scots pine increase of *CCR* transcripts was observed at 15dpi after *H. annosum* (Adomas *et al.*, 2007). *CCR* after necrotroph infection in *Populus* clone 23 and 72 showed increase in local tissues at 1dpi. Results from conifers studies suggest that *CCR* up regulation is shared between these two species, while the exact timing of the increase and the duration of increase may differ between the two and between individuals within each of the species depending on their level of resistance.

In the poplar genome, 21 putative Map Kinases (*Mpks)* have been identified. These serve as signalling molecules that interlink the networks that leads to the nucleus in order to induce

defence related genes (Rathjen & Moffett, 2003; Hamel *et al.*, 2005). *Mpks* are up-regulated in poplar after pathogen infection (Hamel *et al.*, 2005; Boyle *et al.*, 2010).We followed the expression of *Mpk3.1* as this gene is expressed in all tissues of different organs in *Populus* (Nicole *et al.*, 2006). Regulation of *Mpk3.1* in aspen after necrotroph showed indications of a small increase as a local response to infection in both clones (paper III, Fig.1-3), but in the more biotroph resistant clone 72 it also showed systemic upregulation due to wounding only (paper III, Figure s2). The transcript level of a putative map kinase has also been studied in Scots pine at 7dpi after being challenged with *H. annosum,* however, its levels remained largely unchanged (Sun *et al.*, 2011). In loblolly pine*, Mpk6* is involved in phosphorylation of the Myb factors like *Myb1* and *Myb4* that has a role in lignin biosynthesis (Zhao & Dixon, 2011). We infer that *Mpks* are important players in successful plant defence against necrotrophs by (among other roles) facilitating the lignification of cell wall both in angiosperms and gymnosperms.

Genes such as *PAL, C4H3/5* and *HCT1* are also involved in lignin biosynthesis (Whetten *et al.*, 2001) and were followed spatiotemporally in Norway spruce. We found that *H. parviporum* and MeJ induced upregulation of *PAL, C4H3/5* and *HCT1* in Norway spruce that peaked at 6dpi in bark tissues (paper I). In Norway spruce-*H.parviporum* pathosystem *PAL* expression was higher in resistant clones (Karlsson *et al.*, 2007; Likar & Regvar, 2008). In *P. trichocarpa* as many as five isoforms of *PAL* gene have been annotated (Tsai *et al.*, 2006). In aspen *PAL* is found to be positively linked with increased lignin biosynthesis (Kao *et al.*, 2002). In *Populus* inoculated with a biotroph result in down regulation of the *PAL* by 5dpi indicating that the pathogen is dependent on less fortified host cell walls to thrive (Levée *et al.*, 2009). *C4H3/5* catalyses trans-coumaric acid to para-coumaric acid (Kombrink *et al.*, 1995). Transcript level of this gene increased in response to the necrotroph in the relatively resistant Norway spruce clone (paper I) and the results are in line with an earlier finding (Koutaniemi *et al.*, 2007). *HCT* is involved in monolignol biosynthesis (Hoffmann *et al.*, 2003) and was investigated in Norway spruce in this work (paper I). Our results showed that MeJ treatment and pathogen infection induces similar *HCT* related defence responses in the bark of Norway spruce. It has also been reported that *HCT* has great impact on lignin contents and monolignol composition in Monterey pine (*Pinus radiate*) (Wagner *et al.,* 2007). The most striking difference between *Arabidopsis* and *Populus* gene families related to lignification is the considerable expansion of the HCT family in *Populus* (Tsai *et al.*, 2006), possibly indicating diverse roles related also to defence.

PR1 a marker for systemic acquired resistance (SAR) remained at low constitutive levels in healthy controls of bark samples in both aspen clones studied (paper III). Transcript level increased rapidly in aspen after necrotroph infection locally in bark tissues but there is little response to wounding in bark suggesting it may be induced by the SA pathway, and indications of an upregulation both to necrotroph and wounding was detected in the clone 72 (paper III). *PR1* was upregulated systemically in bark tissues of a susceptible clone of Japanese black pine (*Pinus thunbergii*) after pine wood nematode (PWN; *Bursaphelenchus xylophilus*) at 3dpi (Nose & Shiraishi, 2011). Thus *PR1* can be upregulated both locally and systemically in both compatible and incompatible interactions both in spruce and *Populus*, but the timing of its increase is likely the key to its role in resistance.

We quantified *Hevein* (PR4), possibly involved in recognition of pathogen derived chitin, transcript level in the two aspen clones after necrotroph and found and found higher transcripts level in response to the pathogen locally, and the increase was greater with time in compatible interaction with clone23, while only clone 72 showed an increase systemically. Interesting (Rinaldi *et al.*, 2007) reported that *Hevein* was upregulated 2.5 fold in the incompatible *Melampsora-Populus* interaction at 2dpi. *Hevein* was also reported to be upregulated (1 fold) after wounding in local and systemic leaves of *Populus* already at 6hpi but not detectable change at 1dpi (Philippe *et al.*, 2010). Quantification of *Hevein* in Japanese black pine after PWN showed higher upregulation in the resistant clone than in the susceptible clone (Nose & Shiraishi, 2011). These findings indicate that *Heveis* have a role in the defence response to pathogens and wounding in both conifers and *Populus.*

Variation in gene expression of defence related genes in aspen after necrotrophic and biotrophic fungus

Regulation of genes during defence responses to fungal pathogens in gymnosperms and angiosperms trees do share some common general trends as described above. However, due to the genetic variation within a species the level of resistance and corresponding gene expression varies between genotypes therein. The differential gene regulation during defence differs within *Populus* (Rinaldi *et al.*, 2007; Azaiez *et al.*, 2009; Boyle *et al.*, 2010) and Norway spruce (Hietala *et al.*, 2004) reflecting the response of different genotypes. Our microarray results also indicate that the more biotroph resistant aspen clone had higher number of differentially regulated genes after necrotroph, biotroph and wounding treatments than the more susceptible clone after 24 hours (paper II), and an even greater number differ

between them in the healthy state, reflecting that they are different genotypes. The work also shows that the regulation of defence related genes differs depending on whether the invading pathogen is a biotroph or necrotroph (paper II, Fig. 3, 4). Following is an overview of some of the genes that are differentially expressed between responses to biotroph *versus* necrotroph.

O-methyltransferase (*Omt*) are proteins that catalyse the methylation of small molecules such as flavonoids and lignin precusors (Lam *et al.*, 2007). These play important role in lignin biosynthesis and has affect in monolignol contents (Ye *et al.*, 1994; Tsai *et al.*, 2006). *Populus* has 26 Omt genes; most of them are mapped onto the chromosomes of the sequenced genome. The aspen *Omt* studied in paper II is highly similar to the *Omt15* distributed on scaffolds that are yet not assigned place on the physical map of the sequenced *Populus trichocarpa* genome (Barakat *et al.*, 2011). *Omt15* is placed in class I; expresses in leaves grouped in patteren4 on the bases of intron-exon structure of *Populus* Omt genes (for details see (Barakat *et al.*, 2011). The *Omt* was expressed significantly higher in leaves of the more biotroph resistant aspen clone 72 than in susceptible clone 23, and the Omt was upregulated as a systemic response to the biotroph and wounding but not to the necrotroph in clone 72 (paper II). Similarly, an *Omt* was found to be upregulated during incompatible *Melampsora-Populus* interaction at 2dpi (Rinaldi *et al.*, 2007). However, (Azaiez *et al.*, 2009) reported down regulation of class I *Omt* in both compatible and in-compatible *Melampsora* interactions with *Populus* at 6dpi locally in leaf tissues. *Omt* from class I was found to be upregulated after wounding and herbivore treatment at 1dpi (Philippe *et al.*, 2010). We also noticed higher transcript level after wound treatment in present work (paper I). The low transcript level after necrotroph infection than wounding in our work suggests that the necrotroph was able to circumvent the wound related defence measures from the aspen host. The suppression of host defence by necrotrophs and other pathogens is an area of considerable interest (Hammond-Kosack & Rudd, 2008).

In the present study the systemic transcript induction level of *Wrky23-like* in leaves was much lower after necrotroph than corresponding biotroph both in the susceptible clone 23 and resistant aspen clone 72. The increase in *Wrky23-like* transcripts after wounding was observed only in the more biotroph resistant aspen clone 72 and not in the less systemically responsive clone 23 (paper II). Higher level of *Wrky23* transcript has been observed in *Melampsora-Populus* interaction at early time points with decreasing trend at later time points (Levée *et al.*, 2009). We observed similar local early increase in *Wrky23-like* transcripts in bark after necrotroph and then decrease in transcript level at later time points (paper III). This work

shows the clear differences in systemic induction between the more resistant clone and the susceptible toward the biotroph, and also reiterates that host response to the biotroph and necrotroph differ. In addition also for this gene product the necrotroph appears to be able to suppress the host defence response seen toward wounding alone.

Myb gene overexpression improves insect pest resistance and impacts plant growth (Plett et al., 2010) and likely affects the host defence also through its impact on cell wall biosynthesis. In our work, *Myb1* and *Myb2* were found to be upregulated systemically in the more biotroph resistant clone 72, but not in the susceptible clone 23, in response to the biotroph. This increase was not seen in response to the necrotroph, but wounding gave elevated levels in clone 72. The upregulation of Myb genes was observed in herbivore and wound interactions in *Populus* (Ralph et al., 2006).

In the more biotroph resistant aspen clone72 more genes were differentially regulated systemically after biotroph than in response to the necrotroph in array studies (paper II). Interestingly, the majority of differentially regulated genes were upregulated in response to the biotroph while down regulated in response to the necrotroph. We follow up the selected genes with qRT-PCR, these also showed the same trend in gene regulation after biotroph between the two clones. In addition to biotroph, wounding treatment was also induced the up regulation of qRT-PCR studied genes. The differential regulation of the genes was highest at 1dpi in local bark tissues after necrotroph while in systemic tissues this trend was only noticeable in clone72. Clone72 offered higher resistance to the biotroph and this was also reflected at the transcript level in systemic tissues after biotroph inoculations and wounding. The clone72 is rich in tannins and have vigorous growth (Albrectsen *et al.*, 2010) and it grew taller than clone23 as measured six weeks after inoculation in treated and healthy plants. Even though clone 72 was more resistant to the biotroph, the lesion length after necrotroph inoculation did not differ from that in clone 23, and this might be due to suppression of defence system of the host plant by this highly aggressive necrotroph.

Future prospects

The aim of present work has been to contribute to the understanding of defence responses in Norway spruce and *Populus* against biotrophic and necrotrophic fungi. This work, together with previous studies by other research groups collectively have shown that the resistance level within Norway spruce and *Populus* and their response varies greatly towards biotrophic and necrotrophic fungi. By monitoring the regulation of defence related genes in spatio-temporal manner in spruce and poplar clones we found results that support the hypothesis that transcripts level of defence related genes is more rapid systemically in the resistant trees. However, future studies are needed to further establish to what extent the host defences towards biotrophs differ to that towards necrotrophs and wounding in these trees, and to what extent the underlying local and systemic signalling inducing the host defences is shared, between these evolutionary distant trees.

Future progress on comparative studies, and more detailed genomic studies of Norway spruce, is dependent on the ongoing spruce genome projects in Sweden and Canada. Particularly it is important to better characterize the types and number of resistance genes in both species, with a focus not only on the between species variation but possibly even more important for future breeding efforts, the variation within each species. Future studies also need to better characterize what signalling molecules are involved in the local and systemic induction of efficient defences toward biotrophs and towards necrotrophs and wounding. For the intracellular signalling events regulating the defence responses, it is important to study the promoters and transcription factors that act on these to better understand how the trees regulate their expression of downstream genes such as the pathogenesis related proteins.

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Defence-related gene expression in bark and sapwood of Norway spruce in response to *Heterobasidion parviporum* and methyl jasmonate

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Abstract

We compared gene expression in Norway spruce bark and sapwood in response to the pathogen *Heterobasidion parviporum* and methyl jasmonate (MeJ). The pathogen induced systemic up-regulation of *PaPX3, PaPX2, PaACO* and *PaChi4* in both bark and sapwood, whereas responses to MeJ were strongest in the bark. Genes involved in lignin biosynthesis (*PaPAL1, PaPAL2, PaC4H3/5* and *PaHCT1*) were up-regulated locally in the bark, and MeJ induced a stronger and more lasting response than the pathogen. These results demonstrate local and systemic host responses to pathogen infection in bark and sapwood, and reveal similarities in local responses to pathogen and MeJ.

Keywords: qRT–PCR, Gene expression; Resistance; Induced resistance; *Heterobasidion parviporum;* Methyl jasmonate; Necrotroph; Norway spruce

Abbreviations:

qRT-PCR	Quantitative reverse transcription - polymerase chain reaction
H. parviporum.	*Heterobasidion parviporum*
PaC4H3/5	*Cinnamate-4-hydroxylase*
PaHCT1	*Hydroxycinnamoyl:CoA shikimate/quinate hydroxycinnamoyl transferase*
PaPAL	*Phenylalanine ammonia-lyase*
MeJ	Methyl jasmonate
PaACO	*1-aminocyclopropane-1-carboxylate oxidases*
PaACS	1-aminocyclopropane-1-carboxylic acid syntheses
PaChi4	*Chitinase class IV*

1. Introduction

Conifers dominate many temperate regions and Norway spruce (*Picea abies)* is one of the most economically and ecologically important tree species in the northern hemisphere. Trees and other plants are continuously exposed to a variety of abiotic and biotic stresses and have evolved numerous mechanisms to defend themselves. Trees face special challenges in defence due to their often very long lifespan and the fact that most of their biomass is allocated to wood that also must be protected from pathogen attack and decay. Several local and systemic defence responses are initiated in plants upon pathogen recognition. The level of host resistance is determined by how efficiently the plant can coordinate its defences and how quickly the responses can be launched [1-4]. Many plant hormones, such as abscisic acid, salicylic acid, jasmonic acid and ethylene, are involved in plant defence signalling cascades that lead to reinforcement of cell walls and production of hydrolytic enzymes [5]. Conifers have evolved both constitutive and inducible defence systems in the bark that deter, expel or kill invading pathogens [6, 7], but much less is known about the defence reactions in the wood at the molecular level.

Heterobasidion annosum Fr. sensu lato (*H. annosum*), the causal agent of root and butt rot of various conifers, is a well-known necrotroph that is responsible for annual losses of ~800 million € in Europe. The main cause of root and butt rot in Norway spruce is *Heterobasidion parviporum (H. parviporum)* which colonizes trees through bark wounds in the stem or enters fresh stumps and spread to healthy plants through root contact [8]. In response to infection Norway spruce elicits defence responses in the living bark and sapwood, including changes in size and phenolic content of the polyphenolic parenchyma cells [9, 10], and formation of a wound periderm [9, 11]. The anatomical, physiological and chemical aspects of Norway spruce defences in the bark have been examined in recent studies [12-14]. Less is known about defences in the wood, but *H. annosum* spreads further in wood than in bark of Sitka spruce seedlings, indicating that resistance responses are weaker in the wood [15]. Gene expression profiling in the bark has been done to understand the resistance mechanisms in Norway spruce to *H. parviporum* [2, 4], but there have been no attempts to compare the expression profile of putative defence genes in bark and sapwood after *H. parviporum* infection.

Regulation of defence related genes is a key element in all inducible defence mechanisms in plants [16]. Norway spruce upregulates several pathogen related proteins in response to pathogen attack. Chitinases from the host tree hydrolyse chitin, a major

component in the cell walls of all true fungi, and chitinases seem to play important defence roles in many fungus-plant pathosystems, including the release of elicitors [17]. The chitinase *PaChi4* is upregulated in stressed and dying Norway spruce tissues and is a useful marker for local and systemic host response [2]. Lignin, the most abundant polymer in trees after cellulose, also plays important roles in plant defence. Peroxidases that are up-regulated after pathogen invasion in Norway spruce and other conifers are involved in lignification and suberization of host tissues, as well as in production of reactive oxygen species [18-20]. The lignin related peroxidases *PaPX2* and *PaPX3* have a general stress-induced function and are upregulated in Norway spruce bark in response to *H. parviporum* infection [21]. Phenylalanine ammonia-lyase (*PaPAL1* and *PaPAL2*) has a key role in lignin formation, along with cinnamate 4-hydroxylase (*PaC4H*) and hydroxyl cinnamoyl CoA (*PaHCT*) [21-23]. In Norway spruce *PaPAL2* seems to be involved in the formation of defence-related lignin and be responsible for the increased flux of phenylalanine into lignin biosynthesis in stressed trees [21]. The ethylene signalling pathway is another important regulator of plant defence response. Ethylene formation in spruce and other plants is regulated by two key enzymes; 1-aminocyclopropane-1-carboxylate oxidases (ACO) and 1-aminocyclopropane-1-carboxylic acid syntheses (ACS) [24]. The ethylene and MeJ signalling pathways may interact with each other and are involved in both plant development and defence [25]. MeJ-induced ethylene production is responsible for conifer bark defence responses [26].

Here we document differential expression of nine defence related genes in Norway spruce bark and sapwood over a spatial and temporal gradient using qRT-PCR. Because MeJ enhances the resistance of Norway spruce to fungal pathogens [13] it is of interest to compare the expression profiles induced by MeJ treatment and fungal infection. Expression profiling of these key genes will enhance our understanding of their role in local and systemic resistance responses in Norway spruce, and reveal if the systemic defence responses documented in the bark also are detectable in the outer sapwood.

2. *Material and methods*

2.1. Plant material and sampling

Two-year-old Norway spruce saplings generated by somatic embryogenesis from a single clone (AL15886-B10 derived from a single seed from a full-sib family of *Picea abies* (L.) Karst [Cross ♀ #2650 X ♂ #2707] produced at Biri Nursery and Seed Improvement Center, Norway) were selected for this study [27]. This clone is considered highly resistant to fungal

infection, as it shows very short lesion length and recovers quickly from inoculation with *H. parviporum*. Thirty-two genetically identical saplings were kept in growth chambers under optimal temperature (20-22 °C) at ambient day length with 200-250 μmol m^{-2} s^{-1} light conditions.

Saplings were inoculated with *H. parviporum* or painted with methyl jasmonate (MeJ) on the stem. Plants treated with MeJ were kept in a separate chamber under identical conditions, to avoid effects of volatile MeJ on the other plants. The heterokaryotic *H. parviporum* strain 87-257/1 was inoculated into the stem at 5 cm height from the ground by cutting a ~5 mm small bark flap using a scalpel. A small amount of malt agar (1% malt and 1.5% agar) containing actively growing mycelium was placed underneath the bark flap, pressed firmly against the stem, and sealed with parafilm. Unwounded control plants were wrapped with parafilm at the corresponding position of the stem. MeJ was applied by painting a 1 cm section of the stem with 100 mM MeJ in water with the surfactant Tween 20 (0.1%). Water with 0.1% of Tween 20 served as a control for the MeJ treatment. Samples of bark and sapwood were harvested 1, 3, 6 and 13 days after inoculation. Two ramets were harvested at each time point. Samples were quick-frozen in liquid nitrogen and stored at -80 C until analysis. To study local and systemic gene expression in response to fungal inoculation, three sections of bark and sapwood were analysed separately. The first section was 1 cm long and included the inoculation site and ca 0.5 cm above the upper margin of the inoculation wound. The second and third sections were taken 3-5 cm and 6-8 cm above the inoculation site, respectively. The sections were named B1, B2 and B3 for bark and W1, W2 and W3 for sapwood, with position 1 being closest to the inoculation wound. MeJ treated samples were analysed for local responses in the treated 1 cm section of the stem only. The unwounded and tween painted controls were analysed at the corresponding position, and at each sampling position bark and sapwood tissues were analysed separately.

2.2. Total RNA extraction and cDNA synthesis

Tissue samples (100-150 mg) were ground first in liquid nitrogen using a mortar and a pestle and then transferred to 2 ml eppendorf tubes for fine grinding in a Retsch 300 Mill (Retsch Gmbh, Haan, Germany) for 1.5 minutes. The equipment and tissue samples were kept chilled with N_2 throughout the grinding process. Isolation of RNA from bark and shoot tissues was done using the RNAqueous-Midi^{TM} kit (Ambion #1911, Austin, TX, USA) according to the manufacturer's protocol. RNA from sapwood was extracted using the RiboPure^{TM} kit (Ambion, #1924, Austin, TX, USA) according to the manufacturer's guidelines. Isolated

RNA was purified from genomic DNA traces by the DNA-Free^{TM} kit (Ambion #1906) following the manufacturer's protocol. Quantification of obtained RNA was done by spectrophotometer using the RiboGreen® RNA Quantification Kit (R-11490) according to user's manual. Samples were quantified at standard fluorescence wavelengths (excitation ~480 nm, emission ~520 nm). In order to make cDNA, 300 ng RNA was reverse transcribed using Taqman Reverse Transcription Reagents (Applied Biosystems, Foster City, CA) following instructions prescribed by the manufacturer. Reaction mixture was prepared in 50 µl volume and incubated in the thermal cycler at 25 °C for 10 min, then at 48 °C for 30 min, and finally at 95 °C for 5 minutes.

2.3. Transcript quantification by real-time qRT–PCR

Transcript expression levels were determined by qRT-PCR. qRT-PCR reactions were carried out in a 25 µl volume containing 250 nM of each primer, 2µl cDNA and 1 × SYBR®Green PCR master mix (Applied Biosystems, #4309155). The 7500 Real-Time PCR System (Applied Biosystems, #4351106) was used for quantification in a 96-well reaction plate using the parameters recommended by the manufacturer (2 min at 50°C, 10 min at 95°C and 40 cycles at 95°C for 15 sec and 60°C for 1 min). Two technical repeats were run for each gene product to confirm the accuracy of the PCR. We verified the specificity of the amplifications at the end of each PCR-run using the 7500-system SDS (dissociation curve analysis software). Description of PCR primers is given in Table 1. Acquisition and analysis of data were done using the 7500-system SDS software for absolute quantification and MS Excel for final calculations, as described by [28]. Relative transcript amounts were calculated from raw data obtained from real-time PCR in the form of critical threshold cycle values (dCt). The difference between endogenous *α-Tubulin* and corresponding threshold cycles for the transcript to be quantified (TARGET) were calculated as $X = \alpha\text{-}Tubulin - TARGET$ for all genes measured. To obtain better presentation of up- and down-regulation of transcripts, the raw data X were transformed. First, a mean value (Θ) was calculated from all experimental observations of X for a specific target gene. Then a new transformed variable Y_i was made for each sample of each transcript i using the formula $Yi = Xi - \Theta i$. Y is symmetric around its mean value, which is 0. Positive values represent higher and negative values lower transcription than average for control plants, and the unit is number of PCR cycles (Fig.1, 2).

3. Results

3.1. Peroxidases

Transcripts of the class III peroxidases *PaPX2* and *PaPX3* increased significantly in both bark and sapwood following pathogen inoculation, both locally and systemically. Transcript levels were high the first 6 days following inoculation, followed by a dramatic drop to or below constitutive levels by day 13. The greatest systemic increase was seen in the sapwood (Fig. 1a,c).

Local responses to MeJ treatment differed from those seen locally after pathogen inoculation. MeJ induced a more lasting increase of *PaPX3* in the bark and sapwood than pathogen inoculation, and in the bark the response to MeJ was also stronger (Fig. 1c,d). For *PaPX2* MeJ induced a rapid but transient increase in the bark and no increase in the sapwood, indicating differences between MeJ and pathogen induced host responses (Fig. 1a,b).

3.2. PaChi4 induction

PaChi4 was the most highly induced gene in this study (Fig. 2). *PaChi4* was up-regulated locally as well as systemically in both bark and sapwood. Transcript levels were elevated from day 1 in the sapwood and increased until 6 days after fungal infection. Transcript levels in the bark followed the same trend but were lower than in the sapwood. Transcript levels of *PaChi4* decreased by day 13. MeJ induced similar local responses in *PaChi4* as pathogen inoculation, with increased transcript levels in both bark and sapwood indicating similarities in the regulation of *PaChi4* by the two treatments.

3.3. Ethylene signalling

Two genes involved in ethylene signal transduction, *PaACS* and *PaACO*, did not increase locally in the bark or sapwood the first three days after inoculation, but *PaACO* had a transient increase both locally and systemically in the sapwood six days after inoculation (Fig. 3). This increase was most evident further away from the treatment site, suggesting a systemic response in the sapwood after fungal inoculation. A similar systemic increase was not seen in response to MeJ treatment (data not shown). Transcript levels of *PaACO* in the sapwood were below constitutive levels the first three days after inoculation. *PaACS* levels were weakly upregulated by day 13 in both wood and bark after MeJ treatment, with a transient decrease in the wood at day 3 to 6. 3). There was no *PaACS* response after fungal inoculation, neither in the bark (Fig. 3) nor in the sapwood (data not shown).

3.4. Lignin related genes

PaPAL1, PaPAL2, PaC4H3/5 and *PaHCT1* were up-regulated locally in the bark by both treatments, and MeJ treatment resulted in higher expression levels than fungal inoculation (Fig. s1). In general there were no clear responses in the sapwood after treatment. However, MeJ treatment resulted in a modest down-regulation of *PaHCT1* and an early upregulation of *PaPAL1* locally (Fig. s1), and both treatments downregulated *PaC4H3/5* locally at the latest time point (Fig. s2). These responses suggest similarities between the effect of MeJ and pathogen inoculation on lignin related genes.

4. Discussion

This is the first study to compare transcript levels of defence-related genes in bark and sapwood of Norway spruce. We followed spatiotemporal changes in genes of the lignin/phenylpropanoid pathway, the chitinase *PaChi4*, the peroxidases *PaPX3* and *PaPX3*, as well as two ethylene related genes in 2-year-old clonal plants after inoculation with the fungal pathogen *H. parviporum* and MeJ treatment. The results demonstrate that local defence responses to pathogen infection are similar but not identical to those induced by the proposed defense signaling compound MeJ.

A thorough understanding of the regulation of defence-related genes in Norway spruce is important to understand the mechanisms of tree resistance against *H. parviporum* and how MeJ can be used to prime tree resistance. The systemic expression of peroxidases and chitinases suggests the presence of systemic signalling cascades in Norway spruce [2, 18, 29]. Previous studies have established that some Norway spruce trees that are resistant to *H. parviporum* have been found to have very high transcripts levels of defence related genes (e.g. peroxidases, class IV chitinase and others) [2, 30], and they also appear to respond quicker to pathogen attack than susceptible genotypes.

In conifers and other plants induced defence responses to invading organisms are associated with cell wall modification through lignification and suberization [31]. The *PaPX3* and *PaPX2* gene were more highly expressed after inoculation. We found the lignin related peroxidases *PaPX3* and *PaPX2* to be more highly expressed after inoculation, supporting the suggestion that they have general stress-induced and host defence functions [21]. Transcript levels of *PaPX2* were higher in the bark, while *PaPX3* transcripts were more abundant in the sapwood, contributing to an elevated systemic peroxidase response in both tissues. This expression pattern has also been observed by Adomas et al. [32] in *Pinus sylvestris* roots inoculated with *H. annosum.* It confirms a very active participation of peroxidases in host

defence in many tissue types. The quick and strong expression of peroxidases systemically agrees with previous studies in very young Norway spruce seedlings and tissue cultures, where strong upregulation was observed 12-48 hours after inoculation [30, 33, 34]. Kärkönen and co-workers [34] observed peak levels of peroxidases in cell culture of Norway spruce as quickly as 30 min after inoculation with *H. annosum*. Rapid induction of peroxidases can be used as a possible future molecular marker to identify resistant genotypes of Norway spruce.

Chitinases in the tree degrade the chitin in fungal cell walls by breaking the glycosidic bonds, and the resulting oligomers fragments act as elicitors that stimulate plant defence responses [35-37]. Chitinases are upregulated in plant parts where fungal growth is then inhibited by resultant swelling and lysis of fungal hyphal tips [38]. Most previous studies of chitinases in conifers have focused on gene expression in bark tissue, and in Norway spruce differential expression of chitinases has been reported both in barks of mature trees and in callus cultures [2, 30]. We found that early local and systemic increase in transcript levels of the chitinase *PaChi4* were even greater in the sapwood than in the bark, suggesting significant defence response also in the sapwood of these 2-year-old saplings. We may speculate that the higher chitinase level in the sapwood is due to defence signalling from the bark through the rays that run radially trough the bark into the sapwood. Additionally, the faster systemic expression of *PaChi4* in the wood than in the bark suggests that the host defence signal may travel in the xylem, but this remains to be tested. Pathogen infection and MeJ application induced very similar local expression of *PaChi4*, and parallel similarities have been observed in an anatomical study of Norway spruce [13]. It is generally believed that exogenous application of MeJ induces typical defence responses in Norway spruce ([6, 26]. However, the exact mechanisms responsible for enhanced tree resistance after MeJ treatment remain unclear, as we found other defense related genes to be differentially expressed by pathogen infection and MeJ treatment, both locally and systemically.

Reinforcement of cell walls by lignin deposition is a common response to pathogen infection in plants. The first step in lignin biosynthesis is deamination of phenylalanine by the enzyme phenyl alanine ammonia-lyase (PAL) [30, 39, 40], and it is well documented that PAL activity is related to lignification [41, 42]. It is thus of interest to study changes in expression of *PaPAL1, PaPAL2*, and other genes involved in lignin synthesis, such as *PaC4H3/5* and *PaHCT1*, in response to *H. parviporum* infection. Norway spruce clones that are relatively resistant to *H. parviporum* have been found to have higher transcript levels of *PaPAL* than more susceptible clones [30]. Our data show that *PaPAL* transcript levels in the bark rapidly decreased to normal levels after reaching their maximum six days after induction,

and this coincides with the infection being defeated in these plants. These results are in line with Likar & Regvar [43],who found that *PaPAL* transcript levels peaked in Norway spruce shoots five days after inoculation with *H. annosum*, and with Koutaniemi et al. [21] who found significantly up-regulation of *PaPAL2* in Norway spruce bark after *H. annosum* infection. Increased PAL activity after pathogen infection, together with upregulation of peroxidases, suggests a role in cell wall strengthening by increased lignification in Norway spruce.

The second step in the lignin biosynthetic pathway is catalysed by *cinnamate 4-hydroxylase* (*C4H*), which converts trans-coumaric acid to para-coumaric acid [1]. We observed upregulation of *PaC4H* transcript levels locally in the bark, reminiscent of Koutaniemi et al. [21] that found increased transcript levels in the bark of 32-year-old Norway spruce tree 14 days after *H. annosum* inoculation.

The third gene in the lignin pathway that we studied was *PaHCT1*. We observed a 3-fold increase locally in the bark after MeJ treatment, and suppression to below constitutive levels in the wood. Previous work has shown *PaHCT1* to be up-regulated 4-fold in the bark of 32-year-old Norway spruce 14 days after *H. parviporum* inoculation [21]. These data indicate that MeJ treatment and pathogen infection induces similar defence responses in the bark.

Interaction or cross-talk between the ethylene and MeJ signal transduction pathways is important in the defence responses of many plants [25], including *Arabidopsis thaliana* [44] and conifers such as Douglas fir [24]. Similarities between angiosperms and gymnosperm plants, separated by 300 million years of evolution, suggest that these signal transduction pathways have very ancient roots. In conifers ethylene and MeJ seem to induce similar defence responses as wounding, insect attack or fungal infection [26, 45]. We studied two genes in the ethylene pathway, *PaACO* and *PaACS*, but our data do not allow us to draw any firm conclusions about the similarity in the ethylene response of Norway spruce to pathogen infection and MeJ treatment. *PaACO* catalyses the final step in the ethylene biosynthesis pathway and is represented by a single gene in the Pinaceae family [24]. We found no effect of MeJ treatment on *PaACO* expression, whereas fungal inoculation induced a delayed, systemic upregulaion of *PaACO* in the sapwood but not in bark. For the first three days after inoculation there seemed to be a suppression of *PaACO* transcription, followed by an upregulation by day six. This delayed increase agrees with Ralph et al. [46], who studied *PaACO* expression in Sitka spruce after wounding and insect herbivory. *PaACS* on the other hand was upregulated slightly in response to MeJ treatment, but remained close to constitutive levels after fungal inoculation. The non-responsiveness of *PaACS* to fungal inoculation agrees

with Ralph et al. [46], where *PaACS* remained close to constitutive levels in Sitka spruce bark after wounding and insect herbivory. Previously, MeJ has been found to induce high levels of ethylene in Douglas fir [26], where MeJ treated stems had 77-fold higher ethylene production than wounded stems. We observed a much more modest induction of *PaACS* in response to MeJ treatment, with two-fold higher transcripts locally in the bark. The response of *PaACS* in the sapwood was more ambiguous, with an initial increase in transcript levels that was followed by a decrease and then a new increase 13 days after MeJ treatment.

Data from Sitka spruce seedlings suggest that resistance responses to *Heterobasidion* infection are much weaker in the wood than in the bark [15]. However, our results indicate that several local and systemic defence responses are upregulated in the sapwood of Norway spruce saplings resistant to *H. parviporum*, and some transcripts were even upregulated more strongly and for a longer time after infection in the wood than in the bark. This agrees with the observation that 2-year-old Sitka spruce clones with high resistance to *H. annosum* are able to limit the growth of this pathogen not only in the bark but also in the wood [15].

Among the nine genes examined in this study *PaPX2, PaPX3* and *PaChi4* were highly expressed in both bark and sapwood after pathogen inoculation and appeared to play the most important role in defence response both locally and systemically. Genes involved in lignin biosynthesis (such as *PaChi4* and *PaPX3*) were more responsive to MeJ treatment than to inoculation with *H. parviporum*. In general, MeJ treatment induced somewhat higher transcript levels than fungal inoculation in the bark. However, fungal inoculation induced higher levels of *PaChi4, PaACO* and *PaPX2*, as well as systemic responses in the sapwood for *PaPX2, PaPX3, PaChi4* and *PaACO*. In addition to increasing our knowledge of local and systemic defence responses in Norway spruce stem tissues to MeJ and pathogen infection this study may contribute to resistance breeding by providing breeders with possible tools to determine tree resistance at an early age by quantifying gene expression patterns in the bark and sapwood.

Acknowledgements

We thank Lars Dalen for help and support during sample collection, Inger Heldal for help with sample preparation and guidance in the lab. The project was supported by the Research Council of Norway (Grant No 136001).

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Figure captions

Fig. 1. Expression profiles of the peroxidases *PaPX2* and *PaPX3* in the bark (B) and sapwood (W) of Norway spruce 1-13 days after inoculation with *Heterobasidion parviporum* (a, c) or external application of methyl jasmonate (MeJ) (b, d). Numbers following B and W indicate different sampling positions above the treatment site (1 = at treatment site; 2 = 3-5 cm above; 3 = 6-8 cm above). One unit of relative Ct value corresponds to a two-fold difference in transcript level (see materials and method for relative Ct value calculations). Error bars denote SD.

Fig. 2. Transcript increase profiles of *PaChi4* in the bark (B) and sapwood (W) of Norway spruce after inoculation with *Heterobasidion parviporum* or treatment with methyl jasmonate (MeJ). Numbers following B and W indicate different sampling positions above the treatment site (1 = at treatment site; 2 = 3-5 cm above; 3 = 6-8 cm above). One unit of relative Ct value corresponds approximately to a two-fold increase in transcript level (see materials and method for relative Ct value calculations). Error bars denote SD.

Fig. 3. Transcript profiles of *PaACS* and *PaACO* genes in Norway spruce bark (B) and sapwood (W) of Norway spruce after inoculation with *Heterobasidion parviporum* or treatment with methyl jasmonate (MeJ). Numbers following B and W indicate different sampling positions above the treatment site (1 = at treatment site; 2 = 3-5 cm above; 3 = 6-8 cm above). One unit of relative Ct value corresponds approximately to a two-fold increase in transcript level (see materials and method for relative Ct value calculations). Error bars denote SD.

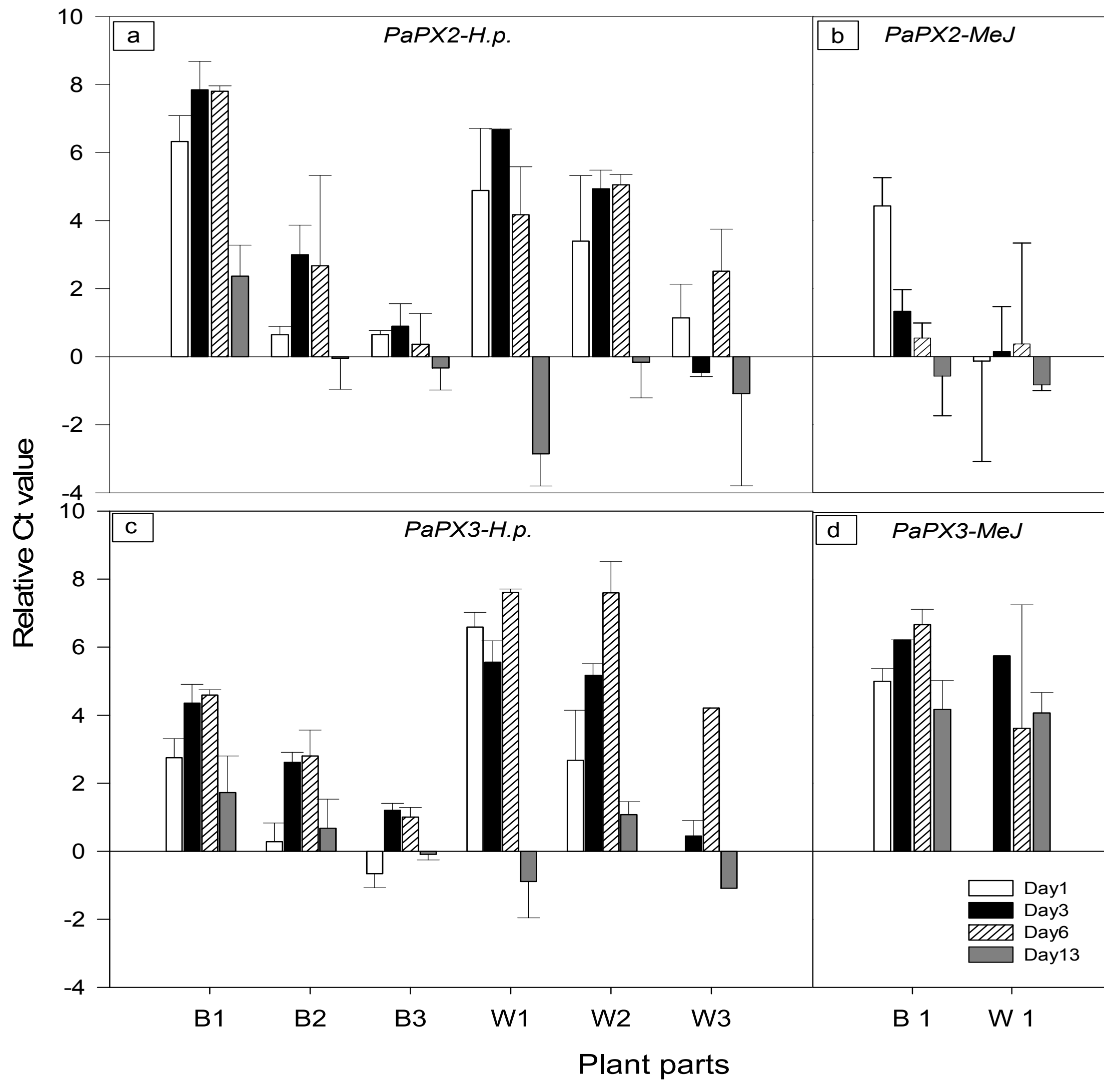

Figure. 1.

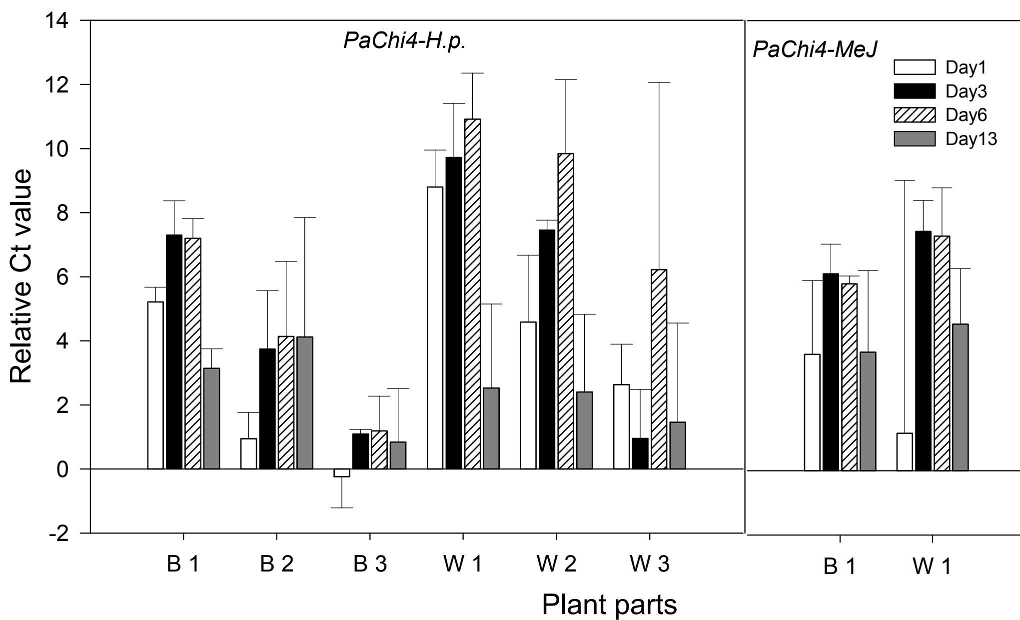

Figure. 2.

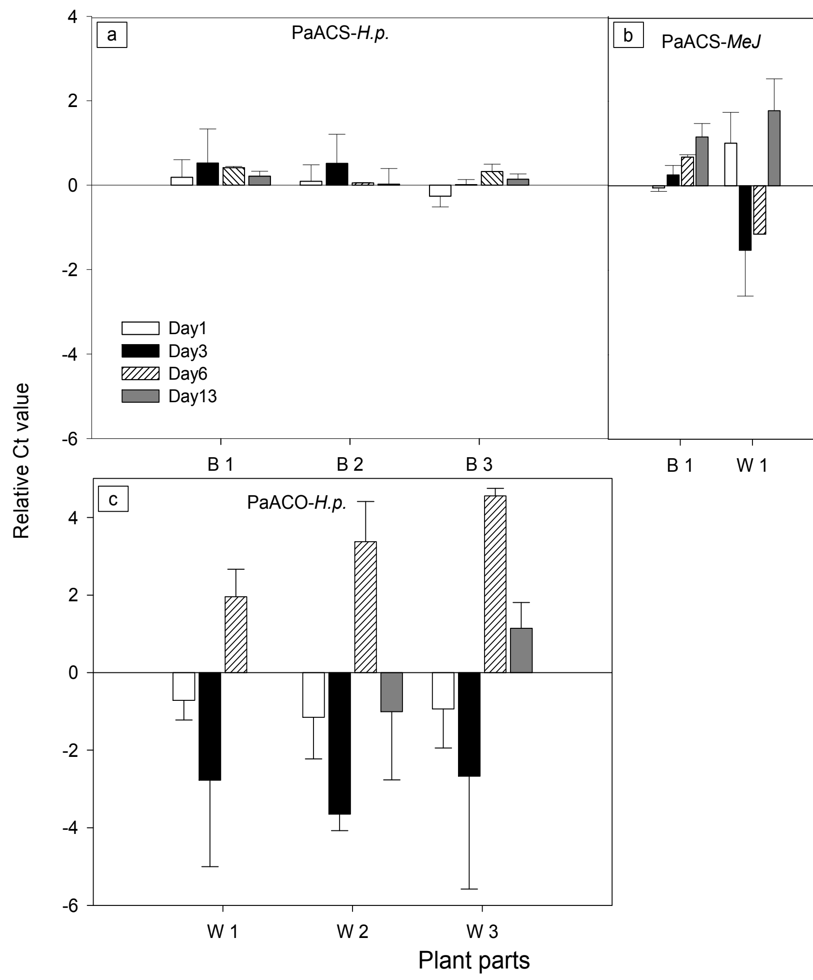

Figure. 3.

Table 1 Candidate defence genes in Norway spruce analysed by real time qRT-PCR

Name	GenBank Accession No.	Predicted Gene	Froward/reverse primers used in qRT-PCR	References
PaPX3	AM170663, AJ566203	*P.abies* Peroxidase (*PaPX3* gene)	ATGGTGGCGCTGTCAATTC/ TGCTGTAGAACGTCCAAGAAAGAC	[21]
PaPX2	AJ566201	*P.abies* mRNA for peroxidase (*PaPX2* gene)	CGCCCAACTTATCGCTGAGA/ TCCACCCGGCTCTTGATG	[21]
PaChi4	AY450924	*P.abies* class IV chitinase (*PaChi4*)	GCGAGGGCAAGGGATTCTAC/ GTGGTGCCAAATCCAGAAA	[2]
PgACS	EF179152	*P.glauca PaACC* synthase (*PaACS4*)	CAAGCAGAATCCCTATGATGCCGAAA/ TCTGGATGAGACTTGAGCCAACCTTC	[46]
PgACO	BT112327	*P.glauca PaACO*-like gene	TGGAACATCGGGCAGTAACAAACG/ GACGGAGCCACCTCTGCATCC	[46]
PaHCT1	AM173211	Hydroxycinnamoyl:CoA shikimate/quinate hydroxycinnamoyl transferase (*PaHCT1*)	TGCGAGTGTAATCCATGAAGCTT/ GGCTGCAATCCCAAATAGTCTAAA	[21]
PaC4H3/5	AM176009, AM173043	*P.abies* Cinnamate-4-hydroxylase	CGCCCCGAGCGATTCT/ GGCTGCAATCCCAAATAGTCTAAA	[21]
PaPAL1	AY952468	*P.abies* phenylalanine ammonia lyase-like protein (*PaPAL1*)	CAGCCCTCTGCCCAACAG/ AGCTGGGTTCTCACGAATTCA	[18]
PaPAL2	AM293549	*P.abies* Phenylalanine ammonia-lyase-like protein (*PaPAL2*)	TTGCTCGTAGGCACCAATAGC/ GCCTTGCCTTCGTTGATAGC	[21]
PaαTub	X57980 S94427	*P.abies* alpha-tubulin	GGCATACCGGCAGCTCTTC/ AAGTTGTTGGCGGCGTCTT	[2]

Appendix

Fig. s1. Expression profiles of genes involved in lignin biosynthesis in bark (B) and sapwood (W) of Norway spruce after inoculation with *Heterobasidion parviporum* or treatment with methyl jasmonate (MeJ). Numbers following B and W indicate different sampling positions above the treatment site (1 = at treatment site; 2 = 3-5 cm above; 3 = 6-8 cm above). One unit of relative Ct value corresponds approximately to a two-fold increase in transcript level (see materials and method for relative Ct value calculations). Error bars denote SD.

Fig. s2. Expression profiles of PaC4H3/5 involved in lignin biosynthesis in bark (B) and sapwood (W) of Norway spruce after inoculation with *Heterobasidion parviporum* or treatment with methyl jasmonate (MeJ). Numbers following B and W indicate different sampling positions above the treatment site (1 = at treatment site; 2 = 3-5 cm above; 3 = 6-8 cm above). One unit of relative Ct value corresponds approximately to a two-fold increase in transcript level (see materials and method for relative Ct value calculations). Error bars denote SD.

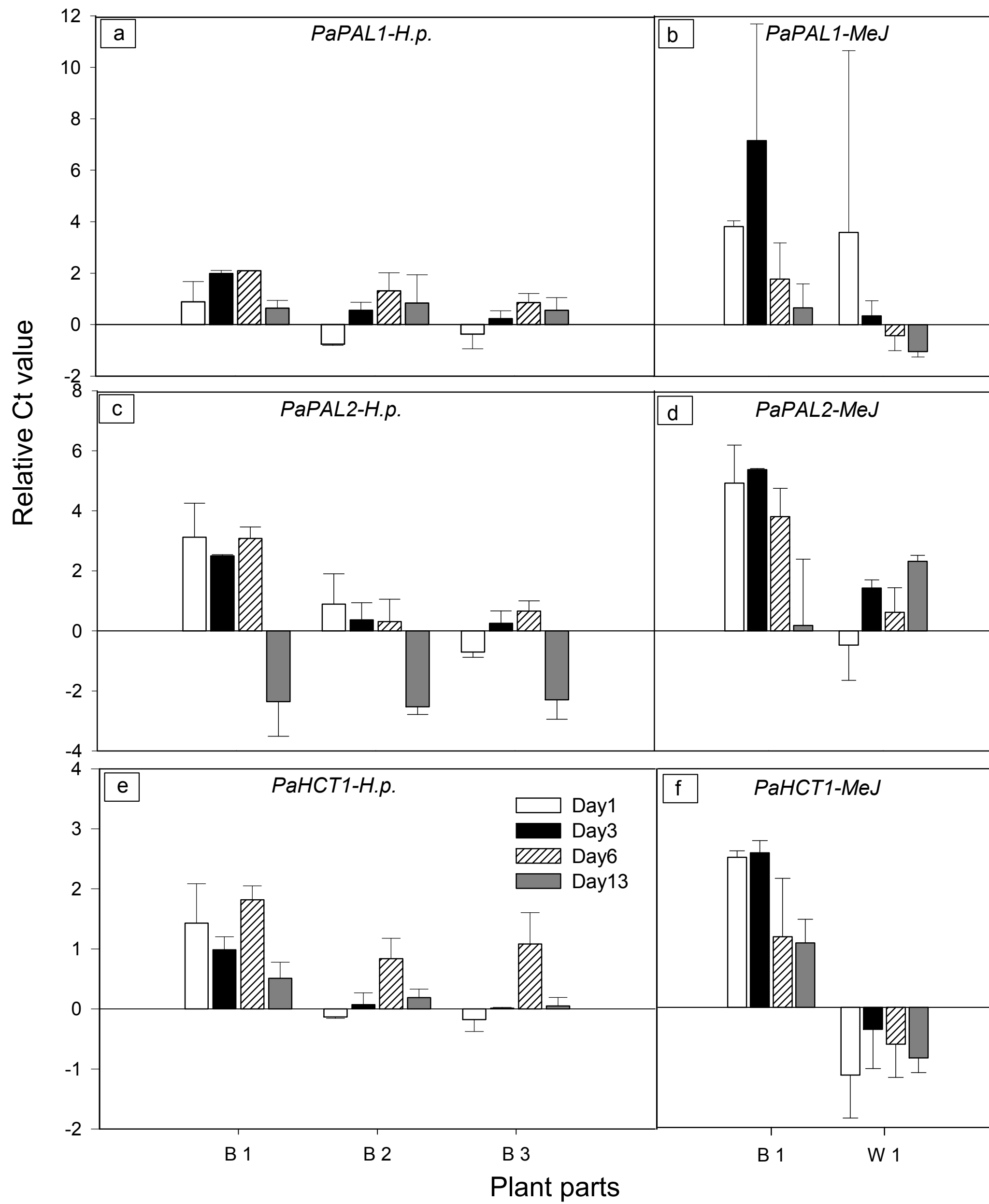

Figure. s1

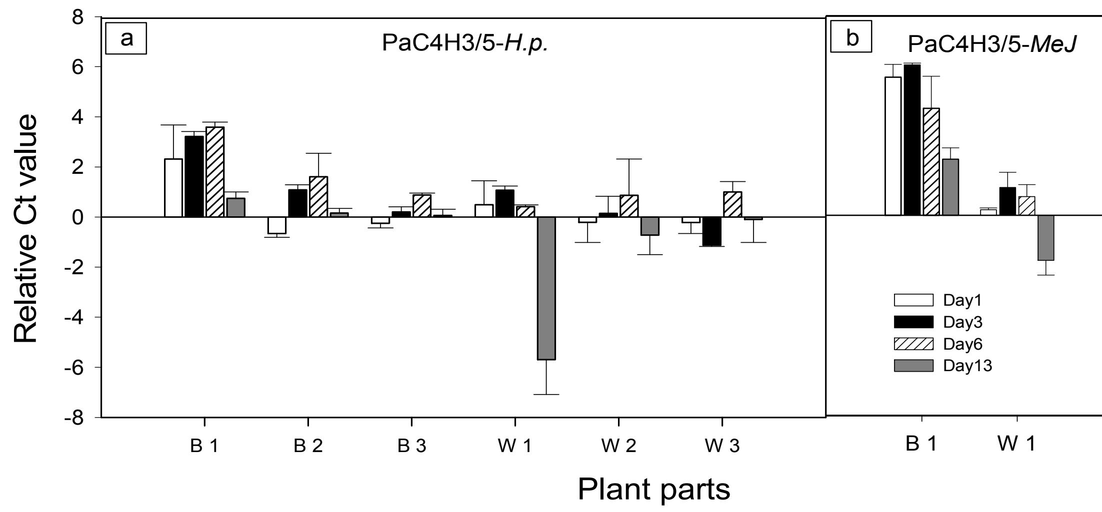

Figure. s2.

Transcriptome differences between two SwAsp aspen clones and their systemic defence response to the biotroph *Melampsora magnusiana*, a novel necrotroph *Ceratocystis* sp. and wounding

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Abstract

Genotypes of a plant species differ in their constitutive gene expression reflecting their basal level of resistance and their phenotype is also reflected in their ability to respond by inducing local and systemic defence gene expression upon exposure to biotic and abiotic stresses. To examine the constitutive and systemically induced defence responses to fungal infection by a biotroph and a highly pathogenic necrotroph, we used microarray for global transcription profiling of two *Populus tremula* genotypes from the SwAsp collection (clone 23 and 72). They have previously expressed diverse growth properties and phenolic profiles and were thus expected to also show differences in their resistance properties to the fungal treatments. In healthy plants, difference in the constitutive gene expression was found between the two clones, among 552 significantly differentially expressed genes in clone 72, 250 were more highly expressed whereas 302 were less expressed as compared to clone 23. The clones differed significantly in their transcriptional response to the biotrophic and necrotrophic pathogens at 24 hours of treatment. Relative to healthy control plants, no differentially expression in response to biotroph treatment was seen in clone 23 whereas 166 genes were differentially regulated in clone 72. Similarly, in response to the necrotroph, only 7 genes were differentially expressed in clone 23 while 118 genes changed their expression in clone 72. Principal Component analysis indicated a wide difference host response at the transcriptome level between the two clones as well as in their systemic response to biotroph and necrotroph infection. Total phenolics and condensed tannins were also quantified from the leaf samples and revealed minor differences. Examination of a larger sample size including a time series by qRT-PCR confirm trends in gene expression observed by the array analysis and here wounded samples were also examined. Clone 23 showed little or no systemic response to all treatments including wounding suggesting a lack or delayed systemic defence signalling. Additionally, the higher level of transcripts induced systemically response to wounding alone in clone 72 as compared to the necrotroph indicates that the pathogen suppress part of the host defence.

Keywords: Microarrays, qRT–PCR, Gene expression; Resistance; Induced resistance; *Melampsora magnusiana;* Necrotroph; *Ceratocystis* sp.; Mechanical wounding; *Populus*; Phenolic

Introduction

The recognition of attacking pathogens locally at site of attack and systemic signalling of the defence responses varies in different genotypes. There is also a constitutive difference in the expression of genes sets in healthy but genetically different individuals of a plant species, reflected in the basal expression of their genes, differences in growth, level of secondary metabolites and ability to respond to external stimuli in their healthy unstressed state. Induced defence responses include physical, chemical, physiological and transcriptional changes in plants. Changes in the host defence gene expression profiles locally at the site of infection and systemically at positions more distally located on the plant may also depend on the type of stress. Therefore; the response of the host plant may differ to mechanical wounding, biotrophs that feed on living host and necrotrophs that kill their host cells and extract energy from dead contents. The induction of defence related genes expression is often quicker in the more resistant plants than in susceptible plants (Fossdal *et al.*, 2006). Both biotrophs and necrotrophs may suppress the defence responses in the host plant (Lambais & Mehdy, 1993; Fossdal *et al.*, 2003). Although, the gene expression changes induced after biotroph infection typically differ from those after necrotroph infection, they still may share a number of genes induced in response to both type of pathogens (Schenk *et al.*, 2000; Glazebrook, 2001; Glazebrook *et al.*, 2003).

With the availability of the whole genome sequence of black cottonwood (*Populus trichocarpa*), poplar species has become a model for perennial woody trees (Tuskan *et al.*, 2006). High throughput tools have been developed for the Populus genus like microarray chips that allow to screen for thousands of gene expressions simultaneously (Jansson & Douglas, 2007). Ralph and co-workers (Ralph *et al.*, 2006) reviewed the response in gene activity to poplar when infected by various fungi, bacteria, viruses and other pests. Many studies in poplars have shown that a suit of constitutive and inducible defences are activated in response to such damage (Philippe & Bohlmann, 2007). Systemic induced defences are also documented in poplars (Parsons *et al.*, 1989; Arimura *et al.*, 2004; Babst *et al.*, 2009).

A large variety of fungal pathogens infect popular leaves both biotrophs (*Melampsora* species) and necrotrophs (*Venturia populina*) (Newcombe, 1996; Callan, 1998). *Melampsora* species are obligate biotrophic fungi that enter the host leaves through stomata. Within 10 days after establishment the fungi produce golden pustules filled with urediniospores. This fungi cause damage to the host through decreased photosynthesis efficiency, early defoliation

(Duplessis *et al.*, 2009) and increased susceptibility to other pests (Newcombe *et al.*, 1994; Gérard *et al.*, 2006). Recent sequencing of the *Melampsora larici-populina* has provided an opportunity to understand poplar-rust interaction at molecular level (Rinaldi *et al.*, 2007; Duplessis *et al.*, 2009). Whole genome transcriptional profile studies using microarray have been performed in order to obtain candidate genes and better understand the defence responses of poplar against *Melampsora* (Miranda *et al.*, 2007; Rinaldi *et al.*, 2007). These studies suggest that a strong gene regulation occurs in incompatible interactions compared to compatibles interactions.

Necrotrophs depend on the killing of host tissue first, and differ from biotrophs that avoid/suppress host defence. Necrotrophs are not studied intensively like biotrophs, mainly because fewer necrotroph are hosted by model plants like *Arabidopsis*. Aspen (*Populus tremula*) has several necrotrophs among others is *Ceratocystis* species. Species of *Ceratocystis* are largely insect-dispersed pathogens of woody plants, infecting their hosts through wounds and are among the fungi responsible for the most serious and second most numerous cases of wood decay in aspen (Schneider *et al.*, 2008).

Beside pathogens, wounding is a continuous threat to plants. It is not only the physical damage but wounds are also an entry point for pathogens. Not only the damage cells are responding to wounding but also neighbouring cells induce host response to wounding inducing gene expression changes. (Leon *et al.*, 2001). Davis and co-workers (Davis *et al.*, 1991) described the wound-induced gene expression in hybrid poplar and isolated genes coding kunitz trypsin inhibitor and chitinases. Wound- and herbivore-stress affects the regulation of gene expression in flavonoid biosynthesis pathway and directly increase the accumulation of proanthocyanidin (Peters & Constabel, 2002). There are several studies giving overview of the transcriptome responses in the damaged leaves and/or insect herbivory (Christopher *et al.*, 2004; Ralph *et al.*, 2006; Miranda *et al.*, 2007).

We describe here the use of microarrays and qRT-PCR validation to investigate the differential constitutive and defence related gene expression during host-pathogen interactions with a biotroph and a necrotroph pathogen in two aspen clones SwAsp 23 and 72. Systemic leaves from two clones were sampled 24 hours after inoculation (hai) with *Melampsora magnusiana*, and a novel necrotroph, *Ceratocystis* sp. Data from microarrays experiment gave the difference in constitutive level of defence in two clones. Pathogens inoculations revealed the differences in gene regulation within and between the two clones. After finding the

differences in genes expression from array, a set of genes was selected to study the expression levels by qRT-PCR. Samples from mechanical wounding were also used in this analysis to investigate the difference of a gene expression level in wounding and necrotroph (since part of the necrotroph treatment was to wound before infection could happen). We also performed chemical analysis to measure total phenolics (TPs) and proanthocyanidins as a measurement of condensed tannins (CTs).

Material and methods

Pathogens used

For biotroph inoculation leaves of *Chelidonium majus* L infected with *M. magnusiana*, Wagner which has aspen as alternate host, collected in Södermanland, Sweden on May 14, 2008 was used. For necrotroph inoculation a novel *Ceratocystis* species (isolate NFLI 2004-466/0501) isolated from aspen was used. This species is an aggressive necrotroph causing long dark coloured lesions on the stem by inoculations (H. Solheim, unpubl.).

Plant material

Two aspen clones (23 and 72) from the SwAsp collection (Luquez *et al.*, 2008) were used for the experiment. The clones were selected from their field expressed levels of tannins which are higher in 72 than in 23, and from the complexity of their salicinoid composition with clone 23 producing the more complex phenolic glycoside 2-cinnamoyl salicortin at elevated levels (Keefover-Ring and Albrectsen in prep). The plants had been propagated from tissue culture in the summer 2007 and kept in greenhouse conditions. In December 2008 the plants entered autumn senescence and defoliated. Buds flush started in mid-April 2009 and the experiment took place in June 2009. Eleven plants from each clone were used in this study. After assigning numbers from 1 to 11, plants were randomly placed on benches in the greenhouse. For each treatment three biological replicates were used, whereas two biological replicates were used for controls.

Pathogens inoculation and wounding treatment procedure

Biotroph inoculation on aspen leaves

A pre-test on single individual clones from the SwAsp collection suggested that *M. magnusiana* developed more pustules on clone 23 and that it was more infectious to this clone

compared to clone 72. For controlled inoculation on the experimental plants, spores were collected in a glass container (50 ml). Six ml of 0.02 mg/ml 6-Bensylamionopurin "Biopur" solution was added to the spores together with 0.6 ml of tween20 (0.6 ml). The solution was well mixed and spore concentration assessed. One drop (0.1 ml) containing 5000 visible spores was used as dose unit.). To infect a plant one drop was placed on the abaxial side of a leaf and spores were spread out on the leaf surface by gently twisting the leaf from side to side. Six adjacent leaves were infected on each plant (leaf numbers: 11-16 from the top leaf). To ensure sufficient humidity and avoid cross contamination in the green house, plastic bags were wrapped around the infected leaves. Sample leaves above infected leaves were harvested after 24 hours.

Wounding and necrotroph treatment on aspen bark

Wounding and necrotroph treatments were applied by making flaps (ca. 1 cm height – cutting bark on the upper side and on both side) at equal heights (Fig. s1, s2). Flaps covered ca 50 % of the circumference of the stem. Treatments were applied at three sites: site 1 just below leaf number 10 from the top; site 2 just below leaf number 16 from the top; and site 3 five cm above the internodium 2007-2008. For inoculation with *Ceratocystis* sp. a small amount of malt agar (1% malt and 1.5% agar) with actively growing mycelium was placed underneath the bark flap, before covered with parafilm. Control plants did not receive any treatment. Sample leaf above infected area was harvested after 24 hours.

Growth and infection assessments

Growth parameters were measured at the beginning of the treatment to ensure equal starting conditions and after 14 days treatment to evaluate treatment effects. To avoid extreme out-liers for infection measurements for *M. magnusiana*, pustules were assessed per leaf using an index that counted exact numbers of lesions on a leaf up to ten and numbers of pustules beyond 10 were set to 11. Lesions in response to wounding and necrotroph treatment were measured as discoloration of the bark, and of the wood under the bark. Size was measured as length of discoloration. Means of growth and infection per individual were then used for clone comparisons in common in Tukey T-tests.

RNA preparation

Leaves were grinded using mortar and pestle cooled by constant addition of liquid nitrogen. Ground samples were weighed to 50 mg and stored at -80ºC until RNA extraction. The

Aurum™ total RNA mini kit (#732-6820) from BioRad (Bio-Rad Laboratories, Inc. USA) was used for RNA isolation by following the manufacturer's protocol. Eluted RNA 80µl was stored at -80ºC for further work. The yield of RNA was measured using a Nanodrop1000 spectrophotometer. In order to make cDNA, 300 ng RNA was reverse transcribed using Taqman Reverse Transcription Reagents (Applied Biosystems, Foster City, CA) following instructions prescribed by the manufacturer. Reaction mixture was prepared in 50 µl volume and incubated in the thermal cycler at 25 °C for 10 min, then at 48 °C for 30 min, and finally at 95 °C for 5 minutes. After completion of PCR reaction 50µl of nuclease free water was added in each reaction mixture to raise its total volume to 100µl for use in qRT-PCR.

Microarray

For this experiment, POP2 microarrays were used (Sterky *et al.*, 2004). Information about arrays, sequences, phytozome poplar gene model numbers, genbank numbers can be found at the *Populus* database www.populus.db.umu.se. The (Fig. 1) Array slide experimental design was organised to compare the biotroph and the necrotroph treatments with undamaged controls for each of the genotypes. The genotype specific constitutive level of expression was measured by comparison of the undamaged controls. For each treatment and control, two RNA samples from two different plants of each clone were prepared in equal amounts. Two biological replicates per clone and treatment were pooled to provide reliable transcript abundance in microarray experiments (for details see(Resman *et al.*, 2010).

Dye hybridization: Prior to hybridizations the RNA was amplified following the prescribed protocol of MessageAmp™ II aRNA Amplification by Ambion®.Later, the RNA was reverse transcribed into aminoallyl-labelled cDNA, using 20µg of total RNA primed with 5µg oligo-dT primer, and superscript II reverse transcriptase (Invitrogen, Carlsbad, CA, USA). Microcon 30 (Millipore, Bedford, MA, USA) was used to purify cDNA following elution in nuclease free water and dried in a Speed-vac centrifugal evaporator. Dried cDNA was re-suspended in 10µl of 0.1M NaHCO3 and was coupled with dyes Cy3 and Cy5 (Amersham Bioscience, Little Chalfont, UK) for two and a half hours at room temperature in dark. 100µl of Cy Dye labelled cDNA was purified by GFX spin column (illustra™ CyScribe™ GFX™ Purification Kit by GE Healthcare). Each sample was reduced to a volume of 40µl with speed-vac at 42°C for 30 minutes (time depending on when the 40 µl volume had been reached). Cy3 and Cy5 dyes labelled cDNA was mixed with hybridization buffer (45µl SSC 5X, 45 µl Formamide 25%, 4µl SDS 0.22%, 25µg tRNA and 10µg of Oligo-dA). Mix was

denatured (at 95°C for 3min), centrifuge (for less than 1 min) and applied to ASP chambers (TeleChem International, Sunnyvale, CA, USA) for at least 16 h at 42°C.

Microarray analysis: Slides were scanned 5 times with predefined increasing laser power and phototube multiplier (PMT) settings using a Scannary scanner (PerkinElmer AB, Sweden). The images were quantified and analysed in Genepix Pro 5.1 (Axon Instruments, CA, USA). On TIFF images weak spots were identified and marked manually. The extracted data was exported to UPSC-BASE, where it was subjected to quality control (Sjödin *et al.*, 2006) and other statistical measurements such as Restricted linear scaling to generate one data set from 4 scans from each wavelength (http://www.umu.se/climi/bact/Microarray/R-libraries.htm), step-wise normalization of spot intensities (Wilson DL., 2003). Raw data from GenPix were also used for principal components analysis. Significantly regulated genes were identified by comparing the transcripts abundance in treated samples with untouched controls. For constitutive regulation difference in two clones were done by comparing untouched samples with each other. A gene was considered significantly regulated when B-statistics was equal or greater than 5, and represented a 99.5% probability of differential expression. A high B-statistics indicated high probability of differential expression of a gene. All raw data were stored in the public microarray poplar database UPSC-BASE and are available with assigned experiment code EXP_0091.

qRT-PCR Analysis

Transcript expression levels were determined by qRT-PCR with SYBR-Green PCR Master kit (PerkineElmer Applied Biosystems) using the 7500 Real-Time PCR System from Applied Biosystems. Each qRT-PCR reaction was carried out in a 25 µl volume containing 250 nM of each primer, 2µl cDNA and 1 × SYBR®Green PCR master mix (Applied Biosystems, #4309155). The 7500 Real-Time PCR System (Applied Biosystems, #4351106) was used for quantification in a 96-well reaction plate using the parameters recommended by the manufacturer (2 min at 50°C , 10 min at 95°C and 40 cycles at 95°C for 15 sec and 60°C for 1 min). Two technical repeats were run for each gene product to confirm the accuracy of the PCR. We verified the specificity of the amplifications at the end of each PCR-run using the 7500-system SDS (dissociation curve analysis). Acquisition and analysis of data were done using the 7500-system SDS software for absolute quantification. All gene expression levels were normalized to the endogenous control *Ubq* (genbank acc. No BU879229; EF148144.1). Relative transcript levels of selected 12 genes were done by standard curve method according

to the User Bulletin 2 (ABI PRISM sequence detection system; PE-Applied Biosystems). Briefly, quantity of transcript of target gene was divided by the quantity of internal reference gene (*Ubq*). Later, a single value is obtained by taking mean of the biological replicates (3 biological repeats for each type of treatment and 2 biological repeats for controls). The primers used in qRT-PCR are listed in table s1.

Statistical analysis of qRT-PCR data

To normalize the data (quantitative qRT-PCR) values of the various genes were divided by the endogenous reference. However, even after standardization the variance tended to increase with the mean. Therefore the data were log transformed after division by the reference gene.

The experiment was designed as a factorial with double repeated measurements taken on the same subject i.e. plant. In this design clone, treatment, and gene were considered fixed factors, whereas plants within clone and treatment were considered a random factor. All interactions between fixed and random factors were also random. The Mixed procedure in the SAS™ system was used for all data analysis. The default REML method was used for estimation of the various covariance components. Planned comparisons of differences between least square means were done by use of the pdiff option.

Extraction of Total phenolics and Condensed tannins

Each leaf sample was ground in liquid nitrogen by hand; from this ca. 50mg (exactly measured) per sample was used to extract total phenolics (TPs) and condensed tannins (CTs). The Folin-Ciocalteu assay (Singleton & Rossi, 1965) was used to measure the concentration of total soluble phenolics. TPs were extracted with 1.8 ml of 80% MeOH in water with constant overtaxing at room temperature for half hour. The mix was centrifuged at 2500g for 10min. 10µl of sample was mixed in 475 µl of Folin-Ciocalteu (diluted 7 times) and incubated at room temperature for 5 min. Further, 475µl of a 7% sodium carbonate was added and kept at 40C for 15 min. Phenolics were detected using spectrophotometer at 724nm. Contents were standardized against cholorogenic acid. The standard curve was linear from 0 to 20µg/µl.

For analysis of CTs the method of (Ossipova *et al.*, 2001) was used. In short samples were suspended in 600µl of 70% aqueous acetone (1% ascorbic acid) and kept at room temperature for 1 hour. Later, the mix was centrifuged for 10 min at 2500g. The pellet was extracted twice with acetone and then dried by speed vacuum. This purified extract was used to determine the

soluble condensed tannins in the samples. In 1 ml of freshly prepared mixture of 1-butanol: hydrochloric acid (95:5), 50µl of populus leaf extract was added. After mixing it well, the mixture was placed in water bath at 95°C for 50min. Prior to absorbance measurement at 550nm; mixture was cool down at room temperature. The standard cure was constructed using epi-catechin (0-80µg/µl).

Results

Transcriptomes differ constitutively in healthy plants between clone 72 and 23

Of the 24,644 unique sequences spotted onto the POP2 microarray, the constitutive transcript level was significantly different for 552 sequences (2.2%) between the two clones. Among these transcripts, 250 were expressed higher in clone 72 and 302 were expressed at a lower level when compared to clone 23. Principal component analysis (PCA) using the complete microarray data also showed that the two clones are clearly separated in their healthy state (Fig. 2). The top 20 highest and lowest constitutively differentially expressed transcripts are shown in tables 1 and 2. The complete list is available in supplemental tables s1, s2. Despite the genotypic and phenotypic differences between the two clones 24111 of the transcripts were expressed at similar levels in both clones.

Transcriptome differ in response to biotroph and necrotroph treatment

The transcriptional level in leaves 24hai by the biotrophic and necrotrophic fungi were compared to the respective levels in healthy control plants. The systemic responses to the biotroph and necrotroph fungi were almost absent in clone 23 i.e. only seven transcripts were significantly differentially regulated (4 up- and 3 down-regulated) after necrotroph treatment and no response above threshold to the biotroph (Table 3). On the other hand, clone 72 that was also more expressive in the uninfected stage responded strongly in leaves to both pathogens. The arrays identified 165 transcripts that were significantly differentially expressed (see description in method) two fold or more between the control and the biotroph infection. Among these 101 transcripts (61%) were up-regulated and 64 (39%) were down-regulated (Tables 4, 5). In the response to the necrotroph 118 genes were differentially expressed; 37 (31%) were up-regulated and 81 (69%) were down regulated (Tables 6, 7). In total 283 transcripts were differentially regulated after pathogens infection but only 46 transcripts were shared in response to both pathogens (16%; 9 up- and 37 down-regulated) in clone 72 (Fig. 3). The PCA also depicts that with the healthy control the two fungi seemed to

induce different gene sets (Fig. 2). There are 69 transcripts up-regulated ≥ 4 fold after biotroph and 24 after necrotroph inoculation in clone 72. However, there are 38 transcripts that are down-regulated ≤4 fold after biotroph and 62 after necrotroph inoculations in clone 72.

Quantification of selected candidate transcripts by qRT-PCR

As both clones differed in their constitutive transcript levels and in their systemic defence response, qRT-PCR was used to get more precise information on selected transcript candidates. We used the microarray results to perform the selection of candidate genes. Because we did not find any common significant transcript behaviour for the two clones 23 and 72 in any comparison, the first criteria of selection were transcripts in clone 72 that were constitutively more expressed in healthy plants than in infected plants [included: *Senescence-associated protein (Senesc), ChitinaseI (ChitiI), Cinnamoyl-CoA reductase (CCR), GH3 auxin responsive promoter (GH3), Methyl-CpG-binding domain (Methyl-CpG), Wrky-like family transcription factor (Wrky)*]. The second criteria of selection were transcripts that expressed a higher level in response to treatment either biotroph, necrotroph or both in clone 72 [included: *Early responsive to dehydration stress (ERD4), Glutaredoxin family protein (Glutaredoxin), Mitochondrial substrate carrier (Mitochondria-Cp), O-methyltransferase (Omt), Myb-like DNA-binding domain (Myb1, 2)*].

The qRT-PCR analysis suggested that clone 72 expressed the selected transcripts at a higher level under all treatments than clone 23 ($P=0.03$), especially in response to biotroph infection and in response to wounding (Fig 4.). No statistically significant increases was found between treatments in clone 23 ($P>0.6$). Interestingly the necrotroph treatment only revealed no statistical difference between clone 23 and 72 ($P<0.5$), but 72 did express a highly elevated response to wounding compared to clone 23. The systemic response to wounding in clone 72 was significantly greater than necrotroph treatment ($P=0.02$). The wounding treatment was not included in the microarray but otherwise the qRT-PCR supported the microarray results. The qRT-PCR analysis suggests that in clone 72 the expression was significantly differentially expressed for *ChitiI, Omt, Wrky* ($P<8.8E-05$) and *CCR* ($P<0.05$) compared to clone 23, all treatments considered. *(* (Fig.3). Constitutively, the transcripts level of *ChitiI* and *Omt* was 118 times or more higher in C72 than in C23. The differentially expressed transcripts *ChitiI, GH3, Wrky, Methyl-CpG, CCR, Senesc* and *Omt* between the two clones

from the microarray analyses showed the same trend except for *GH3* that only showed significant change due to wounding over necrotroph in clone 72 ($P<0.04$).

Clone 72 showed strong systemic response in leaves as a host response to wounding of the stem. The transcripts *ChitiI, CCR, GH3* ($P<0.05$) *Omt*, *Wrky* and *Methyl-CpG* ($P<0.0002$) were at significantly higher level after wounding than to necrotroph in clone 72.

In sum the qRT-PCR data for biotroph and necrotroph inoculations follows similar trend to that obtained in microarray analysis for clone 23 (i.e. no statistical significant changes due to treatments) while for clone 72 the trends were similar for most transcripts with the exception of *Myb1* where qRT-PCR data was clearly not in agreement for the response to the necrotroph from the array.

Chemical analysis of healthy plants

The two clones differ in their chemical contents at the constitutive levels in healthy plants and there was a systemic difference in the leaves after wounding in both clones. There was also an indication of increased levels of TPs and CTs after the necrotroph, particularly in clone 72 (Fig.s3). There was constitutive difference in healthy plants of the two clones for the CTs but no change after the treatments with the exception of its reduced levels in clone 72 after wounding (Fig.s3).

Susceptibility of clones

We knew from previous studies that clone 72 from the SwAsp collection is quite tannin rich when grown under out-door conditions and that it is also characterised by vigorous growth (Albrectsen *et al.*, 2010). Six weeks after inoculation controls of clone 72 were also taller (107.67±3.34 cm, mean±s.e. throughout) than those of clone 23 (89.67±5.20 cm; $t_{10} = 2.91$ $p = 0.015$). Clone 23 is characterised by a higher complexity of its phenolic glycoside composition (it produces the compound 2-cinnamoyl-salicortin), and although clone 23 was more bushy in appearance it did not significantly grow more branches than clone 72 (controls of 23 grew 8.33±1.33 side branches; controls of 72 grew 6.17±1.14; $t_{10} = -1.24$ $p = 0.25$); but leaves developed significantly lower on the stems of clone 23 (23.5±0.62 for position of leaf 10; clone 72: 27.00±1.34; $t_{10} = 2.36$ $p = 0.034$). The number of replicated plants was three per infection treatment, providing a limited power for quantitative evaluation of clone susceptibility and resistance. 14 days after inoculation with *M. magnusiana* a significant difference in susceptibility was found between the clones with clone72 having 1.6±0.45

lesions compared to clone 23 with 3.5±0.38 (t_4= -3.17; p = 0.034). We expected the slower growing clone 23 to be more susceptible to *Melampsora* (Albrectsen *et al.*, 2010), although this expectation is based on studies of mainly *Melampsora pinitorqua* Rostrup infections in all of Sweden, whereas the present experiment used *M. magnusiana* inoculates. No difference was found in lesions following infection by the necrotrophic fungus. Bark lesions were identical on all plants covering exactly the wound = 1 cm. Wood lesions were also identical between the clones (4.2±0.76 cm (clone 23) and 4.3±.83 (clone 72); t_4 = 0.11 p = 0.91.

However, wounding and necrotroph infection affected the clones differently. Whereas growth tended to increase in clone 72 in response to wounding it tended to decrease in clone 23 (Fig. s4) and necrotroph infected reverted this tendency thus suggesting different costs and responses in the two clones.

Discussion

Defence responses in plants are complex and depend on genotype and the level and timing of the expression of relevant genes. The sequenced genome of poplar (Tuskan *et al.*, 2006) offers a key to understand molecular defence responses to biotic and abiotic stresses. The present study evaluated gene activity in relaxed healthy plants (controls) and in plants infected by two fungi of different life style as well as wounded plants. The results suggest that there may be a considerable intraspecific variation in base line gene activity in *aspen*. We tested two genotypes with different properties related to growth, chemical composition and expected resistance properties and found both that the genotypes behaved differently, both in the relaxed state and that they expressed diverse responses to the treatments. That both genotype and environment determines plant responses have been shown in other studies (Osier & Lindroth, 2006) but the difference in base line gene activity that characterise the present experimental clones further suggested that a high base line gene activity indicate an ability of compensatory growth in response to wounding, which may be entirely re-allocated to combat an infection. Thus high gene activity, high growth and elevated defence properties appeared to support each other in this study exemplified in clone 72, whereas clone 23 was much less active, displayed more symptoms of *Melampsora* infection but was also generally combating the infections by continuing its slow gene activity. However not only did the number of active genes differ between the clones 24hai. Some key genes were also differently expressed in the two clones and the genes differed between the biotroph and the necrotroph infection. Interestingly, wounding gave greater systemic increase in the response level of the candidate

genes as compared with the necrotroph infection suggesting that this pathogen has some ability to suppress (Hammond-Kosack & Rudd, 2008) the induction or translocation of the systemic host defence.

Constitutive differences between clones

Previous studies were focused on the induced defence (Ralph *et al.*, 2006; Rinaldi *et al.*, 2007) to biotic and abiotic stresses in poplar and less attention has been given to constitutive or relaxed expressions in plants and studied its association with fitness and resistance. In this work the chemical differences at constitutive and induced levels were studied. The concentration of TPs differ constitutively in different clones of aspen, (Diner *et al.*, 2009) examined 252 trees and found variation in chemicals like TPs and CTs ranging as much as 283% and 392% respectively across clones. Similarly (Lindroth *et al.*, 2007) described variation in foliar concentrations of PGs across 31 clones. Phenolic levels change according to season and ontogeny (Donaldson *et al.*, 2006; Diner *et al.*, 2009). In this study we used 1 year old aspen clones that had been grown under greenhouse conditions (an environment that reduce levels of several phenolics including CTs) and we confirmed that clone72 (Fig.s3) had a higher CT profile compared to clone23. From out-door studies of the same clones we know that the capacity of producing tannins is much enhanced in clone 72, suggesting a potentially more plastic behaviour in phenolic expression. Clone 23 is richer in salicinoid phenolic glycosides, which are much more stable across environments (Abreu *et al*. in press). The plant chemistry is also determined by its genotype (Lindroth & Hwang, 1996) and this was evident from our microarray experiments, in which 552 genes were found to express differently in relaxed healthy plants between the two clones.

Induced differences after biotroph treatment

The transcriptome response to rust infection has been studied by several groups and on the basis of these studies we expected specific gene expressions (Miranda *et al.*, 2007; Rinaldi *et al.*, 2007; Azaiez *et al.*, 2009). These studies covered events of infection from 12 hours to 10days into the infection. Azaiez and colleagues (Azaiez *et al.*, 2009) used two different *Melampsora* species (*M. larici-populina* and *M. medusae f. sp. deltoidae*) on hybrid poplar (*Populus nigra* x *P. maximowiczii*) and found that the two lines initiated differential local transcriptional response in host leaf tissues. (Rinaldi *et al.*, 2007) studied the local differential defence response in hybrid poplar to compatible and incompatible isolates of *Melampsora*.

We describe here for the first time the systemic response in leaf tissues 24 hours after *M. magnusiana* infection in two aspen clones with different constitutive gene expressions and different growth and potential of activating the tannin part of the pehenylpropanoid pathway. We identified 165 (0.7%) poplar transcripts in clone 72 while no transcripts differentially regulated to biotroph above threshold in clone 23. Fewer genes differentially expressed in susceptible clone 23 may be because the biotroph was able to suppress the defence mechanisms of the host as has been proposed in a previous study (Mendgen & Hahn, 2002), or due to lack or slow systemic signalling in this clone. These systemic results are to some extent similar to the local responses previously reported (Miranda *et al.*, 2007) where 0.6% of transcripts were regulated in local defence responses at 24hai (Azaiez *et al.*, 2009) found 276 (0.5%) transcripts after virulent *Melampsora* spp. and 364 (0.7%) after partially virulent *Melampsora* spp at 6 days post inoculation (dpi). In Poplar 2.6% transcript level changed observed in incompatible and 0.4% in compatible *M. larici-populina* infections (Rinaldi *et al.*, 2007). In a study conducted by (Zhang *et al.*, 2007) (15% from 2952) transcripts were deregulated in *Populus deltiodes* on *Marssonina brunnea f. sp. multigermtubi* infection within 2 days.

The higher level of resistance or partial incompatibility of *Melampsora magnusiana* with clone 72 resulted in the greater perturbance in transcriptional change in this study compared to the more heavily colonized clone23. There is some resemblance in the differential expression of the transcripts in present work and the recent studies conducted on rust-poplar pathosystem (Miranda *et al.*, 2007; Rinaldi *et al.*, 2007; Azaiez *et al.*, 2009) where high number of transcripts levels was reported in incompatible infections, but in our case the changes are systemic and thus likely reflect the efficiency of systemic signalling. The minor differences in the transcriptional changes among studies might partly be associated with quantity of inoculum used in each study, for example (Rinaldi *et al.*, 2007) and associates used 100000 urideniospores on three leaves, (Azaiez *et al.*, 2009) sprayed 2000 urediospores/cm^2 similarly (Miranda *et al.*, 2007) also used high number of spores. As we were looking at the systemic defence responses it could be that the lower transcripts level changes detected is directly linked to the strength and speed of the systemic signalling in the plants, as former studies have centred on the more easily detectable local defence responses in the leaves. Formerly it has been suggested that the systemic responses are weaker and narrower version of the local response to infection (Babst *et al.*, 2009). It is also known that the level of transcriptional change varies according to the time after inoculation. In rust-poplar

interactions it has been studied at 3dpi (Zhang *et al.*, 2007) 6 to 9 dpi (Miranda *et al.*, 2007) and 2dpi (Rinaldi *et al.*, 2007). Intact leaves were then detached from plant after 24hours of infection in our case and this intact *in planta* approach can be part of the reason for difference seen compared to previous studies where leafs were removed and then inoculated later in the form of leaf discs (Rinaldi *et al.*, 2007; Azaiez *et al.*, 2009). We do have results more similar to the (Miranda *et al.*, 2007) as they also performed inoculations on intact poplar leaves. In the more susceptible clone 23, differential expression of transcripts was negligible on the array and same trend was observed in qRT-PCR data of selected genes (Fig.4). It indicates the possibility of suppression of host defences in clone23 by the pathogens, however wounding alone did not give elevated levels in the candidate genes either, strongly suggesting a lack of or delay in the recognition of stress or systemic signalling in this clone. In contrast, clone 72 showed rapid and efficient systemic defence signalling reflected in the induction of defence related genes systemically in above leaves (Fig. 4).

The *Wrky* examined by array and qRT-PCR showed significantly ($P<0.003$) higher induction in response to biotroph and wounding but not to necrotroph in the responsive clone 72 that may relate to its participation in defence after biotroph and wounding treatment. *Wrky* genes are is known to be linked to the induction of altered peroxidase activity and lignin deposition in poplar after *Melampsora* infection important for an efficient resistance response (Levée *et al.*, 2009).

Induced differences after necrotroph treatment

An efficient plant response to a necrotroph is likely essentially differ from the defence response to biotroph as a hypersensitive response and host cell death may in fact aid in the invasion of a necrotroph. Therefore it is speculated that plants adopt different mechanisms of defence depending on the type of attacking pathogen. Different sets of genes are known to be expressed on bio-and necrotroph infection in poplar (Morse *et al.*, 2004). The more biotroph susceptible clone 23 showed only minor differentially expressed transcripts; however in clone 72 transcriptome changes were greater (118 transcripts change after necrotroph). Comparing 7 versus 118 differentially expressed genes in clone 23 and 72 respectively suggests that former is either more susceptible or that the systemic host defence response is simply is not induced by the necrotroph in clone 23. Data from average lesion length in the woody stem of the two clones indicating no difference in susceptibility/resistance between the two clones to the necrotroph (and even the difference in resistance to the biotroph is modest in this study).

These differences at the transcript level and morphological data concerning the necrotroph may reflect that in clone 72 the defence is partly suppressed or that the defence response is not sufficient to give any protection toward the necrotroph above that seen in clone 23. Clone 23 on the other hand seem to have no systemic response to the necrotroph and again no response to wounding indicating a general delay or lack in systemic signalling. We propose that the necrotroph is able to overcome the host defence in the woody stem of both clones able to restrict or avoid the response in clone 72, at least at 24hai. However, both clones recover completely from wounding alone indicating that the local machinery to repair wounds is fully functional in both clones.

Host chitinases are known to be express spatiotemporally at high levels in necrotroph resistant plants (Hietala *et al.*, 2004). The level of chitinase (IV) transcripts increased more rapid systemically indicating a more efficient recognition and systemic defence signalling. The aspen *ChitiI* studied by qRT-PCR was highly expressed both constitutively in leaves and stayed high after necrotroph infection and wounding, while all the other candidates appeared suppressed as compared to wounding (and biotroph as well) suggesting that the pathogen is fully able to suppressed the signalling pathway(s) inducing them. The suppression of signalling was also observed in a necrotroph *Mycosphaerella graminicola* and *Triricum aestivum* interactions where host R protein and *Mitogen-activated Protein Kinase* cascade were targeted by necrotroph (Hammond-Kosack & Rudd, 2008). We speculate that the necrotroph is producing effectors negatively affect key players in the systemic defence signalling machinery of the host.

Wounding treatment

Previous studies indicate that aspen and other *Populus* plants respond to the pathogen or insect and wounding treatments differently. It has been shown that wound induced genes can also be regulated on heat and osmotic stress shocks (Reymond *et al.*, 2004). We found the highest levels of transcripts compared to pathogen treatments in the systemic leaves at 24 hours post inoculation. Interestingly, the higher induced transcription levels were found at 24hai in previous studies on hybrid poplars (Constabel *et al.*, 2000; Christopher *et al.*, 2004; Philippe & Bohlmann, 2007; Ma *et al.*, 2011). However, the severity of the wounding and the age of the plants may differ between studies making it difficult to directly compare studies. Most of the studies in poplar were done by damaging leaves. Treatments include leaf margin crushing, light abrasion, sever crushing of leaf lamina and cutting with scissors and suggested

have significant effects on gene expression especially when it is repeated (Major & Constabel, 2008). We wounding by cutting into the bark (see materials and methods; Fig. s2) and at three locations along the stem. It is tempting to suggest that such a sever wound treatment might be the reason for the high systemic transcripts changes seen in clone 72 as compared to the all treatments and control. On the other hand no such systemic response in the leaves was seen in similarly bark wounded clone 23. The trends in transcripts levels in systemic leaves after *Melampsora* and wounding treatment are similar for the qRT-PCR selected transcripts. *Omt* transcript induction was most evident ($P<0.002$) to wounding (and to biotroph) along with *Wrky* ($P<0.003$) being more than 10 fold. *Omt* has a role in defence as it is acting on monomers that are used for lignin biosynthesis that might be important in healing wounds and strengthening cell walls. This gene belongs to group1 of class I in *Omt* family that have been suggested to be preferentially expressed in leaf tissues after wounding and herbivory (Barakat *et al.*, 2011). Blast analysis revealed a close resemblance with genes related to simple phenol production after biotic and abiotic stresses (Barakat *et al.*, 2011) also in our chemical analysis we see that plants tend to produce higher amount of phenols after the wound treatment.

Conclusion

Defence to pathogens attack is complex and likely consist of inter regulatory mechanisms and signalling pathways in host that can antagonize or enhance each other (Grant & Jones, 2009; Pieterse *et al.*, 2009). Few of the differentially expressed genes in clone 72 were common between the responses to the two very different pathogens applied, and indicate that cross talk or sharing of signal transduction pathways is not easy observable in our material beyond the similarity in response between wounding and biotroph treatments. This lack of similarity in induction between the two pathogens may be partly be due to the efficient repression mechanism or manipulation of the defence signalling in the host employed by the necrotroph that is not seen in response to the biotroph.

Acknowledgments

We thank Inger Heldal and Mari Kjos for their help in the lab and Harald Kvaalen for conducting statistical analysis on the data. Carl Tryggers Foundation. Professor Lars Ericson kindly provided spores of *Melampsora magnusiana*. This project was funded by Norwegian Research Council via grant NFR 178576.

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Figure legends

Figure 1. Design of microarray experiment. In each treatment, RNA of the two biological repeats was pooled. Fourteen microarray slides were used to compare the relative transcriptional levels for treatments with relevant control and between controls of two clones. B from plants inoculated with the biotroph, *Melampsora megnosiana*, N from plants inoculated with the necrotroph, *Ceratocystis* sp. and C are healthy controls.

Figure 2. Principal component analysis for clones 23 and 72 at 24 hours after inoculation. Every point shows the average of 2 biological replicates), healthy control plants (C) infected with biotroph (B) or infected with necrotroph (N).

Figure.3. Venn diagram showing numbers of unique and overlaping changes in gene expression. In each circle, the number above the line indicates the number of upregulated genes, and the number below the line indicates the number of downregulated genes. Host response to biotroph (B) and necrotroph (N).

Figure 4. qRT-PCR of transcripts *Senesc, ChitiI, CCR, GH3, Methyl-CpG, Wrky, Myb1, 2, ERD4, monothiol, mitochondria-Cp*, and *Omt* in aspen clones 23 and 72 in response to the biotroph (B), necrotroph (N) and wounding (W) 24 hours after inoculation. The untouched controls of respective clones are also shown (C). Data was normalized to internal reference gene *Ubq* by dividing quantity of target gene by quantity of internal refereance gene. n=3 for treatments and n=2 for controls; bars±SE

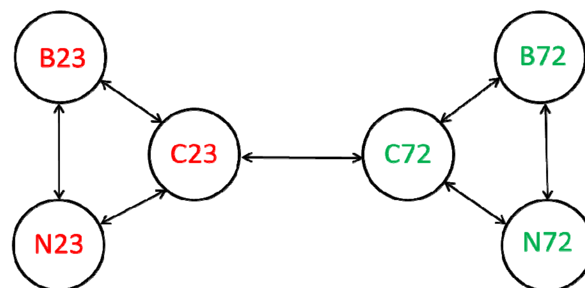

Figure 1.

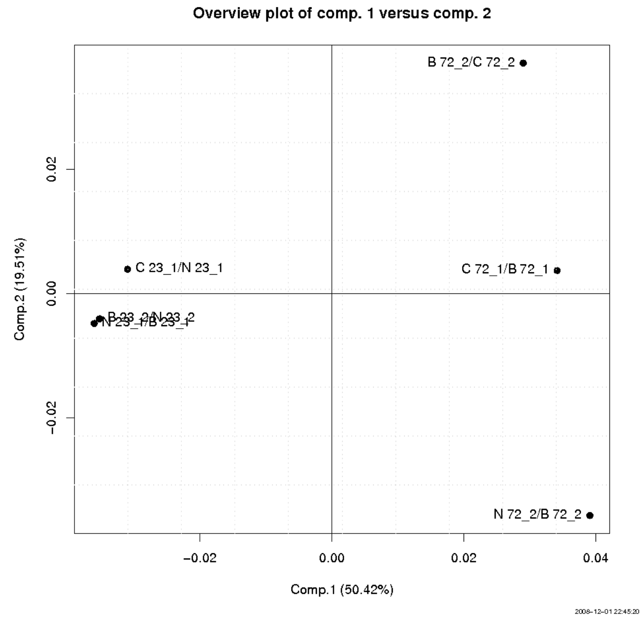

Figure 2.

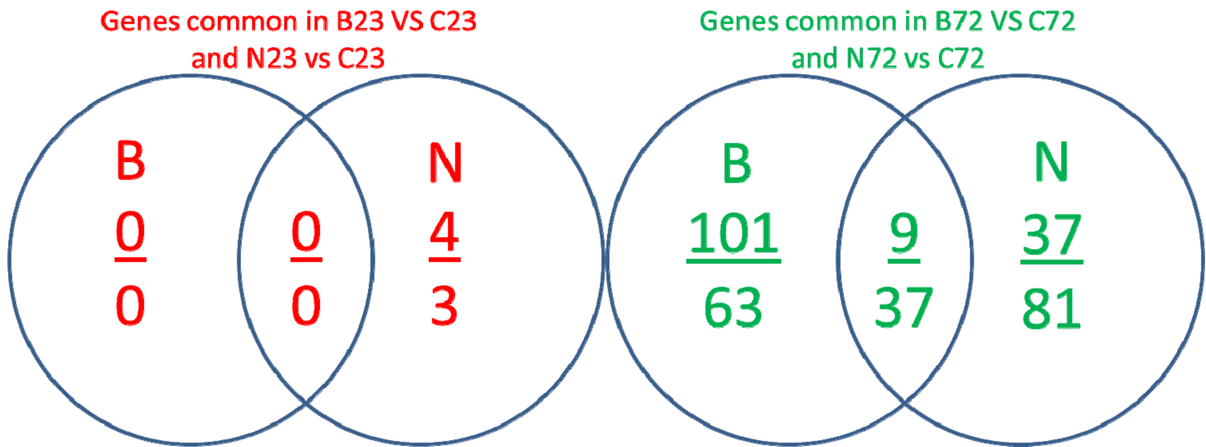

Figure 3.

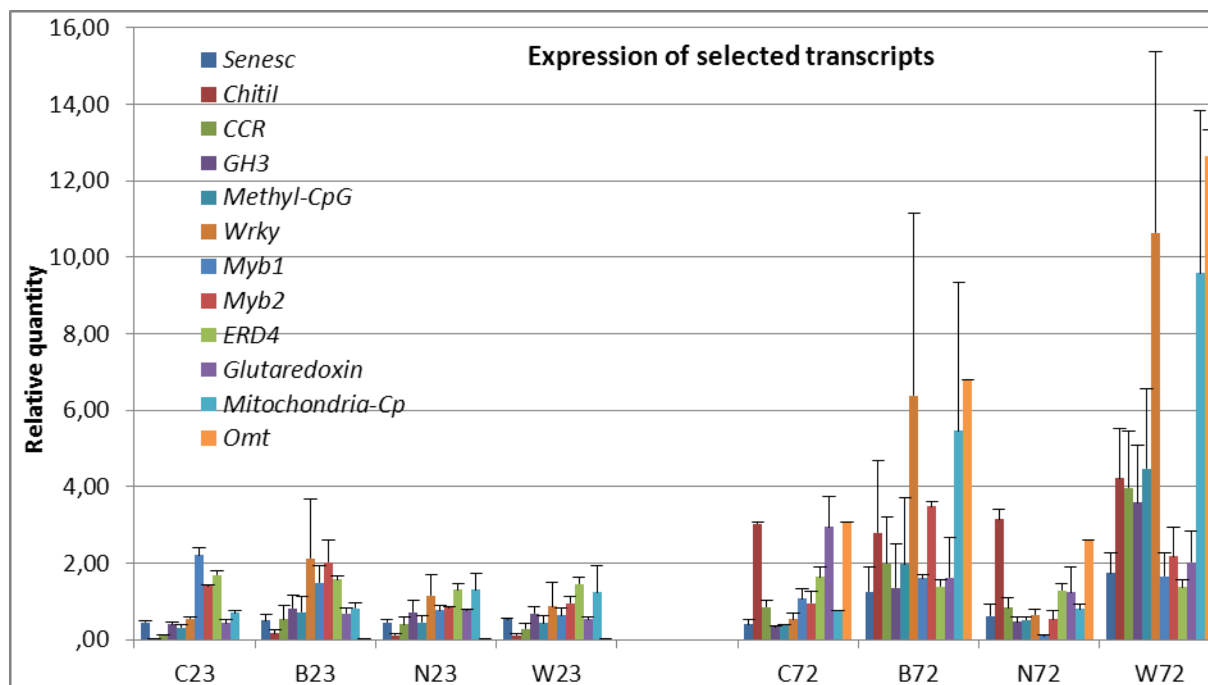

Figure 4.

Table1. Top 20 most constitutively higher transcripts in leaves of healthy plants of clone 72 relative to clone 23 in the microarray analysis. PU_ID numbers represent gene identifiers in *Populus* database (http://www.populus.db.umu.se/). M = $\log_2$ R/G. An absolute M-value of 1 corresponds to a 2-fold change in expression, since we are using logs to the base 2. On this scale, M = 0 represents equal expression, M = 2 represents a 4-fold change, and so on. R = Red channel (Cy3), G = Green channel (Cy5).

reporterId	reference	annotation	M	A	*T test*	P,Value	*B* stat
PU11019	At4g21390	S-locus lectin protein kinase family protein	4,16	11,50	11,02	1,30E-04	7,04
PU11280	At1g74170	leucine-rich repeat family protein	4,06	13,38	13,14	2,04E-06	12,45
PU23259	At4g03030	kelch repeat -containing F-box family protein	4,02	9,58	11,08	7,62E-04	4,75
PU12269	At2g45420	LOB domain protein 18	3,92	9,40	9,32	2,71E-04	6,23
PU30445	At4g35150	O-methyltransferase family 2 protein	3,70	11,84	9,31	9,09E-05	8,13
PU04363	At4g04955	amidohydrolase family protein	3,53	10,79	17,50	2,10E-07	14,88
PU27442	At3g52870	calmodulin-binding family protein	3,14	12,69	7,90	3,24E-04	6,11
PU02101		No Hit	3,14	11,39	8,64	1,63E-04	7,19
PU03656	At3g54420	class IV chitinase (CHIV)	3,03	13,56	9,73	5,71E-05	8,68
PU13423	At4g18960	floral homeotic protein AGAMOUS (AG)	3,02	10,98	9,70	1,30E-04	7,18
PU01657	At3g07040	disease resistance protein RPM1	2,98	10,63	8,10	2,71E-04	6,41
PU11156	At5g40990	GDSL-motif lipase/hydrolase family protein	2,93	9,70	5,53	1,32E-02	0,98
PU02560	At4g35160	O-methyltransferase family 2 protein	2,91	13,33	4,54	1,80E-02	0,15
PU05953	At1g19670	coronatine-responsive protein	2,82	10,97	6,58	1,30E-03	3,96
PU26670	At2g33590	cinnamoyl-CoA reductase family	2,73	11,15	12,15	4,24E-06	11,47
PU10916	At1g28560	snRNA activating complex family protein	2,69	11,59	5,37	8,51E-03	1,46
PU00467	At4g27390	expressed protein hypothetical protein	2,66	11,63	7,46	1,32E-02	1,17
PU12629	At2g14960	auxin-responsive GH3 family protein	2,39	14,00	8,48	1,89E-04	6,96

Table2. Top 20 most constitutively lower transcripts in leaves of healthy plants of clone 72 relative to clone 23 in the microarray analysis. PU_ID numbers represent gene identifiers in *Populus* database (http://www.populus.db.umu.se/). M = $\log_2$ R/G. An absolute M-value of 1 corresponds to a 2-fold change in expression, since we are using logs to the base 2. On this scale, M = 0 represents equal expression, M = 2 represents a 4-fold change, and so on. R = Red channel (Cy3), G = Green channel (Cy5).

reporterId	reference	annotation	M	A	T test	P,Value	B stat
PU01033	At3g13230	expressed protein	-6,39	12,46	-23,15	5,51E-09	18,89
PU08363	At5g60900	lectin protein kinase family protein	-5,79	12,43	-23,04	1,14E-08	17,60
PU00011	At4g37110	expressed protein	-5,35	12,28	-22,79	1,30E-07	14,94
PU29395	At1g72470	exocyst subunit EXO70 family protein	-4,30	15,59	-14,26	8,69E-07	13,46
PU29409		No Hit	-4,21	15,92	-13,90	1,11E-06	13,15
PU04441	At5g59810	subtilase family protein subtilisin-like protease AIR3	-4,18	12,02	-10,78	9,12E-05	7,55
PU30583		No Hit	-4,11	11,81	-14,59	7,14E-07	13,74
PU21779		No Hit	-4,00	15,68	-12,85	2,19E-06	12,18
PU21744	At3g23020	pentatricopeptide (PPR) repeat-containing protein	-3,96	17,13	-12,90	2,19E-06	12,23
PU29246		No Hit	-3,91	14,68	-15,33	4,08E-07	14,34
PU10957	At5g58070	lipocalin, putative similar to temperature stress-induced	-3,88	16,71	-13,24	2,01E-06	12,55
PU04028	At1g50400	porin family protein	-3,80	10,15	-11,04	4,39E-05	8,44
PU29816	At1g78520	glycosyl hydrolase family protein 17	-3,74	18,03	-9,25	9,10E-05	8,05
PU30489	At4g14730	transmembrane protein	-3,71	17,81	-13,06	2,04E-06	12,38
PU21867	At3g09735	DNA-binding S1FA family protein	-3,63	12,06	-17,04	1,30E-07	15,59
PU21539	At1g45201	lipase class 3 family protein	-3,55	13,68	-9,59	6,59E-05	8,51
PU02535	At1g04040	acid phosphatase class B family protein	-3,52	9,86	-7,16	3,03E-03	2,98

Table3. Systemic up and down regulation of transcripts in microarray analysis in leaves of clone 23 in response to the necrotroph inoculation at 24 hours after inoculation. PU_ID numbers represent gene identifiers in *Populus* database (http://www.populus.db.umu.se/). M = $\log_2$ R/G. An absolute M-value of 1 corresponds to a 2-fold change in expression, since we are using logs to the base 2. On this scale, M = 0 represents equal expression, M = 2 represents a 4-fold change, and so on. R = Red channel (Cy3), G = Green channel (Cy5).

reporterId	reference	annotation	M	A	T test	P,Value	B stat
PU12604	At1g08170	histone H2B family protein	1,66	11,63	-5,39	3,42E-01	0,26
PU07373	At4g03210	xyloglucan:xyloglucosyl transferase	1,40	11,22	-6,38	1,24E-01	1,43
PU20211	At1g64660	Met metabolism pyridoxal-phosphate-dependent enzyme	1,21	10,59	-5,68	2,79E-01	0,55
PU10330	At3g57270	glycosyl hydrolase family 17 protein	0,99	12,25	-5,21	3,42E-01	0,42
PU24096	At4g24150	expressed protein ; expression supported by MPSS	-0,83	11,00	5,10	3,72E-01	0,13
PU06679	At4g32940	vacuolar processing enzyme	-0,97	14,26	5,70	2,37E-01	0,97
PU20978	At1g31320	LOB domain protein 4	-1,33	10,25	5,70	2,46E-01	0,78

Table4. Top 20 most highly systemically up regulated transcripts in microarray analysis in leaves of clone 72 in response to the biotroph inoculation at 24 hours after inoculation. PU_ID numbers represent gene identifiers in *Populus* database (http://www.populus.db.umu.se/). M = log2 R/G. An absolute M-value of 1 corresponds to a 2-fold change in expression, since we are using logs to the base 2. On this scale, M = 0 represents equal expression, M = 2 represents a 4-fold change, and so on. R = Red channel (Cy3), G = Green channel (Cy5).

reporterId	reference	annotation	M	A	T test	P,Value	B stat
PU05442	At1g75250	myb family transcription factor	2,57	11,44	5,83	2,10E-02	2,39
PU00202	At3g19130	RNA-binding protein	2,28	8,98	5,76	4,80E-02	0,67
PU30449	At3g02040	glycerophosphoryl diester phosphodiesterase	1,90	11,41	6,64	1,05E-02	3,96
PU09734	At1g22160	senescence-associated protein	1,66	11,20	4,36	8,01E-02	0,00
PU28190	At2g47500	kinesin motor protein-related	1,52	15,85	7,81	7,41E-03	5,67
PU28105	At2g19580	senescence-associated protein	1,52	15,55	7,13	7,83E-03	4,70
PU20116	At2g31945	expressed protein	1,51	12,98	6,07	1,41E-02	3,05
PU28637	At1g16390	organic cation transporter	1,49	14,13	7,49	7,41E-03	5,23
PU22674	At3g52300	ATP synthase D chain	1,48	15,93	7,19	7,83E-03	4,79
PU26573	At3g28990	hypothetical protein	1,46	15,92	5,30	3,51E-02	1,72
PU22639	At1g60490	phosphatidylinositol 3-kinase (PI3K)	1,44	14,28	4,98	4,77E-02	1,16
PU22505	At4g18670	leucine-rich repeat family protein	1,42	14,43	6,06	1,41E-02	3,02
PU22579	At5g50790	nodulin MtN3 family protein	1,41	16,15	6,44	1,10E-02	3,65
PU29089	At1g73260	trypsin and protease inhibitor family protein	1,40	8,95	6,40	2,24E-02	2,34
PU29747	At3g46030	histone H2B	1,38	12,71	4,48	7,15E-02	0,23
PU28892	At1g73180	eukaryotic translation initiation factor	1,37	16,25	6,99	8,36E-03	4,50
PU22335	At5g28650	WRKY family transcription factor	1,35	13,35	6,30	1,12E-02	3,42
PU28007	At5g47060	senescence-associated protein	1,10	11,03	5,15	4,00E-02	1,47

Table5. Top 20 most highly systemically down regulated transcripts in microarray analysis in leaves of clone 72 in response to the biotroph inoculation at 24 hours after inoculation. PU_ID numbers represent gene identifiers in *Populus* database (http://www.populus.db.umu.se/). M = log2 R/G. An absolute M-value of 1 corresponds to a 2-fold change in expression, since we are using logs to the base 2. On this scale, M = 0 represents equal expression, M = 2 represents a 4-fold change, and so on. R = Red channel (Cy3), G = Green channel (Cy5).

reporterId	reference	annotation	M	A	T test	P,Value	B stat
PU03284	At4g24340	phosphorylase family protein	-4,17	12,99	-5,17	3,93E-02	1,49
PU03743	At4g24340	phosphorylase family protein	-4,11	12,18	-5,08	4,34E-02	1,33
PU03824	At4g24340	phosphorylase family protein	-4,10	14,32	-4,86	5,47E-02	0,92
PU25155	At4g24340	phosphorylase family protein	-4,03	14,48	-4,59	6,51E-02	0,44
PU25134	At4g24340	phosphorylase family protein	-3,97	14,94	-4,70	6,05E-02	0,64
PU03548	At4g24340	phosphorylase family protein	-3,95	13,79	-4,72	6,05E-02	0,68
PU25144	At4g24340	phosphorylase family protein	-3,89	15,10	-4,44	7,43E-02	0,15
PU25143	At4g24340	phosphorylase family protein	-3,88	13,05	-4,52	6,85E-02	0,30
PU25167	At4g24340	phosphorylase family protein	-3,88	14,40	-4,49	7,12E-02	0,25
PU03820	At4g24340	phosphorylase family protein	-3,87	14,75	-4,78	5,65E-02	0,78
PU25136	At4g24340	phosphorylase family protein	-3,82	14,88	-4,59	6,51E-02	0,43
PU03510	At4g24340	phosphorylase family protein	-3,82	14,14	-4,64	6,16E-02	0,53
PU03394	At4g24340	phosphorylase family protein	-3,82	12,88	-4,51	6,90E-02	0,29
PU03139	At4g24340	phosphorylase family protein	-3,80	12,76	-4,49	7,15E-02	0,24
PU03784	At4g24340	phosphorylase family protein	-3,57	14,25	-4,41	7,43E-02	0,10
PU21994	At3g62930	glutaredoxin family protein	-2,42	13,17	-4,58	6,51E-02	0,42
PU03656	At3g54420	class IV chitinase (CHIV)	-1,40	13,56	-5,50	2,63E-02	2,08
PU03202	At1g30360	early-responsive to dehydration stress protein (ERD4)	-0,95	14,57	-5,71	2,06E-02	2,43

Table6. Top 20 most highly systemically up regulated transcripts in microarray analysis in leaves of clone 72 in response to the necrotroph inoculation at 24hours after inoculation. PU_ID numbers represent gene identifiers in *Populus* database (http://www.populus.db.umu.se/). M = log2 R/G. An absolute M-value of 1 corresponds to a 2-fold change in expression, since we are using logs to the base 2. On this scale, M = 0 represents equal expression, M = 2 represents a 4-fold change, and so on. R = Red channel (Cy3), G = Green channel (Cy5).

reporterId	reference	annotation	M	A	T test	P,Value	B stat
PU23259	At4g03030	kelch repeat-containing F-box	4,02	9,58	11,08	2,86E-02	2,40
PU00467	At4g27390	expressed protein	2,66	11,63	7,46	7,78E-02	0,05
PU26084	At2g21650	myb family transcription factor	2,19	12,48	5,51	4,75E-02	2,08
PU03729	At5g10140	MADS-box protein flowering locus F	1,96	11,69	5,66	4,59E-02	2,35
PU00529	At4g35830	aconitate hydratase	1,69	13,83	6,26	3,70E-02	3,33
PU01150	At5g51050	mitochondrial substrate carrier family protein	1,32	9,68	4,66	1,03E-01	0,13
PU26390	At1g75030	pathogenesis-related thaumatin	1,31	10,38	5,04	6,03E-02	1,26
PU20342	At3g09390	metallothionein protein	1,28	14,07	4,66	8,28E-02	0,57
PU21132	At5g18650	zinc finger (C3HC4-type RING finger)	1,25	10,61	4,45	1,03E-01	0,06
PU23434	At1g64380	AP2 domain	1,24	12,26	6,65	2,86E-02	3,95
PU29741	At4g25200	23.6 kDa mitochondrial small heat shock protein	1,12	13,85	4,73	8,09E-02	0,69
PU00203	At1g77120	alcohol dehydrogenase (ADH)	1,12	12,25	6,07	4,40E-02	3,02
PU21821	At3g58520	expressed protein	1,09	14,60	4,75	8,09E-02	0,73
PU09593	At5g51660	cleavage and polyadenylation specificity factor	1,08	11,08	6,21	3,70E-02	3,27
PU09017	At1g65010	expressed protein	1,08	12,62	5,38	4,96E-02	1,85
PU00667	At2g34090	expressed protein	1,05	13,41	5,48	4,80E-02	2,04
PU23800	At5g38760	expressed protein	1,03	16,84	4,61	8,28E-02	0,48
PU09258	At3g26650	glyceraldehyde 3-phosphate dehydrogenase A, chloroplast	1,02	12,37	4,64	8,28E-02	0,53

Table7. Top 20 most highly systemically down regulated transcripts in microarray analysis in leaves of clone 72 in response to the necrotroph inoculation at 24hours after inoculation. PU_ID numbers represent gene identifiers in *Populus* database (http://www.populus.db.umu.se/). M = log2 R/G. An absolute M-value of 1 corresponds to a 2-fold change in expression, since we are using logs to the base 2. On this scale, M = 0 represents equal expression, M = 2 represents a 4-fold change, and so on. R = Red channel (Cy3), G = Green channel (Cy5).

reporterId	reference	annotation	M	A	*T test*	P,Value	B stat
PU06405	At3g23390	60S ribosomal protein L36a/L44 (RPL36aA)	-3,47	8,44	-9,73	4,59E-02	0,70
PU04297	At4g32480	expressed protein	-2,50	10,89	-8,94	1,30E-02	5,98
PU22024	At4g30460	glycine-rich protein	-2,17	9,85	-6,96	2,86E-02	4,43
PU24168	At5g57450	DNA repair family protein	-2,15	9,16	-6,99	4,59E-02	1,25
PU02345	At1g20850	cysteine endopeptidase, papain-type (XCP2)	-2,15	10,48	-7,43	2,86E-02	3,91
PU02379	At4g03210	xyloglucan:xyloglucosyl transferase	-1,86	12,79	-4,69	8,28E-02	0,62
PU03004	At5g15230	gibberellin-regulated protein 4 (GASA4)	-1,77	9,97	-6,06	4,59E-02	1,95
PU30776	At1g65890	acyl-activating enzyme 12 (AAE12)	-1,63	11,13	-5,70	4,59E-02	2,19
PU05426	At1g52190	proton-dependent oligopeptide transport (POT)	-1,60	12,67	-5,64	4,59E-02	2,31
PU04667	At4g24250	seven transmembrane MLO family protein	-1,59	9,50	-4,90	1,09E-01	0,00
PU29457	At4g15910	drought-responsive protein	-1,55	13,98	-4,93	6,69E-02	1,06
PU05694	At4g15910	drought-responsive protein	-1,48	13,66	-4,76	8,09E-02	0,75
PU28084	At4g30460	glycine-rich protein	-1,45	11,04	-5,16	5,59E-02	1,47
PU20440	At4g10780	disease resistance protein (CC-NBS-LRR class)	-1,45	11,05	-4,68	9,23E-02	0,32
PU04847	At1g27660	ethylene-responsive protein	-1,41	9,39	-6,41	4,59E-02	2,05
PU27587	At4g15910	drought-responsive protein	-1,40	12,63	-4,61	8,28E-02	0,48
PU07286	At1g20850	cysteine endopeptidase, papain-type (XCP2)	-1,39	11,40	-4,52	9,19E-02	0,31
PU01278	At3g46570	glycosyl hydrolase family 17 protein	-1,37	9,71	-5,42	5,23E-02	1,74

Appendix

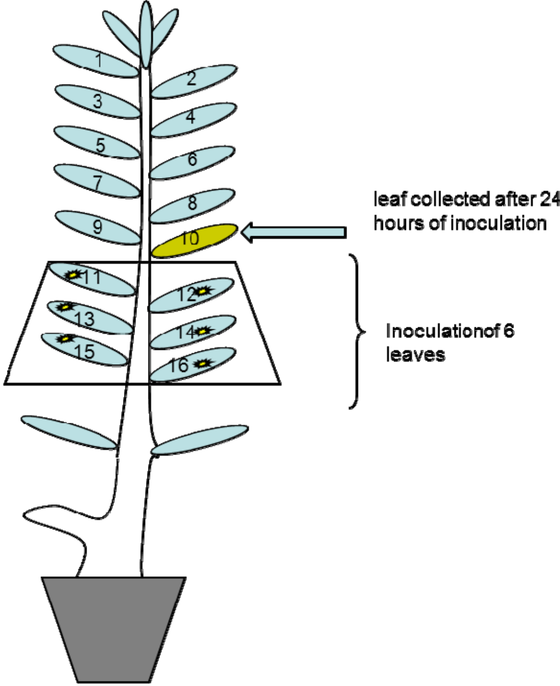

Figure s1. Inoculation of 6 leaves with spores of biotroph. Each leaf with 0.1ml and covered with transparent polythene bag. Leaf#10 was collected from immediate above the bag at 24 hours after inoculation.

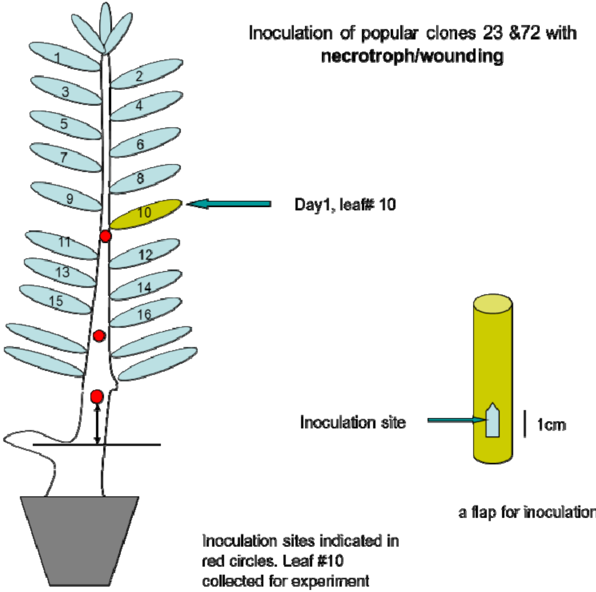

Figure.s2. Inoculation and sample collection for necrotroph and wounding in aspen in this experiment.

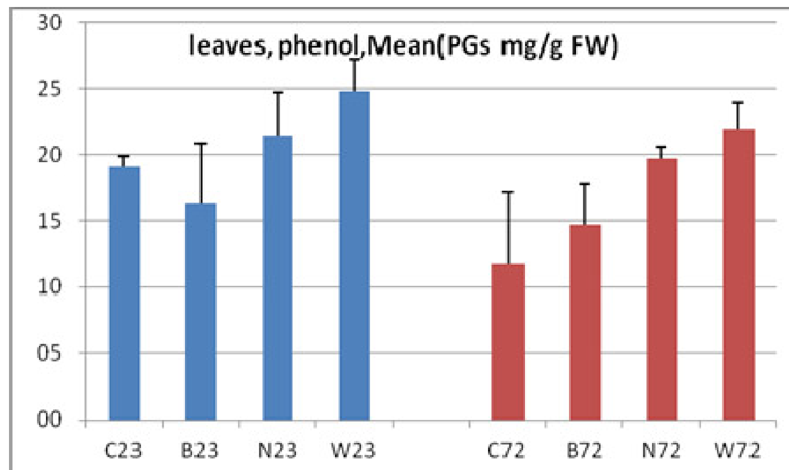

Figure s3. Content of total soluble phenolics in the systemic leaves of aspen clones 23 and 72 after biotroph (B), necrotroph (N) and mechanical wounding after 24 hours after inoculation. For treatments n=3 and controls N=2 and bars are ±SD.

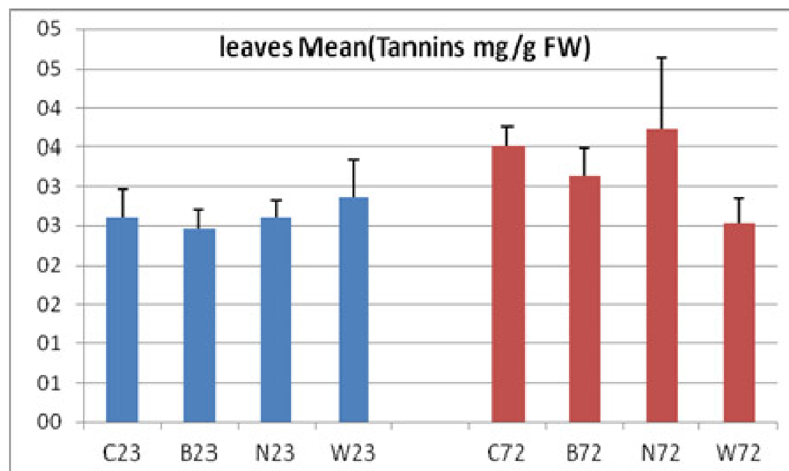

Figure s4. Content of soluble condensed tannins in the systemic leaves of aspen clones 23 and 72 after biotroph (B), necrotroph (N) and mechanical wounding after 24 hours after inoculation. For treatments n=3 and controls N=2 and bars are ±SD.

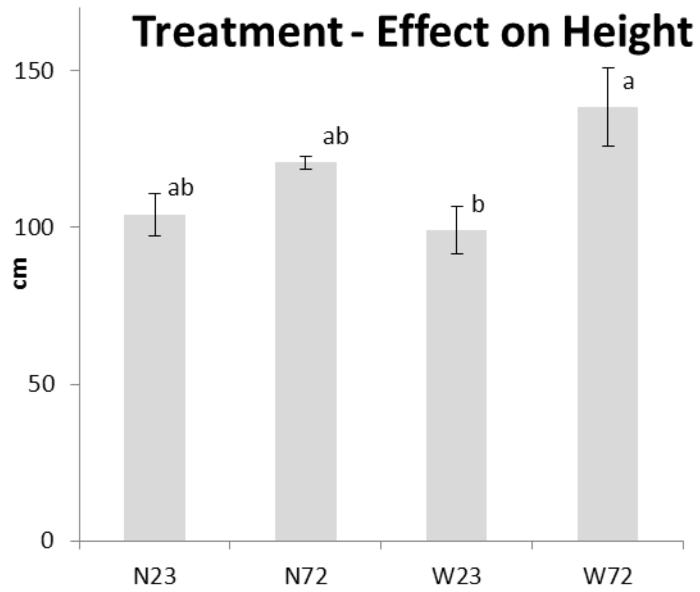

Figure s5. Average heights for wounded and necrotroph infected plants. Different letters indicate significant differences in height (Tukey test). W= wounded, N=infected with necrotroph, clone 23 and 72 respectively.

Table s1. Candidate defence genes in Norway spruce analysed by real time qRT-PCR.

Name	GenBank	Predicted Gene	Froward/reverse primers used in qRT-PCR
Senesc	XM_002329508.1	Senescence-associated protein	GGCTGTTAGATCCTGATCTGGCCTTG/GCCTTCACTGTTCAAGCGTGACTCC
ChitiI	XM_002326005.1	ChitinaseI	CAACAGGCAATACCCATGTGTTGCAG/CTCCTTCCGGCTGGCCCATA
CCR	XM_002300584.1	Cinnamoyl-CoA reductase	CGGGTTCCACTGTCTAGTGTTTCCA/GGGCCTTTGTTGCAGCCAACC
GH3	XM_002300212.1	GH3 auxin responsive promoter	GGCAAAGCTGGGCTGAAATGC/TGCAATTTCAAGAGGGGCATCCA
Methyl-CpG	CU232284.1	Methyl-CpG-binding domain	GTCGTGACCGTGCCGCTCTG/TGTCTTTGCTGGCTGCAACATGAA
Wrky	XM_002319046.1	WRKY family transcription factor	GAGCATGGATGGTGCTGTGT/TCAGATGAGTGAGGCATACCAATC
Myb1	XM_002303091.1	Myb-like DNA-binding domain	TGAAGACGTCCAGCAGATTGAA/GTTGCTAGCTCCAGATCTTCTAGTGTAG
Myb2	XM_002310776.1	Myb-like DNA-binding domain	CACCCCTGACCGCTGGCATA/TGCCAGACTCAATTTCCCTGACATCC
ERD4	CU229417.1	Early responsive to dehydration stress	GCAAGACCGAAGCGGAGTTA/GGAATCCGGGTTGCATACC
Glutaredoxin	XM_002319046.1	Glutaredoxin family protein	CAGCTAGGATGTCAGCCAAGTG/CATGACTTGCTTATCACCACCAA
Mitoch-Cp	CU222755.1	Mitochondrial substrate carrier	CTGGAGCCGCTTCTCGTAGT/CATACAAGCACGGGTAGTCTGAAC
Omt	XP_002335435	O-methyltransferase	TGTCGGGGGAGGCTCAGGAA/TCAGAAACCACACGCGGGAGA
Ubq	FJ438462	Ubiquitin-like protein	GTTGATTTTTGCTGGGAAGC/GATCTTGGCCTTCACGTTGT

Table s2. Visible lesions lengths at 14th day of sample collection after wounding and necrotroph treatments. N=3.

Clone	Plant #	Treatment	Bark lesion length	Wood lesion length
23	average of N		1	4,2
72	average of N		1	4,3

Local and systemic host response in aspen *(Populus tremula)* to necrotroph *Ceretocystis* sp. and wounding

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Abstract

Two aspen SwAsp clones 72 and 23 were inoculated in the phloem with a newly discovered and highly aggressive necrotrophic fungus and wounded to examine the local and systemic host response. The pathogen was able to colonize both clones but these were able to recover from wounding alone. The lesion length caused by the necrotroph was 3 to 4 times longer in xylem than in phloem, suggesting that the bark defence is stronger than that of sapwood. Height growth was better in clone 72 indicating that it was more vigorous. There were similar levels and changes in total phenols and tannins during the experiment for the two clones. The level of total phenols transiently dropped systemically in both clones, while a local and transient drop for the tannins was more prominent in clone 23. The local effect to inoculation and wounding in the bark was similar between the two clones in most transcripts examined. However, the local response to the necrotroph in clone 23 was in general stronger than clone 72. The systemic effect in leaves was also examined 24 hours after treatments and revealed that clone 72 had a significantly more effective systemic signalling inducing increased transcript levels than clone 23. Clone 23 showed a general lack of systemic induction despite the clear local response. Furthermore, some transcripts increased to a higher level systemically in leaves of clone 72 in response to wounding than to the pathogen, suggesting that the pathogen has some ability to repress the induction and translocation of host systemic defence signalling.

Keywords: qRT–PCR, Gene expression; Induced resistance; Necrotroph; *Ceratocystis sp*.; Mechanical wounding; *Populus,* Phenolic

Introduction

Necrotrophic fungi are causing the most damaging diseases of forest trees and are responsible for enormous losses to forestry worldwide. These pathogens enter living or dead plant parts through wounds or natural openings, kill the host tissues, produce enzymes that cause breakdown of membranes, cell contents and cell wall components, and absorb the resulting nutrients. Plant resistance against necrotrophs differ to that toward biotrophs that need to feed on living cells. Resistance to necrotroph can be monogenic, but in trees it is usually complex, involves many mechanisms leading to resistance and is under polygenic control (Laluk *et al.*, 2010). Effective defence against necrotrophic pathogens may involve both local and systemic defence signalling, production of secondary metabolites, lignification of cell walls and formation of pathogenesis-related proteins.

Plant defence mechanisms against biotroph that include hypersensitive response (HR), rapid cell death at the site of pathogen attack and other biochemical barriers offer no proper resistance to the invading necrotrophs instead, and most likely need to be operated oppositely for effective against necrotrophic pathogens (Kliebenstein & Rowe, 2008). Secondary metabolites are part of the defence responses to mechanical wounding and may have similarities to that toward necrotrophs. On the other hand the necrotrophic pathogen may also be able to suppress the wounding related host defences. A balance may exists between salicylic acid- (SA) and methyl jasmonic acid- (JA) mediated host defence signalling for an effective resistance to biotrophic versus necrotrophic pathogens and that needed for recovery from wounding (Dodds & Rathjen, 2010; Deller *et al.*, 2011).

Plants have evolved numerous defence mechanisms induced after pathogen attack. These defence responses include transcriptional, chemical, physiological and anatomical changes in plants. Induced defence responses may occur in locally attacked tissues or systemically in healthy tissues. Molecular mechanisms underlying the induced defences are exceedingly complex (Glazebrook, 2005) usually involving several signalling pathways (Babst *et al.*, 2009) and vary depending on the pathogen type.

Populus trichocarpa (black cottonwood) was the first tree species with fully sequenced genome. Its relatively small genome (ca.485 Mb) organised into 19 chromosomes (Tuskan *et al.*, 2006), its rapid growth, easy clonal propagation and established protocol for transformation has made it the model tree of choice. Genus *Populus* consists of 30-40 species among them *Populus tremula* (European aspen) widely distributed in the Northern

hemisphere. Resistant aspen genotypes perceive and limit the pathogen and thus spread more effectively than susceptible ones (Bucciarelli *et al.*, 1998). Aspen genotypes has been described to show remarkable differences for induced resistance at biochemical level for example (Osier & Lindroth, 2006) showed that phenolic glycoside concentrations are genotypes dependent in *P. termuloides*.

Induced defences have been studied in local tissues in *Populus* at transcriptomic and metabolomics levels. Secondary metabolites especially from the phenylpropanoid and flavonoid pathways has been in focus for host defence after wounding, herbivory and rust infection (Arimura *et al.*, 2004; Miranda *et al.*, 2007) . Genes involved in regulation of these metabolites has also been under investigations for their expression in local and systemic tissues (Miranda *et al.*, 2007; Babst *et al.*, 2009). Most of the transcriptome studies related to *Populus*-rust interactions have focused on the local defence responses (Miranda *et al.*, 2007; Rinaldi *et al.*, 2007; Azaiez *et al.*, 2009). Temporal and spatial patterns of local and systemic defence responses in poplar have also been partly addressed in a few studies (Boyle *et al.*, 2010; Philippe *et al.*, 2010). In *Populus*, local and systemic defence studies based on leaf tissues suggest that the systemic response is a weaker version of the local host response, and that the intensity is believed to depend on the amount of invading pathogen, age of the leaves and distance from the inoculation site (Babst *et al.*, 2009).

Populus interaction with biotrophs, herbivores and wounding are quite well studied in leaf tissues but studies addressing *Populus*-necrotroph interaction at the molecular level is scarce. Our microarray experiments suggest differential host defence responses in systemic leaf tissues towards necrotroph (Yaqoob *et al.,* in prep). We found 118 differentially regulated genes after necrotroph attack in aspen clone 72 showing some resistance toward the biotroph *Melampsora magnusiana*. In a contrast the aspen clone (23) more susceptible to *M. magnusiana*, showed only 7 genes were differentially regulated after necrotroph inoculation. The differential behaviour of the two clones towards necrotroph inoculation was followed over the period of two weeks locally in bark as well as systemically in leaves in present study to evaluate the local and systemic regulation of selected defence related transcripts.

Genes that play a role in active host defence have previously been identified in aspen and other *Populus* species, e.g., Chitinases cause hydrolysis of the chitin in fungal cell wall and are known to highly up-regulated in resistant trees after infections (Noël *et al.*, 2005). *Cinnamyl CoA reductase* (*CCR*) is a key gene that regulate the carbon flux towards lignin

deposition in cell walls and has a positive role in *Populus* defence responses against pathogens (Piquemal *et al.*, 1998). Poplar has 192 Myb genes, several of these are induced by wounding and pathogen infection and participate in regulation of the phenylpropanoid pathway (Duplessis *et al.*, 2009). Plant Aconitase functions as an RNA-binding protein and plays a role in regulating resistance to oxidative stress and hypersensitive cell death (Moeder *et al.*, 2007).

Signal perception and transduction is important in defence responses. In the *Populus* genome 21 putative Mitogen-activated protein kinases (Mpks) genes have been identified. Mpks interlink the regulatory networks that lead to the nucleus in order to induce defence related genes (Rathjen & Moffett, 2003; Hamel *et al.*, 2005). Putative *IAA-amido synthetase* (*GH3*) is an early response class of genes to auxins that are up-regulated after pathogen attack in *Arabidopsis* (Zhang *et al.*, 2007). The *Populus* genome has 105 *Wrky* genes (Zhu *et al.*, 2007) and are known to be up-regulated quickly and strongly in response to wounding, pathogen infection and abiotic factors in many plants (Eulgem & Somssich, 2007). *Hevein* and pathogen related proteins (PR) such as *PR1* (van Loon et al., 2006) play roles in natural defence to pathogenic fungi in plants and served as marker genes for host defence responses such as the so-called immune response in plants, hypersensitive response (HR) and systemic acquired resistance (SAR). Increase of *PR1* is a classical sign of an induced defence response, but its increase is seen in both incompatible and compatible interactions of poplar with rusts (Miranda *et al.*, 2007; Duplessis *et al.*, 2009). We also wanted to examine the expression of a Senescence (*Senesc)* related conserved gene with unknown function (XM_002329508), a drought-stress induced *Methyl-CpG-like* gene (CU232284) and a Mitochondrial substrate carrier that belongs to a superfamily of calcium sensors and calcium signal modulators (*Mitoch-Cp*) genes was also monitored. The mitochondrial carrier protein family is known to be over-represented among identified stress-responsive genes in *Arabidopsis*, suggesting that stress induces altered needs for transport across the mitochondrial inner membrane (Van Aken *et al.*, 2009) and also *Wrky* transcription factors are involved in stress and defence and are likely candidates for regulating the mitochondrial stress response (Van Aken *et al.*, 2009). To study the local and systemic host response we inoculated and wounded two SwAsp Aspen clones. The local host response in the woody stem and the systemic response in the leaves to the necrotroph and wounding of the stem were examined. The inoculations were performed with a newly discovered and highly aggressive necrotroph fungus *Ceratocystis* sp. (NFLI 2004-466/501) from Norway, and compared to the response to mechanical wounding and to

healthy untreated plants of the same clones. We measured the differential expression of the candidate genes, total phenolics (TPs) and proanthocyanidins as a measurement of condensed tannins (CTs) locally in bark and systemically in leaf tissues.

Material and Methods

Plant material

Two aspen clones (23 and 72) from the SwAsp collection (Luquez *et al.*, 2008) were used for the experiment. The plants were propagated from tissue culture in the summer 2007 and kept in greenhouse under natural light conditions with sufficient water and fertilizer. In December 2008 the plants lost their leaves and overwintered. The plants started bud flush in mid-April and the inoculations and later sampling took place starting at June 10 2008. 12 plants from each clone were used in this study. After assigning numbers from 1 to 12, the plants were randomly placed on benches in the green house. For each treatment (biotroph, necrotroph, wounding) three biological plants (replicates) were used for each time point for each treatment, whereas two biological replicates were used for controls.

Procedure of wounding and necrotroph treatments on aspen bark for local host response

Wounding and necrotroph treatments were applied by making flaps (ca 1 cm height – cutting bark on the upper side and on both side) at the same height. Both flaps cover ca 50 % of the circumference of the stem. Treatments were applied at three sites: site 1 below leaf 10 from the top; site 2: below six leaves from site 1; and site 3: 5cm up from the internodium 2007-2008. Wounding was done first, starting from site 3, then 2 and at last at the site1. We continued with fungal inoculation, first those at site 1, then those at site 2 and at last the site3. A small amount of malt agar (1%malt and 1.5% agar) was placed underneath the bark flap, then covered with parafilm in wounding treatment whereas for fungal treatment malt agar had actively growing mycelium of a novel aggressive necrotroph. At each time point two plants from each clone were used as control plants, additionally, control plants from day1 served as Ex-controls (control plants that healthy leaf was collected from at day 1) and Ex-Ex-controls at day3 and 14 respectively. These Ex- and Ex-Ex- controls was used to see effect of removing the leaves from healthy plants alone. At the time of sample collection, a 7cm stem piece was cut starting from 1cm below the inoculation wound and up. After separating the bark from wood, samples were frozen in liquid nitrogen and stored at -80C until use. The bark and leaf samples were collected at 1, 3, and 14 days post inoculation (dpi).

RNA preparation

For systemic host defence response the leaves were grinded in mortar and pistil. Samples were kept cold by liquid nitrogen throughout. To investigate local host responses, 1 cm bark piece covering whole inoculation site was cut from the collected 7cm bark sample and ground with metal beads for 2 minutes in cylinders cooled with liquid nitrogen. The resulting ground powder was aliquot to 50 mg and stored at -80ºC until RNA extraction. The Aurum™ total RNA mini kit (#732-6820) from BioRad (Bio-Rad Laboratories, Inc. USA) was used for RNA isolation by following the manufacturer's protocol. Eluted RNA 80µl was stored at -80ºC for further work. The yield of RNA was measured using a Nanodrop1000 spectrophotometer. In order to make cDNA, 300ng RNA was reverse transcribed using Taqman Reverse Transcription Reagents (Applied Biosystems, Foster City, CA) following instructions prescribed by the manufacturer. RT reaction mixture was prepared in 50 µl volume and incubated in the thermal cycler at 25 °C for 10 min, then at 48 °C for 30 min, and finally at 95 °C for 5 minutes. After completion of RT reaction 50µl of the nuclease free water was added in each reaction mixture to raise its total volume to 100µl before further use in qRT-PCR.

qRT-PCR Analysis

We followed the induced host defence response both locally in bark (secondary phloem) and systemically in leaves of the SwAsp clones 23 and 72 over the period of 1, 3 and 14 days post inoculation and wounding by qRT-PCR. We selected a set of transcripts [included: *Senescence-associated protein (Senesc), ChitinaseI (ChitiI), Cinnamoyl-CoA reductase (CCR),* Auxin responsive GH3 family protein *(GH3), Methyl-CpG-binding domain (Methyl-CpG), Wrky-like family transcription factor (Wrky), Aconitase, Myb-like DNA-binding domain containing protein (Myb1), Mitochondria-Carrier protein (Mitoch-Cp*)] that were differentially expressed in leaves of clone 72 in a microarray experiment (Yaqoob *et al.,* in prep;(table s1)). We also followed the target transcripts [(*Pathogenesis related protein* (*PR1), Hevein, Mitogen-activated protein kinase Mpk3.1*)] that have been established as markers for induced defence after pathogen and wounding (Levée *et al.*, 2009).

Transcript expression levels were determined by qRT-PCR using the SYBR-Green PCR Master kit (PerkineElmer Applied Biosystems) using the 7500 Real-Time PCR System from Applied Biosystems. Each qRT-PCR reactions was carried out in a 25 µl volume containing 250 nM of each primer, 2µl cDNA and 1 × SYBR®Green PCR master mix (Applied Biosystems, #4309155). The 7500 Real-Time PCR System (Applied Biosystems, #4351106)

was used for quantification in a 96-well reaction plate using the parameters recommended by the manufacturer (10 min at 95°C and 40 cycles at 95°C for 15 sec and 60°C for 1 min). Two technical repeats were run for each gene product independently to confirm the accuracy of the PCR. We verified the specificity of the amplifications at the end of each PCR-run using the 7500-system SDS (dissociation curve analysis). Description of the PCR primers used is given in table s2. Acquisition and analysis of data were done using the 7500-system SDS software for absolute quantification. All gene expression levels of target transcripts were normalized to the endogenous control *Cdc2* previously established as an endogenous reference gene in poplar (Nicole *et al.*, 2006). Boyle and associates (Boyle *et al.*, 2010) (2010), looked the expression of the *Cdc2* gene expression in wounded and fungal pathogen inoculated aspen over the period of 10 days and found no significant difference in the expression patterns. The relative transcript level of selected target 12 genes was performed by the standard curve method according to the User Bulletin 2 (ABI PRISM sequence detection system; PE-Applied Biosystems). Briefly, quantitative values of target gene were divided by quantitative value of the endogenous reference gene. Later, the final value for each time point was obtained by taking mean of 3 biological replicates.

Statistical analysis of qRT-PCR data

To normalize the data (quantitative qRT-PCR) values of the various genes were divided by the endogenous reference. However, even after standardization the variance tended to increase with the mean. Therefore the data were log transformed after division by the reference gene.

The experiment was designed as a factorial with double repeated measurements (gene and time) taken on the same subject i.e. plant. In this design clone, treatment, time, and gene were considered fixed factors, whereas plants within clone and treatment were considered a random factor. All interactions between fixed and random factors were also random. The Mixed procedure in the SAS™ system was used for all data analysis. The default REML method was used for estimation of the various covariance components. Planned comparisons of differences between least square means were done by use of the pdiff option.

Extraction of total phenols and condensed soluble tannins

Each leaf sample was ground in liquid nitrogen by hand; from this ca. 50mg (exactly measured) per sample was used to extract total phenolics (TPs) and condensed tannins (CTs). The Folin-Ciocalteu assay (Singleton & Rossi, 1965) was used to measure the concentration

of total soluble phenolics. TPs were extracted with 1.8 ml of 80% MeOH in water with constant overtaxing at room temperature for half hour. The mix was centrifuged at 2500g for 10min. 10µl of sample was mixed in 475 µl of Folin-Ciocalteu (diluted 7 times) and incubated at room temperature for 5 min. Further, 475µl of a 7% sodium carbonate was added and kept at 40C for 15 min. Phenolics were detected using spectrophotometer at 724nm. Contents were standardized against cholorogenic acid. The standard curve was linear from 0 to 20µg/µl.

For analysis of CTs the method of Ossipova and associates (Ossipova *et al.*, 2001) was used. In short samples were suspended in 600µl of 70% aqueous acetone (1% asbcrobic acid) and kept at room temperature for 1 hour. Later, the mix was centrifuged for 10 min at 2500g. The pellet was extracted twice with acetone and then dried by speed vacuum. This purified extract was used to determine the soluble condensed tannins in the samples. In 1 ml of freshly prepared mixture of 1-butanol: hydrochloric acid (95:5), 50µl of aspen leaf extract was added. After mixing it well, the mixture was placed in water bath at 95C for 50min. Prior to absorbance measurement at 550nm, mixture was cool down at room temperature. The standard cure was constructed using epi-catechin (0-80µg/µl).

Results

Local defence response in bark

The constitutive levels of some of the selected candidates in healthy bark tissue differed between the two clones. The transcript level of *Wrky-like family transcription factor (Wrky), Aconitase* and *Hevein* was higher in healthy bark tissue of clone 72 while *Myb-like (Myb1)* and auxin responsive GH3 family protein (*GH3*) were constitutively higher in clone 23.

At day1 in Clone 23, the local host response to necrotroph inoculation induced higher transcript levels for most transcripts, while indications of a decrease for the *Wrky* and *Methyl-CpG*. However, in response to wounding clone 23 shows only minor, reduced or no local increase, and the overall local host response is significantly higher to necrotroph inoculation then to wounding alone ($P<0.0008$). In clone 72 the levels of *Wrky* are reduced by both wounding and necrotroph inoculation, but remain at all times higher than in clone 23, while *Aconitase* start out constitutively higher in 72 and then drop in response to treatments to levels less than in clone 23. Of the remaining transcripts none tend to increase more to wounding than necrotroph locally in bark of clone 72, and the *ChitinaseI* (*ChitiI)* and particularly *PR1*

($P<9E-05$) are highly induced locally to the necrotroph while little response are seen to wounding for these two gene products. In sum the local response to the necrotroph is on average greater in clone 23 ($P<0.0003$) but the response to the necrotroph is also highly significant for clone 72 ($P<0.0005$). While up- and down-regulation due to the wounding is more pronounced locally in bark of clone 72 at this first time. At day3 the transcripts levels were also measured locally in bark and showed large variation between biological replicates for most of the gene products at this time. However, in both clones the *PR1* level increased significantly as local response to the necrotroph ($P<0.001$) and a minor but significant response is detected also to wounding ($P<0.003$). The *Hevein* increased dramatically in clone 23 after necrotroph inoculation and there was a smaller but still significant effect of wounding ($P<3E-06$), while in clone 72 the *Hevein* remained at lower levels in all treatments at 3dpi. The *ChitiI* increased in response to necrotroph and wounding in clone 72 while in clone 23 there was an increased only to the necrotroph ($P<0.002$). For *Wrky, Mpk3.1, Myb1, Senesc*, *Methyl-CpG, Mitochondrial-Cp, Aconitase* and *GH3* there is no clear pattern of changes between treatments, with the exception of the reduction due to both necrotroph inoculation and wounding observed in clone 23 for *Mitochondrial-Cp* ($P<0.0002$) and the apparent increase in *Aconitase* to wounding and the necrotroph was not statistically significant. However, there was a tendency of an increase in *CCR* transcripts in both clones after necrotroph inoculation and wounding, but the increase was only statistically significant for the clone 72 response to the necrotroph ($P<0.02$). The excision control (C-Ex), i.e. the control plants from day one the healthy bark sample was collected from, shows changes similar to that of wounding for both clones.

The last time point for local changes in transcripts level analysed was 14dpi. At this time there was also a statistical significant increase in *PR1* not only to the necrotroph ($P<0.0005$) but also to wounding ($P<0.02$) in both clones. Necrotroph and wounding treatments gave now higher CCR levels in both clones, but was significant only for clone 72 to necrotroph ($P<0.006$) and wounding ($P<0.04$). For *ChitiI* both clones showed a significant local increase in response to the necrotroph ($P<0.03$) but not to wounding alone. *GH3* was significantly reduced in response to wounding and necrotroph in both clones ($P<0.02$) compared to the control. In clone 23 the *Methyl-CpG, Wrky*, *Myb1, Senesc, Aconitase and Mpk3.1* levels showed only minor changes and in clone 72 the *Mitoch-Cp, Hevein and Mpl* showed no clear changes and increase in *Myb1* to treatments was not significant. However, clone 23 showed

again higher *Hevein* levels and the increase was statistically significant toward the necrotroph ($P<0.02$).

Overall, differential transcription as a local host response to wounding and inoculation of the bark was significant relative to their respective healthy controls for both clones. Considering the effect of treatment for all gene products for the duration of the experiment clone 72 showed statistically stronger effect of inoculation and wounding ($P<6E-05$, $P<0.004$) than clone 23 ($P<0.0002$, $P<0.04$), and only in clone 23 the local host response to necrotroph was overall significantly greater than wounding ($P<0.04$).

Systemic defence response in leaves

Induced defence in plants have also a systemic dimension, for comparison of the systemic host defence response to that locally in bark at day 1 we selected the genes (*ChitiI, Senesc, GH3, Methyl-CpG, CCR, Aconitase, Hevein, Senesc-Apr2, Mpk3.1, PR1, KASIII* and *Kinesin*) that were quantified at the transcripts level in leave samples from both clones (Fig 4, s1).

Transcripts levels were found higher locally in bark than systemically in leaves for clone 23 to wounding and necrotroph for *ChitiI, Senesc, GH3, Methyl-CpG and CCR,* while *Aconitase* showed higher levels in leaves, and only decreases and minor systemic increases overall was found for clone 23. In sharp contrast, the systemic response to wounding in leaves was higher than the local host response in bark for clone 72 for all gene products besides the *ChitiI* and *PR1* that showed similar increases to both treatments in leaves. Overall, clone 72 showed a larger and more significant systemic response in leaves to wounding ($P<0.002$) and necrotroph inoculation ($P<0.02$) than clone 23. The *ChitiI* expression level in leaves of 72 was constitutively higher ($P<6E-06$) and was further induced by both treatments, while in clone 23 the levels remained low in both treatments and control. *CCR* was always higher and increased due to wounding in clone 72, and a similar increase was seen in response to wounding in clone 72 for *GH3* relative to clone 23. *Hevein* increased in clone 72 and dropped in clone 23 in response to both treatments. *PR1* levels tended to increase in clone 72 while reduced in clone 23 by wounding and necrotroph inoculation.

Quantification of local and systemic CTs and TPs after necrotroph and mechanical wounding

Condensed tannins (CTs) and phenolic glycosides (TPs) are known to produce in stress conditions in poplar and their quantity indicates the defence response. TPs and CTs were

produced more in leaves than bark. We observed higher amount of CTs in bark and leaves for clone 72 than in clone 23.

For CTs the local response of clone 23 to the necrotroph was first a reduction at day 1 and later a maximum was reached at 14dpi, while in clone 72 there was no clear pattern of changes. Systemically in leaves little change in CTs with time was found for the two clones (Fig.5).

The quantity of TPs found locally and systemically was similar in the two clones regardless of time and treatment. Locally in bark only minor changes were observed, with the highest levels found after wounding in clone 72. Systemically in leaves there was first an increase due to wounding and inoculation followed by a reduction by day 14. In clone 72 starting from a lower level the systemic increase were evident at day 1 and day 3, also clone 23 showed the same trend but had higher average values and more variation between samples (Fig.6).

Morphology parameters

The pathogen was able to colonize both clones. At day 14, the lesion length was measured and no visible lesions were found in the wounded samples, except for a 0,5cm lesion in both bark and wood one of the three clone 23 samples. This indicates that both clones recover well from the wounding treatment. In contrast, the necrotroph caused dark visible lesions that extended up to 1cm up and down the inoculation site in the bark (secondary phloem) and 3 to 6 times longer in the wood (developing xylem) of both clones at day 14s, indicating that the host defence in the bark is stronger than that in the sapwood. After harvesting samples from wounding and pathogen treatments, plants were left to grow for another 6 weeks and found that clone 72 grew taller than clone 23 suggesting recovery, while no statistically significant differences in branching was observed between the two clones.

Discussion

This is the first study to compare transcripts levels of defence related genes in local and systemic tissues of susceptible and resistant clones of *Populus* after necrotroph and wounding treatments. We followed the changes in expression of the selected candidate genes (*ChitiI, CCR, Myb1, Aconitase, Senesc, GH3, Methyl-CpG, Wrky, Mitoch-Cp, PR-I, Hevein, Mpk3.1*) over the period of two weeks. We found that the two clones had similar local responses to the necrotroph, but clone 72 showed a greater response to wounding and significantly greater systemic change in transcript changes than clone 23. The similar local response to the

necrotroph was also mirrored by similar lesion length indicative of rapid fungal colonization in both clones. However, the greater systemic response in clone 72 and the greater systemic response to wounding alone than to necrotroph suggests that the pathogen inhibit or suppress part of the systemic defence response in clone 72. The lack of systemic induction of transcription to both necrotroph and wounding in clone 23 and significantly less local response to wounding in clone 23 indicates that clone 23 has a slower or deficient systemic host defence signalling system than clone 72.

The downstream defence related genes such as *Chiti1* is a part of a molecular network that is impacted by Myb transcript level in poplar (Plett *et al.*, 2010). The expression of our *Myb1* and the *Chiti1* is not fully compatible as the *Myb1* levels are higher in clone 23 while the *ChitiI* levels are higher in clone 72 suggesting a different or more complex signalling pathway relationship for the *Myb1* studied. Plant chitinases catalyse the hydrolysis of chitin, which is an integral part of the fungal cell wall and this not only impacts negatively on the growth of the pathogen but release elicitors that further induce defence responses in the host. Noël and coworkers (Noël *et al.*, 2005) have demonstrated that transgenic poplars with increased chitinase level had better resistance to the *Melampsora medusa*. At least 3 distinct types of chitinases are up-regulates in rust-infected leaves of poplar. Rinaldi (Rinaldi *et al.*, 2007) found that the *ChitiI* was most responsive in incompatible rust-poplar interaction at 2dpi, giving an 6.3fold increase. We also found that *ChitiI* transcript level increased after necrotroph inoculation and this 3 fold increase was more rapidly in bark of clone 72. The increase in transcript level was consistent at all-time points after necrotrophic pathogen; however accumulation of these transcripts was lower for wounding locally in bark, while the levels systemically in aspen leaves after pathogen and wounding at 1dpi were similar in clone 72 while remaining at lower levels both tissues in clone 23 at this time (Fig.4). Thus, clone 72 may have a higher constitutive level of defence in both bark and leaves and more efficient systemic signalling system to respond to necrotrophs and wounding or that the clone 23 is simply deficient in its defence signalling capabilities or allocating its resources towards increased growth. The later argument is unlikely since clone 72 grows equally well or better than clone 23 under the conditions and treatments tested. The difference in type of tissues for local and systemic response plays a role in interpreting the responses seen as well, but the constitutive and induced levels between tissues for most gene products are more similar than they are dissimilar to conclude on any tissue specific effects in this study. The transcripts accumulation in local bark of clone23 close to double at all-time points as compared to a

previous study where it was 0.75 fold at 1dpi locally in leaves compatible rust-poplar interaction (Miranda *et al.*, 2007), but again the two tissues are different and more importantly the pathogen is an necrotroph in our case so local host responses cannot be expected to be identical. Furthermore, even in clone 72 that appear to have an efficient local and systemic signalling system the necrotroph is fully able to colonize the tissues.

Cinnamoyl-CoA reductase (*CCR*) catalyses the conversion of hydroxyl cinnamoyl-CoA esters into their corresponding cinnamyl aldehydes. *CCR* is related to lignification and involved in development as well as defence responses in plants. Deregulation of the *CCR* gene may result in the 50% reduction of lignin contents in *Populus* cell wall (Leplé *et al.*, 2007). A single noncoding two-state marker in *CCR* is reported to strongly linked with lignin composition gene (Wegrzyn *et al.*, 2010). We noticed a 3 times up-regulation of *CCR* gene locally in both clones in response the pathogen and a lower increase to wounding. A similar trend in *CCR* expression after compatible (2.14fold) and incompatible (2.61fold) rust-poplar interactions has been reported at 6dpi (Azaiez *et al.*, 2009). It seems that expression of *CCR* gene after necrotroph and biotroph shown similarities but is rapid followed by a less intense increase by 3 to 13 days in response to the necrotroph in our pathosystem.

The Myb genes in poplar are involved in cell wall biosynthesis. *PtrMyb3* and *PtrMyb20a* are involved in the activation of cell wall biosynthesis in hybrid aspen, similarly overexpression of *PtrMyb186* improved the poplar resistance against pests (Plett *et al.*, 2010). Expression of Myb also has influence on phenotype, e.g. over expression of *Myb186* resulted in the increase in trichomes in hybrid *Populus* (Plett *et al.*, 2010). The *Myb1* gene has so far no described function and is more highly expressed constitutively in clone 23 and tend to increase at 1dpi after necrotroph and wounding in clone 72, however it is difficult to find a clear pattern of expression of this gene over time course of the experiment and the changes detected are modest and rather hinting at a constitutive role, making it difficult to establish a relationship with a role in resistance for *Myb1* beside it being transiently down-regulated by wounding in clone 23 at day 3 and up-regulated in response to wounding in clone72 at day1 (Fig.1). It has been shown that wounding lead to higher level of transcripts of Mybs (*Myb134,Myb097, Myb183,Myb46, Myb83,Myb3, Myb20*) and leads to up-regulation of phenylpropanoid and flavonoid genes in poplar (Mellway *et al.*, 2009; McCarthy *et al.*, 2010). Myb genes are also reported to up-regulated 3 fold in *Populus* at 1 day after herbivore in microarray studies (Ralph *et al.*, 2006). It is believed that Myb factors are involved in regulation of CTs in *Populus* through phenylpropanoid and flavonoid pathway under stress conditions like rust,

wounding, ultra violet light and herbivores. However, it is unlikely that *Myb1* has such function as its levels I not correlated with the level of CTs measured.

Mpks are up-regulated in poplar after elicitor chitiosan treatment (Hamel *et al.*, 2005) and pathogen infection (Hamel *et al.*, 2005; Boyle *et al.*, 2010). In RT-PCR of 17 different Mpks from different poplar tissues, *Mpk3.1* gene expressed was shown to be expressed in a number of different organs (Nicole *et al.*, 2006). *Mpk3.1* is up-regulated in *M. medusae*-poplar interaction (incompatible interaction) and its transcripts levels were highest at 5dpi, however in response to a virulent strain of *Melampsora larici-populina* transcript level was highest already at 1-2dpi (Boyle *et al.*, 2010). Regulation of *Mpk3.1* in aspen clones 23 and 72 after necrotroph shows highest transcript accumulation in clone 23. In contrast, the *Mpk3.1* increase systemically in clone 72 at day 1 in response to wounding and is down-regulated in response to or actively suppressed by the necrotroph. This result may indicate that clone 23 may initially recognize and initiated a local defence response toward the necrotroph but fail at later stage of defence, possibly partially due to the lack of efficient systemic defence signalling. While *Mpk3.1* is down-regulated or suppressed in interaction with the necrotroph. *Wrky* is known to involve in the early events of defence responses to biotic and abiotic stresses. A model proposed by (Eulgem & Somssich, 2007) suggests *Wrky* activation by *Mpk3.1* gene that convey defence signals from the plasma membrane to the nucleus. Wrky's regulates a number of genes related to defence responses. Transcriptome studies with wounding and rust-poplar pathosystem are unanimous in their findings that *Wrky23* is up regulated at early time points. Overexpression of this gene resulted in deregulation of 600 genes that involved in stress responses (Levée *et al.*, 2009). The *Wrky* factor used in this study is highly similar to the uncharacterized *Wrky* transcription factor protein from *Populus. trichocarpa*. The constitutive values were higher in clone 72 and remained constitutively higher than in clone 23 despite the minor reduction as a response to pathogen as well as wounding at day 1. Clone 23 had much lower level of *Wrky* in both treatments and at all-time points. This is partly in agreement with (Levée *et al.*, 2009) where highest response was nearly 2fold after rust infection and decreased at later time points in compatible pathogen interaction. Similarly *AtWrky23* in *Arabidopsis* is induces strongly and rapidly (Grunewald *et al.*, 2008). In this study necrotroph induced similar regulation of *Wrky* to wounding in both clones, and thus likely this gene product is likely not actively suppressed by the pathogen but is regulated in response to wounding in both clones, and considered a wound regulated protein

We found indications of increased *GH3* transcript levels in bark of clone23 (3.2fold) in response to the necrotroph after wounding at 1dpi, but after that time there was in general local down-regulation of this protein in both clones, while systemically in leaves there was increased level in response to wounding alone at day 1. The higher transcript level at 1dpi makes that we cannot rule out that this gene is involved in early defence response. *GH3* in aspen (Teichmann *et al.*, 2008) and *Arabidopsis* (Zhang *et al.*, 2007) is associated to early responses to plant- pathogen interaction and involved in IAA homeostasis (Fu *et al.*, 2011) and we can expect that it has similar role in aspen and other *Populus* species in general.

PR1 a marker for systemic acquired resistance (SAR) and remain at low constitutive levels in healthy controls of bark samples in both clones. The transcript level increased after pathogen infection at day 1, reaching to its maximum increase (7-8 fold), before finally decreased to its constitutive level at 14dpi as a local response in bark of both clones, while showing a variable increase in leaves of clone 72 and a reduction below constitutive levels in clone 23 in response to both wounding and inoculation. (Miranda *et al.*, 2007) observed 3-10 fold increase in *PR1* transcripts at 3 and 9dpi respectively in poplar leaf discs infected with rust. While (Levée *et al.*, 2009) reported more than 50 fold increase in *PR1* transcripts at 10dpi in *M. medusae-Populus* pathosystem. Boyle and associates (Boyle *et al.*, 2010) observed that two rust species *M. medusae* (partial compatible) and *M. larici-populina* (compatible) yield different levels of transcripts in the host *P. nigra x P. maximowiczii* NM6. Although *PR1* transcripts level remain low (1fold increase) and equal in partial and complete compatible interactions until 3dpi but former ended up with >100 fold increase and the later with 10 fold change by10dpi. Up to 350fold increase of *PR1* transcripts was seen in incompatible *Melamapsora-Populus* interactions at 2dpi compared to 200 fold increase in compatible interactions with *Melampsora* (Rinaldi *et al.*, 2007). There is consensus from previous studies that in compatible interactions increase in *PR1* transcripts is typically lower than incompatible interactions, but this might be true only for interactions with classical biotrophs. The present results from necrotroph-aspen pathosystem reflect the ubiquitous nature of increased *PR1* transcript in local and systemic tissues regardless whether the interaction is incompatible or not. In this study stronger systemic response was observed than local in resistant clone. It seems that necrotroph is not efficiently able to suppress the systemic *PR1* regulation in clone 72, suggesting the *PR1* and mimic the expression seen for the *Chiti1*, possibly sharing fungal induced promoter and regulatory induction pathways; however there is some induction by wounding alone locally in both clones and more evidently systemically in clone 72.

The *Hevein* gene (EF055879) studied is highly similar to a class I *PR-4* gene and induced by wounding (Ponstein *et al.*, 1994). Pathogens, ethylene and O_3 are also known *Hevein* inducers ((Guevara-Morato *et al.*, 2010) and references therein). The protein contains a Barwin family domain and ChtBD1 Chitin binding domain, involved in recognition of chitin subunits and occurs in plant proteins that bind N-acetylglucosamine and wound-induced proteins. The *Hevein* transcript level in the *Populus* hybrid *P. tremula X alba* is known to increase 3 fold after wounding at 1dpi, followed by an increase to25 fold after *M .medusae* infection at 10dpi and is used as a marker for induced infection together with *PR1* and *Mpk3.1* (Levée *et al.*, 2009). We quantified the *Hevein* transcript level and found higher transcripts level in the clone23 bark at all-time points with peak level of 26 fold higher at 3dpi in response to the necrotroph alone, with the notable exception of higher levels in clone 72 after wounding and systemically in leaves at day 1. The local increase in bark of *Hevein* transcripts 1 day after necrotroph inoculation was 3 and 2 fold in clone 23 and 72 respectively. The necrotroph-*Populus* pathosystem appears to partly mimic the previously described biotroph-*Populus* pathosystem where compatible interactions resulted (paradoxically) in accumulation of *Hevein* transcript at higher level in susceptible clones. In a recent report, *Hevein* was up-regulated (1 fold) after wounding in both local and systemic leaves of *Populus* at 6hpi but transcript were undetected at 1dpi (Philippe *et al.*, 2010). No studies are available from aspen or other *Populus* pathosystem to compare the results obtained after necrotroph treatment, but evidence points to the protein being induced upon wounding and that the protein accumulates in compatible interactions and in situations where the tissues are heavily damaged as is the case with highly necrotizing pathogens. Clone 72 showed significant host response both locally and systemically to wounding and inoculations, and the greater systemic response to wounding than the necrotroph suggest that the pathogen is able to inhibit the host systemic signalling system. This could potentially be the action of effectors or other pathogen factors that negatively regulate the defence signalling in the host. The necrotrophic *Ceratocystis sp.* used in this study was isolated from discoloured spots in the wood aspen in Norway, and gave lesion lengths of 68 mm in bark 181 mm in wood of aspen upon artificial inoculations within one season. This highly necrotrophic isolate belong to the *Ceratocystis fimbriata* complex. Fungi in this complex are known to produce cerato-platanin (CP), a pathogen associated molecular pattern (PAMP) protein that stimulated poplar leaf tissues to activate defence responses, so far found in 5 species of the genus *Ceratocystis*, including *C. fimbriata* known to be pathogenic to plants with considerable importance in agriculture, forestry, and as ornamental plants (Comparini *et al.*, 2009). One may speculate that such a PAMP may also be

present in our system causing the induction of the host responses observed or if it or another molecule(s) is acting as effector with positive or negative impact on host defence signalling in our system. This work further suggest that clone 23 has a local response to the necrotroph and either a delayed or impaired systemic signalling inducing transcriptional changes in tissues away from the site of infection, and that this impairment not only entails the response to a necrotroph but also towards wounding. These hypotheses can be tested in future experiments on clone 23 and 72 by examining possible induction of priming or induced resistance toward a challenge (with compatible and incompatible fungi) or wounding followed by a later challenge with the pathogens to examine for any altered or long lasting (i.e. SAR and other forms of acquired resistance) speed and level of host resistance.

Acknowledgments

We thank Inger Heldal and Mari Kjos for their help in the lab and Harald Kvaalen for conducting statistical analysis on the data. Carl Tryggers Foundation. Professor Lars Ericson kindly provided spores of *Melampsora magnusiana*. This project was funded by Norwegian Research Council via grant NFR 178576.

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Figure legends

Figure 1. qRT-PCR of transcripts *Senescence, ChitiI, CCR, GH3, Methyl-CpG, Wrky, Myb1, Aconitase, Mitochondria-Cp*, *Hevein, PR1and Mpk3.1* in aspen clones 23 and 72 in response to the necrotroph (N) and wounding (W) at 1day post inoculation. The untouched controls of respective clones are also shown (C). Data was normalized to internal reference gene *Cdc2* by dividing quantity of target gene by quantity of internal reference gene. N=3 for treatments and n=2 for controls; bars±SD

Figure 2. Local change in transcripts levels in bark at 3dpi. qRT-PCR of transcripts *Senescence, ChitiI, CCR, GH3, Methyl-CpG, Wrky, Myb1, Aconitase, Mitochondria-Cp*, *Hevein, PR1and Mpk3.1* in aspen clones 23 and 72 in response to the necrotroph (N) and wounding (W) at 3day post inoculation. The untouched controls of respective clones are also shown (C). Data was normalized to internal reference gene *Cdc2* by dividing quantity of target gene by quantity of internal reference gene. n=3 for treatments and n=2 for controls; bars±SD

Figure 3. Local change in transcripts levels in bark at 14dpi. qRT-PCR of transcripts *Senescence, ChitiI CCR, GH3, Methyl-CpG, Wrky, Myb1, Aconitase, mitochondria-Cp*, *Hevein, PR1and Mpk3.1* in aspen clones 23 and 72 in response to the necrotroph (N) and wounding (W) at 14day post inoculation. The untouched controls of respective clones are also shown (C). Data was normalized to internal reference gene *Cdc2* by dividing quantity of target gene by quantity of internal reference gene. N=3 for treatments and n=2 for controls; bars±SD

Figure 4. Systemic change in transcripts levels in leaves at 1dpi. qRT-PCR of transcripts *Senescence, ChitiI, CCR, GH3, Methyl-CpG,* and *Aconitase* in aspen clones 23 and 72 in response to the necrotroph (N) and wounding (W). The untouched controls of respective clones are also shown (C). Data was normalized to internal reference gene *Cdc2* by dividing quantity of target gene by quantity of internal reference gene. N=3 for treatments and n=2 for controls; bars±SD

Figure 5. Contents of condensed tannins (CTs) in the local bark and systemic leaves of aspen clones 23 and 72 after necrotroph (N) and mechanical wounding at 1, 3, and 14days post inoculation. For treatments n=3 and controls n=2 and bars are ±SD

Figure 6. Contents of phenolic glycosides (TPs) in the local bark and systemic leaves of aspen clones 23 and 72 after necrotroph (N) and mechanical wounding at 1, 3, and 14days post inoculation. For treatments n=3 and controls n=2 and bars are ±SD

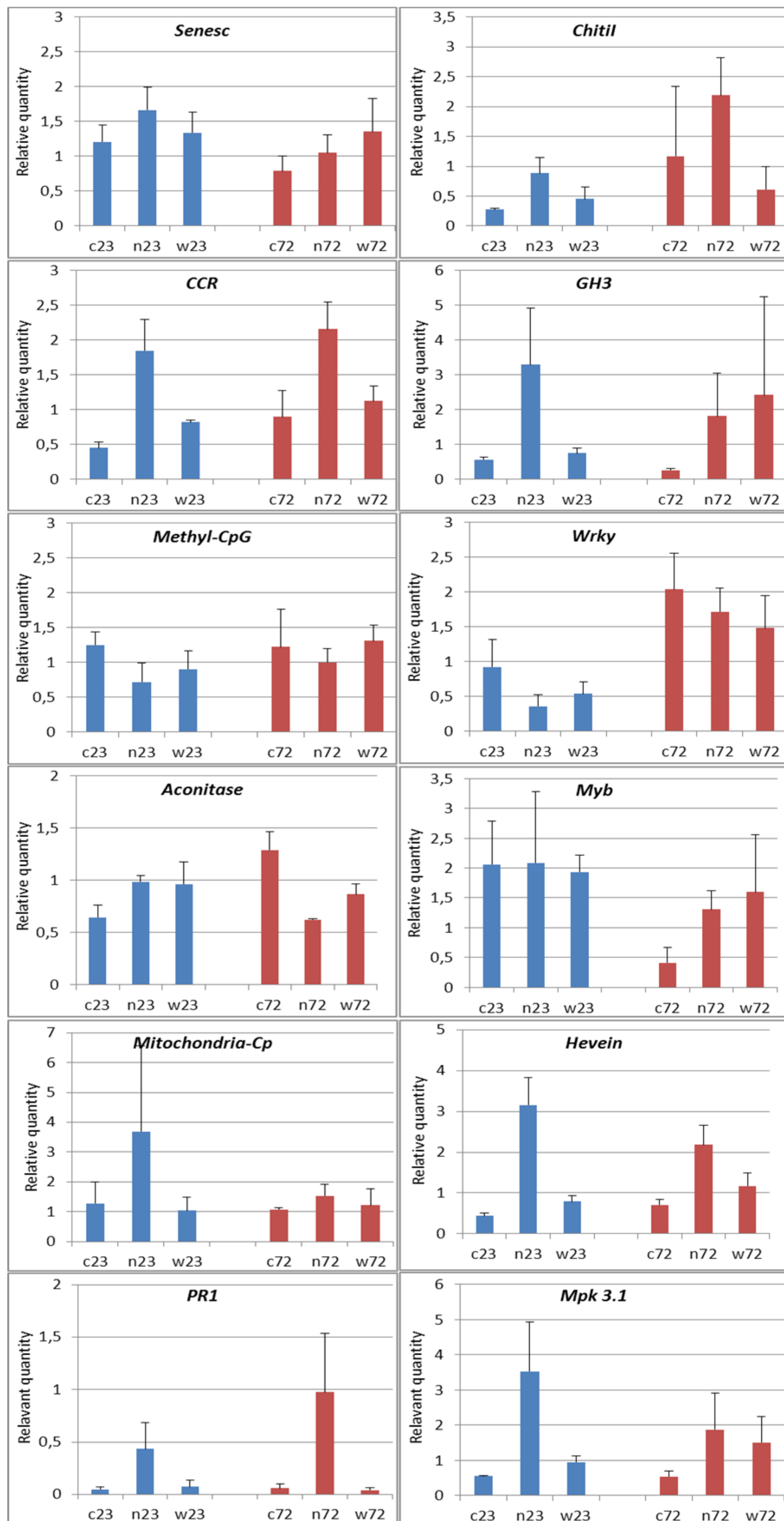

Figure 1.

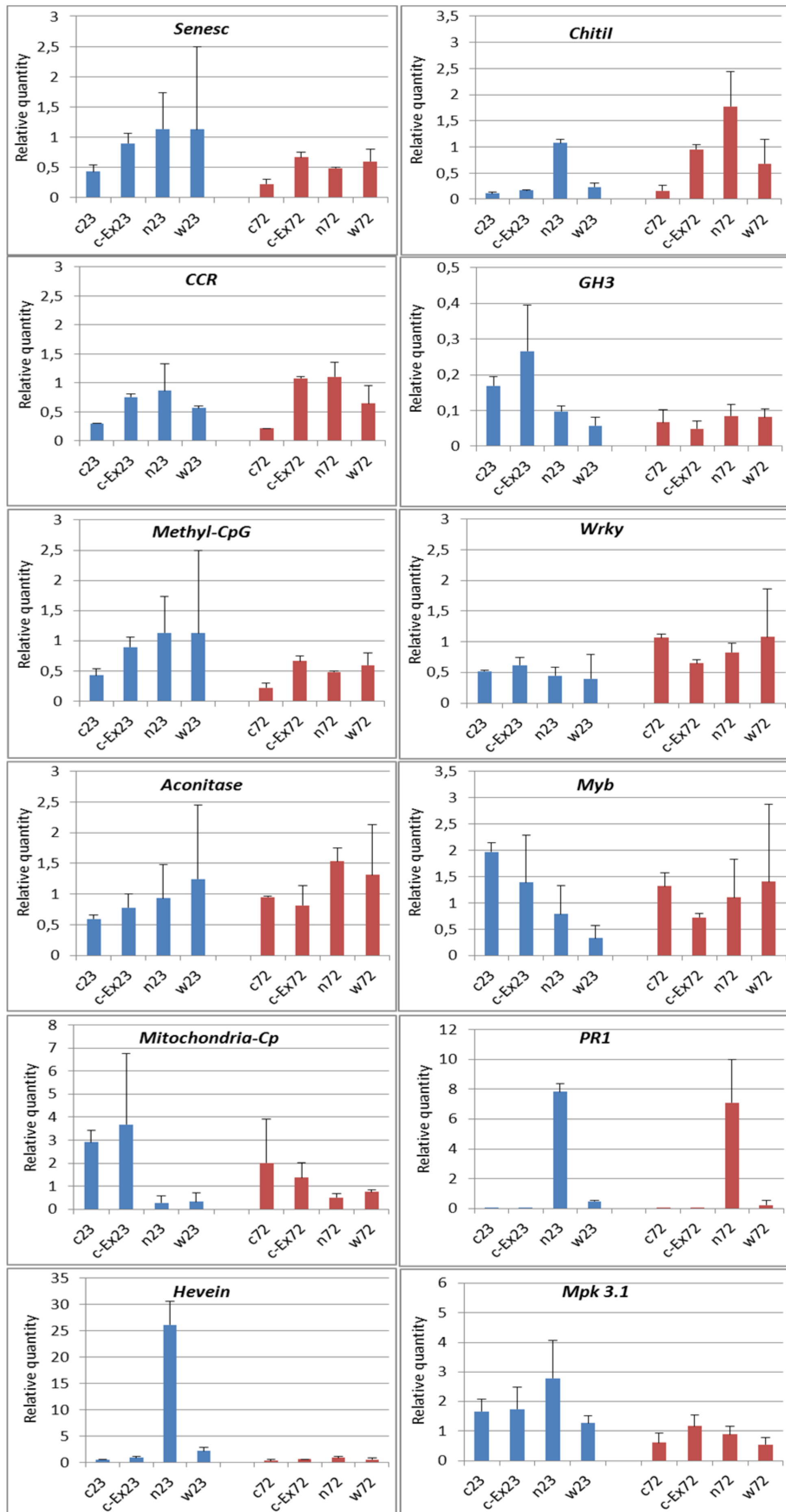

Figure 2

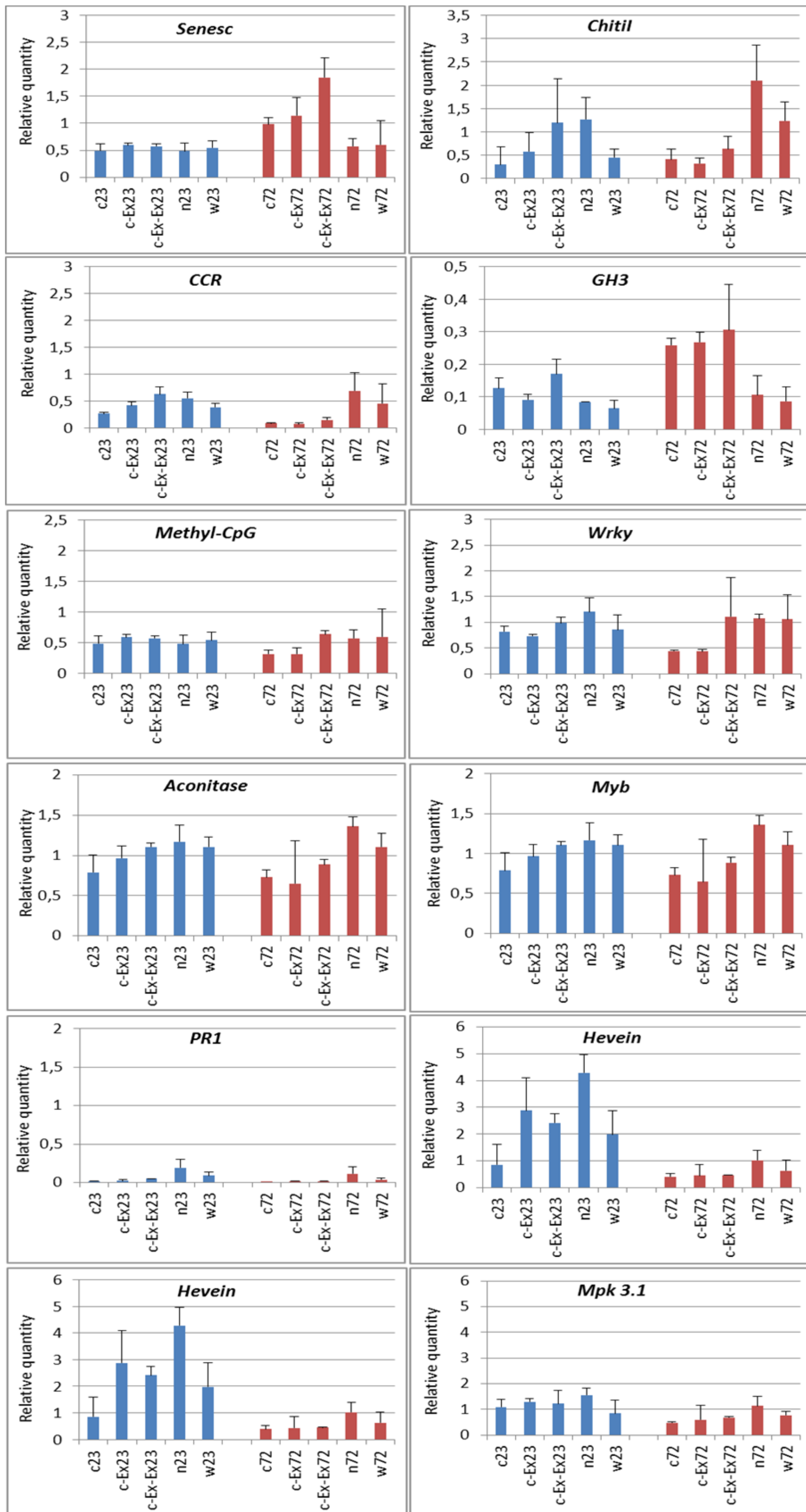

Figure 3

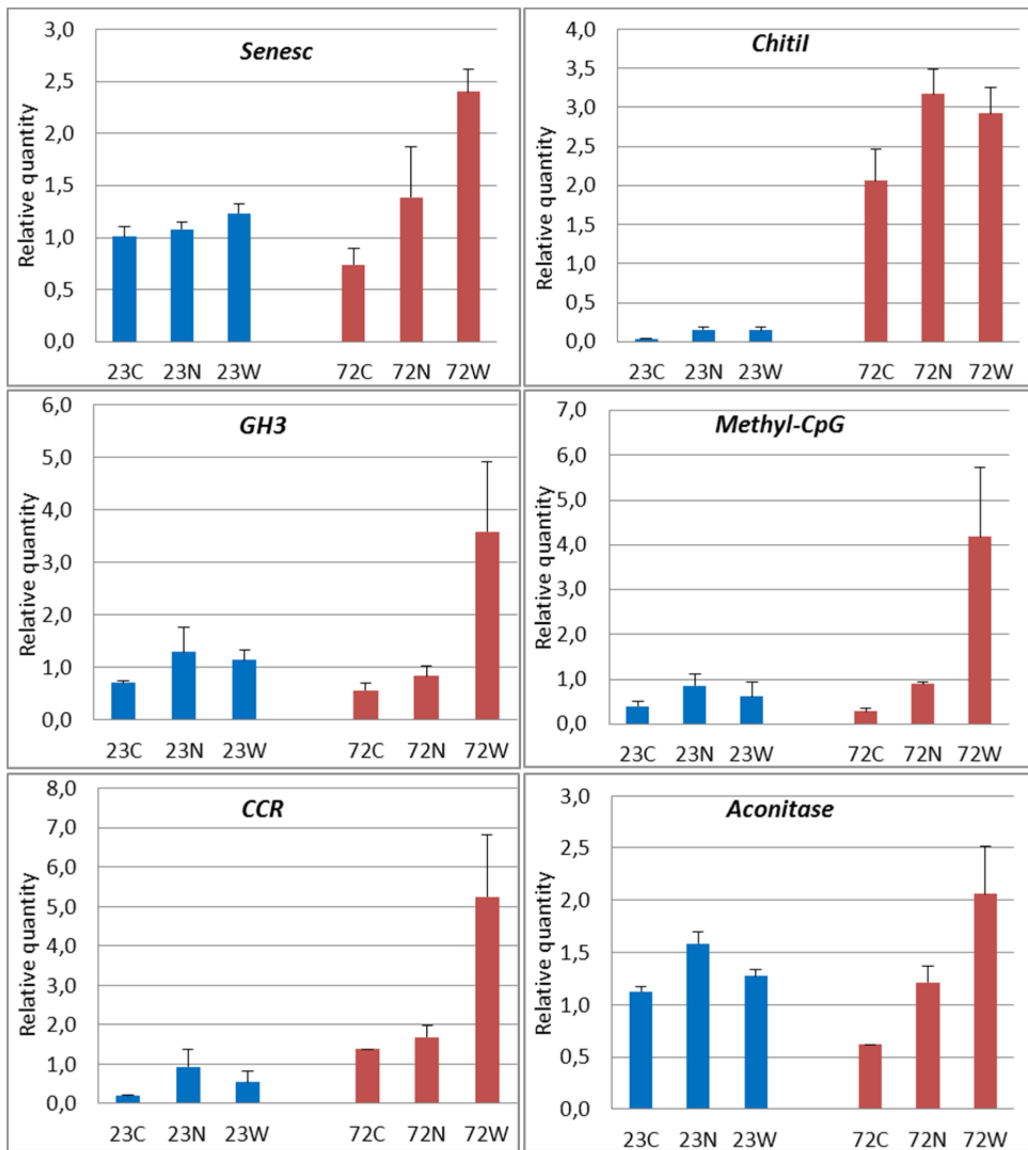

Figure 4.

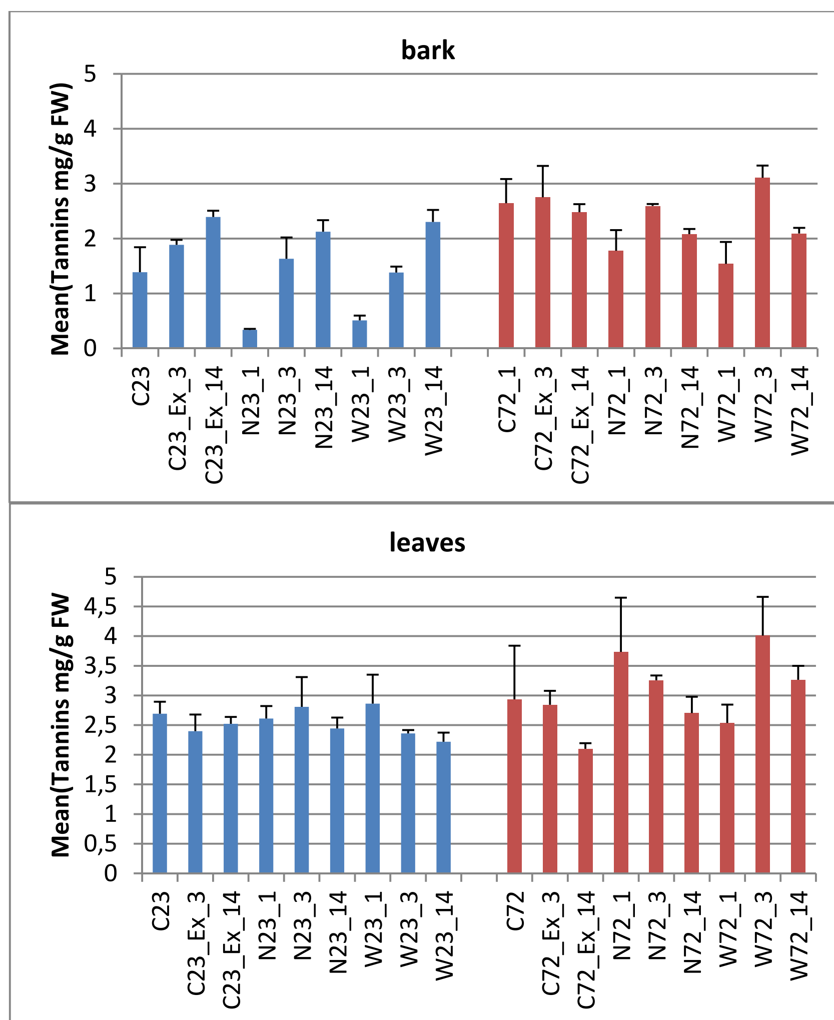

Figure 5.

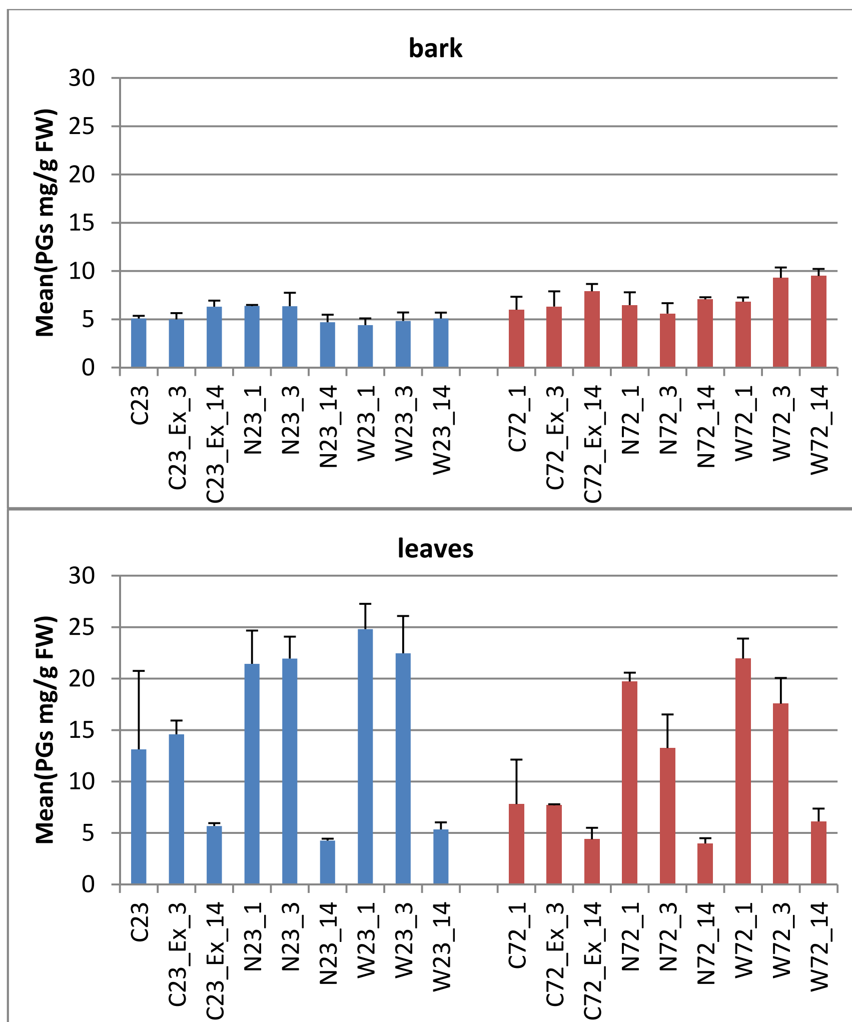

Figure 6.

Appendix

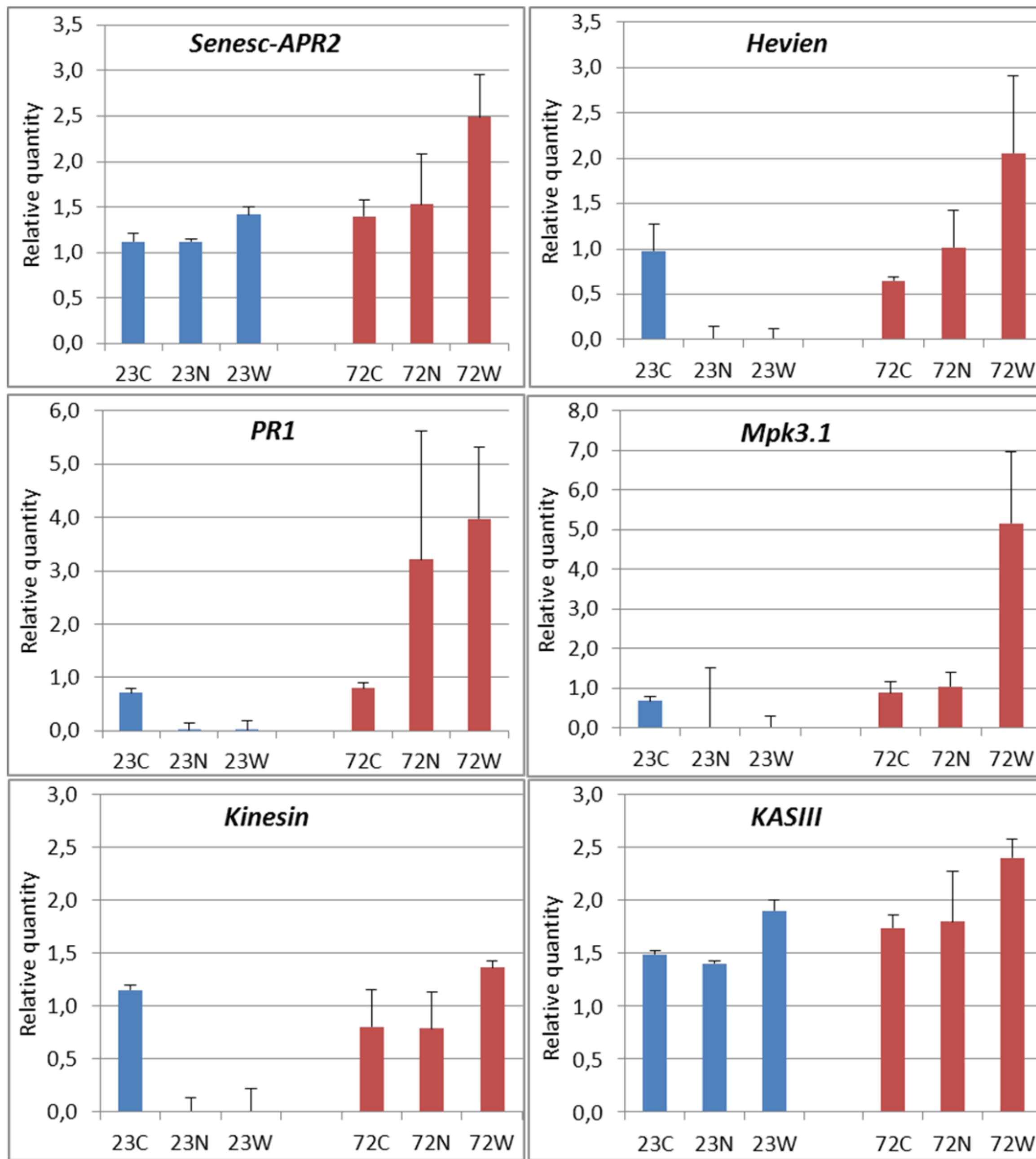

Figure s1. Systemic qRT-PCR of transcripts *SenescAPR2, Hevein, PR1, Mpk3.1, Kinesin* and *KASIII* in aspen clones 23 and 72 in response to the necrotroph (N) and wounding (W) at 1dpi. The untouched controls of respective clones are also shown (C). Data was normalized to internal reference gene *Ubq* by dividing quantity of target gene by quantity of internal reference gene. N=3 for treatments and n=2 for controls; bars±SD

Table s1. Selected gene products from microarray experiments (Yaqoob *et al.*, in prep) and literature

reporterId	Gene Model	reference	annotation	M	A	t	P,Value	B
PU03656	POPTR_0019s12390.1	At3g54420	class IV chitinase (CHIV)	3,00	13,56	9,73E+00	0,00E+00	8,68
PU28007	POPTR_0003s03400.1	At5g47060	senescence-associated protein	-1,20	11,03	-4,77E+00	1,00E-02	0,6
PU22335	POPTR_0013s03990.1	At5g28650	WRKY family transcription factor	-1,60	13,35	-6,16E+00	0,00E+00	3,21
PU22826	POPTR_0010s05320.1	At4g00416	methyl-CpG-binding domain	1,50	11,46	5,68E+00	0,00E+00	2,35
PU12629	POPTR_0001s30560.1	At2g14960	auxin-responsive GH3	2,40	14,00	8,48E+00	0,00E+00	6,96
PU26670	POPTR_0002s00600.1	At2g33590	cinnamoyl-CoA reductase	2,70	11,15	1,22E+01	0,00E+00	11,47
PU00529	POPTR_0005s10990.1	At4g35830	aconitate hydratase	1,70	13,83	6,26E+00	4,00E-02	3,33
PU26084	POPTR_0002s26160.1	At2g21650	myb family transcription factor	2,20	12,48	5,51E+00	5,00E-02	2,08
PU01150	POPTR_0012s11250.1	At5g51050	mitochondrial substrate carrier family prot	1,30	9,68	4,66E+00	1,00E-01	0,13
	POPTR_0001s29530.1	At2g14610	PR-1					
PU10439	POPTR_0009s07050.1	At3g45640	PtMPK3.1					
PU07591	POPTR_0013s03890.1	At3g04720	PtHEVEIN					
PU06293	POPTR_0004s14080.1	At3g48750	Ptcdc2					

Table s2. Candidate defence genes in Norway spruce analysed by real time qRT-PCR

Name	GenBankAccession No.	Predicted Gene	Froward/reverse primers used in qRT-PCR
CCR	XM_002329508.1	Senescence-associated protein	CGGGTTCCACTGTCTAGTGTTTCCA/GGGCCTTTGTTGCAGCCAACC
GH3	XM_002300212.1	GH3 auxin responsive promoter	GGCAAAGCTGGGCTGAAATGC/TGCAATTTCAAGAGGGGCATCCA
Methyl-CpG	CU232284.1	Methyl-CpG-binding domain	GTCGTGACCGTGCCGCTCTG/TGTCTTTGCTGGCTGCAACATGAA
WrkyY	XM_002319046.1	WRKY family transcription factor	GCCGGGCAACCCCTCAGACT/TGCCATTGTTCAAAGAGGAGGACACTG
Senesc	XM_002329508.1	Senescence-associated protein	GGCTGTTAGATCCTGATCTGGCCTTG/GCCTTCACTGTTCAAGCGTGACTCC
ChitiI	XM_002326005.1	ChitinaseI	CTCCTTCCGGCTGGCCCATA/CAACAGGCAATACCCATGTGTTGCAG
Aconitase	XM_002327692.1	Aconitase	TGCCAAGGGTCCAATGCTA/CGGTGAATTCGCTCAAAGCT
Myb1	XM_002303091.1	Myb-like DNA-binding domain	TGAAGACGTCCAGCAGATTGAA/GTTGCTAGCTCCAGATCTTCTAGTGTAG
Mitoch-Cp	CU222755.1	Mitochondrial substrate carrier	CTGGAGCCGCTTCTCGTAGT/CATACAAGCACGGGTAGTCTGAAC
Hevein	EF055879	Hevein	GACGGAGATGGATACGCAAAAGG/TCGACGATAGAGAGCAGAGGTTTAG
Mpk3.1	XM_002313981.1	Mitogen-activated protein kinase	CCATAAGCACTACCAGTCC/TCAGATACAGAGGAGTGC
PR1	eugene3.07480004	Pathogen related protein1	TGGGTTGATGAGAAACCAAAGTATG/GCTGCACCTTGCTTTAGCAC
Cdc2	AF194820	Cyclin dependent kinase2	ATTCCCCAAGTGGCCTTCTAAG/TATTCATGCTCCAAAGCACTCC

Comparison of the local and systemic change in NB-LRR expression and microRNA targeting NB-LRRs in Norway spruce after inoculation with *Ceratocystis polonica* and wounding

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Abstract

NB-LRR resistance proteins are involved in recognizing pathogens and other exogenous signals. NBS-LRRs may thus be the first step in inducing the defence response and are therefore of great interest to us. MicroRNAs (miRNA) are often involved in post-transcriptional regulating. We examined the expression of five Norway spruce miRNA putatively targeting putative NB-LRR related transcripts in secondary phloem (bark) of a resistance clone after wounding and inoculation with the necrotrophic blue stain fungus *Ceratocystis polonica*. We found local and systemic induction of the resistance marker genes *PaChi4, PaPAL* and *PaPX3* indicative of induced host defence response. There was also local and systemic change in the expression of the 5 miRNAs and 21 NB-LRRs in bark between healthy and treated plants. However, only the putative NB-LRRs PaLRR3 and Pa LRR13 showed greater than two fold changes. The miRNAs showed a rapid local and systemic down regulation at day 1 that was followed by a later increase to and beyond the constitutive levels. Overall the changes in expression both locally at site of treatment and systemically away from the site of treatment were minor for the miRNAs and predicted NB-LRR targets studied, suggesting that the expression of NB-LRR related genes are likely kept close to their constitutive levels and are possibly under tight transcriptional control in both stressed and healthy plants.

Keywords: qRT–PCR; Resistance; *Ceratocystis polonica;* Necrotroph; *Picea abies;* MicroRNA

Abbreviations:

qRT-PCR	Quantitative reverse transcription - polymerase chain reaction
C. polonica	*Ceratocystis polonica*
PaChi4	Chitinases class IV

1. Introduction

The recognition of the invading pathogen may be specific or indirect and the regulation of the resulting local and systemic plant defence responses likely to be complex and multi layered. Most disease resistance genes in plants encode NBS-LRR (NB-LRR) proteins, and they may comprise one of the larger gene families in plants [1]. The direct role and function of NB-LRRs in resistance, turning on the downstream defence genes needed for resistance have been shown in angiosperms, but NB-LRRs are also found in gymnosperms such as spruce and pine, and have been tightly linked to resistance to white pine blister rust [2, 3]. MicroRNAs (miRNAs) have been linked to regulation of plant defence response in *Arabidopsis* [4] and miRNA putatively targeting NB-LRRs have been found in Norway spruce [5].

In the NB-LRR genes, the LRR domain interacts with the products of pathogen AVR genes directly or indirectly and hence is thought to function primarily in the recognition of the presence of pathogens [6, 7]. More specifically, putatively solvent exposed residues in the b-sheet in the LRR domain are considered to interact with the pathogen gene products [8, 9]. The ability of each individual plant to recognize pathogens depends on what kinds of R-genes it has [10]. There have been significant recent duplications in perennial angiosperm species, compared with those of annuals rice and *Arabidopsis*, suggesting that NB-encoding gene expansion that may compensate for the longer generation time in woody perennial species [11]. The *Populus trichocarpa* genome contains over 400 NBS class genes, at least twice the complement of *Arabidopsis* [12]. Kohler and co-workers [12] have subdivided the *Populus* NBS into subfamilies with distinct domain organizations containing 119 CCC-NB-LRR genes, 64 TIR-NB-LRR genes, 34 BED-NB-LRR, and unusual NBS- and NBS-LRR-containing genes. They found that out of all these NB-LRR transcripts only 34 NBS-LRR genes were detected in rust-infected and non-infected leaves using a whole-genome oligoarray but none of these showed an altered expression levels two days post inoculation. The BED motif in BED-NB-LRRs was identified in poplar but its role, importance or presence in other trees is not yet clear [13]. However, the exact numbers of NBS-encoding genes in conifers and other gymnosperms besides the effort in pine (*Pinus monticola)* is far from established. Noteworthy, not only has Liu and Ekramoddoullah[2] found a great number of TIR-NB-LRRs (also called TNLs) in pine but also 61 of the CC-NB-LRR (CNLs) subfamily in the conifer *Pinus monticola* [3]. Also here is a wealth of transcript sequences available from several spruce species that allow identification of these proteins based on *in silico* based similarity

searches. However, genome- and deep transcriptome sequencing of gymnosperms following inoculations and also surveys of the within species variation in the different NB-LRRs is needed to get a firm grip of the actual number of and variation in these important genes.

miRNAs involved in pathogen associated molecular pattern (PAMP) triggered plant innate immunity have been identified in Arabidopsis [4]. miRNAs are post-transcriptional regulators that bind to complementary sequences on target messenger RNA transcripts usually resulting in translational repression and gene silencing. miRNAs are short ribonucleic acid molecules, and are on average found to be only 21 nucleotides long in pine and Norway spruce [5, 14]. miRNAs have been identified in loblolly pine associated with the fusiform rust gall disease [14]. In small RNA libraries from Norway spruce to discover miRNA involved in epigenetic modifications important for climatic adaptation, miRNAs targeting stress related genes were overrepresented and among these were miRNA that putatively targets NB-LRR transcripts [5].

The response of Norway spruce trees to wounding and necrotrophic pathogens show similarities. Norway spruce trees pre-treated with mechanical bark wounds exhibit strongly enhanced resistance to subsequent challenge inoculation to the necrotrophic blue stain fungus *Ceratocystis polonica* (Siem.) C. Moreau, a virulent associate of the Eurasian spruce bark beetle *Ips typographus* L. [15]. Similarly, induced local acquired resistance has been observed in response to a previous inoculations, resulting in smaller lesion length and less damage upon challenge with *C. polonica* [16].

Regulation of defence related genes is a key element in inducible defence mechanisms, and NB-LRRs or other host receptors recognizing the invader likely trigger the downstream induction of defence related genes. In Norway spruce a number of pathogenesis proteins are upregulated in response to pathogen attack and stress [17, 18]. .The chitinase *PaChi4* is a useful marker for local and systemic host response in Norway spruce [19]. Increased lignification as part of cell wall strengthening to combat the invading pathogen is a defence strategy likely employed by trees. Lignin related peroxidases are up-regulated after pathogen invasion in conifers and are involved in lignification and suberization of host tissues, as well as in production of reactive oxygen species [20-22]. The lignin related *PaPX3* have a general stress-induced function and are upregulated in Norway spruce bark in response to *H.*

parviporum infection [23]. *Phenylalanine ammonia-lyase* (*PaPAL*) has a key role in lignin and phenylpropanoid formation [23-25].

Here we follow the differential expression of 21 NB-LRR related genes and 5 putative miRNA targeting these as well as the defence response markers *PaChi4, PaPX3* and *PaPAL* in Norway spruce bark over a spatial and temporal gradient. Expression profiling of these key genes will enhance our understanding of whether NB-LRR transcriptional or miRNA changes may have a role in local and systemic resistance responses in Norway spruce, and reveal if there is differentially expression to wounding and pathogen stress.

2. Material and methods

2.1. Plant material and sampling

Two-year-old Norway spruce saplings generated by somatic embryogenesis from a single clone (AL15886-B10 derived from a single seed from a full-sib family of *Picea abies* (L.) Karst. [Cross ♀ #2650 X ♂ #2707] produced at Biri Nursery and Seed Improvement Centre, Norway) were selected for this study [26]. This clone has relatively high resistance to fungal infection, as it shows very short lesion length and recovers within a month from bark inoculations with both the necrotrophs *Heterobasidion Parviporum* and *Ceratocystis polonica*. The genetically identical saplings were kept in growth chambers under optimal temperature (20-22 °C) at ambient day length with 200-250 μmol m^{-2} s^{-1} light conditions and were well watered with sufficient fertilizers throughout the experiment.

Saplings were inoculated with *C. polonica* or wounded on the stem and control plants were left untouched. The *C. polonica* was inoculated into the stem at 5 cm height from the ground by cutting a ~5 mm small bark flap using a scalpel, wounding was identical treatment but without the fungus. A small amount of malt agar (1% malt and 1.5% agar) containing actively growing mycelium was placed underneath the bark flap, pressed firmly against the stem, and sealed with parafilm. Unwounded control plants were also wrapped with parafilm at the corresponding position of the stem. Local and distal samples of bark were harvested 1, 3 and 6 days after inoculation. At least two ramets were harvested for each treatment at each time point. Samples were quick-frozen in liquid nitrogen and stored at -80 C until analysis. To study the local and systemic gene expression in response to fungal inoculation and bark, local and more distal sections of bark were analysed separately. The first section was 1 cm long and

included the inoculation site and ca 0.5 cm above the upper margin of the inoculation wound. The second were taken 2-3 cm above the inoculation site. The sections were named W1 and W2 for wounding ramets and Cp1 and Cp2 for fungal infected ramets, with position 1 being closest to the inoculation wound. The unwounded controls were analysed at the corresponding position, and at each sampling position samples were analysed separately.

2.2 LRR gene annotation, peptide structure and phylogenetic analyses

Initial searches for LRR family genes were performed with the use of the LRR family conserved catalytic domain derived from a range of LRR gene sequences available from National Centre for Biotechnology Information (NCBI) GenBank (http://www.ncbi.nlm.nih.gov/) to probe the *Picea* (taxid:3328) sequences at the NCBI Database using BLASTP algorithms. Further all obtained unique sequences were verified using NCBI blast server with BLASTP search against the NCBI GenBank and the Conserved Domain Database (CDD) [27] (Supplement 5). Gene models containing LRR motif that were chosen for further examination (Table s3).

Phylogenetic and molecular evolutionary analyses for the translated amino acid sequences of the selected LRR gene models presented in supplemental figure 2 were conducted using the MEGA4 [28]. The evolutionary history was inferred using the Neighbour-Joining method. The bootstrap consensus tree inferred from 500 replicates is taken to represent the evolutionary history of the taxa analysed. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The phylogenetic tree was linearized assuming equal evolutionary rates in all lineages. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Poisson correction method and are in the units of the number of amino acid substitutions per site. All positions containing alignment gaps and missing data were eliminated only in pairwise sequence comparisons (Pairwise deletion option).

2.3 Selection of putative NB-LRR targeting miRNAs

The 5 putative NB-LRR targeting miRNAs PaLRR25, PaLRR26, PaLRR27, PaLRR28 and PaLRR29 in table s1.2 followed were identified and found to have all the *in silico* characteristics of microRNA in an earlier work to identify miRNA involved in epigenetic regulation and climatic adaptation [5].

2.4 Total RNA extractions and cDNA synthesis

Tissue samples (100-150 mg) were ground first in liquid nitrogen using a mortar and a pestle and then transferred to 2 ml eppendorf tubes for fine grinding in a Retsch 300 Mill (Retsch Gmbh, Haan, Germany) for 1.5 minutes. The equipment and tissue samples were kept chilled with N_2 throughout the grinding process. Total RNA (including small RNA fraction) was extracted from 100 mg powder of the pulverized samples using the Master Pure™ RNA Purification Kit (Epicentre, Madison, WI, USA, #MCR85102) following manufacturer recommendation and stored at -80°C until further use. RNA was quantified using micro-volume spectrophotometer Nano Drop 2000 (Thermo Scientific, Wilmington, DE, USA).

2.5 cDNA synthesis and real-time RT-PCR

Gene specific primers were designed using Primer3 [29] and following criteria: melting temperature 70°C and product size inferior to 120 bp. The list of the studied gene NBS-LRR like homologs and their primer sequences can be found in table s1.1. The specificity of all primers were tested by running the PCR product on 2% agarose gel after using RT-PCR amplification parameters. Only primers producing clear one-band pattern on gel were used for RT-PCR experiment (Supplement 4). The primers used for quantification of the *PaChi4, PaPAL* and *PaPX3* are as listed in Deflorio and co-workers [30]. Total RNA was reverse transcribed (1000 ng per reaction) using the TaqMan Reverse Transcription kit (Applied Biosystems, Carlsbad, CA, USA, # 8080234) in 50-µl-reaction volume and diluted up to 200 µl. PCR amplification was performed in a 25-µl-reaction volume, using 2 µl of diluted cDNA solution as template, 12.5 µl of 1x SYBR Green master mix and 200 nM of each primer. RT-PCRs were performed using the 7500 Fast Real-time PCR System (Applied Biosystems, Carlsbad CA, USA) with standard cycling parameters. All reactions were done in triplicate and no-template control was run for each primer pair. For data analysis, the arithmetic mean of two biological replicates was calculated. Target gene expression was normalized to transcript level of actin (*PaAct*).Absolute quantification was performed using 7500-system SDS software. Data were further processed in MS Excel.

2.6 Quantification of miRNAs

Transcript abundance of the selected miRNAs was determined with relative real-time RT-PCR as described by Yakovlev and coworkers (2010). cDNAs were synthesized from 600 ng

of small RNA with the NCode miRNA First-Strand cDNA Synthesis Kit (MIRC-50; Invitrogen) following the manufacturer recommendations. Real-time RT-PCR amplification was performed using NCode SYBR GreenER miRNA qRT-PCR Kit (MIRQER-100; Invitrogen) in a 25 µl reaction volume, using 2 µl of a diluted cDNA solution already described as template and 200 nM of each primer. Reactions were conducted on the 7500 Real-time PCR System (Applied Biosystems, Foster City, CA, USA) using the Invitrogen recommended cycling conditions. After PCR, dissociation curves were carried out to verify the specificity of the amplification. There were two technical and two biological replicates for each sample. All expression levels were normalized to geometric mean of three selected ribosomal and transfer RNA genes. Forward primers were designed based on mature miRNA sequence. If Tm of mature miRNA was < 60 °C, it had been adjusted by adding Gs and Cs to the 5-end and ⁄or as to the 3-end of the miRNA sequence. To verify the specificity of the miRNA amplification, we analysed several PCR samples for each miRNAs on 2% agarose gels with ethidium bromide (EtBr) visualization of bands. Reverse primer was supplied with the NCode miRNA First-Strand cDNA Synthesis Kit (MIRC-50; Invitrogen). miRNA primers used are listed in supplemental table s1.2 and table s21.
The relative ddCT quantification method using the critical cycle threshold values was performed for the miRNAs using 7500-system SDS software. Data were further processed in MS Excel.

3. *Results*

3.1. Host defence response induction

Transcripts level of the class III peroxidase *PaPX3 and the class IV chitinases PaPaChi4* increased both as a local (W1, Cp1) and systemic (W2, Cp2) host response in bark following both the necrotrophic pathogen *C. polonica* (Cp) and wounding (W). The *Phenylalanine ammonia-lyase PaPAL* increased as a local response to necrotroph and wounding, but not systemically. The local increase peaked by day 3 and fell toward constitutive levels by 6 days. The local increase was more rapid toward the necrotroph than wounding. In contrast, the systemic response was more evident toward wounding for both *PaChi4* and *PaPX3*, and the systemic increase was lower and delayed compared to the local response (Fig.1).

3.2. Putative NB-LRRs and Putative NBS-LRR targeting miRNAs

Using the procedures described in the material and methods we found NB-LRR related spruce sequences that clustered into 6 groups; one RI family, NB-LRRs, CC-NB-LRRs, TIR-P-loop—LRRs, LRR receptor-like kinase, Plant intracellular Ras-group-related LRR. No BED-finger-NB-LRRs were discovered in our search among available spruce sequences. The five *Putative NBS-LRR targeting miRNAs* PamiR950, PamiR9501, PamiR3693, PamiR3697 and PamiR3705 were identified in an earlier work [5], and their targets based on similarity determined to be PaLRR25, PaLRR26, PaLRR27, PaLRR28 and PaLRR29. Of these five, PaLRR27 is predicted to be a CC-NB-LRR, while the remaining four are predicted TIR-NB-LRRs (Table s2). All these NB-LRRs and miRNAs were followed by real-time based quantification and the primers pairs used gave only one single sized PCR product with corresponding single peak melting point for each corresponding target (Fig. s4).

3.3. Differential expression of the putative NB-LRRs in response to necrotroph and wounding

The PaLRRs 1, 2, 6, 8, 12, 14, 16, 19, 26, 28 and 29 showed minor or close to constitutive level in all treatments and only *PaLRR3*, *PaLRR13* and PaLRR25 showed more than twofold changes (Fig. 1). In the TIR-NB-LRR related PaLRR5 there was a rapid twofold reduction in response to wounding and necrotroph locally at site of infection and systemically at the distal site, and a weak but similar trend was seen also for PaLRRs 6-8. PaLRR25 was down regulated only in response to the necrotroph both locally and systemically. The RI-like subfamily related PaLRR13 increased locally in response to wounding and less so to the necrotroph, and then levels returned to constitutive level by day 6. The CC-NB-LRR related PaLRR3 showed an opposite pattern or change between wood and necrotroph and the greatest difference was the large and rapid systemic increase in response to the necrotroph. The TIR-NB-LRR related PaLRR27 show first a rapid drop followed by an increase at day 3 before returning to constitutive levels at day 6. For PaLRRs 11, 15, 17, 18, 19 and 26 there is a tendency of a minor systemic increase due to both wounding and necrotroph treatments at the last time point.

3.4. Differential expression of the miRNAS in response to necrotroph and wounding

For the microRNAs the values are all relative and given as PCR cycle differences (ddCt, so here a values of 1 equals 2^1) while absolute quantitative values are given for the NB-LRR-like transcripts above. The miRNAs show a similar trends of expression in both wood and bark with the exception of PamiR951 that show a more than twofold increase as a systemic response to the necrotroph only (Fig. 1). The most noticeable pattern is the small but rapid

downregulation of PamiR950, PamiR3693, PamiR3697 and PamiR3705 in both wounded and necrotroph inoculated samples. By day 3 the levels are close to constitutive and at day 6 there is a systemic increase due to both wounding and necrotroph for PamiR950, PamiR3693 and PamiR3705. The presumed miRNA targets PaLRRs 25 to 29 do not show patterns of expression varying as much as and not consistently in opposite direction to the corresponding miRNAs.

4. Discussion

This is the first study to compare transcript levels of putative NB-LRR targeting miRNA in wounded and necrotroph inoculated Norway spruce and the first to look at their expression at different distances from the site of treatment. We found differential expression of the induced defence response marker genes *PaPAL, PaChi4* and *PaPX3*, miRNAs and NB-LRR-related transcripts locally at the site of treatment and distally as a systemic host response. However the level of differential expression changes were small for most NB-LRRs and all miRNAs followed.

Previous studies have established that some Norway spruce trees that are more resistant to *H. parviporum* have been found to have rapidly induced defence related genes (e.g. peroxidases, class IV chitinase and others) [19, 31], and they also appear to have a more efficient systemic defence signalling in response to pathogen attack than susceptible genotypes. In this work with a relatively resistant Norway spruce clone we find indications that not only the downstream genes such as *PaPX3* and *PaChi4* but also individual NB-LRRs-like transcripts, and miRNAs targeting these, potentially involved in the initiation of defence responses are differentially regulated during the first day as local and systemic responses to both the necrotroph and wounding. Little is so far know about how NB-LRR and related genes are regulatedin response to pathogens and other stresses and how they are expressed in trees, and this even includes the *Populus* species [1]. This can partly be due to the large size of this gene family and the high degree of sequence similarity between these genes. To establish a link between the miRNA expression of PamiR950, PamiR951, PamiR3693, PamiR3697 and PamiR3705, the NB-LRR candidates 25-29 and the downstream defence induction markers in Norway is obviously a very difficult task based on the transcriptional data obtained here, but the rapid and small but consistent down regulation of the PamiR3693, PamiR3697 and PamiR3705 would be expected to allow for greater expression of their putative targets. There could be several reason for the lack of correspondence between the miRNA and predicted

NB-LRR target sequences; the change in miRNA levels detected here are simply too small to have a significant impact on the targets, the targets act preferentially on other paralogues than the ones identified here, the identified targets could be also the origin of the miRNAs complicating the matter even further, the expression of the targets genes is under tight control of positive and negative regulators that bind their promoters or that these miRNA might inhibit only the translation of and not cause breakdown of their putatively bound targets. Of the different explanations, we find it most likely that the small changes in miRNA levels seen here may not be enough to overrule any regulatory changes by transcription factors at the promoter of these genes. It is also possible that looking at changes in NB-LRRs and miRNA that control these should have been performed at a much earlier time after treatments, and preferentially only individual host cells at the site of inoculation and wounding (at least for looking at the actual recognition of the pathogen). However, the combined results suggest that miRNA might be involved in the local and systemic defence response in Norway spruce, but a much larger set of small RNAs extending to other targets than NB-LRRs need to be studied in combination with corresponding transcriptome studies of all targets by way of last generation sequencing methods.

Western white pine shows genetic variation in disease resistance to white pine blister rust and in their TIR-NB-LRR and CC-NB-LEE genes [2, 3]. Liu and Ekramoddoullah [2] proposed that a large and diverse NB-LRR gene family be functional in conifers and that conifer resistance genes share a common origin with R genes from angiosperms. NB-LRRs have been reported in the gymnosperms *Pinus taeda* (L.) [32], *Cryptomeria japonica* [33], *Picea abies* (L.) [34], *Pinus lambertiana*, and *P. monticola* [35]. There is now also available large numbers of transcripts from spruces and other conifers. We found 259 LRR matching sequences at the DFCI Spruce Gene Index (Sgi, Release 5.0, dt. 30.03.2011) (http://compbio.dfci.harvard.edu/cgi-bin/tgi/gimain.pl?gudb=spruce). *In silico* analysis of these confirm a reasonable repertoire of defence and resistance-like genes in spruce, in our search we found all types of LRR genes: NBS/LRR, CC/NBS/LRR, TIR/P-loop/LRR, F-box/LRR, L-zip/NBS/LRR, LRR receptor-like kinase, Plant intracellular Ras-group-related LRR, etc. We did not find any BED-finger LRR protein genes among available spruce ESTs.

In White pine the TIR-NB-LRR homologue PmTNL1 is a resistance gene that is linked with partial resistance to white pine blister rust (WPBR). The *PmTNL1* transcript is expressed at low basal levels in different tissues and exhibited similar low level expression patterns during

compatible and incompatible interactions of *P. monticola* with *C. ribicola* at early stages of post inoculation, but higher levels are found in plants that shown WPBR symptoms at later stages of interaction [36]. The PaLRR15 belonging to the LRR containing IR class show a similar increase at the latest time point to both wounding and the pathogen. However, no similar tight linkage between NB-LRR genes to that seen in pine have been found so far in spruce, but we do see the low and close to stable levels of expression of most NB-LRR related genes also in our interaction between *C. polonica* and Norway spruce within the six days monitored. This fits with notion that resistance genes such as NB-LRRs genes are typically expressed constitutively at low basal levels and without increase after pathogen challenge [36, 37]. The results in Norway spruce thus support that most NB-LRRs are kept close to their constitutive levels, and agrees with the work on *Populus* as well. Kohler and coworkers [12] examined the diversity of NB-LRR genes was examined in the *Populus trichocarpa* genome and of the 400 homologues found that only 34 NBS-LRR genes expressed in rust-infected and non-infected leaves using a whole-genome oligoarray, and none of these showed an altered expression two days post inoculation. However, for individual TIR-NB-LRR homologues such as PaLRR3 we saw up to six times increase systemically following inoculation at day one after infection so we cannot rule out that at least some R-like gene are regulated at the transcriptional or posttranscriptional level within hours after attacked and also that the constitutive level of individual NB-LRRs may be heightened in primed plants[38].

Acknowledgements

We thank Lars Dalen for support during the sample collection, Inger Heldal for help with sample preparation, and Harald Kvaalen for providing the clonal plants. The project was supported by the Research Council of Norway (Grant No 136001).

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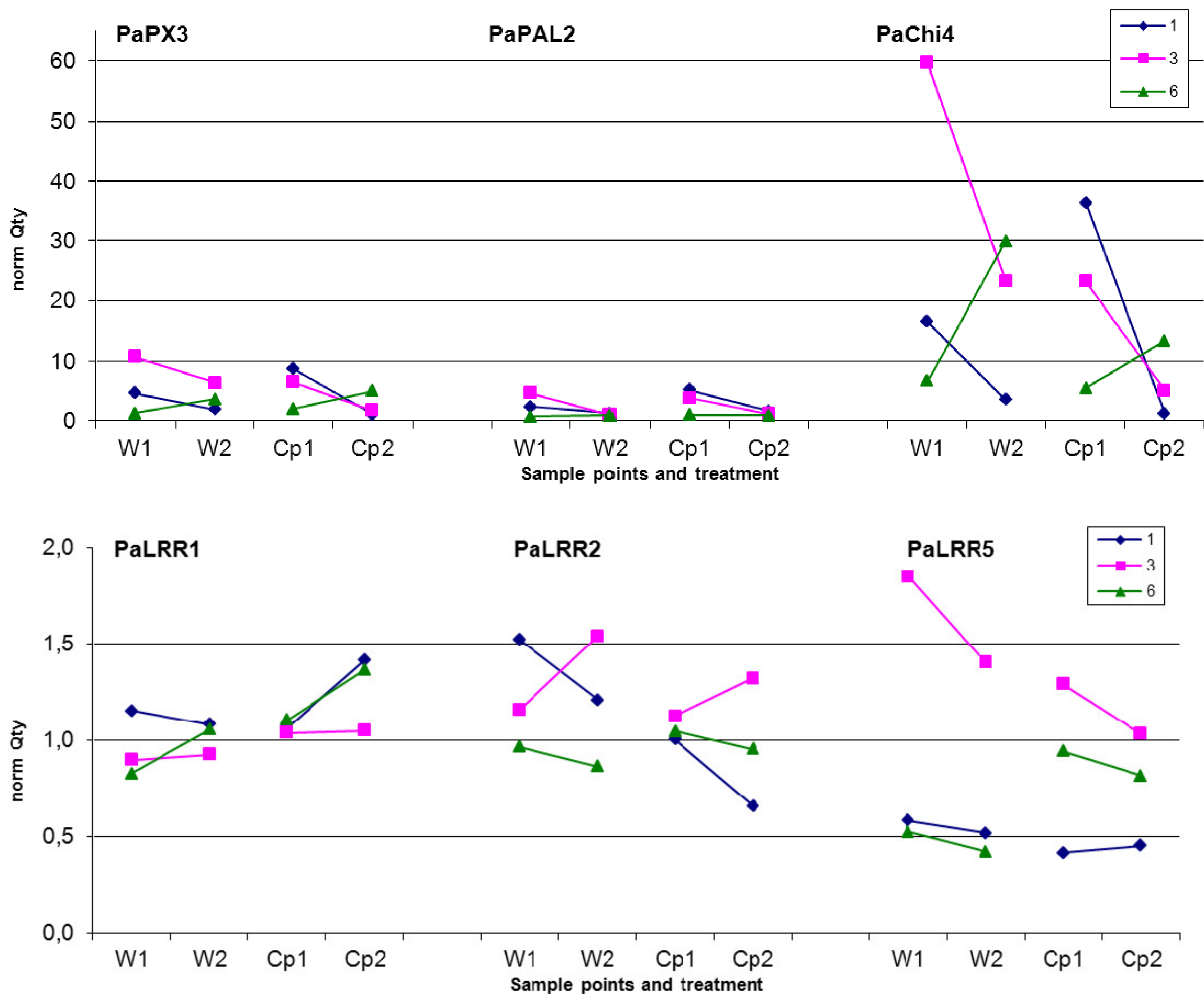
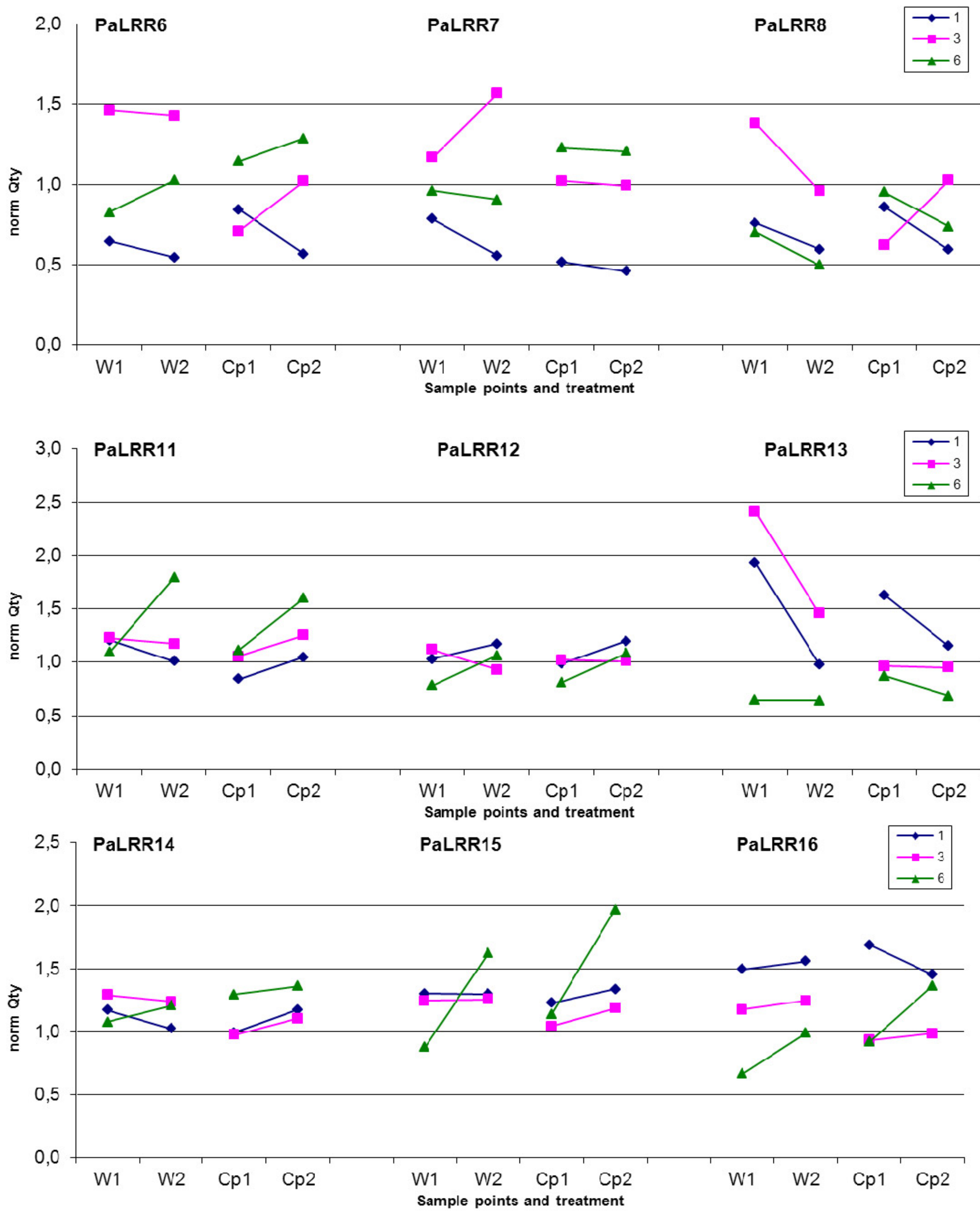
Figure captions

Figure 1. Expression profiles of the induced defence markers, NB-LRRs and miRNAsin the bark (of Norway spruce 1-6 days after inoculation with the necrotroph *C. polonica* and wounding) (Cp, W). Numbers following B and W indicate different sampling positions above the treatment site (1 = at treatment site; 2 = 2-3 cm above). The quantitative values indicate change relative to the healthy controls. One unit of relative Ct value corresponds to a two-fold difference in transcript level (see materials and method for relative Ct value calculations).





PaLRR6
PaLRR7
PaLRR8
1
3
6
2,0
1,5
1,0
0,5
0,0
norm Qty
W1
W2
Cp1
Cp2
Sample points and treatment
PaLRR11
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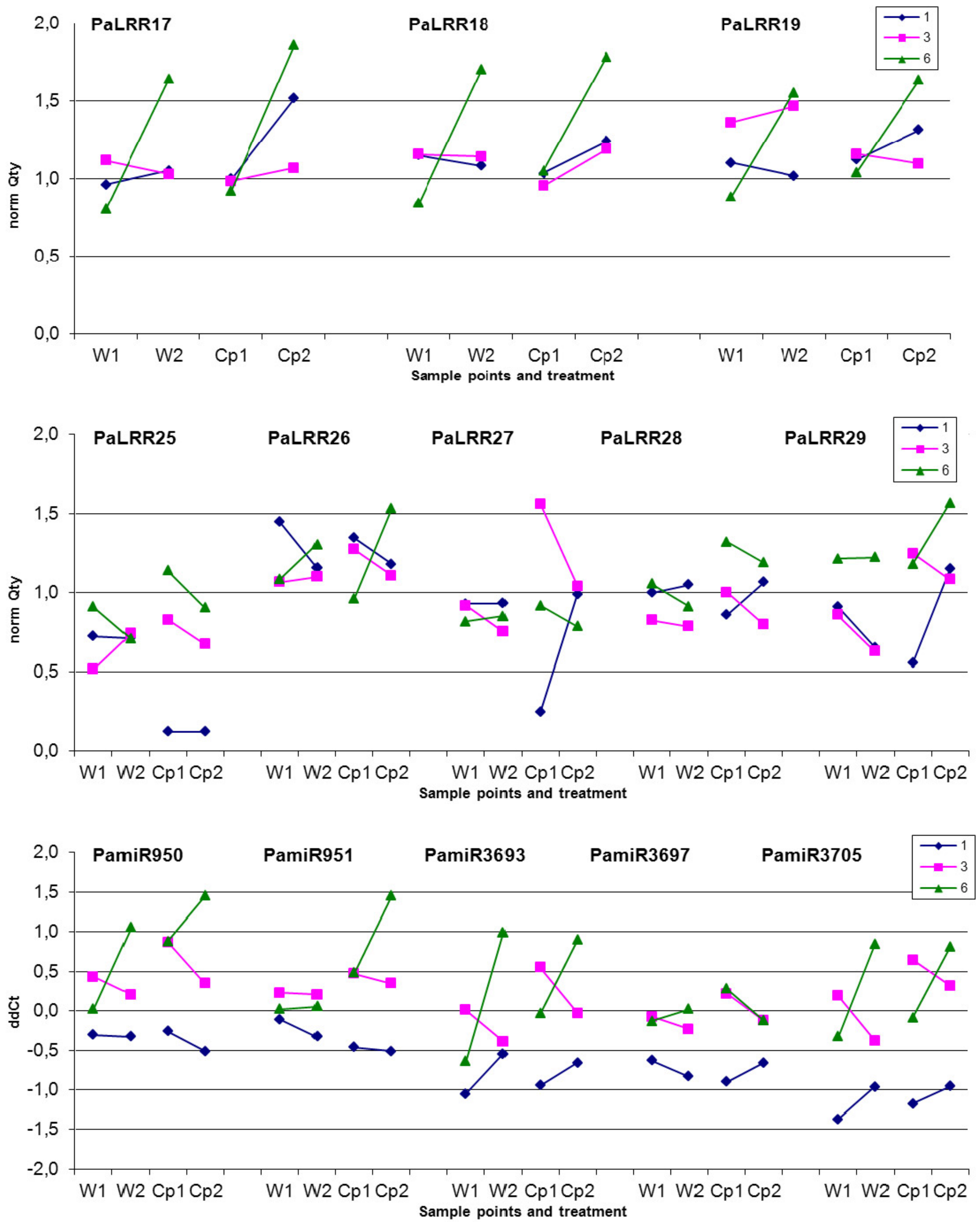
2,5
PaLRR14
PaLRR15
PaLRR16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PaLRR18
PaLRR19
1
3
6
2,0
1,5
1,0
0,5
0,0
norm Qty
W1
W2
Cp1
Cp2
Sample points and treatment
PaLRR25
PaLRR26
PaLRR27
PaLRR28
PaLRR29
PamiR950
PamiR951
PamiR3693
PamiR3697
PamiR3705
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-1,0
-1,5
-2,0
ddCt

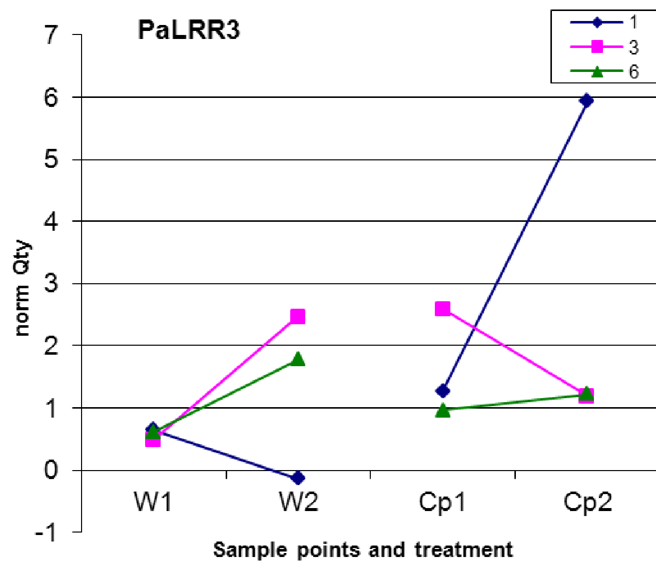


Figure 1

Appendix

Supplement 1 LRR genes spruce_primers. The two tables give the description of primers used for real-time RT-PCR. NB-LRRs in upper table and miRNAs plus their putative NBs-LRR targets in the lower.

Supplement 2. Cluster analysis of NB-LRRs

Supplement 3_Cluster description. . Description of motifs found in transcripts used in cluster analysis in Supplement 2.

Supplement 4. Gel showing primer test of the identified NB-LRRs. Only primer pairs giving a single band was further used for real-time RT-PCR. Therefore, NB-LRRs 22, 20, 10, 8 and 4 were not studied by qRT-PCR.

Supplement 5. Description of motifs used to identify spruce NB-LRR like transcripts.

Supplement 1

Table s1.1 Selected LRR family genes in Norway spruce analysed by real time qRT-PCR

Cluster	Group		Protein ID	Nucleotide source sequence	Gene name	Primers (5′→ 3′), F/ R	Product length, bp
1	1	1	ABK24805	gi_116788244	PaLRR2	TGGCAAGCCGGAAGATGAGG/ CCAGCAGGGTTTCCAGCAACG	120
2	2	3	ABF73316	gi_104642234	PaLRR11	GCACAAGCGTCCTGCCCATT/ ATCCTTGCCCGTTGCCTTGG	101
		4	ABR18304	gi_148910456	PaLRR12	AAAGTGGCCTTGCTTTGCACTGA/ TGCGACAAAGTATTTCATCATGCACCTT	113
	3	19	ACN41041	gi_224286674	PaLRR13	TGTGGGCCAATCCCAAGAGGAA/ CCACAGGGATGTAATGGCAAACC	97
	4	21	ABR17329	gi_148908431	PaLRR17	CATTGGCTTGACCCGATCCTCTC/ GCTTCAAATGCTTACTCCATGGTTCACA	95
3	5	26	ACN40981	gi_224286550	PaLRR9	GGAGTGCTCAGGATCGAAGAAGATGC/ GCTTTGATGGACAACCACGCTCCTA	111
		27	ABR16233	gi_148906150	PaLRR10	GCCTGATGACCTGGGCAAGTTAAGG/ TCGCAAATGGAAGCTGGAAGCA	90
	6	30	ABK24822	gi_116788283	PaLRR3	CACATTGATCTTCAGGGATGCCACA/ CGGCAGCCATTCCAAATCGT	114
		32	ABR18026	gi_148909879	PaLRR18	GCACAACCGTTCTACCTGTGAGAGAG/ CTGGCTGCTCCTCAAATCTTCCA	106
	7	36	ABR18473	gi_148910813	PaLRR6	TGGTGGAGGATCTCCCGCAAG/ TGCAATTGTGGAAACTCGCAAATACAA	115
		37	ABR18096	gi_148910025	PaLRR7	TGCAATGAGTTGATGAAATTGGACTTG/ TGTACCAAATGGTCCTGTCATGCTCTC	111
4	8	39	ACN39861	gi_224284250	PaLRR8	TGCAAGAACTCAATTTCCAAGGTTGC/ TTTCGCAAGAGGATAAATCCAATATTCTCA	102
	9	40	ABR16849	gi_148907432	PaLRR20	TGGTGCAGCTGACAGGACTTCG/ TTCCGACACCCACCGGCATT	118
	10	47	ABR17027	gi_148907802	PaLRR21	TCCTCCGTTGCCAATGCTCA/ GGGCAGACACCTTCACCAAAGGA	104
	11	54	ADE76430	gi_294461742	PaLRR22	TGCCAAAGTCCCTCGGCAAC/	114

						CCCGCAACTCTTCCAGCGATAACA	
	12	56	ABR17471	gi_148908727	PaLRR23	CGCCCTACGGTTGGAGGCTGA/ CGCAGTGCATTCTCCCGCTTG	93
5	13	57	ABK24857	gi_116788373	PaLRR19	AAACTCTGATCCCGTGAAGGCAAAC/ CAACATTTGGCCGGGTATGGAA	100
		58	ABK24560	gi_116787569	PaLRR24	TCGACTCCGGGAATGCCCTCT/ AGCGTACTTCCATATCCTCGGCCTTG	90
	14	60	ABR18087	gi_148910004	PaLRR1	TTCCAAGGCAGTGAAGCGTTAGCAG/ CCCGAATGGAACCAGAGCCAAG	90
		62	ABK25485	gi_116790053	PaLRR16	TGGGCTTTCCGTGCTCTCCTT/ AGATAGCCAGGTCTCCAGGGTTTGA	120
		63	ADE76117	gi_294461100	PaLRR14	TGCTCTGCTGGATTGCGAGAGTT/ ACTGCACCCACGGGCTTCCA	106
		65	ABR17804	gi_148909411	PaLRR15	CGACAGTAAGTTGTGGGTGTTCTCAAGG/ CAAACTCGGAATGTTGAATGGCTGGT	103
6	15	72	ABR17008	gi_148907764	PaLRR4	GGGATCGGCGCAAGCAACA/ GCTCTTCAGATTCACACATCCGTCCA	100
		73	ABR18194	gi_148910225	PaLRR5	TGCCTGGTGGAGGTGGACAGG/ GCGCCAAAGACGACACGGATT	108

Table s1.2. Selected miRNAs and their putative target LRR family genes in Norway spruce analysed by real time qRT-PCR

MiRNA ID	Nucleotide source sequence	Gene name	Primers (5′→ 3′), F/ R	Product length, bp	miRNA primer sequence (F)
pab-miR950-3p	ES261905	PaLRR25	TCTCCGTCAGGCGGGTGTTT/ TTGAGGAAGGCAGTTTCAAGCAGAG	80	TCTGGGCCCCGGTGGTTTATGA
pab-miR951	DR563813	PaLRR26	TGCAGAGCGTATTGGATCTTGCTTCT/ GGAGTGGGATGTGGACGAGGATG	88	TGTTCTTGACGTCTGGACCACG
pab-MIR3693 (pab-miR029)	EX326419	PaLRR27	GGAGCTGCTCGTAGGTGAGGGATG/ GAAGTTTGCTCCATTTGAGCGCTTGT	97	AGAGGGTGCTCATGAACTGCTC
pab-MIR3697 (pab-miR065)	EX427311	PaLRR28	CCCTCGCATCCTCCTCATCCA/ TGGCTGGCGAGACCTTTCTTTACATC	106	TAGCCCCTGACTTCAACATGAG
pab-MIR3705 (pab-miR119)	DR499063	PaLRR29	CCGTTAAACTTGCCGCCATCCA/ CAGCCACGATATGACCCCGACA	101	GTAAGTGGTTATGATCTGGAC

Table s2. Isolation and identification of conserved and novel Norway spruce specific miRNAs and their putative targets (miRNA precursors are shown in Yakovlev et al., 2010)

Name	Sequence	Length, nt	Arm	miRNA containing EST	Target	Target gene function	Score/ E-value
pab-miR950-3p (pab-miR02m)	TCTGGGCCCCGGTGGTTTATGA	22	5′	ES261905	DR543641	gi|321530320|ADW94527.1 putative TIR-NBS-LRR protein [Pinus monticola]	58.5 bits (140)/ 3e-07
pab-miR951 (pab-miR02*l*)	TGTTCTTGACGTCTGGACCACG	22	5′	DR563813	EX417324 (4 mismatches)	gb|AAM28917.1| putative TIR/NBS/LRR disease resistance protein [Pinus taeda]	157(396)/ 6e-37
pab-MIR3693 (pab-miR029)	AGAGGGTGCTCATGAACTGCTC	22	5′	EX326419	DR492284 (2 mismatches)	gb|AAY78890.1| CC-NBS-LRR resistance-like protein [Pinus lambertiana]	311(796)/ 5e-83
pab-MIR3697 (pab-miR065)	TAGCCCCTGACTTCAACATGAG	22	5′	EX427311	EX417859 (3 mismatches)	gb|AAM28917.1| putative TIR/NBS/LRR disease resistance protein [Pinus taeda]	147(372)/ 2e-42
pab-MIR3705 (pab-miR119)	GTAAGTGGTTATGATCTGGAC	21	5′	DR499063	DR491334 (1 mismatch)	gb|AAM28917.1| putative TIR/NBS/LRR disease resistance protein [Pinus taeda]	212(539)/ 3e-53

Supplement 3

<table>
<tr><td>1</td><td>1</td><td>cd00116: LRR_RI - Leucine-rich repeats (LRRs), ribonuclease inhibitor (RI)-like subfamily - 11-residue segments of the Leucine-Rich Repeats (LxxLxLxxN/CxL)</td><td>leucine-rich repeat receptor-like protein kinase, RI- like subfamily</td></tr>
<tr><td rowspan="3">2</td><td>2</td><td>PLN00113[PLN00113], leucine-rich repeat receptor-like protein kinase
Pkinase[pfam00069], Protein kinase domain;
Including LRR_RI[cd00116], Leucine-rich repeats (LRRs), ribonuclease inhibitor (RI)-like subfamily.</td><td rowspan="3">leucine-rich repeat receptor-like protein kinase, RI- like subfamily</td></tr>
<tr><td>3</td><td>PLN00113[PLN00113], leucine-rich repeat receptor-like protein kinase
Including LRR_RI[cd00116], Leucine-rich repeats (LRRs), ribonuclease inhibitor (RI)-like subfamily</td></tr>
<tr><td>4</td><td>PLN00113[PLN00113], leucine-rich repeat receptor-like protein kinase
Including LRR_RI[cd00116], Leucine-rich repeats (LRRs), ribonuclease inhibitor (RI)-like subfamily</td></tr>
<tr><td rowspan="3">3</td><td>5</td><td>CC-NBS-LRR resistance-like protein, contain NB-ARC[pfam00931], NB-ARC domain and COG4886[COG4886], Leucine-rich repeat (LRR) protein</td><td rowspan="3">CC-NBS-LRR resistance-like</td></tr>
<tr><td>6</td><td>PLN00113[PLN00113], leucine-rich repeat receptor-like protein kinase, including COG4886[COG4886], Leucine-rich repeat (LRR) protein</td></tr>
<tr><td>7</td><td>CC-NBS-LRR resistance-like protein, contain NB-ARC[pfam00931], NB-ARC domain; COG4886[COG4886], Leucine-rich repeat (LRR) protein PLN00113[PLN00113], leucine-rich repeat receptor-like protein kinase</td></tr>
<tr><td rowspan="5">4</td><td>8</td><td>TIR-NBS-LRR class</td><td rowspan="5">TIR-NBS-LRR class; leucine-rich repeat receptor-like protein kinase</td></tr>
<tr><td>9</td><td>TIR/P-loop/LRR</td></tr>
<tr><td>10</td><td>PLN00113[PLN00113], leucine-rich repeat receptor-like protein kinase</td></tr>
<tr><td>11</td><td>PLN00113[PLN00113], leucine-rich repeat receptor-like protein kinase</td></tr>
<tr><td>12</td><td>COG4886[COG4886], Leucine-rich repeat (LRR) protein; PLN00113[PLN00113], leucine-rich repeat receptor-like protein kinase</td></tr>
<tr><td rowspan="2">5</td><td>13</td><td>F-box/LRR-repeat protein; Ran GTPase/Leucine-rich repeats (LRRs), ribonuclease inhibitor(RI)-like subfamily</td><td rowspan="2">Ran GTPase/Leucine-rich repeats (LRRs), ribonuclease inhibitor(RI)-like</td></tr>
<tr><td>14</td><td>Leucine-rich repeats (LRRs), ribonuclease inhibitor(RI)-like subfamily</td></tr>
<tr><td rowspan="2">6</td><td>15</td><td>TIR[pfam01582], TIR domain; The Toll/interleukin-1 receptor (TIR) homology domain; NB-ARC[pfam00931], NB-ARC domain; PLN00113[PLN00113], leucine-rich repeat receptor-like protein kinase</td><td rowspan="2">TIR-NBS-LRR class; leucine-rich repeat receptor-like protein kinase</td></tr>
<tr><td>16</td><td>LRRNT_2[pfam08263], Leucine rich repeat N-terminal domain; LRR_RI[cd00116], Leucine-rich repeats (LRRs), ribonuclease inhibitor (RI)-like subfamily; PLN00113[PLN00113], leucine-rich repeat receptor-like protein kinase</td></tr>
</table>

TIR[pfam01582]; The Toll/interleukin-1 receptor (TIR) homology domain is an intracellular signalling domain found in MyD88, interleukin 1 receptor and the Toll receptor. It contains three highly-conserved regions, and mediates protein-protein interactions between the Toll-like receptors (TLRs) and signal-transduction components. TIR-like motifs are also found in plant proteins thought to be involved in resistance to disease. When activated, TIR domains recruit cytoplasmic adaptor proteins MyD88 and TOLLIP (Toll interacting protein). In turn, these associate with various kinases to set off signalling cascades.

LRR_RI[cd00116] – Leucine-rich repeats (LRRs), ribonuclease inhibitor (RI)-like subfamily. LRRs are 20-29 residue sequence motifs present in many proteins that participate in protein-protein interactions and have different functions and cellular locations. LRRs correspond to structural units consisting of a beta strand (LxxLxLxxN/CxL conserved pattern) and an alpha helix. This alignment contains 12 strands corresponding to 11 full repeats, consistent with the extent observed in the subfamily acting as Ran GTPase Activating Proteins (RanGAP1).

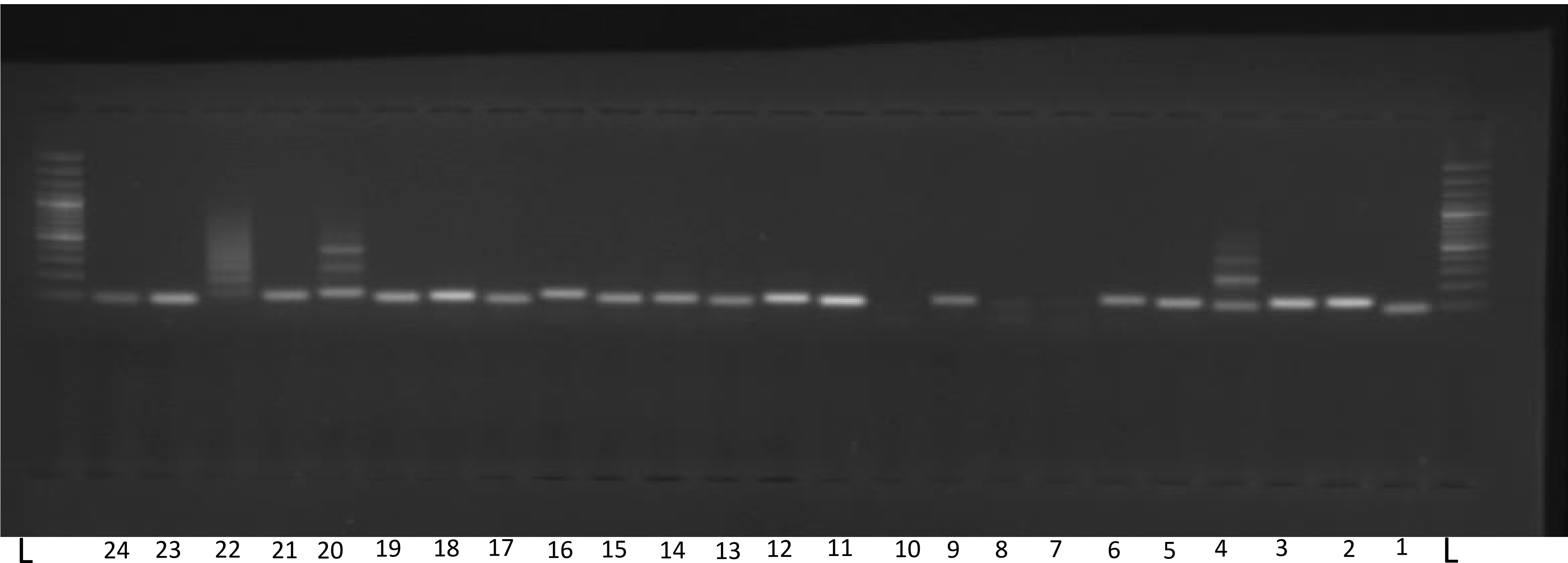
LRRNT_2[pfam08263], Leucine rich repeat N-terminal domain; Leucine rich repeat N-terminal domain; Leucine Rich Repeats pfam00560 are short sequence motifs present in a number of proteins with diverse functions and cellular locations. Leucine Rich Repeats are often flanked by cysteine rich domains. This domain is often found at the N-terminus of tandem leucine rich repeats.

PLN00113[PLN00113], leucine-rich repeat receptor-like protein kinase

COG4886[COG4886], Leucine-rich repeat (LRR) protein

Pkinase[pfam00069], Protein kinase domain

Supplement 4



Supplement 5

TIR domain

```
                                  10        20        30        40        50
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1fyva   ( 625 )    nipleelqrnlqfhAFISySghdsfwVkneLLpnLeke--gqIÇLhernf
                                bbbb      aaaaa

                              60        70        80        90       100
1fywa   ( 680 )    ipgkwiidniidsiekShktVfVLSenFvkseW-kyeldfshfrlfdenn
1fyva   ( 674 )    vpgksivenIitÇIekSYKSIFVLSpnFVqseWchYelyFahhnlFhegs
                        aaaaaaaaaaa  bbbbbb aaaaaa

                              110       120       130       140       150
1fywa   ( 730 )    daailIllepIekkaIpqrf-kLrkintktylewp-deaqregFWvnLra
1fyva   ( 724 )    nsLiLIlLepIpqysIpssyhkLksLarrtylewpkekskrglFwanLra
                      bbbb     333                bb     333   aaaaaaaa

                              160
1fywa   ( 781 )    aIks
1fyva   ( 775 )    aIniklteqak
                   aa
```

VFVSFRGKSRE---ASSSDDRDTFVSHLLKELEKQ---+GINLFIDDRDELPGESI+E

Pinus taeda (Loblolly pine)

>Q8L8J4

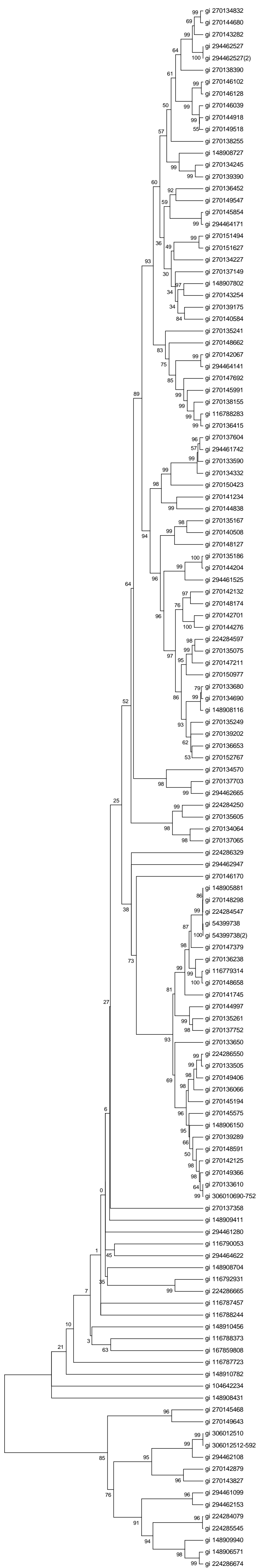
VFLNHRGKDVKNTLASNLYYRLRHLGLRVFFDKEEMQKGSRIDFVIENVIKATPLHIAIFSAGYAESEWCMDE LLLMIESGSIIIAVYYNLNPAEMWSNPVDGTNGVYAEALRLLEEEKAFDPQNHQERPRYKSSTIEKWKTALMEVTGR EGFKLDTYDGNEGKLLDMIVQEVVKYKSIGRRGKKIVKNNVRRTSKQSSCLTQIN

Table S 4. Major Motifs in Predicted Arabidopsis CNL and TNL Proteins Domain (Sub)Group Motif Sequence

Domain	(Sub)Group	Motif	Sequence
TIR	TNL	TIR-1	DVFPSFRGEDVRKTFLSHLLKEF
	TNL	TIR-2	IGPELIQAIRESRIAIVVLSKNYASSSWCLDELVEIMKC
	TNL	TIR-3	ELGQIVMPIFYGVDPSDVRKQ
	TNL	TIR-4	WRKALTDVANIAGEHS
TN linker	TNL		NxTPSRDFDDLVGIEAHLEKMKSLLCLES
NBS	TNL	P-loop	VGIWGPPGIGKTTIARALF
	CNL	P-loop	VGIYGMGGVGKTTLARQIF
	TNL	RNBS-A	DYGMKLHLQEQFLSEILNQKDIKIxHLGV
	CNL	RNBS-A	VKxGFDIVIWVVVSQEFTLKKIQQDILEK
	TNL	Kinase 2	RLKDKKVLIVLDDVD
	CNL	Kinase 2	KRFLLVLDDIW
	TNL	RNBS-B	QLDALAGETxWFGPGSRIIVTTEDK
	CNL	RNBS-B	NGCKVLFTTRSEEVC
	TNL	RNBS-C	NHIYEVxFPSxEEALQIFCQYAFGQNSPP
	CNL	RNBS-C	KVECLTPEEAWELFQRKV
	TNL	GLPL	EVAxLAGGLPLGLKVL
	CNL	GLPL	EVAKKCGGLPLALKVI
	TNL	RNBS-D	EDKDLFLHIACFFNG
	CNL	RNBS-D	CFLYCALFPEDYEIxKEKLIDYWIAEGFI
	TNL	MHDV	MHNLLQQLGREIV
	CNL	MHDV	VKMHDVVREMALWIA
NL linker	TNL	NL	QFLVDAEDICDVLTDDTGTEK(x)_13ELxISEKAFKGMRNLRFLKIY(x)_18PPKLRLLHWDAYPLKSLPxxF_NPENLVELNMPYSKLEKLWE
	CNL-B	NL	SDFGKQKENCIVQAGVGLREIPKVKNWGAVRRMSLMNNQIEHITCSPECPELTTLFLQYNQ
	CNL-C/D	NL	KEENFLQITSDPTSTANIQSQxxxTSRRFVYHYPTTLHVEGDINNPKLRSLVV
LR	TNL	Motif 1	MDLSYSRNLKELPDLSNATNLERLDLSYCSSLVELPSSI

R		(LDL)		
	CNL		Motif 1	IGNLVHLRYLDLSYTGITHLPYGLGNLKKLIYLNL
		(LDL)		
	TNL		Motif 4	LHWLDLKGCRKLVSLPQLPDSLQYLDAHGCESLETVACP
		(end)		
	CNL		Motif 8	LHTITIWNCPKLKKLPDGICF
		(end)		
C terminus	TNL			
	CNL-B		CT	EPEWIERVEWEDEATKNRFLP
	CNL-C/D		CT	WKERLSEGGEDYYKVQHIPSV

X - indicates a nonconserved residue.



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