Understanding virulence of *Heterobasidion annosum s.l.*, a root rot pathogen of conifers

Yang Hu
Faculty of Forest Sciences
Department of Forest Mycology and Plant Pathology
Uppsala

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Abstract

Heterobasidion annosum sensu lato (*s.l.*) species are destructive pathogens causing root and butt rot in conifers. The species complex consists of five species: *H. annosum sensu stricto* (*s.s.*), *H. abietinum*, *H. parviporum*, *H. irregulare* and *H. occidentale*. The aim of this thesis was to improve the understanding of fungal virulence in this species complex. The comparison of *H. irregulare* and *H. occidentale* transcriptomes revealed differences in the consistently significant up-regulated genes (CUGs) in Norway spruce (*Picea abies*) bark. It appears that more CUGs involved in detoxification and in the production of secondary metabolites are activated in *H. irregulare*. By contrast, *H. occidentale* emphasizes carbohydrate degradation. This enrichment of CUGs in particular gene ontology terms may be driven by their host preferences and by their evolutionary history.

In *H. irregulare*, an endo-rhamnogalacturonase gene (*HIRHG*) from a virulence QTL was up-regulated during infection and the protein was mainly produced during growth on complex carbon sources. Although the *HIRHG* gene had been lost in most of the biotrophic and hemibiotrophic plant pathogens investigated, it was common in the necrotrophic pathogens and saprotrophs. Expression of *HIRHG* in *Magnaporthe oryzae* increased its capacity to grow on pectin, but did not significantly affect its virulence in our experimental set up.

In parallel, the evolution of RNA interference (RNAi) was investigated to lay a foundation for the establishment of reverse genetics study tools. Dicer and argonaute are central to the functioning of the RNAi machinery required for gene silencing applications. The evolution of argonaute- and dicer-encoding genes in 43 fungal genomes indicated an ancient duplication of dicer and argonaute genes concurrent with the early diversification of the Basidiomycota, followed by additional species-specific duplications and losses of a more recent origin. The quelling pathway possibly exists in most Basidiomycota; however, to date, no evidence for the meiotic silencing (MSUD) pathway has been found. Given that both argonaute and dicer are present, it should be possible to apply RNAi to study virulence genes in *H. annosum s.l.*

Keywords: Heterobasidion annosum sensu lato (s.l.), virulence, transcriptomes, RNAi, endo-rhamnogalacturonase

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

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Contents

List of Publications

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

I Hu, Y., Stenlid, J., Elfstrand, M., Durling M. and Olson, Å. The conifer root rot pathogens *Heterobasidion irregulare* and *Heterobasidion occidentale* employ different strategies to infect Norway spruce (manuscript).

II Hu, Y., Stenlid, J., Elfstrand, M. and Olson, Å. 2013. Evolution of RNA interference proteins dicer and argonaute in Basidiomycota. *Mycologia,* 105**,** 1489-1498.

III Hu, Y., Kim S., Jeon J., Elfstrand M., Stenlid J., Lee Y. and Olson, Å. Endo-rhamnogalacturonase associated with the *in planta* growth of *Heterobasidion irregulare* (manuscript).

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The contribution of Yang Hu to the papers included in this thesis was as follows:

I Participated in experimental design, performed the experiments, analysed the data and wrote the paper in cooperation with co-authors.

II Participated in experimental design, analysis data and wrote the paper in cooperation with co-authors.

III Participated in experimental design, performed the experiments, analysed the data and wrote the paper in cooperation with co-authors.

Abbreviations

CE	Carbohydrate esterase
CUGs	Consistently significant up-regulated genes
DEGs	Significant differentially expressed genes
dsRNA	Double-stranded RNAs
ESTs	Expressed sequence tags
ETI	Effector-triggered immunity
ETS	Effector-triggered susceptibility
GFP	Green fluorescence protein
GHs	Glycoside hydrolases
GO	Gene ontology
GWA	Genome-wide association
HR	Hypersensitive response
HIRHG	*Heterobasidion irregulare* endo-rhamnogalacturonase
LTR	Long terminal repeat
M/PAMPs	Microbial/pathogen-associated molecular patterns
MSUD	Meiotic silencing of unpaired DNA
PCA	Principal component analysis
PCD	Programmed cell death
PL	Polysaccharide lyase
PRRs	Pattern recognition receptors
PTI	Patterns-triggered immunity
QTL	Quantitative trait loci
RISC	RNA-induced silencing complex
RNAi	RNA interference
RNA-seq	RNA sequencing
s.l.	*sensu lato*
s.s.	*sensu stricto*
sRNA	Small noncoding RNAs
TE	Transposable element

1 Introduction

1.1 The annosus root and butt rot

1.1.1 Economic and ecological impact of annosus root and butt rot

Heterobasidion annosum sensu lato (*s.l.*), which causes root and butt rot in conifers, is one of the most devastating pathogens in the boreal and temperate coniferous forests of the northern hemisphere (Woodward *et al.*, 1998). In modern forestry, the forests are usually established by plantation and intensively managed, and site productivity can be severely affected by *H. annosum s.l.* The overall effects of *H. annosum s.l.* infection include host mortality, losses due to decay as well as an overall reduction in the diameter growth of infected trees, wind-throw and a reduction in the resistance of stands to storm damage, which may be significant in certain places (Garbelotto & Gonthier, 2013). The estimated financial losses caused by annosus root and butt rot in the European Union were estimated in 1998 to be about 790 million euro per year (Woodward *et al.*, 1998). In the Southeast USA, up to 30% of trees can be killed by *H. annosum s.l.* in severely infected stands. In the United Kingdom, the incidence of decay has been reported to be as high as 68% in some Sitka spruce (*Picea sitchensis*) stands, with a loss in value of 43% (Pratt, 1979). In Alpine Norway spruce forests, financial losses derived from *H. annosum s.l.* infection have been estimated to be between 18% and 34%, and the local disease incidence could be as high as 71% (Gonthier *et al.*, 2012). In Sweden, up to 15% of Norway spruce [*Picea abies* (L.) Karst.] trees have been found to be decayed at harvest, mostly by *H. annosum s.l.*, and an increase over time has been observed (Thor *et al.*, 2005; Stenlid & Wasterlund, 1986). Losses due to decayed wood cost the Swedish forestry industry about 250 million SEK annually, together with growth losses and increased management costs of the same order of magnitude (Bendz-Hellgren & Stenlid, 1998).

In addition to being a necrotrophic plant pathogen, *H. annosum s.l.* also plays a role in the ecosystem as a saprotrophic wood decayer. The fungus

contributes substantially to nutrient recycling by returning nutrients locked up in woody tissues back to the soil (Woodward *et al.*, 1998). *H. annosum s.l.* also influences species composition, ecosystem diversity, stand structure, stand density, and the direction and rate of forest succession. Annosus root rot has been reported to affect succession patterns of forest development by selectively killing certain tree species (Woodward *et al.*, 1998). Furthermore, mortality caused by root rot creates gaps in the forest canopy, which change the light conditions, moisture and temperature in the forest and, thus, increases the biodiversity range of the forest ecosystem.

1.1.2 Distribution and evolution of *Heterobasidion annosum s.l.* species

Until the occurrence of intersterile groups (ISGs) was dicovered, *H. annosum s.l.* had long been regarded as a single species (Capretti *et al.*, 1990; Korhonen, 1978). Currently, three European and two North American ISGs are formally described as five species: *H. annosum sensu stricto* (*s.s.*), *H. abietinum*, *H. parviporum*, *H. irregulare* and *H. occidentale*. The species were defined on the basis of partial reproductive isolation and morphology, and were further supported by phylogenetic analyses (Otrosina & Garbelotto, 2010; Niemelä & Korhonen, 1998; Dalman *et al.*, 2010). The species in the complex have an overlapping geographic distribution and are found in the coniferous forests of the Northern Hemisphere. The species *H. annosum s.s.* occurs all over Europe (except in the very northern regions) and extends east to the Altai region in southern Siberia. *H. parviporum* is found from northern Europe to the southern Alps and from western Europe to east Asia (Dalman *et al.*, 2010; Otrosina & Garbelotto, 2010; Dai *et al.*, 2006; Korhonen *et al.*, 1998; Korhonen & Stenlid, 1998). *H. abietinum* is restricted to central and southern Europe and the Mediterranean Basin (Luchi *et al.*, 2011; Sanchez *et al.*, 2007; Dogmus-Lehtijarvi *et al.*, 2006; Korhonen *et al.*, 1998). The North American *H. occidentale* has only been reported from the western parts (Garbelotto & Chapela, 2000), whereas *H. irregulare* can be found from the western to the eastern North American forests (Otrosina & Garbelotto, 2010). In addition, *H. irregulare* was introduced into central Italy during World War II and has become established in Italian stone pine (*Pinus pinea* L.) stands (Gonthier *et al.*, 2007; Gonthier *et al.*, 2004).

In general, the *H. annosum s.l.* species show different host preferences. The European *H. annosum s.s.* mostly attacks pines (*Pinus* spp.), especially Scots pine (*Pinus sylvestris* L.), but can be associated with several other conifers, including Norway spruce, and even some broad-leaved tree species. *H. parviporum* is mostly associated with Norway spruce but has also been found on *Abies siberica*. *H. abietinum* is commonly associated with silver fir (*Abies*

alba Mill.) and other species of the genus *Abies* (Hüttermann & Woodward, 1998). In North America, *H. irregulare* generally attacks pines, junipers (*Juniperus* spp.), and incense cedar [*Calocedrus decurrens* (Torr.) Florin], whereas *H. occidentale* shows a broader host range and can be found on species in the genera *Abies*, *Picea*, *Tsuga*, *Pseudotsuga* and *Sequoiadendron* (Otrosina & Garbelotto, 2010). In general, the host preference of *H. annosum s.l.* species can be separated into two groups: the pine-infecting species, which include *H. annosum s.s.* and *H. irregulare*, and the non-pine-infecting species, which include *H. parviporum*, *H. abietinum* and *H. occidentale* (Dalman *et al.*, 2010).

1.1.3 Epidemiology

The infection cycle of *H. annosum s.l.* in nature involves a primary and secondary infection phase. Primary infection is mediated by basidiospores that land on freshly exposed stump surfaces or on wounds on the roots or stem (Redfern & Stenlid, 1998). Spore production in *H. annosum s.l.* is affected by temperature and humidity, and once released into the air, spores can travel hundreds of kilometres; however, effective dispersal has been estimated to be between 98 and 1,255 m (Redfern & Stenlid, 1998; Moykkynen *et al.*, 1997; Stenlid *et al.*, 1994; Kallio, 1970). The fungus establishes much less frequently when the temperature drops below +5 °C, (Meredith, 1959) or exceeds +35 °C (Ross, 1973). The role of conidiospores in the spread of the fungus in nature is unclear but might be important for transmission in substrates or when being vectored by insects (Korhonen & Stenlid, 1998). Once established, *H. annosum s.l.* spreads and infects uninjured trees by vegetative growth of the mycelium through root contacts or grafts (secondary infection).

1.1.4 Management

In *H. annosum s.l.* can remain active in dead stumps and in root systems for decades. It is virtually impossible to eradicate the fungus completely after it has become established in a stand (Greig & Pratt, 1976). Stump removal could be a strategy for controlling *H. annosum s.l.* root and butt rots (Cleary *et al.*, 2013; Vasaitis *et al.*, 2008; Asiegbu *et al.*, 2005; Stenlid, 1987) but it is time consuming and costly and, therefore, unsuitable for most forest stands (Walmsley & Godbold, 2010). Treating the stump surface with sodium tetraborate decahydrate (borax), disodium octaborate tetrahydrate (DOT), urea, or the biological control fungus *Phlebiopsis gigantea* (Fr.) Jülich at the time of logging have all proved to be effective methods of restricting the establishment of *H. annosum s.l.* that can be used in practical forestry (Oliva *et al.*, 2010; Vasaitis *et al.*, 2008; Nicolotti & Gonthier, 2005; Pratt *et al.*, 1998; Thor &

Stenlid, 1998). Borates have a direct effect on fungal metabolism; urea inhibits spore germination (Johansson *et al.*, 2002); and *Phlebiopsis gigantea* competes for the substrate and causes hyphal interference (Holdenrieder & Greig, 1998). Integrated disease management combining different approaches is generally more effective and even cheaper than the use of a single control method (Gonthier & Thor, 2013).

However, to find more efficient ways of controlling this disease, it is important to understand the processes that determine the outcome of the interactions between the pathogen and the host tree. Understanding the virulence of the fungus could enable the design of tailored control measures against the pathogen.

1.2 Host–pathogen interaction

The epidemiology of a plant disease is affected by the host, the environment and the pathogen; all three factors are often jointly referred to as the disease triangle. Changes in one or more will influence the outcome of the interaction. Plants protect themselves from disease with the aid of pre-existing structural defences, anti-microbial chemicals and pathogen inhibitors. They are also able to induce defence reactions resulting in structural and biochemical responses such as callose formation, thickened cell walls, the hypersensitive cell death response (HR) and by phytoalexin production (Franceschi *et al.*, 2005). To be successful, a pathogen needs virulence factors to prevent activation of, or interaction with, host defences in order to enter the host tissue and acquire nutrients for its development and reproduction.

1.2.1 Models for plant immunity to their pathogens

The theory of plant–microbe interaction has to a large extent been driven by its development in the agricultural system, with some similarities and differences dependent of the trophic strategy of the pathogen. Pathogens can be divided into: biotrophs, which live and acquire nutrients from the living cells of their hosts; necrotrophs, which kill the host cells and feed on the dead tissue; and hemibiotrophs, which begin with a biotrophic phase at the start of the infection but then continue their parasitic life in a similar way to necrotrophs. Acquiring information about the inheritance patterns of the flax rust pathosystem, and the pathogen avirulence (*avr)* and host resistant (*R*) genes, lead Flor (1942) to propose the gene-for-gene hypothesis, which has been widely applied both in plant pathology and ecology (Gassmann & Bhattacharjee, 2012; Flor, 1942). This idea was developed further by Jones and Dangl (2006) who proposed a “zigzag” model to describe a simplified scheme for the molecular interaction

between the pathogen and the plant host immune system. In the "zigzag" model, small secreted proteins that could modulate the host cells/or induce host programmed cell death (PCD) were defined as effectors. Effector molecules are important virulence factors of biotrophic or hemibiotrophic plant pathogenic bacteria, oomycetes and fungi. When the pathogen enters the plant apoplast, the microbial/pathogen-associated molecular patterns (M/PAMPs) are recognized by host pattern recognition receptors (PRRs), resulting in patterns-triggered immunity (PTI) in the host. Successful pathogens deploy effectors that interfere with PTI, resulting in effector-triggered susceptibility (ETS), which enables pathogen development. A given effector may be specifically recognized by one of the host's R proteins (nucleotide-binding site leucine-rich repeat (NBS-LRR) proteins), subsequently triggering effector-triggered immunity (ETI). ETI is essentially an accelerated and amplified PTI response, resulting in disease resistance and, usually, a HR at the infection site. Natural selection results in pathogens either discarding or diversifying the recognized effector gene to avoid recognition, or acquiring additional effectors that suppress ETI. In the host, new resistance specificities develop so that ETI can be triggered again (Jones & Dangl, 2006). However, this elegant model has its limitations in that it cannot fully explain the interaction between necrotrophic pathogens and their hosts. Also, the model does not include virulence aspects outside of the interaction with the plant immune system.

Necrotrophic pathogens acquire nutrients for growth and reproduction from dead cells. Therefore, necrotrophs might tolerate the HR response or even benefit from host PCD, which is considered a very important feature of the host resistant response to biotrophs and hemibiotrophs (Mengiste, 2012). The necrotrophic pathogens can be further divided into host-specific necrotrophs and broad-host-range necrotrophs. In host-specific necrotrophic fungi, such as *Pyrenophora tritici-repentis*, *Stagonospora nodorum* and *Cochliobolus carbonum*, host-specific toxin Ptr(Sn)Tox (proteinaceous) and HC-toxin have been demonstrated as effectors that induce toxicity, killing host cells, thereby enhancing virulence (Friesen *et al.,* 2006; Kuo *et al.,* 1970). This type of interaction has also been described as an inverse gene-for-gene model. A successful infection by host-specific necrotrophs will only occur when effectors recognize their sensitivity receptor; no disease occurs when the host sensitivity receptor or fungal effector is absent or altered (Ciuffetti *et al.*, 2010; Tan *et al.,* 2010). The broad-host-range necrotrophs have also been shown to have a complex interaction with their hosts (Kabbage *et al.,* 2013; Shlezinger *et al.,* 2011; Williams *et al.,* 2011). Some of the broad-host-range necrotrophs have been shown to produce diverse MAMPs, such as cerato-platanin from *Botrytis*, or damage-associated molecular pattern molecules (DAMPs), that

activate the plant immune response (Frias *et al.*, 2011). Unlike biotrophs and hemibiotrophs, some necrotrophs are able to hijack part of the immune signalling (Rahman *et al.*, 2012; El Oirdi *et al.*, 2011) to induce host PCD which benefit to pathogens but to suppress other defence responses such as callose formation and host oxidative burst, which might be harmful to the pathogens. The necrotrophs suppress these defence responses by secreting toxins from secondary metabolism instead of effector proteins (Kabbage *et al.*, 2013; Mengiste, 2012; Williams *et al.*, 2011; Bartz *et al.*, 2013). The broad-host-range necrotrophic plant pathogen *Sclerotinia sclerotiorum* is an example of such an interaction. During infection, it triggers a HR-like response in host plants, typified by the oxidative burst that should restrict pathogen growth (Williams *et al.*, 2011). The pathogen produces the non-specific phytotoxin oxalic acid, which suppresses the host defence responses, including callose deposition and modulation of the host redox environment. Furthermore, once infection is established, this necrotrophic pathogen is able to promote and spread PCD of host tissue, the result of which is of direct benefit to the pathogen (Mengiste, 2012; Williams *et al.*, 2011).

1.2.2 The virulence factors beside the interaction with the host immune system

Virulence factors can be much more than just mechanisms to overcome the host immune system. One example of virulence factors not manipulating the host immune system is the capability of pathogens to macerate tissue and acquire nutrients to grow, develop and reproduce. As more and more fungal plant pathogen genomes have been sequenced, the number of gene families encoding carbohydrate active enzymes (CAZys) in necrotrophs has expanded. Enzymes that are capable of hydrolytically cleaving glycosidic bonds in oligo- or polysaccharides are generally summarized under the term glycoside hydrolases (GHs). Fungal species with individual *GH* genes deleted might not directly show reduced virulence owing to the redundancy of function provided by another enzyme or suite of enzymes. However, some GHs have been shown to impair virulence, such as GH11s, GH12s and GH28s (Ma *et al.*, 2015; Brito *et al.*, 2006; Oeser *et al.*, 2002).

How well the pathogen tolerates the host response is another key virulence factor for many necrotrophs. The mechanism of a given pathogen's tolerance of the host response could be based on detoxification of phytoalexins or avoidance of PCD. Production of antimicrobial compounds is induced in plants following infection by pathogens (Bednarek *et al.*, 2009; Kliebenstein *et al.*, 2005). Such compounds have been shown to kill fungal pathogens by inducing fungal PCD (Lazniewska *et al.*, 2010). Pathogens can employ enzymes to metabolize the plant defence compounds or drug transporters to pump the

compounds out of their cells to avoid harm. Detoxing by xenobiotic metabolism is important for necrotrophic pathogens such as *Fusarium verticillioides* (Glenn *et al.*, 2002) and *S. sclerotiorum* (Sexton *et al.*, 2009). The pea (*Pisum sativum*) pathogen *Nectria haematococca* utilizes a cytochrome P-450 monooxygenase to detoxify the phytoalexin pisatin. Expression of this cytochrome P-450 monooxygenase from *N. haematococca* can make the maize pathogen *Cochliobolus heterostrophus* adapt to pea as a new host (Schafer *et al.*, 1989). The bark beetle-associated Norway spruce pathogen *Ceratocystis polonica* can circumvent the antifungal activity of stilbenes, which are synthesized by Norway spruce as part of its chemical defence during bark infection (Hammerbacher *et al.*, 2013). The rapid biotransformation of stilbenes resulting in the formation of ring-opened lactones in Norway spruce bark is associated with greater levels of fungal virulence (Hammerbacher *et al.*, 2013). The transporters contribute to pathogen virulence by both secretion of pathogen toxin and efflux of the molecules produced by the host. ATP-binding cassette transporters and other drug transporters have been shown to provide *B. cinerea*, *Gibberella pulicaris*, *N. haematococca* and the biocontrol fungus *Clonostachys rosea* with xenobiotic tolerance (Dubey *et al.*, 2014; Fleissner *et al.*, 2002; Han *et al.*, 2001; Schoonbeek *et al.*, 2001). During the early stage of *B. cinerea* infection, massive pathogen cell death occurs because of the anti-fungal compounds produced as part of the host defence. This is followed by recovery of the fungus from the surviving cells, which were protected by fungal anti-apoptotic machinery. This might be a common strategy in many necrotrophic pathogens because many pathogens in this class have been shown to tolerate host defences (Shlezinger *et al.*, 2011). These phenomena suggest that during the battle between broad-host-range necrotrophic fungal plant pathogens and their hosts, avoiding being killed might be as important as killing host cells (Figure 1).

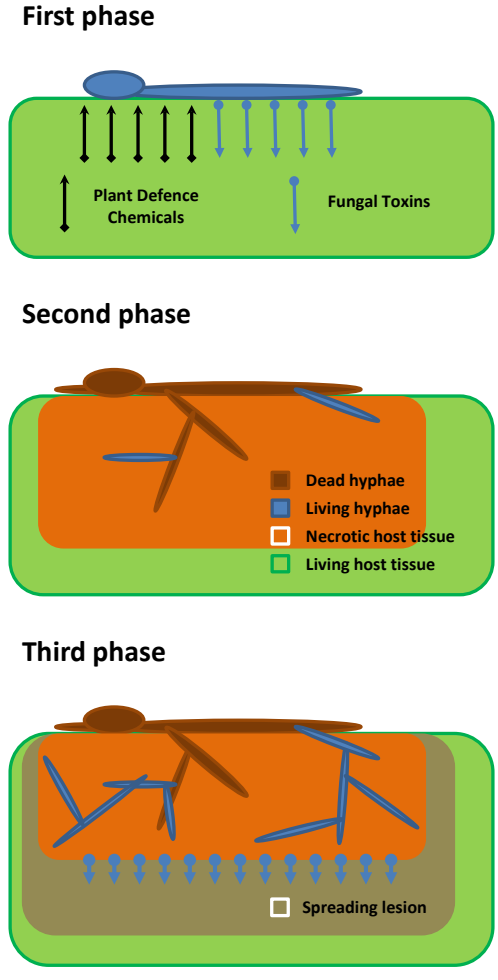

Figure 1. A model showing the contribution of detoxification and anti-apoptotic machinery to fungal pathogen virulence (adapted from a model of *B. cinerea* infection) (Shlezinger et al., 2011). First phase: the fungal pathogen reaches the host tissue secreting necrosis-inducing factors. Second phase: anti-fungal plant products attack the fungal cells. Fungal detoxification and anti-apoptotic machinery prevents the complete elimination of the fungus at this stage. Third phase: viable fungal cells retained within the necrotized plant tissue are protected from the host toxic molecules. These fungal cells give rise to new hyphae, which secrete molecules that induce and promote the spread of the lesion into the surrounding plant tissue.

1.2.3 Studies of plant–pathogen interactions in the -omics era

Making a successful broad-host-range necrotrophic pathogen requires multiple genetic factors, especially in polygenic genetic control of the interaction (Lindhout, 2002). With the development of sequencing technology, more and more pathogen genomes have been sequenced in recent years, allowing us to look at the bigger picture of pathogen virulence during plant–pathogen interactions in addition to single gene molecular genetics. The first global analyses of pathogen transcriptomes were provided by microarray-based studies. Today, high-throughput RNA-seq has rapidly developed to become an important tool and is widely applied. RNA-seq provides several advantages over the microarray-based approach (Westermann *et al.*, 2012). Firstly, RNA-seq is a digital quantification method and, therefore, has a higher (and theoretically infinite) dynamic range and is much more sensitive compared with the array-based analogue quantification method. The linear dynamic range for RNA-seq can already approach the upper limit of changes in gene expression in eukaryotic cells. Secondly, RNA-seq does not require a sequenced genome and can help to identify novel transcripts. Furthermore, RNA-seq has allowed the refinement of gene structure through the accurate determination of transcript borders, alternative splicing and processing events. Elucidation of the transcriptome changes of the pathogen in *planta* using the RNA-seq approach has been carried out on a number of fungal plant pathogens, such as *Colletotrichum graminicola*, *Colletotrichum higginsianum*, *Fusarium graminearum*, *Magnaporthe oryzae* and powdery mildews (Wicker *et al.*, 2013; Kawahara *et al.*, 2012; O'Connell *et al.*, 2012; Zhang *et al.*, 2012). A comparison of *F. graminearum* transcriptomes showed that hundreds of genes are differentially expressed during infection (Zhang *et al.*, 2012); with deeper sequencing, more differentially expressed genes might be identified. However, to date, relatively few studies have been carried out on necrotrophic fungal pathogens interacting with their hosts. Furthermore, the intraspecific variation affecting virulence-related traits are less clear compared with the well-studied common intraspecific variation in biotrophic and hemibiotrophic pathogens. The transcriptomes of different *H. annosum s.l.* species during saprotrophic and necrotrophic life stages should contribute to our general understanding of the interaction between necrotrophic plant pathogens and their hosts.

1.3 Understanding the virulence of *Heterobasidion annosum s.l.*

1.3.1 Lifestyle of *Heterobasidion annosum* s.l.

The Both saprotrophic wood decay and a necrotrophic interaction with the host are necessary for the *H. annosum s.l.* to complete its infection cycle (Olson *et al.*, 2012). When *H. annosum s.l.* initially infects the host, it establishes as a saprotroph on a stump or on exposed woody tissue, beginning the infection cycle with saprotrophic wood decay. During the development of the disease, the fungus attacks host bark in root contacts like a typical necrotroph. When the phelloderm of four-year-old Norway spruce seedlings was inoculated with *H. annosum s.l.*, the fungi initially induced extensive necrosis of about 50 cell layers in depth, involving brown staining and thickening of cell walls and the death of ray cells (Asiegbu *et al.*, 1998). Hyphal growth then followed the expansion of necrosis, and the hyphae were always one or two cell layers behind the contact with the living cells (Asiegbu *et al.*, 1998). It has also been shown that *H. annosum s.l.* have the capacity to tolerate HR (Asiegbu *et al.*, 1994). Both *H. annosum s.l.* and *Fusarium* spp. hyphal material induced death in 20–50 cell layers in living bark of spruce without lignin formation (Asiegbu *et al.*, 1998). By contrast, the saprotrophs *Phlebiopsis gigantea* and *Resinicium bicolor* induced lignified necrosis in only two to five cell layers (Asiegbu *et al.*, 1998). This type of interaction was very similar to the wild-type *S. sclerotiorum* interaction with its host, in which the pathogen induced and spread cell death of the host tissue, whereas an oxalic acid (toxin)-deficient mutant induced restricted programmed cell death and callose formation (Williams *et al.*, 2011). A similar mechanism might be operating in the *H. annosum s.l.*–conifer pathosystems.

1.3.2 Previous approaches used to study *Heterobasidion annosum s.l.* virulence

To understand the genetic background of the virulence in *H. annosum s.l*, Olson and Stenlid (Olson & Stenlid, 2001) created heterokaryon hybrids by mating homokaryon strains of *H. irregulare* and *H. occidentale*. The virulence of the parental homokaryons and hybrid heterokaryons was analysed *in vitro* by visually scoring the proportion of infected pine seedlings that suffered mortality. Fungal virulence was observed to correlate significantly with the mitochondrial type acquired by the hybrids. The exact reason for the correlation between mitochondrial origin and virulence is not clear. The genome sequencing of *H. irregulare* mitochondria indicated that factors that might influence *H. annosum s.l.* virulence are likely to be the exchangeable parts of the mitochondrial genome containing the homing endonuclease in the

intron or the plasmid-integrated genes rather than the core genes of the mitochondrial genome (Himmelstrand *et al.*, 2014).

As well as the mitochondrial factor, nuclear factors have also been shown to contribute to virulence. (Olson *et al.*, 2005) showed that the hybrid progeny isolates from the AO8 heterokaryon, which is a cross between the homokaryons *H. irregulare* TC32-1 and *H. occidentale* TC122-12, showed a wide range of virulence on pine and spruce and segregated with a continuous variation that indicated a polygenetic control of virulence (Olson *et al.*, 2005). Therefore, a quantitative trait loci (QTL) mapping study was performed to identify the loci for virulence factors and for future map-based cloning of virulence factors by measuring lesion length and fungal growth in sapwood using 102 progeny isolates from a previous virulence experiment (Olson *et al.*, 2005), and to associate the traits with 358 AFLP markers. Three major loci for virulence on Norway spruce and Scots pine were identified and assigned to specific regions in the fungus. The virulence towards Norway spruce was controlled by either a few or closely situated regions (Lind *et al.*, 2007). After the whole genome of *H. irregulare* TC32-1 had been sequenced, three major QTL regions important for pathogenic interactions with Norway spruce and Scots pine were placed on the physical genome by re-mapping virulence data from (Lind *et al.*, 2007). One QTL region was located on chromosome 1 and two QTL regions were located on chromosome 12 (Olson *et al.*, 2012). The pathogenicity QTLs were located in parts of the genome that had a higher density of transposable elements (TEs) than average, which is similar to the findings reported for many other pathogen genomes (Haas *et al.*, 2009; Cuomo *et al.*, 2007). The sequence of the QTL regions has low sequence similarity with other Basidiomycota genomes, and a higher frequency of orphan genes than other parts of the genome (53% relative to 34%). These QTL regions included 178, 142 and 299 predicted gene models (Olson *et al.*, 2012). Gene models such as a sugar transporter, putative flavin-containing Baeyer-Villiger monooxygenase and pectinase were identified and were shown to be significantly up-regulated during interactions with pine, making them very strong pathogenicity candidates. In addition, two overlapping secondary metabolite clusters harbouring 43 gene models in total were located in the QTL region of scaffold 12. The clusters included three non-ribosomal peptide synthetase-like (NRPS-like) enzymes, several oxidative enzymes and transport proteins (Olson *et al.*, 2012).

Genome-wide association (GWA) is another genetic approach to link a phenotypic trait to the genotype. By finding single nucleotide polymorphisms (SNPs), markers that are linked to the phenotype of interest, it is possible to identify the genes associated with the traits. A GWA study was conducted in a

H. annosum s.s. population with the objective of identifying the *H. annosum s.s.* virulence factors (Dalman *et al.*, 2013). To this end, a set of 33,018 SNPs was generated by sequencing the genomes of the population, which comprised 23 haploid isolates. Twelve SNP markers distributed on seven contigs were associated with virulence, some of which were found very close to or directly overlapping with previous known virulence QTLs (Dalman *et al.*, 2013).

1.3.3 Secondary metabolism

A wide range of toxins secreted by *H. annosum s.l.* are produced by secondary metabolism pathways, including fomannoxin, fomannosin, fomannoxin acid, oosponol and oospoglycol, which are believed to be important during the infection process (Asiegbu *et al.*, 2005; Sonnenbichler *et al.*, 1989; Donnelly *et al.*, 1988; Holdenrieder, 1982). Recently, a total of 33 compounds have been identified from *H. annosum s.l.* (Hansson *et al.*, 2014). Among them, six new sesquiterpenes belonging to the fomannosin class of compounds; seven fomannoxin-like compounds that were previously unknown or that had not been described from nature; and also fomajorins, drimanes, tryptophan and an indole-containing compound were identified (Hansson *et al.*, 2014; Hansson *et al.*, 2012). The secondary metabolite profile of the five species of *H. annosum s.l.* was not identical (Hansson *et al.*, 2014). The five species could be separated according to their host preference, pine infecting and non-pine infecting, and by their phylogeny (Hansson *et al.*, 2014). One of the compounds, fomannoxin, has been isolated from the uninfected zone in front of the invading hyphae, which indicated that it might be released into host tissue preceding infection (Heslin *et al.*, 1983), and it has been shown to be biosynthesized by a combination of the mevalonic acid (MVA) pathway and the shikimic acid pathway (Hansson *et al.*, 2014; Hansson *et al.*, 2012). Fomannosin is one of the toxins that shows both antifungal and antibacterial activity, and has been shown to cause decolouration and cell death of *Chlorella pyrenoidosa* (Heslin *et al.*, 1983), as well as inducing local necrosis and systemic killing of loblolly pine (*Pinus taeda*) (Sonnenbichler *et al.*, 1989).

1.3.4 Enzymes for host material degradation

To penetrate host tissue and to access and obtain nutrients locked up in the polysaccharides and lignified tree tissue, *H. annosum s.l.* secretes a wide range of extracellular enzymes for degrading sugars, polysaccharides and lignin, and for detoxifying the phenolic compounds (Woodward *et al.*, 1998). In earlier studies, laccase has received special attention. Laccases are copper-containing enzymes that presumably contribute to lignin degradation and detoxification of the host's defence chemicals and structure (ten Have & Teunissen, 2001).

Eighteen laccases have been found in the genome of *H. irregulare* (Olson *et al.*, 2012), orthologues to eight of the laccases were found to be up-regulated in *H. annosum s.s* during interactions with scots pine seedlings, which suggests that they may be involved in virulence (Kuo *et al.*, 2015). Very few of the enzymes related to cell wall degradation have been thoroughly studied in *H. annosum s.l.* (Asiegbu *et al.*, 2005). The genome and transcriptomes of *H. irregulare* provide a primary view of these enzymes. When comparing the CAZyme profile of *H. irregulare* with eight other fungi, including seven basidiomycetes (*Ustilago maydis*, *Postia placenta*, *Phanerochaete chrysosporium*, *Laccaria bicolor*, *Coprinopsis cinerea*, *Schizophyllum commune*, *Cryptococcus neoformans*) and the ascomycete plant pathogen *M. oryzae*, *H. irregulare* appears to have all the enzymatic equipment to digest cellulose (enzymes from families GH5, GH6, GH7 and GH45), xyloglucan and its side chains (GH27, GH29, GH12 and GH74), and pectin and its side chains (GH28, GH43, GH51, GH53, GH78, GH88, GH105, PL1, PL4, CE8 and CE12) (Olson *et al.*, 2012). In particular, *H. irregulare* has more than twice as many enzymes that are active in pectin degradation as those found in the other basidiomycetes and the pathogenic *M. oryzae* (Olson *et al.*, 2012). Earlier studies of *H. annosum s.l.* showed that the isozyme pattern of proteins encoded by the GH28 family differed between the different species previously known as intersterility groups. Intersterility group P (*H. annosum s.s.*) showed stronger and more diverse GH28 activity than *H. parviporum* (Comparini *et al.*, 2000; Karlsson & Stenlid, 1991; Johansson, 1988).

1.3.5 Previous gene expression studies

Karlsson *et al.* (2003) studied the transcriptome of *H. irregulare* during the early stage of infection of Scots pine seedlings by constructing a library of expressed sequence tags (ESTs). From ESTs, the genes encoding hydrophobins, cytochrome P450 monooxygenase, arabinose, farnesyl-pyrophosphate synthetase and genes involved in handling oxidative stress, such as superoxide dismutase were found to be up-regulated. A *SOD1* gene, which encodes a manganese-type superoxide dismutase, has been further investigated from the early infection stage between *H. irregulare* and Scots pine (Karlsson *et al.*, 2005). Karlsson *et al.* (2007) also found that the *H. parviporum* genes encoding putative glutathione-*S*-transferases, laccase, cellulase, cytochrome P450 and superoxide dismutase were expressed during infection of Norway spruce tissue cultures. The sequenced genome of *H. irregulare* allowed the global gene expression patterns of *H. irregulare* to be profiled under different conditions in a microarray study. Comparison of the global transcript profiles of *H. irregulare* growing in different cultures, wood and the cambial zone of

pine revealed that the genes induced during saprotrophic wood degradation, but not during interaction with living host tissue, represented a trade-off between the two trophic strategies (Olson *et al.*, 2012). Gene expression patterns during saprotrophic growth on wood showed high correlations with gene expression patterns during growth on cellulose and lignin, but lower correlations with gene expression during growth in the cambial zone of pine. The distinct pattern of gene expression during growth in the cambial zone of pine indicated that interaction with living tissue is very different from the other growth conditions analysed. The genes with a higher level of expression during growth in the cambial zone of pine included genes encoding pectinolytic enzymes (Olson *et al.*, 2012). Transcriptome analyses combined with the QTL approach are a powerful way of reducing the number of candidate virulence genes in the QTL regions for virulence. Strong candidate genes are significantly up-regulated during pathogenic interaction with the host and are present in the QTL regions for virulence (Olson *et al.*, 2012). The same *H. irregulare* microarray was used to investigate the transcriptomic response of *H. annosum s.s.* exposed to several environmental stresses (high and low temperature, osmotic stress, oxidative stress and nutrient starvation) and during growth on specific pine wood compartments (bark, sapwood and heartwood). (Raffaello *et al.*, 2014) The global gene expression changes provide a picture of *H. annosum s.s.* balanced between sensing and survival when under abiotic stress; nutrient uptake during saprotrophic growth might be associated with the induction of a variety of different gene sets and pathways. Dual transcriptomes for *H. annosum s.s.* and Norway spruce showed similar gene induction patterns as those seen in *H. irregulare* (Lunden *et al.*, 2015).

1.4 RNA interference (RNAi) in fungi and its potential application

1.4.1 RNAi in fungi

RNA interference (RNAi) originally referred to the phenomenon in the nematode *Caenorhabditis elegans* were exogenously introduced double-stranded RNA (dsRNA) molecules can silence the expression of homologous genes (Fire et al. 1998). It is mechanistically related to a number of conserved pathways mediated by small noncoding RNAs (snRNAs or sRNAs) (Carthew & Sontheimer, 2009; Ghildiyal & Zamore, 2009; Moazed, 2009). The main pathway of RNAi relies on the dicer and argonaute proteins: dicers generate the small RNA duplexes from dsRNA precursors, and then load the dsRNA duplexes onto the RNA-induced silencing complex (RISC) in which argonaute functions as the core catalytic component. The RISC actively removes the

passenger strand of the dsRNA duplex and uses the remaining single-stranded molecule as a guide to silence the target homologue messenger RNAs (mRNAs) (Jinek & Doudna, 2009; Maiti *et al.*, 2007; Tomari & Zamore, 2005; Meister & Tuschl, 2004; Bernstein *et al.*, 2001). In filamentous fungi, RNAi pathways have been most extensively studied in *Neurospora crassa* (Li *et al.*, 2010). Quelling and meiotic silencing of unpaired DNA (MSUD) are the two best-understood RNAi pathways. Either the quelling or the MSUD pathways synthesize the dsRNAs by dicers, which then go through the argonaute protein qde-2 (quelling) or sms-2 (MSUD), respectively (Li *et al.*, 2010). Quelling is a potent mechanism that represses the expression and expansion of transposons. It silences the transgenes by detecting and targeting the transgenic DNA (Chicas *et al.*, 2005; Nolan *et al.*, 2005). MSUD may function as a mechanism for silencing transposon expansion and generating unpaired DNA during meiosis (Dang *et al.*, 2011).

1.4.2 RNAi contribution to fungal virulence

Small RNAs perform important functions in the host–pathogen interaction. The sRNAs contribution to virulence could be direct or indirect. Many sRNAs are associated with TEs and are generated from the TE-rich region in the genome, which is also often the location of pathogen effectors (Weiberg *et al.*, 2014; Haas *et al.*, 2009; Cuomo *et al.*, 2007). Long terminal repeat (LTR) retrotransposons have been shown to regulate the expression of fungal and oomycete effectors (Weiberg *et al.*, 2014; Raman *et al.*, 2013). For example, in *M. oryzae*, LTR-associated sRNA levels are increased during invasive growth, and regulate the effector gene ACE1 (Fudal *et al.*, 2007). In *Phytophthora infestans*, numerous sRNAs can be mapped to the TEs and RxLRs or crinkler (CRN) (Vetukuri *et al.*, 2012). RxLRs and crinkler (CRN) are two major classes of effectors that are well-known virulence factors of Oomycetes. The RxLR effector gene *PiAvr3a*, which suppresses plant programmed cell death for virulence, has been shown to be under the regulation of sRNAs (Vetukuri *et al.*, 2012). Evidence for the direct contribution of pathogen sRNAs to virulence has been found in *B. cinerea* (Weiberg *et al.*, 2013). RNA sequencing of *B. cinerea* sRNAs showed that a number of them could be mapped to the host defence genes, including genes encoding targeted *Arabidopsis* mitogen-activated protein kinase MPK1 and MPK2, a cell wall-associated kinase (WAK), a peroxiredoxin (PRXIIF) and the tomato MPK-kinase 4 (MAPKKK4). The *B. cinerea* sRNAs were able to suppress these host defence genes during infection by loading into a host argonaute protein and hijacking the host RNAi pathway (Weiberg *et al.*, 2013).

1.4.3 Potential of applying RNAi as a biotechnological tool in *Heterobasidion annosum s.l.*

RNAi has been applied in several fungi as a reverse genetics tool, but there are relatively few examples from the Basidiomycota. Insight into the diversification of RNA silencing pathways during the evolution of Basidiomycota offers a deeper understanding of the mechanism of RNAi and could eventually lead to its application in research. Specifically, investigating whether orthologues to genes involved in quelling and MSUD are present in Basidiomycota would indicate the existence of similar pathways in Basidiomycota as has previously been described in other organisms. An established transformation system is required for applying RNAi in *H. annosum s.l.* to study the function of virulence genes. The first time that *H. annosum s.l.* was successfully transformed was by particle bombardment (Asiegbu, 2000). The selective marker hygromycin B resistance gene (*hph*) was successfully introduced into *H. annosum s.l.* (FSE-7). However, the frequencies of transformation were lower than those reported from other transformed fungi and the resistance phenotype of transformants were lost after a growth period on non-selective medium (Asiegbu, 2000). Samils *et al.* (2006) developed a rapid and simple *Agrobacterium tumefaciens*-mediated method to improve the transformation system for *H. annosum s.l.* The *hph* gene and green fluorescence protein (GFP) were successfully introduced into *H. irregulare* conidia; however, the transformants were not stable over time. One possible explanation for the instability is that the *H. annosum s.l.* conidia generally contain multiple nuclei (Korhonen & Stenlid, 1998). The introduced DNA might be integrated into only one of the nuclei in the germinating conidia and the isolate would then represent a chimera, with both nuclei that carry the integrated DNA and nuclei that do not (Samils *et al.*, 2006).

2 Aims and hypotheses

2.1 Main aim

The aim of this thesis was to better understand the virulence of *H. annosum s.l.* by acquiring knowledge of the gene expression changes associated with infection; and to develop a system for functional studies of candidate virulence genes. Such knowledge could provide a way of designing tailored control measures against the pathogen in the future.

2.2 Main hypotheses

- Pathogenicity in the *H. annosum s.l.* complex is associated with common gene expression modules during host infection
- The speciation in the *H. annosum s.l.* complex is associated with the differentiation of gene expression patterns during host infection.
- *H. annosum s.l.* possesses all the necessary components of functional RNAi machinery.
- HIRHG is one of the virulence factors of *H. annosum s.l.*, and endo-rhamnogalacturonase is associated with the necrotrophic lifestyle of the fungal pathogen.

3 Materials and methods

3.1 Biological material

The *H. irregulare* strain TC32-1 and *H. occidentale* strain TC122-12 were used in this study and maintained on Hagem agar (HA) medium (Stenlid, 1985) at 25 °C in darkness. The *M. oryzae* wild-type strain KJ201 was obtained from the Center for Fungal Genetic Resources (Korea) and was maintained on oatmeal agar medium or V8 juice agar medium at 25 °C under constant fluorescent light.

One-year-old Norway spruce plants were grown in the greenhouse at 20 °C for one month before inoculation. For study I, eight-year-old ramets were used, each ramet was from two progenies in the Norway spruce family S21H9820005 (Arnerup *et al.*, 2010). Rice seedlings (*Oryza sativa* cv. Nakdongbyeo) were grown in a growth chamber with a temperature of 25 °C, 80% humidity, fluorescent lights and a photoperiod of 16 h.

3.2 Phylogenetic studies

The protein and transcript sequences of argonaute and dicer were obtained by searching selected fungal genome databases at the Joint Genome Institute, US Department of Energy, and the Candida Database at the Broad Institute using default BLASTp searching with filtered proteins database. For argonautes and dicers, the conserved domains of all the sequences were examined using the online programme SMART (Letunic *et al.*, 2012); proteins containing both PAZ and PIWI domains were identified as argonaute and proteins containing at least two entire RNaseIII domains were identified as dicer. The fungal endo-rhamnogalacturonases were identified based on sequence features described by Markovič and Janeček (2001). For the phylogenetic study, the PAZ and PIWI domains of argonaute, two entire RNaseIII domains of dicer and whole protein

sequences of fungal endo-rhamnogalacturonases were aligned with MUSCLE (Edgar, 2004). Manual editing and the software Gblocks were used to remove amino acid columns of uncertain parts of the alignment (Talavera & Castresana, 2007). For Bayesian analysis, the protein alignment was converted to DNA alignment by the RevTrans 1.4 Server according to the predicted transcript sequences and further processed by Gblocks. ProtTest 3.2 was used to select best-fit models of amino acid replacement for the data. Maximum likelihood (ML) phylogenetic trees of protein sequences were generated by PhyML (Guindon *et al.*, 2010) using the models selected by ProtTest 3.2 (Darriba *et al.*, 2011). For Bayesian analysis, the evolutionary model was set to the GTR substitution model with gamma-distributed rate variation across sites and a proportion of invariable sites. The sample and print frequency was set to 500, the diagnostic frequency to 5000, and the run length to 1,000,000. To summarize the trees, the same burn-in was used as the mcmc command when the final standard deviation of split frequencies was less than 0.01.

NOTUNG and COUNT were used to analyse gene loss and duplication events. NOTUNG employs a parsimony approach to reconcile the gene tree with the species tree (Chen *et al.*, 2000). The weakly supported branches in the gene tree (edges weighted below the user-specified Edge Weight Threshold, EWT) were rearranged by the programme to minimize penalty scores for gene duplications and losses. COUNT was used to analyse the gain and loss of endo-rhamnogalacturonase genes using Dollo and Wagner parsimony (Csuroes, 2010). A phylogenetic tree of fungal species was synthesized with information from several publications and used in this study (Hu *et al.*, 2013; Floudas *et al.*, 2012; Binder *et al.*, 2010; Hibbett, 2006).

3.3 RNAseq and data analysis

The two isolates TC32-1 and TC122-12 were grown in liquid Hagem medium for two and four weeks, and spruce barks infected with the two isolates were harvested for RNA isolation after two, four or six weeks. Total RNA was isolated as described by (Chang *et al.*, 1993) and stored at −80 °C. The RNA 6000 Nano Kit (Agilent Technologies) was used to evaluate the quantity and integrity of the total RNA using the Bio-analyzer 2001. Total RNA was treated with DNase I (Sigma-Aldrich) to eliminate contamination of genomic DNA. Library construction and cDNA synthesis were performed at the SNP&SEQ Technology Platform of Uppsala University Hospital. High-throughput sequencing was performed using the Illumina Hiseq (Illumina, San Diego, CA, USA) according to standard protocols. The samples were sequenced for ‘paired-end’ reads. The software Nesoni v1.0 was used to filter the Illumina

reads and all the filtered reads were mapped to *H. irregulare* and *H. occidentale* genomes (Lind *et al.*, 2012; Olson *et al.*, 2012) by Tophat v2.0 with a setting to disallow any mismatch, and the software MAKER was used to re-annotate gene models. Differential expression analysis was processed by the software package Cufflinks v2.0 and visualized by CummeRbund (Trapnell *et al.*, 2012). BLAST2GO v3.10 (Conesa *et al.*, 2005) was used to annotate *H. irregulare* and *H. occidentale* transcripts as well as assessing Gene Ontology (GO) term enrichment. Reciprocal BLAST analysis was used to search for One-to-one orthologous genes between *H. irregulare* and *H. occidentale*.

3.4 qPCR

For qPCR, an iScript™ cDNA synthesis kit (Bio-Rad) was used to reverse transcribe RNA. Transcript levels were quantified by RT-qPCR using the iQ5 qPCR System (Bio-Rad, Hercules, CA). PCR was performed using a SsoFast™ EvaGreen® Supermix kit (Bio-Rad). Primers were designed using the Primer3 software (Untergrasser *et al.*, 2000) with a melting temperature (Tm) between 58 and 62 °C. Amplification of a single product was confirmed by melt curve analysis, and the PCR efficiency was measured using a linear plasmid standard curve. Transcript abundance was normalized to the constitutively expressed genes encoding actin, L-kynurenine hydrolase (Tryp metab) and RNA polymerase III transcription factor (RNA Pol3 TF) (Raffaello & Asiegbu, 2013), which all showed low variation among samples by best-ref. The relative expression was calculated using the Pfaffl method (Pfaffl *et al.*, 2002). Transcript levels were determined in at least three biological replicates, each based on three technical replicates.

3.5 Western blot

The antibody targeting HIRHG was produced by GenScript HK Limited based on the synthetic peptides mixture with sequences of GTVGPTTKLSAKGHTC, LDYGGKVGSTDIGP and NWDGEVVDGVQRAP, which specifically targeted the HIRHG protein in *H. irregulare*. Total protein extraction and western blot were carried out according to (Elfstrand *et al.*, 2002). 30 g protein samples were loaded for SDS-PAGE. After gel electrophoresis, protein was transferred to a polyvinylidene difluoride (PVDF) membrane using the trans-blot turbo transfer system (Bio-Rad). HIRHG protein was detected using one μg/mL primary antibody and an anti-rabbit horseradish peroxidase-conjugated secondary antibody from donkey (GE Healthcare). The membranes were

developed using an ECL Prime kit (Amersham, GE Healthcare) and detected in an LAS-3000 luminescent image analyser (Fujifilm, Fuji Photo Film).

3.6 Heterologous gene expression in *Magnaporthe oryzae*

HIRHG coding sequence was PCR-amplified from TC32-1 cDNA used in RT-qPCR and inserted into the pCB1004 vector downstream of the Ptrpc promoter to generate pCB1004-Ptrpc::HIRHG and pCB1004-Ptrpc::HIRHG-mcherry fusion. Polyethylene glycol (PEG)-mediated transformation was performed using *M. oryzae* wild-type strain KJ201 protoplasts and hygromycin-resistant transformants were selected. A quick and accurate PCR-based screening method was used to screen the mutants to confirm that the target genes were inserted into the *M. oryzae* wild-type strain KJ201 genome (Park *et al.*, 2014). Reverse transcription PCR was used to confirm the expression of the target gene in the mutants. Mycelial growth was quantified according to Jeon *et al.* (2014) and a pathogenicity assay was performed according to Kim *et al.* (2009).

3.7 Statistical analysis

Data were analysed using Minitab 16 (Minitab Inc.) by performing analysis of variance (one way ANOVA) using a general linear model; pairwise comparisons were made using Tukey's test at the 95% significance level.

4 Results

4.1 Virulence of *H .irregulare* and *H. occidentale* on Norway spruce

Both *H. occidentale* and *H. irregulare* were able to induce necrosis and colonise the sapwood of the four-year-old branches of Norway spruce. The success rate of infections was slightly higher for *H. occidentale* (87%) than for *H. irregulare* inoculations (73%). There was no significant difference in the growth of *H. occidentale* and *H. irregulare* in the sapwood or in the expansion of lesions in the inner bark (Table 1). However, both species showed significantly more growth in the sapwood and greater lesion expansion in the inner bark at 4 and 6 weeks compared with at two weeks (Tukey's test, $P<0.05$).

Table 1. *Virulence of H. irregulare and H. occidentale measured as fungal growth in the spruce sapwood, and lesion expansion in the inner bark. (Growth = Growth in the sapwood, lesion = lesion length in the inner bark, 2w, 4w and 6w = 2, 4 and 6 weeks after inoculation)*

	Growth (mm)			Lesion (mm)		
	2w	4w	6w	2w	4w	6w
H. occidentale	16.7±12.6	42.0±28.4	41.0±36.0	7.7±4.2	21.2±16.6	28.2±11.0
H. irregulare	23.8±11.1	58.3±7.6	62.5±20.6	9.5±7.6	11.0±5.0	20.5±7.2

4.2 *H .irregulare* and *H. occidentale* have different gene expression patterns *in planta*

4.2.1 Genome annotation and orthologous gene identification showed the similarity between *H. irregulare* and *H. occidentale* genomes

The genome sequences of *H. irregulare* and *H. occidentale* were acquired from previous published data (Lind *et al.*, 2012; Olson *et al.*, 2012) and were re-assembled and re-annotated using MAKER so that they could be used as reference genomes for RNA sequencing. In total, we identified 9462 gene models in *H. irregulare* and 10,295 in *H. occidentale.* Our annotation of the *H. irregulare* genome and the published annotation of the *H. irregulare* genome available at the Joint Genome Institute website (http://genome.jgi.doe.gov/Hetan2/Hetan2.home.html) shared 8412 (89%) gene models. The two species, *H. irregulare* and *H. occidentale*, shared 7545 one-to-one orthologous genes. To further identify similar genes, the gene models from both two genomes were grouped together by orthoMCL into 8306 groups.

4.2.2 Numbers of *H. irregulare* and *H. occidentale* genes are consistently induced during infection

After filtering the pure fungal (liquid culture) samples, we obtained around 10 million–14 million reads per sample. The fungal tree interaction samples, which were sequenced much deeper, yielded between 30.5 million and 154.8 million reads per sample. From the fungal tree interaction samples we achieved more than 500,000 mapped aligned pairs except from one of three replicates of one sample for which we obtained 229,570 mapped aligned pairs (Table 2). In *H. irregulare*, 2081 genes were significantly differentially expressed between any two treatments [grown in liquid culture (L), 2-weeks in bark (2w), 4-weeks in bark (4w) and 6-weeks in bark (6w)] compared with 2360 genes in *H. occidentale*. PCA analysis of the expression of the four samples of each species suggested that the L sample was very different from the samples of fungal growth in bark (Fig. 2). By comparing the significant differentially expressed genes (DEGs) of the 2w, 4w and 6w treatments versus the L treatment, 385 *H. irregulare* genes and 407 *H. occidentale* genes were shown to be consistently up-regulated (CUGs) and 222 *H. irregulare* genes and 310 *H. occidentale* genes were consistently down-regulated (CDGs) (Figure 2).

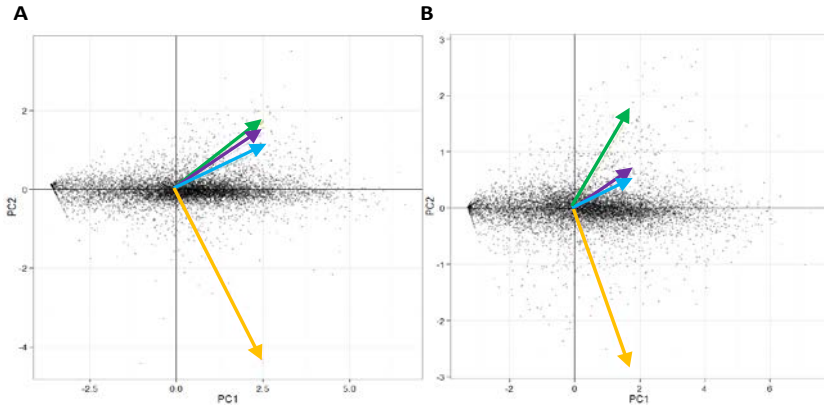

Figure 2. PCA analysis of the gene expression of (A) *H. irregulare* and (B) *H. occidentale* when grown in liquid culture (yellow), 2-weeks in bark (green), 4-weeks in bark (purple) and 6-weeks in bark (blue).

Table 2. Summary of RNA sequencing data. The numbers of Total reads were obtained after filtering with Nesoni. The aligned pairs were mapped by Tophat2 based on no base pair mismatch. (L = liquid culture, 2w, 4w and 6w = 2 weeks, 4 weeks and 6 weeks after inoculating Norway spruce bark with the fungus)

Sample names	Total reads	Aligned pairs	Mapped pairs(%)
TC32-1-L-rep1	11809378	9114879	77.2%
TC32-1-L-rep2	10555531	8764947	83.0%
TC32-1-L-rep3	13240830	11124807	84.0%
TC32-1-2w-rep1	39748040	1148233	2.9%
TC32-1-2w-rep2	43180655	1637664	3.8%
TC32-1-2w-rep3	40750502	1282462	3.1%
TC32-1-4w-rep1	121996688	583954	0.5%
TC32-1-4w-rep2	49434129	1241663	2.5%
TC32-1-4w-rep3	95247997	3219921	3.4%
TC32-1-6w-rep1	58357874	229570	0.4%
TC32-1-6w-rep2	53266103	681649	1.3%
TC32-1-6w-rep3	55675183	975852	1.8%
TC122-12-L-rep1	13661132	11135225	81.5%
TC122-12-L-rep2	12389419	10207752	82.4%
TC122-12-L-rep3	11098200	9092986	81.9%
TC122-12-2w-rep1	30863928	6764247	21.9%
TC122-12-2w-rep2	39222132	1928245	4.9%
TC122-12-2w-rep3	33431531	2346657	7.0%
TC122-12-4w-rep1	154786107	1519679	1.0%

TC122-12-4w-rep2	78321161	1714337	2.2%
TC122-12-4w-rep3	32287019	4234747	13.1%
TC122-12-6w-rep1	30472557	5817262	19.1%
TC122-12-6w-rep2	73805957	3078694	4.2%
TC122-12-6w-rep3	142671388	3277956	2.3%

4.2.3 *H .irregulare* and *H. occidentale* have both common and different enriched GO terms for *in planta* growth

In total, 60% of the gene models of the *H. irregulare* genome and 56% of the *H. occidentale* gene models were assigned to the GO terms. CUGs were enriched in 17 GO terms in *H. irregulare* and *H. occidentale*, but the GO terms were different between the species. The alpha-amino acid catabolic process (GO:1901606), drug transport (GO:0015893), benzoate metabolic process (GO:0018874) and xenobiotic catabolic process (GO:1901606) were uniquely enriched in *H. irregulare*; whereas carbohydrate transport (GO:0008643), polysaccharide metabolic process (GO:0005976), cellular carbohydrate metabolic process (GO:0044262) and cell wall organization (GO:0071555) were specifically enriched in *H. occidentale*. Far fewer GO categories were enriched among the consistently down-regulated genes in the samples of fungal growth in bark. The expression patterns of genes assigned to the enriched GO terms compared with their orthologues further confirmed the specificity of the expression of those genes to the species (Figure 3).

A

	HI			HO			
JGI-proteins-ID	L-2w	L-4w	L-6w	L-2w	L-4w	L-6w	SeqDesc
305989	1.56	1.48	1.86	0.60	0.52	0.63	glycine dehydrogenase
471015	2.63	3.18	2.21	-1.23	-0.08	0.25	3-oxoacid -transferase
380456	1.18	1.40	0.95	-1.03	-0.18	-0.39	arginase
157104	3.07	3.21	2.72	2.33	4.33	3.57	homogentisate -dioxygenase
384796	2.08	2.42	1.50	2.23	2.14	1.63	flavocytochrome c
103954	1.32	1.10	1.35	1.19	1.54	1.13	glycine cleavage system t protein
126494	5.44	5.18	5.63	0.86	2.89	1.95	mop flippase
429046	2.61	2.89	1.98	-1.09	0.71	0.19	aromatic compound dioxygenase
454399	2.33	3.39	1.69	-0.12	0.29	0.92	aldehyde dehydrogenase
66124	4.47	5.96	4.34	3.11	3.90	3.73	abc transporter
330547	1.84	2.07	1.89	0.32	0.95	0.61	3-hydroxyanthranilic acid dioxygenase
456302	1.95	1.89	1.53	0.86	0.52	0.51	3-hydroxyacyl-CoA dehydrogenase
423519	2.20	2.85	2.01	-1.59	-1.56	-1.01	phenylalanine ammonia-lyase

B

	HI			HO			
JGI-proteins-ID	L-2w	L-4w	L-6w	L-2w	L-4w	L-6w	SeqDesc
42076	5.04	2.38	4.02	3.28	2.28	2.16	glycoside hydrolase family 28 protein
121313	1.35	-0.38	1.01	4.58	1.92	1.69	glycoside hydrolase family 28 protein
407618	0.43	0.23	0.27	1.55	1.09	1.51	3-hydroxyisobutyrate dehydrogenase
60203	0.52	-0.08	0.42	1.55	1.11	1.25	udp-galactose transporter
471495	0.82	0.56	0.74	1.43	1.30	1.23	myo-inositol-1-phosphate synthase
122126	0.37	-1.41	1.06	4.85	3.43	3.35	dak1-domain-containing protein
123490	0.92	0.24	0.64	2.09	0.96	1.20	glycoside hydrolase family 3 protein
62767	2.04	1.49	2.09	3.03	3.57	2.77	mfs general substrate transporter
46597	0.93	0.00	0.82	3.32	1.41	1.78	glycoside hydrolase family 3 protein
157934	1.00	-0.47	1.08	3.48	1.88	2.34	sugar transporter
172978	3.38	-0.44	2.64	7.36	1.84	1.69	glycoside hydrolase family 43 protein
45732	5.24	-0.48	4.60	6.98	2.46	2.56	carbohydrate esterase family 8 protein
61998	1.51	0.96	1.03	3.82	3.75	2.81	mfs sugar transporter
48830	4.52	3.12	4.69	4.85	3.45	3.49	general substrate transporter
67107	5.34	1.19	4.92	8.25	6.39	6.81	general substrate transporter
325916	0.41	0.72	0.69	1.64	1.96	1.54	duf706-domain-containing protein
12581	0.09	0.19	0.44	1.57	1.70	0.98	mfs sugar partial
Not available	-0.25	-0.87	-0.22	2.11	1.21	1.62	pin domain-like protein
157457	2.86	1.94	1.59	1.00	1.78	1.20	hexose transporter
152014	4.68	-0.19	4.34	7.00	2.43	2.84	glycoside hydrolase family 28 protein-PG1
53076	1.86	-0.90	1.24	2.76	1.64	1.82	glycoside hydrolase family 5 protein
37838	1.75	-0.01	1.61	5.30	3.00	3.22	general substrate transporter
148374	-2.88	-3.36	-1.13	4.11	1.23	1.96	general substrate transporter
106809	4.10	3.35	3.60	3.07	3.80	2.17	general substrate transporter
164687	2.50	1.99	1.56	3.98	2.29	1.96	glycoside hydrolase family 12 protein
331340	3.97	1.09	2.41	4.55	1.21	1.66	endo-beta-xylanase
12392	3.64	-1.43	2.97	8.70	4.42	4.59	glycoside hydrolase family 28 protein

Figure 3. Detailed expression patterns of genes assigned to the enriched GO terms, which are specific in *H. irregulare* (A) and *H. occidentale* (B) compare to their orthologues. The number and colour indicate the log2 fold change. (L-2w, -4w and –w = liquid culture compared with 2 weeks, 4 weeks and 6 weeks fungal growth in spruce bark)

4.2.4 Common and different CUGs from *H .irregulare* and *H. occidentale*

Of the CUGs, 369 genes in *H. irregulare* and 380 in *H. occidentale* had an orthologous gene in the other genome; however, only 143 gene models with orthologues were consistently up-regulated in bark in both species (Figure 4A). Among the consistently down-regulated genes in bark there were 70 orthologous gene pairs (Figure 4B). In addition, there were nine *H. irregulare* genes that corresponded to 11 *H. occidentale* genes that did not have reciprocal orthologues but were in the same gene families and were also among the shared up-regulated genes. The 143 CUGs in both species included a number of genes encoding host material degradation enzymes, transmembrane transporters, and genes involved in metabolism.

Among the CUGs identified specifically in *H. irregulare* or *H. occidentale*, 226 and 237, respectively, had a reciprocal BLAST hit in the other species were the gene was not significantly up-regulated. In addition, 15 *H. irregulare* and 22 *H. occidentale* CUGs were specifically induced in their respective species that did not have a reciprocal BLAST hit (Figure 4). A set of 241 *H. irregulare* genes and 259 *H. occidentale* genes, corresponding to two thirds of the CUGs, were species specific induced. The Kyoto Encyclopedia of Genes and Genomes (KEGG) Automatic Annotation Server (KAAS) BLAST assigned 91 of the 241 *H. irregulare* genes with KEGG orthology (KO) terms and were mapped to 102 KEGG pathways, and 87 of the 259 *H. occidentale* genes were assigned with KO terms and were mapped to 85 KEGG pathways. Approximately half of the KEGG pathways identified in *H. irregulare* and *H. occidentale* were the same.

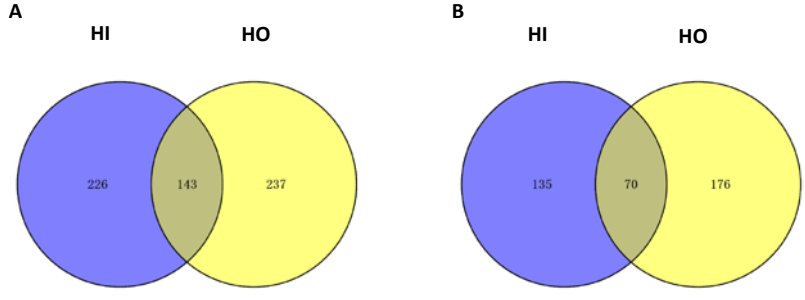

Figure 4. The reciprocal BLAST revealed large differences in the amount of *H. irregulare* and *H. occidentale* CUGs (A) and CDGs (B). (The numbers indicate the number of gene models, HI = *H. irregulare*, HO = *H. occidentale*).

4.3 Evolution of argonaute and dicer in Basidiomycota

4.3.1 Argonaute and dicer are widely represented and relatively conserved in Basidiomycota

In total, 194 argonaute- and 104 dicer-encoding genes were identified from 43 fungal genomes. The copy number of both argonaute and dicer genes differed substantially between species. Generally, the copy numbers of argonaute and dicer were significantly greater in Agaricomycetes (6 ± 1.3 and 3 ± 0.7, respectively) than in other Basidiomycota species (1.6 ± 1.4 and 1.2 ± 0.7), or in the Ascomycota (1.8 ±1.2 and 1.2 ± 0.8). All the fungal argonaute proteins had a conserved structure containing a PAZ domain and a PIWI domain. Fungal dicers were composed of a DExH box, a RNA helicase domain (HELICc), a dsRNA binding domain, a PAZ domain, two RNaseIII domains

and another dsRNA binding domain, organized from N-terminal to C-terminal. However, a number of dicer proteins did not contain all domains.

4.3.2 Phylogenetic analysis of argonaute and dicer

The Basidiomycota argonaute proteins, excluding the Tremellales, were divided into two groups with weak support that from now on will be referred to as ago-A and -B. The ago-B was a Basidiomycota-specific group whereas the ago-A group contained proteins from both Ascomycota and Basidiomycota species. The Basidiomycota dicer proteins were divided into three groups, referred to as dcl-A, -B, and -C. The dcl-C group only contained proteins from Basidiomycota species whereas dcl-B also included one group of Ascomycota dicer. Although the phylogeny of both dcl-B and dcl-C subgroups followed the taxonomic diversification of fungal groups, dcl-A did not. The Tremellales argonautes and dicers were a diversified group that was separate from the other Basidiomycota dicers, and seemed to be related to the Ascomycota dicers.

Gene duplication of argonaute and dicer happened in parallel in the Zygomycota and Dikarya. In Dikarya, gene duplication of dicers essentially happened in two steps: first it occurred during early diversification of the Dikarya and Basidiomycota, which resulted in the three dicer groups; and then it occurred much more recently and in parallel in several genera. Frequent duplications and losses of argonautes were found in different groups within the Agaricomycotina, except in the Tremellales where reduced copies of argonaute were found.

4.4 The endo-rhamnogalacturonase of *H. irregulare* (HIRHG)

4.4.1 Expression of the *HIRHG* gene and production of the HIRHG protein

There was only one copy of the endo-rhamnogalacturonase gene (*RHG*) in the *H. irregulare* genome, and it was located in the pathogenicity QTL region. Transcript levels of *HIRHG* were significantly elevated during infection in bark. This finding had been indicated by a previous microarray experiment (Olson *et al*., 2012) and was confirmed by the RNAseq data. The RNAseq data showed that both *HIRHG* and its orthologous gene in *H. occidentale* were up-regulated in spruce bark; however the *HIRHG* showed a higher fold change at two weeks and six weeks in bark (Figure 5A). The expression of *HIRHG* was validated by Q-PCR, which showed that it increased 25- and 19-fold in spruce bark after 2- and 4-weeks growth, respectively (Figure 5B). *H. irregulare* grew faster on pectin culture medium than on the media containing other sugar sources, which was manifested as a larger colony radius, and the protein

extracted from the mycelia gave the strongest signal using western blot (Figure 5 C and D).

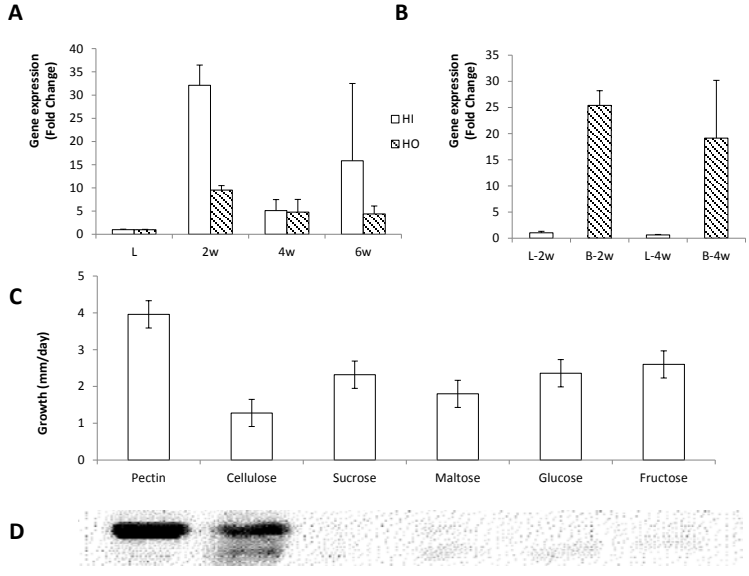

Figure 5. H. annosum s.l. RHG gene regulation during infection of *Picea abies* and *H. irregulare* growth rate and HIRHG protein production on different carbon sources. (A) Gene expression of *HIRHG* and its orthologue in *H. occidentale* measured by RNA sequencing compared by fold change. (B) *HIRHG* gene expression was profiled using quantitative reverse transcription-polymerase chain reaction (qRT-PCR) throughout 2 and 4 weeks in liquid culture and after infection. Expression of the *H. irregulare* actin, RNA polymerase II transcription factor, and tryptophan catabolism genes were used as constitutively expressed endogenous gene controls, and the level of *HIRHG* expression was determined relative to these three endogenous gene controls. Three to five biological replicates, each containing three technical replicates for each sample, were performed. Statistical significance was analysed using one-way ANOVA followed by Tukey's test. (C) The mean radius of *H. irregulare* colonies after one week of growth on plates. (D) Western blot of *H. irregulare* HIRHG in extracts from mycelium grown on different carbon sources.

4.4.2 Distribution of fungal endo-rhamnogalacturonase

Maximum likelihood analysis of the amino acid sequences revealed that the fungal endo-rhamnogalacturonase genes have a very similar phylogenetic relationship (Figure 6). The phylogeny of endo-rhamnogalacturonases in 59 published fungal genomes representing fungal pathogens and fungi with other trophic modes was investigated. Most of the 22 biotrophic and hemibiotrophic plant pathogens did not have any endo-rhamnogalacturonase genes, the exceptions being the hemibiotrophic plant pathogen *Mycosphaerella graminicola*, the biotrophic plant pathogens *Puccinia graminis* and

Melampsora laricis-populina and the mycorrhizal *Tuber melanosporum*. Most of the necrotrophic plant pathogens and saprotrophs had between one and six gene copies of the *RHG* gene in their genomes. The gene families were expanded in some *Aspergillus* spp. The main group of identified endo-rhamnogalacturonase genes (52 of the 58) were genes from necrotrophic plant pathogens and saprotrophs. In this cluster, the phylogeny of the genes basically followed the species phylogeny; the only exception was the *Armillaria mellea* gene (Armme1/425), which clustered together with the Ascomycota instead of the Basidiomycota.

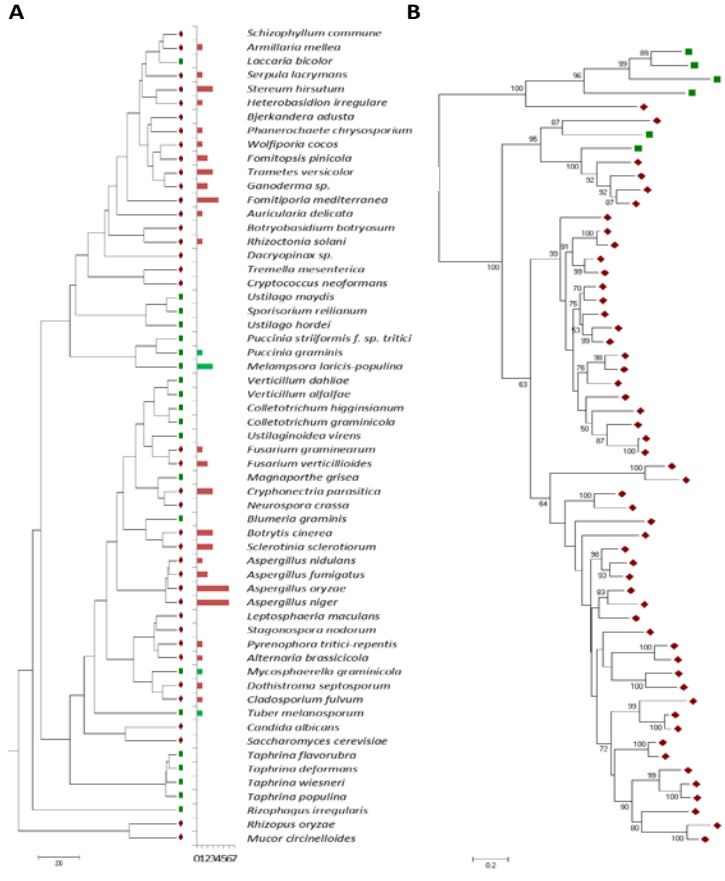

Figure 6. Diversity and distribution of fungal RHG is associated with the fungal life style. (A) The species phylogeny of 59 fungi with published genome sequences: the green squares indicate biotrophs and hemibiotrophs, the brown oblique squares indicate necrotrophs and saprotrophs. The columns under the phylogenetic tree indicate the number of gene copies. (B) The maximum likelihood tree of fungal RHG protein sequences. Green squares indicate genes from biotrophs and hemibiotrophs, and brown oblique squares indicate necrotrophs and saprotrophs.

4.4.3 Expressing *HIRHG* in *M. oryzae* improves growth on pectin

A *M. oryzae* wild-type strain (KJ201) was transformed by PEG-mediated fungal transformation with *HIRHG* and *HIRHG::mcherry*. The two *HIRHG* expression strains C7 and C8 and two *HIRHG::mcherry* fusion expression strains 1-6 and 2-20 showed no significant difference in conidiation, conidial morphology, conidial germination and appressorium formation. The growth rates of transformants on pectin agarose medium were found to be significantly greater than that of the wild type ($P<0.05$, Tukey's test): the mean radius of the colonies of transformants increased by between 3.08 mm and 3.22 mm per day, whereas the radius of the wild type increased by 2.44 mm/day (Figure 7). The transformants were also inoculated on rice and showed no significant change in virulence compared with the wild type.

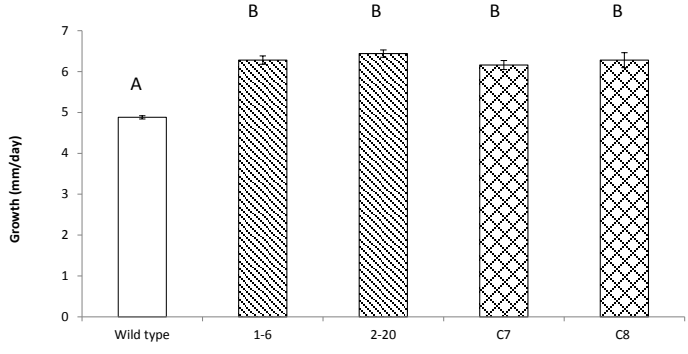

Figure 7. Growth rate of the rice blast fungus *M. oryzae* expressing *HIRHG* and *HIRHG::mCherry* fusion on apple pectin media. Strains 1-6 and 2-20 were *M. oryzae*-expressed *HIRHG::mCherry* fusion, C7 and C8 were *M. oryzae*-expressed *HIRHG*. The different letters (A and B) indicate a significant difference in growth rate.

5 Discussion

As one of the most devastating forest pathogens, *H. annosum s.l.* has been studied intensively. Genetic mappings, combined with genomic and transcriptomic studies, have accumulated information on potential virulence factors. However, the differences in global gene expression between the species within the *H. annosum s.l.* species complex, and how that relates to their evolutionary history and host preference is poorly understood. Furthermore, the molecular functions of identified candidate virulence genes need to be investigated for a deeper understanding of virulence. In this thesis, deep RNA sequencing was used to explore the common and different transcriptomes as well as to generate more candidate virulence genes for future molecular characterization (Paper I). In parallel, the evolution of RNA interference was investigated to establish the RNAi-based gene-silencing tool for studying virulence candidates in basidiomycetes in general and *H. annosum s.l.* in particular (Paper II). Finally, one of the previously identified candidate virulence genes, an endo-rhamnogalacturonase (*RHG*) gene from the GH28 family, was chosen for further characterization (Paper III).

Despite being closely related conifer pathogens, *H. irregulare* and *H. occidentale* apparently with different infection strategies (Paper I). The pine specialist *H. irregulare* induced more genes active in detoxification when growing within Norway spruce tissue than *H. occidentale*. To colonize their host successfully, pathogens have to overcome the antimicrobial effects of chemicals such as terpenes, phenolic and nitrogen-containing compounds (Franceschi *et al.*, 2005) by employing their xenobiotic metabolizing enzymes (Lah *et al.*, 2011; Sexton *et al.*, 2009). Dioxygenase, an aromatic compound, was up-regulated during the interaction of *H. irregulare* with spruce. Dioxygenase catalyses the oxidative ring cleavage of catechol, which might be involved in the detoxification of phenolic compounds produced by the host. In *H. annosum s.s.*-infected tissues, stilbenes that have been converted to ring-opened, deglycosylated, and dimeric products have been found (Danielsson *et*

al., 2011). Analyses of *Ceratocystis polonica* protein and metabolite extracts have shown that these stilbene metabolites arise from fungal enzyme activities (Hammerbacher *et al.*, 2013). Possibly, *H. irregulare* uses the aromatic compound dioxygenase to generate linearized stilbene metabolites for nutrition. The efflux of antimicrobial compounds out of cells is another important detoxification mechanism. One Multidrug/Oligosaccharidyl-lipid/Polysaccharide (MOP) flippase is induced in *H. irregulare* significantly. Several members of the MOP flippases have been functionally characterized and have been shown to export diverse drugs out of *Escherichia coli* (Mortier-Barriere *et al.*, 1998). Perhaps MOP flippases have the same function in *H. irregulare*, exporting the Norway spruce defence compounds.

Another key finding of the differences between *H. irregulare* and *H. occidentale* during infection was that *H. irregulare* induced more biosynthetic genes. Production of low-molecular weight toxins is proposed to be an important virulence factor of many necrotrophic plant pathogens (Bartz *et al.*, 2013). *H. annosum s.l.* is known to produce toxins such as oosponol, fomannosin, fomannoxin, and the fomajorins (Asiegbu *et al.*, 2005). The CUGs of *H. irregulare* and *H. occidentale* mapped to very diverse KEGG pathways, which indicates that diverse toxin production is induced during pathogen-growth in spruce bark. The secondary metabolite profile of the pine-infecting species is very different from that of the non-pine-infecting species of *H. annosum s.l.* (Hansson *et al.*, 2014). Here, the patterns of gene expression mapped to different KEGG pathways in *H. irregulare and H. occidentale*, suggesting that the secondary metabolites produced in spruce bark also differed between the two species. In particular, the relatively large number of biosynthetic genes up-regulated in *H. irregulare* should reflect a better capacity to produce a wider arsenal of toxins in spruce bark.

Interestingly, we also found that the glyoxylate cycle was only induced during *in planta* growth in *H. irregulare*, which is reminiscent of the induction of the glyoxylate cycle in *Fusarium graminearum* growing in wheat (Zhang *et al.*, 2012). The glyoxylate cycle requires mitochondrial inner membrane carriers to transport isocitrate to cytosol. Three mitochondrial carriers and one mitochondrial inner membrane carrier were up-regulated in *H. irregulare*. When one of the mitochondrial carrier genes (*CIC1* or *FOW1*) was knocked out in *F. graminearum* or *Fusarium oxysporum* to disrupt the glyoxylate cycle, the mutants showed normal growth *in vitro*; however, the size of the lesions in infected coleoptiles were reduced to approximately one third, whereas the lesion size induced by the complemented strain were similar to that of the wild type (Zhang *et al.*, 2012; Inoue *et al.*, 2002). The glyoxylate cycle has also been shown to be important for virulence in *Candida albicans* (Lorenz & Fink,

2001). Furthermore, the importance of the glyoxylate cycle for *H. irregulare* growing in the host tree could be correlated to a previous observation of mitochondrial inference of *H. annosum s.l.* virulence (Olson & Stenlid, 2001). Perhaps, the activation of the glyoxylate cycle relies on the interaction of the tricarboxylic acid cycle (TCA cycle) within mitochondria, reflecting the observation that mitochondria control virulence.

Genes in different GH families are often found to be up-regulated in saprotrophic wood decay (Floudas *et al.*, 2012). Although there were genes of GH families that were significantly up-regulated in both *H. irregulare* and *H. occidentale*, far more of these genes were highly expressed in *H. occidentale*, especially at two weeks after the infection. This indicates that *H. occidentale* is likely to employ these enzymes for the destruction of the host cell structure and at an earlier infection stage than *H. irregulare* does. It also suggests that *H. occidentale* uses degraded plant material as a carbon source during this phase of the interaction. This type of virulence has also been shown in other necrotrophic pathogens. The GH28s are cell wall-degrading enzymes, for which there is some evidence that they function as virulence factors in *B. cinerea* (ten Have et al., 1998), *Alternaria citri* (Isshiki *et al.*, 2001) and *Aspergillus flavus* (Shieh *et al.*, 1997) by causing cell wall decomposition and tissue maceration. Importantly, a *Claviceps purpurea* strain carrying a deletion of two GH28 genes is nearly non-pathogenic on rye without affecting its vegetative properties (Oeser *et al.*, 2002). Here, one GH28 was found to be up-regulated in both *H. irregulare* and *H. occidentale* when growing in the host, and three other GH28s and a number of additional GHs for cellulose- and hemi-cellulose degradation were highly induced in *H. occidentale*, which indicates that GH28 might play a more important role in *H. occidentale* survival and spread throughout woody tissues than it does in *H. irregulare*.

Being closely related species in a species complex, *H. irregulare* and *H. occidentale* share the majority of their gene models. However, when inoculated on the same host species (which is not the natural host for either species) they showed divergent gene expression patterns. Approximately two thirds of the CUGs during growth in bark are specifically induced in that species. The evolutionary history of *H. annosum s.l.* shows that the separation of *H. irregulare* and *H. occidentale* from the last common ancestor to modern species is associated with the host preference for pine infection and non-pine infection (Dalman *et al.*, 2010). If the difference in gene expression between the two species were a consequence of their separate evolutionary history the genes that are differentially expressed would be random. However, the differences between the differentially expressed genes in the two species are not random, which is reflected by the difference between *H. irregulare* and *H.*

occidentale in enriched GO terms. This indicates that the differences found are likely to be as a result of adaptive selection to their respective host tree.

Even though *H. irregulare* and *H. occidentale* showed a large difference in their gene expression pattern during infection, they appear to share some common features needed for virulence. There are a number of transporters that were up-regulated in both species. These transporters might be important for the pathogen to acquire nutrients from the host tissue as well as for transporting out molecules that may be harmful for the pathogen. Another common feature was the induction of degrading enzymes. Laccases, which have traditionally been considered as virulence factors of tree pathogens (Kuo *et al.*, 2015; Asiegbu *et al.*, 2005), were induced in both species and expressed at high levels. Laccases are multi-copper-containing enzymes that catalyse the oxidation of phenolic compounds, including the bioconversion of lignin and degrading phenolic compounds (Mayer & Staples, 2002; Pezet *et al.*, 1992). In addition, clavaminate synthase-like proteins, which belong to the alpha-ketoglutarate-dependent oxygenases, were highly expressed and induced. Clavaminate synthase-like proteins have been observed in the interaction between *H. annosum s.s* and Norway spruce, and have been predicted to be involved in secondary metabolism for fungal toxin production (Lunden *et al.*, 2015). One of the *GH28* genes that were commonly up-regulated in both species was an *RHG* gene. *RHG* is located in one of the virulence QTLs, and it is considered to be an important virulence factor for both *H. irregulare* (Olson et al., 2012) and *H. occidentale*. *HIRHG*, the *H. irregulare* endo-rhamnogalacturonase gene was selected for functional characterization (Paper III).

The RNAseq study (Paper I) together with earlier genetic mapping using QTLs (Olson *et al.*, 2012; Lind *et al.*, 2007) and a GWA study (Dalman *et al.*, 2013) identified a number of candidate virulence genes. To investigate the function of a particular candidate gene will be the next step to understanding the virulence of *H. annosum s.l.* Reverse genetics such as targeted gene disruption and gene silencing by RNAi are appreciated as seminal tools for investigating gene products. Gene silencing by RNAi has been used to reduce the expression of a target gene in several other organisms to analyse their loss-of-function phenotype. Compared with the complete deletion or disruption of a target gene, RNAi allows analysis of genes involved in a conserved biological process, without which the organisms might not survive after deletion or disruption. Many of the candidate virulence genes of *H. annosum s.l.* encode enzymes involved in metabolic processes (Olson *et al.*, 2012); RNAi could be a suitable technique to further investigate their function.

Phylogenetic analysis of argonaute and dicer proteins gives insights into the diversification of RNA silencing pathways during the evolution of the Basidiomycota. It also offers the potential for a deeper understanding of the mechanism of RNAi in the Basidiomycota and might eventually lead to its application in research. Specifically, we investigated whether orthologues to genes involved in quelling and MSUD are present in the Basidiomycota, which would indicate the existence of similar pathways in the Basidiomycota as those that have previously been described in other organisms. *N. crassa* harbours two dicer-encoding genes (dcl-1 and dcl-2) and the argonaute-encoding gene qde-2, which is involved in the quelling pathway (Li *et al.*, 2010). Both *N. crassa* dcl-1 and dcl-2 are able to process dsRNA in the quelling pathway; however, dcl-2 is the major dsRNA processing enzyme (Li *et al.*, 2010; Catalanotto *et al.*, 2004). In our phylogeny, *N. crassa* dcl-2 clustered together with the Basidiomycota-specific clade dcl-B, and qde-2 clustered together with the ago-A clade. *Dacryopinax*, Tremellales and *M. laricis-populina* all lack members of both dcl-B and ago-A, which might indicate that the quelling pathway does not exist in these fungi. The species *Botryobasidium botryosum*, *Auricularia delicata* and *Fomitiporia mediterranea* lack the dcl-B protein but they have proteins represented in both the ago-A and -B clades. Perhaps their quelling pathway could be maintained by dcl-A proteins given that dcl-1 and dcl-2 have been shown to be partially functionally redundant in *N. crassa* (Chang *et al.*, 2012). MSUD, which only functions during meiosis, relies on *N. crassa* dcl-1 and not dcl-2 for processing the RNA; the small RNAs are then loaded onto a sms-2-based RISC complex. Neither dcl-1 nor sms-2 cluster together with any of the dicer or argonaute groups containing proteins from the Basidiomycota. This may indicate that the MSUD pathway is missing in Basidiomycota, although other as yet unidentified pathways might operate during the sexual cycle. There are some additional RNAi pathways found in *N. crassa* that all share a core component, the qde-2-associated RISC (Li *et al.*, 2010). Given that the Basidiomycota ago-1 clustered with the *N. crassa* qde-2 and is present in all Basidiomycota except *Dacryopinax*, Tremellales and *Melampsora laricis-populina*, similar pathways might also exist in the Basidiomycota. Very interestingly, one unique dicer (dcl-C) cluster and one unique argonaute (ago-B) cluster were found in the Basidiomycota. The presence of these unique clusters may indicate that the Basidiomycota could possess as yet undiscovered RNAi pathways. The RNAi proteins in Tremellales seem to be distinctively diversified from other Basidiomycota. An RNAi mechanism was discovered in *C. neoformans* that is specifically activated during sexual reproduction and, hence, was named sex-induced silencing (SIS) (Wang *et al.*, 2010).

RNAi gene silencing has been developed as a biotechnological tool for modifying gene expression and functional studies in many Ascomycota species (Salame *et al.*, 2011). To date, RNAi gene silencing has only been successfully applied to six species of Basidiomycota: *Phanerochaete chrysosporium*, *Agaricus bisporus*, *Laccaria bicolor*, *C. neoformans*, *Coprinopsis cinereus* and *M. lini* (Kemppainen & Pardo, 2010; Costa *et al.*, 2009; Kemppainen *et al.*, 2009; Panepinto *et al.*, 2009; Costa *et al.*, 2008; Matityahu *et al.*, 2008; Namekawa *et al.*, 2005; Liu *et al.*, 2002). Given that both argonaute and dicer are present in almost all Basidiomycota; our results indicate that it should be possible to develop RNAi as a tool for the functional study of genes in most Basidiomycota species, including *H. annosum s.l.* However, the RNAi gene silencing technology is relaying on the fungal transformation and a well-established transformation system is required for using RNAi gene silencing to study the function of candidate virulence genes by reverse genetics.

Based on the RNA sequencing data (Paper I), and virulence QTLs, the *H. irregulare HIRHG* gene was selected as the candidate virulence gene for further characterization. Although, more GH28s proteins were up-regulated in *H. occidentale*, *HIRHG* was found to be up-regulated in both *H. irregulare* and *H. occidentale*. Therefore, it could be considered to be a common virulence factor for *H. annosum s.l.* However, in some host–pathogen systems, polygalacturonases been shown to act as MAMPs (Zhang *et al.*, 2014), underlining their importance for the colonization ability of the pathogen. A distinct group of GH28s, the RHG proteins, have not been studied in the pathogen–host interaction. In order to get an overview of fungal RHG, we investigated *RHG* genes in 59 published fungal genomes. The results suggested that loss of RHG was associated with adaption to biotrophy and hemibiotrophy, and expansion of the gene family was associated with necrotrophy and saprotrophy. However, a few *RHG*-like genes are present in the rust fungi *M. laricis-populina* and *P. graminis*; however, they have very divergent sequence features, thus indicating divergent functions. The loss of RHG in biotrophs and hemibiotrophs is possibly as a result of purifying selection to avoid recognition by the plant innate immune system. The *Botrytis* PG BcPG3 is recognized by the *Arabidopsis* receptor RBPG1 resulting in a HR response (Zhang *et al.*, 2014). Oligoglucans (OGs) generated by PGs have also been shown to act as elicitors (DAMPs) inducing a wide range of defence responses in several plant species. The host PCD response has been reported to be harmful to biotrophs and hemibiotrophs, but tolerable for necrotrophs such as *H. annosum s.l.* (Asiegbu *et al.*, 1994; Asiegbu *et al.*, 1993). Therefore, we hypothesize that the loss of the *RHG* genes in biotrophs and hemibiotrophs may be due to the potential of the proteins to induce PTI in the host, which

might be better tolerated by certain necrotrophic pathogens. We investigated the effect of expressing *HIRHG* in the hemibiotroph *M. oryzae*, anticipating that HIRHG-transformed *M. oryzae* would show reduced virulence if *HIRHG* activated PTI in rice. An alternative outcome of the experiment might have been an increase in the virulence of *M. oryzae* as a result of enhanced degradation of pectin in rice. However, we did not observe any significant change in the virulence of *M. oryzae* in the strain expressing the *HIRHG* gene, indicating either that *HIRHG* does not activate PTI in rice, perhaps because of the lack of an appropriate receptor (Zhang *et al.*, 2014) to detect HIRHG as a MAMP or the potential OGs produced as DAMPs, or that *M. oryzae* has effectors that are able to suppress the PTI induced by *HIRHG*. In the hemibiotrophic oomycete pathogen *Phytophthora sojae*, another GH12 protein, XEG1, which also acts as a cell wall-degrading enzyme, can activate PTI in different plants and many *P. sojae* effectors can also suppress defence responses induced by XEG1 (Ma *et al.*, 2015). Moreover, the faster growth rate found on pectin medium in *HIRHG*-expressing *M. oryzae* transformants was not reflected in the virulence of the transgenic strains. It is possible that the trophic strategy of *M. oryzae in planta* would not benefit from RHG activity, whereas *H. irregulare*, which uses both necrotrophism and saprotrophism in its interaction with the host, would benefit. Unlike biotrophs, necrotrophs rely on the degradation of host material for the acquisition of energy and nutrients. This strategy appears to be reflected in their genome because necrotrophs generally have a more diverse arsenal of plant cell wall-degrading enzymes than pathogens with other trophic strategies, and many biotrophic plant pathogens show a strong reduction of genome size and total number of gene models (Schirawski *et al.*, 2010; Spanu *et al.*, 2010).

HIRHG and other RHGs can contribute to carbon acquisition by degrading pectin. Heterologous expression of the *HIRHG* gene in *M. oryzae* resulted in a faster growth rate than the wild type on pectin medium, which provides indirect evidence that HIRHG has pectinase activity and that the activity of RHGs contribute to fungal growth. The *Botrytis* RHGs have also been identified in pectin liquid culture, but not when grown on sucrose, suggesting that RHGs play an active role in pectin-degradation and fungal carbon acquisition (Shah *et al.*, 2009). Western blot of HIRHG from *H. irregulare* grown on different carbon sources supports a similar role for HIRHG; the level of HIRHG protein was much higher when *H. irregulare* was grown on pectin medium compared with on other carbon sources. The expression levels of *HIRHG* and its orthologues were also higher *in planta* compared with *in vitro*. The plant cell walls, which are composed of pectin and cellulose, are the main carbon sources for necrotrophic pathogens. Degradation of host cell walls to

provide carbon is important for *H. annosum s.l.* growing *in planta*. The relatively higher induced levels of HIRHG compared with that of its orthologues in *H. occidentale* could be because there are more GH28s and plant cell wall-degrading enzymes induced during the interaction, which might complement the function of *HIRHG*.

6 Conclusions and future prospects

6.1 Conclusions

Transcriptome studies showed that the virulence of *H. annosum s.l.* is controlled by multitude of genetic factors. There were large differences found between different species within the complex. Most likely *H. irregulare* and *H. occidentale* have different strategies to infect Norway spruce. Together with previous studies, a number of candidate virulence factors were identified. Further study is required to exploit the details of virulence of *H. annosum s.l.* However, it was shown that most likely RNAi is present in *H. irregul*are and could be developed for functional characterization of candidate virulence genes. Such a reverse-genetic tool would still relays on a well-established genetic transformation system. The *H. irregulare endo-rhamnogalacturonase* gene (*HIRHG*) was selected to be characterization in this study. The results suggest that it important for *H. annosum s.l.* growing *in planta* due to its contribution to decomposition of pectin of host cell wall.

6.2 Future prospects

The genetic differences between *H. annosum s.l.* species have been intensively studied; however, further investigation is needed to determine virulence factor differences between *H. annosum s.l.* species and to understand the mechanisms involved in virulence. The genomic comparison of *H. annosum s.l.* species is still ongoing. In addition to genomic comparison studies, a comparison of the gene expression pattern of the heterokaryon hybrid AO8 with that of *H. irregulare* and *H. occidentale*, and an investigation of how the homokaryon parents effect heterokaryon hybrid gene expression could be used as an approach to understand the evolution of gene expression and virulence in the pathogen.

The genetic transformation of *H. annosum s.l.* is rather difficult compared with the transformation of many other plant pathogens. However, the development of new technologies, such as the CRISPR system (Ran *et al.*, 2013), might prove valuable for transforming *H. annosum s.l.* In addition, using a well-established model system to avoid complicated difficult work might be a good strategy. Many of the virulence candidates such as transporters, secondary metabolism enzymes and secreted proteins could be characterized in a heterologous model system.

The development of theory about the necrotrophic pathogen–host interaction that is relatable to the *H. annosum s.l.*–conifer system would be an important approach for us to understand the molecular mechanism, evolution and ecology of *H. annosum s.l.* and the virulence of fungal pathogens in general.

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