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Pathogenomics of the *Heterobasidion* species: Functional analysis of the *HaHOG1* MAP kinase

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ACADEMIC DISSERTATION

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Cover: Conifer trunk infected by *Heterobasdion* (left); germinated spores and mycelium of *Heterobasidion annosum* s.s. grown on wood water extracts (upper right); primary germ tube of *Heterobasidion annosum* s.s. (bottom centre), conidiophore of *Heterobasidion annosum* s.s. (bottom right)

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“Above all, don’t fear difficult moments.
The best comes from them.”

Rita Levi Montalcini

CONTENTS

ABBREVIATIONS 6

LIST OF ORIGINAL PUBLICATIONS AND SUBMITTED MANUSCRIPTS 8

ABSTRACT 10

1. INTRODUCTION 12

1.1. Taxonomy of the *Heterobasidion* species complex 12

1.2. Infection cycle and growth in wood 13

1.3. Survival in infected stands and in trees 14

1.4. Interaction with the host and environmental stimuli 15

1.5. The role of fungal intracellular signalling in sensing environmental stimuli 16

1.6. The wood degradation capacity of white-rot fungi 18

2. AIMS OF THE STUDY 22

3. MATERIALS AND METHODS 23

4. RESULTS AND DISCUSSION 24

4.1. *H. irregulare* genome (I) 24

4.1.1. The pheromone pathway *FUS3/KSS1* (I) 25

4.1.2. The high osmolarity glycerol pathway *HOG1* (I) 27

4.1.3. The *MPK1* cell integrity pathway (I) 28

4.1.4. The Ca^{2+} pathway (I) 29

4.1.5. The cAMP-PKA pathway (I) 29

4.2. Gene expression and adaptation of *H. annosum* s.s. exposed to abiotic stresses and during saprotrophic growth on pine wood materials (II) 31

4.2.1. Cluster analysis and gene expression (II) 31

4.2.2. Response of *H. annosum* s.s. during temperature variation (II) 32

4.2.3. Response of *H. annosum* s.s. to salt osmotic stress (II) 33

4.2.4. Response of *H. annosum* s.s. to oxidative stress and starvation (II) 34

4.2.5. *H. annosum* s.s. adaptation during saprotrophic growth on pine wood (II) 35

4.3. Functional study of the MAPK *HaHOG1* and its involvement in stress response (III) 39

4.3.1. *H. annosum* s.s. growth in high osmotic and oxidative conditions (III) 39

4.3.2. Up-regulation of genes involved in the osmotic stress response (III) 40

4.3.3. Cloning and functional study of the *HaHOG1* gene (III) 41

4.3.4. Phosphorylation of the MAPK HaHog1p (III) 42

4.3.5. Cellular localisation of HaHog1p in *S. cerevisiae* (III) 43
4.4. Validation of reference genes for gene expression studies in *H. annosum* s.s. (IV)......... 44
5. SUMMARY AND CONCLUSIONS 47
6. FUTURE PERSPECTIVES 49
ACKNOWLEDGMENTS 50
REFERENCES 52

ABBREVIATIONS

Ab	antibody
ABC	ATP binding cassette
AC	adenylate cyclase
ATP	adenosine triphosphate
BLAST	basic local alignment search tool
cAMP	cyclic adenosine monophosphate
CAZy	carbohydrate active enzymes
CDH	cellobiose dehydrogenase
cDNA	complementary DNA
CE	carboxyl esterase
DNA	deoxyribonucleic acid
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
gDNA	genomic deoxyribonucleic acid
GDP	guanosine diphosphate
GFP	green fluorescent protein
GH	glycosyl hydrolase
GPI	glycosyl-phosphatidylinositol
GPD	glyceraldehyde-3-phosphate dehydrogenase
GTP	guanosine triphosphate
HK	histidine kinase
HOG	high osmolarity glycerol
ISG	intersterile group
ITS	internal transcribed spacer
LiP	lignin peroxidase
MAPK	mitogen activated protein kinase
MAP2K	mitogen activated protein kinase kinase
MAP3K	mitogen activated protein kinase kinase kinase
MB	mega base pairs
MFS-1	major facilitator superfamily 1
MnP	manganese peroxidase
mRNA	messenger RNA

PCR	polymerase chain reaction
PDE	phosphate diesterase
PIP5K	phosphatidylinositol-4-phosphate 5-kinase
PKA	protein kinase A
qPCR	real time quantitative PCR
SAPK	stress activated protein kinase
SH3	SRC Homology 3
ST	sugar transporter
STL	glycerol proton symporter of the plasma membrane
TE	transposable element
TF	transcription factor
UV	ultraviolet
VP	versatile peroxidase

LIST OF ORIGINAL PUBLICATIONS AND SUBMITTED MANUSCRIPTS

The doctoral thesis is based on the following publications, which are referred to in the text by their roman numerals.

I. Olson, A., Aerts, A., Asiegbu, F., Belbahri, L., Bouzid, O., Broberg, A., Canback, B., Coutinho, P.M., Cullen, D., Dalman, K., Deflorio, G., van Diepen, L.T.A., Dunand, C., Duplessis, S., Durling, M., Gonthier, P., Grimwood, J., Fossdal, C.G., Hansson, D., Henrissat, B., Hietala, A., Himmelstrand, K., Hoffmeister, D., Hogberg, N., James, T.Y., Karlsson, M., Kohler, A., Kuees, U., Lee, Y., Lin, Y., Lind, M., Lindquist, E., Lombard, V., Lucas, S., Lunden, K., Morin, E., Murat, C., Park, J., **Raffaello, T.**, Rouze, P., Salamov, A., Schmutz, J., Solheim, H., Stahlberg, J., Velez, H., de Vries, R.P., Wiebenga, A., Woodward, S., Yakovlev, I., Garbelotto, M., Martin, F., Grigoriev, I.V. & Stenlid, J. 2012, "Insight into trade-off between wood decay and parasitism from the genome of a fungal forest pathogen", *New Phytologist,* vol. 194, no. 4, pp. 1001-1013. DOI: 10.1111/j.1469-8137.2012.04128.x

II. **Raffaello, T.**, Chen, H., Kohler, A. & Asiegbu, F.O. 2013, "Transcriptomic profiles of *Heterobasidion annosum* under abiotic stresses and during saprotrophic growth in bark, sapwood and heartwood". (Submitted)

III. **Raffaello, T.**, Keriö, S. & Asiegbu, F.O. 2012, "Role of the HaHOG1 MAP kinase in response of the conifer root and butt rot pathogen (*Heterobasidion annosum*) to osmotic and oxidative stress", *Plos One,* vol. 7, no. 2, pp. e31186. DOI:10.1371/journal.pone.0031186

IV. **Raffaello, T.** & Asiegbu, F.O. 2013, "Evaluation of potential reference genes for use in gene expression studies in the conifer pathogen (*Heterobasidion annosum*)", *Molecular biology reports,* vol. 40, no. 7, pp: 4605-4611. DOI: 10.1007/s11033-013-2553-z

Other articles not included in this thesis:

- Kovalchuk, A., Keriö, S., Oghenekaro, A.O., Jaber, E., **Raffaello, T.**, & Asiegbu, F.O. 2013, "Antimicrobial defenses and resistance in forest trees: challenges and perspectives in a genomic era", *Annu. Rev. Phytopathol*, vol. 51, pp: 221-244. DOI: 10.1146/annurev-phyto-082712-102307

Author contribution:

I. The author participated in the manual annotation of genes in the *H. irregulare* genome consortium. The author manually annotated all the conserved components of five intracellular pathways: the pheromone pathway *FUS3/KSS1*, the high osmolarity pathway *HOG1*, the cell integrity pathway *MPK1*, the calcium/calcineurin signalling pathway, and the cAMP pathway.
II. The author planned the experiment and did the laboratory work together with HC. The author analysed the data, interpreted the results and wrote the article. AK contributed to the statistical analysis of the microarray data. FOA conceived the study and contributed to the experimental design and drafting of the article.
III. The author planned the experiment and did the laboratory work. SK helped in quantifying the fungal growth in different growth conditions. The author analysed the data, interpreted the results and wrote the article. FOA conceived the study and contributed to the experimental design and drafting of the article.
IV. The author planned the experiment and did the laboratory work. The author analysed the data, interpreted the results and wrote the article. FOA contributed to the experimental design and drafting of the article.

ABSTRACT

The basidiomycete white-rot fungus *Heterobasidion annosum* sensu lato (s.l.) is a species complex comprising five species considered to be the most economically important pathogens of conifer trees in the northern hemisphere. The infection of new wood substrate is mediated by basidiospores, which land on the stump surface of a felled tree. After spore germination, the fungal mycelia actively colonise the stump and spread to new healthy trees by root-to-root contact. To start a new infection cycle, *H. annosum* s.l. must counteract the adverse environmental factors (abiotic stresses) at the stump surface. Moreover, active wood degradation requires the ability to detoxify the high levels of fungistatic and fungitoxic compounds (such as phenolics) that naturally accumulate in the tree wood tissue as a defence against pathogen attack.

The availability of the genome sequence of the *H. irregulare* species allowed us to investigate the conservation of the intracellular pathways that are responsible for the abiotic stress response and cellular adaptation and proliferation. Using *Saccharomyces cerevisiae* as a model organism in which many of these pathways have been well characterised, we annotated all the conserved components of the mitogen activated protein kinase (MAPK) pathways in *H. irregulare*, namely, those involving the pheromone *FUS3/KSS1*, the high osmolarity gene *HOG1*, the cell integrity gene *MPK1*, calcium/calcineurin signalling, and the cAMP pathway.

To better understand the *H. annosum* sensu stricto (s.s.) adaptation during abiotic stress and wood degradation, we investigated the general transcriptional profiles under several abiotic stresses (osmotic, oxidative, temperature, and nutrient starvation) and during growth on different pine woody materials (pine bark, sapwood, and heartwood). The results for abiotic stresses indicated the activation of genes involved in signalling (for example, protein kinase and transcription factors during starvation) but also genes involved in toxic substance detoxification and membrane transporters (cytochrome P450 and Major Facilitator Superfamily, MFS-1, respectively, in cold stress). During saprotrophic growth on different pine wood materials, a dramatic induction of several glycosyl hydrolase (GH) genes was observed. Some of these genes (for example, GH61) were specifically induced, mainly in pine heartwood, while others demonstrated less tissue specificity and were generally expressed during saprotrophic growth in all woody materials. During saprotrophic growth on pine lignocellulose material, several genes

involved in lignin degradation, such as multi-copper oxidases (MCOs) and oxidoreductases, were also strongly induced.

The central MAPK of one of the pathways involved in adaptation to abiotic stress (the HOG pathway) was further characterised. The *H. annosum* s.s. *HaHOG1* gene was cloned and functionally studied to investigate its role in osmotic and oxidative stress response in this fungus. The *HaHOG1* gene restored the function of the homologous *HOG1*, and the protein translocated to the cell nucleus under osmotic conditions in the *S. cerevisiae* heterologous host. Furthermore, HaHog1p was strongly phosphorylated in the presence of high concentrations of NaCl, KCl, and H_2O_2. These results suggest that the HOG pathway is activated when *H. annosum* s.s. is challenged with osmotic and oxidative stressors.

This study sheds light on some adaptive mechanisms that characterise the growth of *H. annosum* s.s. under several conditions. Finally, this work provides new data at the transcriptome level to help identify genes that are activated during wood degradation and response to abiotic stresses.

1. INTRODUCTION

1.1. Taxonomy of the *Heterobasidion* species complex

The *Heterobasidion annosum* sensu lato (s.l.) species complex comprises several fungal species of the genus *Heterobasidion*, which are considered to be the most economically important forest pathogens in the northern hemisphere (Asiegbu, Adomas & Stenlid 2005, Garbelotto, Gonthie 2013). Initially, mating experiments revealed the existence of several intersterile groups (ISGs), including the P-, S-, and F-type, depending on their host preferences (Scots pine, Norway spruce, and silver fir, respectively). Subsequently, new names for the European species were given: *H. annosum* sensu stricto (s.s.) (P ISG), *H. parviporum* (S ISG), and *H. abietinum* (F ISG) (Korhonen 1978, Capretti et al. 1990). The *H. parviporum* species preferentially infects Norway spruce, and it is commonly present in northern Europe. In Finland, the vast majority of the *H. annosum* s.l. isolated from infected spruce trees belongs to the *H. parviporum* species (Piri, Korhonen 2001). Moreover, the high prevalence of *H. parviporum* in infected stands regenerated with Norway spruce has been documented (Piri 1996). *H. abietinum* is mainly distributed in the Mediterranean area and prefers trees of the genus *Abies* (Niemelä, Korhonen 1998). Finally, *H. annosum* s.s. is also present in Europe. Scots pine is its preferred host, although it has broader host specificity compared to *H. parviporum* and *H. abietinum* because it can also infect other gymnosperms (for example, *Picea*, *Juniperus*, etc.) (Niemelä, Korhonen 1998). Two other species are present in North America, which originally were named North American S and P ISG because they showed a high degree of interfertility with the former European representatives of P and S ISG (Filip, Morrison 1998). However, based on morphometric analysis (pore size, density, and shape) and genetic analysis (ITS sequences), the North American P and S ISG names were replaced by *H. irregulare* and *H. occidentale*, respectively (Otrosina, Garbelotto 2010). Interestingly, non-pathogenic species of the genus *Heterobasidion* exist; these species include *H. araucariae* (Australia, New Zealand, Papua New Guinea, and Fiji Islands), *H. insulare* (Southern and Eastern Asia), and *H. rutilantiforme* (Tropical America), which live as saprotrophic fungi on dead wood material (Niemelä, Korhonen 1998).

1.2. Infection cycle and growth in wood

The most supported infection model indicates that the fungus spreads from the colonised stump to the root system and then to near healthy trees by root-to-root contact (Hodges 1969) (Figure 1). A primary source of inoculum, such as pre-colonised roots and wood material, is necessary for the fungus to infect new trees as *H. annosum* s.l. is not capable of growing efficiently in soil (Stenlid, Redfern 1998). *H. annosum* s.l. basidiospores land on the stump surface, and the infection cycle starts with stump colonisation, mediated mainly by basidiospores (Stenlid, Redfern 1998) (Figure 1). Based on infection models, the fungus spreads within the root to a varying extent depending on the resistance of the tree, root development or site factors that influence the virulence of the fungus (Sinclair 1962, Asiegbu, Daniel & Johansson 1995, Towers, Stambaug 1968).

The ability of *H. annosum* s.l. to actively colonise a stump is also affected by abiotic stresses (temperature, UV radiation, variation in pH, etc.), which are numerous in the open wound of the stump surface and which threaten fungal survival. The capacity to overcome tree immunity, which is still active for a certain period of time after the tree is felled, is also important for successful stump colonisation (Cooke, Rayner 1984). In particular, the wood moisture content can influence the pattern of stump colonisation by *H. annosum* s.l. in *Picea sitchensis* (Redfern 1993). Pine heartwood is more resistant to *H. annosum* s.l. decay than sapwood, which is characterised by higher moisture content. However, when rainfall leads to higher water content, *P. sitchensis* heartwood stumps are preferred to sapwood, which becomes too wet for optimal fungal growth (Redfern 1993).

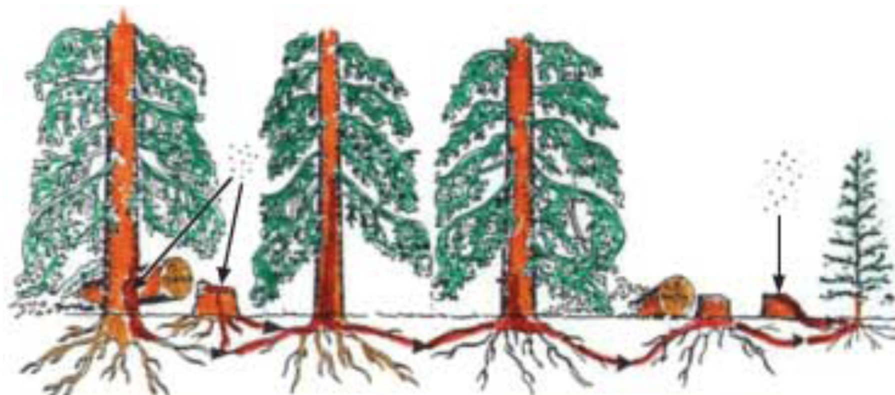

Figure 1: *Heterobasidion annosum* s.l. infection cycle in a conifer stand (Asiegbu, Adomas & Stenlid 2005)

From this point of view, the ability of *H. annosum* s.l. to induce decay is influenced not only by environmental abiotic factors but also by the physical and chemical properties of the stump wood (Redfern, Stenlid 1998). In normal conditions, with a moderate inoculum load, *H. annosum* s.l. does not penetrate intact bark. However, a high inoculum load can support mycelial bark penetration and subsequent ectotrophic colonisation of the underlying layers. Superficial wounds (caused by animals, insects, abiotic factors, or artificial means) may facilitate the necrotrophic invasion of phelloderm and phloem bark, where the tree defence response is characterised by both the chemical and mechanical properties of Norway spruce (Lindberg, Johansson 1991).

Once inside the root system, *H. annosum* s.l. grows within the tree stem to a varying extent that depends on the tree species. For example, the decay column can reach the remarkable height of 12 m in *P. abies* (Stenlid, Wasterlund 1986). However, different levels of decay are reported in different surveys, ranging from 2.2 m to 10.4 m, with a possible correlation between the height and width of the decay column (Kallio, Tamminen 1974, Tamminen 1985, Stenlid, Redfern 1998). *H. annosum* s.l. growing in heartwood tends to spread into sapwood in the living tree, but this colonisation is restricted by the tree´s immune response. Additionally, the high levels of fungistatic or fungitoxic molecules in sapwood and the low rate of oxygen diffusion caused by the high water content slow the invasive growth of the fungus (Stenlid, Redfern 1998). However, *H. annosum* s.l. produces several enzymes that detoxify these molecules that restrain fungal growth, which is also supported by the high sugar content of sapwood.

1.3. Survival in infected stands and in trees

Several factors affect the survival of the fungus in the environment, infected trees, and stumps (Garbelotto, Gonthie 2013). Some of these factors can be abiotic, for example, soil pH and composition. The rate of fungal transmission from stumps to surrounding trees is higher in mineral soils compared with peat soil, where, for some reasons, the spread to healthy trees through root-to-root contact is inhibited (Redfern 1998). While pH can limit fungal infection, other factors, such as soil texture and organic content, may be more important than pH variation (Alexander, Skelly & Morris 1975). The optimal pH for *H. annosum* s.l. growth is 4.0 - 5.7 (Korhonen, Stenlid 1998), and conidia germination is unaffected in the pH 3 to 6 range (Avis et al. 2009).

Drought can significantly increase the rate of *H. annosum* s.l. infection compared with wetter soils, which tend to accumulate organic matter and to form peat in cold and moist areas, which inhibit the *H. annosum* s.l. infection (Laine 1976). Apparently, drought stimulates infection by creating more favourable conditions for fungal growth in trees (Stenlid, Redfern 1998). Decreases in water content make the xylem more susceptible to infection in Norway spruce seedlings inoculated with *H. parviporum*. In this context, wounding damages the tracheids, leaving a dry zone in the xylem tissue, which increases susceptibility (Lindberg, Johansson 1992). However, data related to fungal survival and longevity in standing trees can be affected by the type of experiment. Direct inoculation into the tree stem leads to a high rate of infection failure in *P. abies*, most likely because of the defences mounted in the sapwood area. In contrast, live *H. annosum* s.l. has been found in the decay columns of infected *P. sitchensis* 10 years after the infection of adjacent stumps (Stenlid, Redfern 1998).

1.4. Interaction with the host and environmental stimuli

Fungi are constantly exposed to a plethora of different environmental stimuli. (Bahn et al. 2007). Pathogenic fungi in particular have to counteract simultaneous environmental abiotic stresses and host immune responses to be able to successfully start an infection cycle (Jones, Dangl 2006). During the initial stages of infection, the fungus must recognise specific molecules or properties on the host surface to trigger intracellular pathways involved in fungal penetration. After host penetration, a broad variety of molecules and proteins are produced to actively support fungal growth. *H. parviporum* expresses a wide range of genes involved in protein synthesis, DNA processing, cell cycle, transport, defence, and detoxification during the colonisation of Norway spruce (Yakovlev et al. 2008). Similar patterns of *H. annosum* s.l. gene expression are observed during infection of Scots pine (Karlsson, Olson & Stenlid 2003, Asiegbu, Nahalkova & Li 2005) and conidia germination (Abu, Li & Asiegbu 2004). During its interaction with pine roots, *H. annosum* s.l. up-regulates a glutaredoxin-encoding gene, which is responsible for DNA synthesis, and cytochrome P450, which is responsible for detoxification of plant-derived molecules. In contrast, the fungus down-regulates a protein involved in chitin biosynthesis and a proton-pumping vacuolar ATPase (Karlsson, Olson & Stenlid 2003). The effects of cell wall phenolic compounds and different sugars on *H. annosum* s.s. growth have been studied *in vitro* (Asiegbu 2000). Phenolic

compounds, including ferulic acid, caffeic acid, and *p*-coumaric acid, inhibit fungal growth, and this effect is reversed in the presence of sugars, such as glucose, fructose, sucrose, and cellobiose (Asiegbu 2000). Cytochrome P450 is overexpressed when *H. parviporum* is grown on ferulic acid and oxalic acid (produced by the fungus during the wood colonisation), indicating the possible role of cytochrome P450 during the natural fungal growth inside the tree (Abu, Li & Asiegbu 2004).

Investigation of the *H. annosum* s.l. transcriptome during its growth on different substrates or during important stages of the fungal lifestyle, such as saprotrophic or necrotrophic growth, is constrained by the lack of a suitable transformation system and of information on its genome sequence. Recently, the complete genome sequence of *H. irregulare* TC 32-1 was determined (Olson et al. 2012), and this information will broaden the possibilities and the approaches used to study the fundamental molecular biology of this necrotrophic fungus.

1.5. The role of fungal intracellular signalling in sensing environmental stimuli

All living organisms must face and respond to changes in environmental conditions to survive and to successfully colonise their specific ecological niche. Fungi, including *H. annosum* s.l., are naturally exposed to harsh environments, so they have developed specific mechanisms to counteract these challenges. In particular, abiotic stresses, including changes in osmolarity, temperature, UV radiation, and oxidative stress, significantly affect fungal fitness and survival. Fungi rely on a complicated network of intracellular signalling pathways to respond to environmental cues sensed at the cell surface. In eukaryotes, these pathways are characterised by a highly conserved module of three mitogen activated protein kinase (MAPK) proteins that are activated by phosphorylation (Widmann et al. 1999) (Figure 2). The module is composed of a MAP3K that phosphorylates an intermediate MAP2K, which finally activates a MAPK effector that usually translocates into the fungal nucleus (Widmann et al. 1999).

In the model organism *Saccharomyces cerevisiae,* the mechanisms that control osmotic adaptation have been well studied (Hohmann 2002). In yeast, a key component of the intracellular pathway responsible for osmotic adaptation is the MAPK *HOG1* gene, which was first described in the early nineties (Brewster et al. 1993). Since then, more

experimental data relative to the role of *HOG1* in osmotic adaptation in yeast have been reported (Schüller et al. 1994, Albertyn et al. 1994, Posas et al. 1996). Analysis of fungal genomes has elucidated the high degree of conservation of HOG and other pathways that are involved in starvation tolerance, cell integrity, or hypotonic shock (Rispail et al. 2009, Hamel et al. 2012).

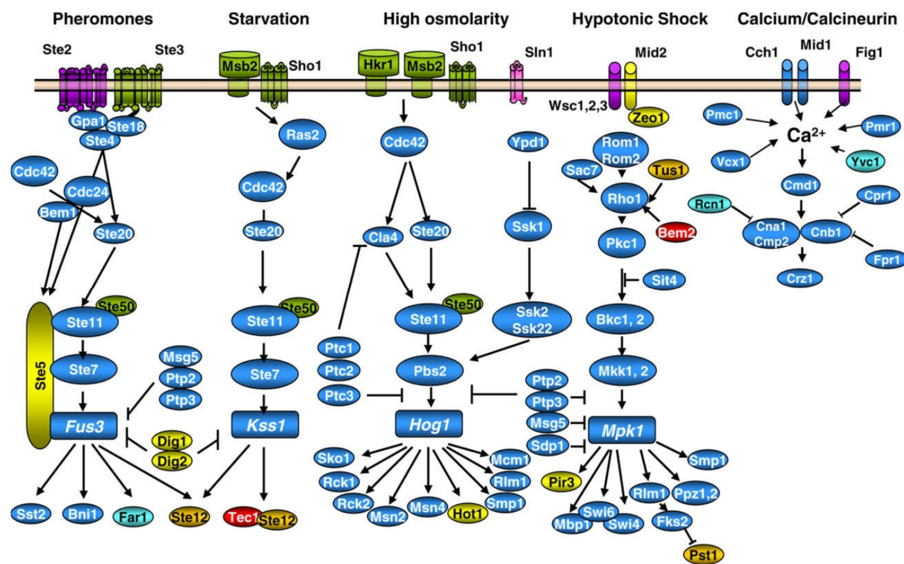

Figure 2: Diagram of conserved signalling MAPK pathways in fungi. Blue, components detected in all species studied; orange, all except basidiomycetes; cyan, all except zygomycetes and archiascomycetes; pink, all except basidiomycetes and archiascomycetes; red, all except euascomycetes; purple, only ascomycetes; yellow: only hemiascomycetes (Rispail et al. 2009).

These intracellular pathways are characterised by a central core of MAPKs, which mediate signal transduction from the cell surface to the nucleus. MAPK proteins are stress-activated protein kinases (SAPKs) that are activated by phosphorylation upon stress induction (Widmann et al. 1999). In *S. cerevisiae*, the core of the HOG stress pathway is an upstream MAPKKK (Ste11p) that activates an intermediate MAPKK (Pbs2p), which finally activates a MAPK effector (Hog1p) that translocates to the nucleus to induce the expression of specific stress related genes (Widmann et al. 1999). The HOG pathway is activated by the receptors Sho1p and Mbs2p, which stimulate the dissociation of the α-subunit of GTPase Cdc42p. This subunit activates Ste20p, which in turn stimulates the interaction between Ste50p and Ste11p, leading to HOG pathway activation. There is extensive cross talk between the most conserved pathways in fungi; for example, Sho1p/Mbs2p, Cdc42p, Ste20p, and Ste50p are also involved in the nutrient starvation pathway (Hamel et al. 2012). In addition to the high osmolarity and nutrient starvation

pathways, MAPKKK Ste11p is also involved in the pheromone pathway (Hamel et al. 2012).

Functional analysis of these MAPK modules is important to characterise how fungi respond to general abiotic stresses, but it is also crucial to investigate their roles in plant pathogenic fungi. One of the best characterised MAPKs in pathogenic fungi is Mor-Kss1 (formerly named Pmk1, as in pathogenicity MAP kinase 1) in the rice blast fungus *Magnaporthe oryzae* (Xu, Hamer 1996). The *M. oryzae* Δ*kss1* mutant fails to accumulate glycerol into the infection structure appressorium, thus inhibiting penetration of the rice leaf surface. In the maize pathogen *Ustilago maydis*, a MAP kinase called *Kpp6* (homologous to *S. cerevisiae FUS3/KSS1*) is also indispensable for penetration of the plant surface (Brachmann et al. 2003). In the citrus pathogen *Alternaria alternata*, the *FUS3/KSS1* homologue *AaFUS3* is required for leaf penetration, melanin production, resistance to chemicals, and expression of hydrolytic enzymes important for fungal pathogenicity (Lin et al. 2010). In soil-born *Fusarium oxysporum*, the *FMK1* gene is indispensable for pathogenesis in tomatoes. The Δ*fmk1* mutant does not produce an essential hydrolytic enzyme (the pectate lyase *pl1*), but in contrast to *A. alteranta*, the vegetative mycelia and conidia remain normal in culture (Di Pietro et al. 2001).

The experimental data in the literature indicate that despite the relatively high degree of conservation of these MAPKs, their functions can be different and can reflect specific adaptations to diverse ecological niches and environments.

1.6. The wood degradation capacity of white-rot fungi

Among the fungi, white-rotters possess the extraordinary ability to degrade all major wood constituents, including lignin, cellulose, and hemicellulose (Blanchette 1991, Lundell, Mäkelä & Hildén 2010). The middle lamella in the wood tracheids and fibres in either hardwood (angiosperms and deciduous trees) or softwood (gymnosperms) have the highest lignin content (Eriksson, Blanchette & Ander 1990). Lignin is an aromatic and amorphous polymer that strengthens the plant cell wall and makes it more resistant to mechanical stress and pathogen attack. The main constituents of lignin are *p*-coumaryl, coniferyl, and sinapyl alcohol, and the amount of lignin varies from 20-25% in hardwood to 25-33% in softwood (Adler 1977). The term “white-rot” is used to describe the appearance of delignified decayed wood in which the white cellulose remains after the lignin has been removed. The ability to depolymerise lignin varies among white-rotters.

Some fully remove lignin from fallen trees, while others create small pockets of delignified cellulose (Blanchette 1991). White-rot fungi depolymerise lignin by oxidative reactions mediated by class II heme peroxidases, including lignin peroxidases (LiPs), manganese peroxidases (MnPs), hybrid manganese peroxidases (hMNP), and versatile peroxidases (VPs) (Lundell, Mäkelä & Hildén 2010), and by laccases (Higuchi 2006, Martínez 2002, Lundell, Mäkelä & Hildén 2010).

LiPs are peroxidases harbouring an Fe^{3+} moiety pentacoordinated to four heme nitrogens and a histidine (Hammel, Cullen 2008). These peroxidases use hydrogen peroxide as a two-electron acceptor; the reaction forms Compound I, which is characterised by the oxidised Fe^{4+} and a free radical in the pyrrolic ring. Compound I preferentially oxidises non-phenolic aromatic compounds in lignin (Martínez 2002). MnPs are different in that they oxidise Mn^{2+} to Mn^{3+}, a potent and highly diffusible ion that oxidises the phenolic residues of lignin. Because phenolic residues do not comprise the major part of the lignin, Mn^{3+} may generate other oxidants that oxidise non-phenolic lignin residues (Hammel, Cullen 2008). hMNPs, which have overlapping physiological functions with MNPs, are characterised by three acidic amino acids involved in Mn^{2+} binding, although they lack the typical Trp residue of LiPs (Lundell, Mäkelä & Hildén 2010). Finally, VPs share enzymatic properties with LiPs and MnPs. However, the differences in the enzyme structure and catalytic properties allow the description of VPs as a diverse enzyme class (Ruiz-Dueñas et al. 2001). Fungal laccases (multi-copper oxidases, MCOs) are also involved in lignin degradation. Using oxygen as an oxidant, laccases oxidise a variety of phenolic compounds but not tyrosine (oxidised by tyrosinases) (Baldrian 2006). However, the absence of conventional laccases in the genome of the white-rot fungus *Phanerochaete chrysosporium* (Martinez et al. 2004) and the fact that laccases are involved in many diverse cellular processes (pigment biosynthesis, conidiation, fruiting body formation, etc.) could indicate their multifunctional roles (Lundell, Mäkelä & Hildén 2010).

The removal of highly recalcitrant and resistant lignin exposes cellulose, a highly polymerised molecule with β-1,4-glucoside bonds, which comprises 35-50% of the total plant dry mass (Lynd et al. 2002). Cellulose is efficiently broken down via an extraordinarily large repertoire of carbohydrate active enzymes (CAZy, http://www.cazy.org/) (Cantarel et al. 2009), and many of these enzymes are produced by white-rot fungi. These hydrolytic enzymes belong to three major families: endoglucanases (endo-β-1,4-glucanases), which cleave internal glycosidic bonds in the

cellulose polymere, exoglucanases (exo-β-1,4-glucanases), which cleave cellulose (amorphous or crystalline) from the non-reducing end of the polysaccharide chain (Davies, Henrissat 1995), and cellobiose dehydrogenases (CDH), which oxidise mainly cellobiose (also cellodextrins, mannodextrins, and lactose) to the lactone form (Baldrian, Valášková 2008). Finally, cellobiose is hydrolysed into glucose monomers by β-glucanases (Baldrian, Valášková 2008).

The genome of the white-rot fungus *P. chrysosporium* has elucidated the enzyme repertoire involved in degrading lignocellulose wood material (Martinez et al. 2004). The genome encodes 16 class II peroxidases, 5 multi-copper oxidases, and more than 240 putative CAZy enzymes, such as glycosyl hydrolase, carbohydrate esterases and glycosyltransferases (Martinez et al. 2004, Floudas et al. 2012). Other white- and brown-rot wood decayers belonging to the Basidiomycota have been sequenced. *Postia placenta* is the first brown-rot to be sequenced. Genome analysis revealed the absence of class II lignin peroxidases (only one gene was detected by Floudas and co-authors) (Floudas et al. 2012) but the presences of a few MCOs (5 members) and 242 predicted CAZy genes (Martinez et al. 2009, Floudas et al. 2012). Interestingly, the carbohydrate binding module 1 (CBM1), a common domain among other wood decayers that is fused to a variety of GH enzymes, is missing from the *P. placenta* genome, and large families of glycosyl hydrolases, including GH6, GH7, GH11, and GH74, are also absent (Martinez et al. 2009, Floudas et al. 2012). The genome of the white-rot fungus *Schizophyllum commune* has been analysed in terms of its lignocellulose-degrading enzymes. Compared with other sequenced white-rot genomes, *S. commune* has the highest number of CAZy enzymes, with a large number of genes in the GH93 (hemicellulose degradation), GH43 (hemicellulose and pectin degradation, 12 members), PL1, PL3 and PL4 (pectin degradation) families (Ohm et al. 2010, Floudas et al. 2012). Despite being a white-rot fungus, *S. commune* lacks any members of the class II fungal peroxidases (Floudas et al. 2012). Recently, the availability of genomes from other wood decay basidiomycetes has allowed a broader comparison of the lignocellulose-degrading enzyme repertoire. In *Serpula lacrymans*, for example, the contraction of specific gene families involved in lignolysis characterises the evolutionary divergence of brown-rot and white-rot fungi. The energetically expensive lignolysis apparatus of white-rot decayers is replaced by a more efficient lignocellulose breakdown mechanism, which uses hydroxide radicals to initially degrade the substrate (Eastwood et al. 2011, Floudas et al. 2012).

By analysing an increasing number of genome sequences, we will better understand the intricate enzymatic and chemical mechanisms used by these fungi in the important processes of wood decomposition and carbon recycling.

2. AIMS OF THE STUDY

H. annosum s.l. is one of the most important conifer pathogens in the northern hemisphere. The ecological impact and behaviour of this basidiomycete are well characterised. However, the lack of suitable molecular tools to investigate the functions of its genes and proteins during survival in the environment and during pathogenesis has limited our knowledge at the molecular and genetic levels. The recently published genome sequence of a closely related species, *H. irregulare*, has made it possible to investigate the key mechanisms that control this fungus.

The specific aims of this study are as follows:

A. To manually curate the components of the MAPK pathways in the *H. irregulare* genome.
B. To investigate the transcriptome profiles of *H. annosum* s.s. exposed to abiotic stresses and during saprotrophic growth on different pine woody materials.
C. To investigate the role of the *HOG1* gene homologue in *H. annosum* s.s. (*HaHOG1*) and the activation of this MAPK in different osmotic and oxidative conditions.
D. To assess the stability of putative reference genes used in gene expression studies (RT-qPCR) in *H. annosum* s.s.

3. MATERIALS AND METHODS

The materials, methods, and fungal strains used in this study are summarised in Table 1 and Table 2:

Table 1: Materials and Methods used in this study

Materials and methods	***Publications***
Fungal strains and growth conditions	I, II, III, IV
gDNA isolation	I
gDNA sequencing and data analysis	I
qPCR conditions and data analysis	II, III, IV
Primers design	II, III, IV
PCR conditions	III, IV
Gene cloning	III, IV
S. cerevisiae transformation and complementation	III
RNA isolation	I, II, III, IV
cDNA synthesis	II, III, IV
RNA amplification	II, IV
Protein extraction and western blot	III
GFP fusion protein	III
Microscopy	III
Microarray preparation and data analysis	I, II

Table 2: Fungal strains used in this study

Fungal strains	***Strain/Genotype***	***Publications***
Heterobasidion irregulare	TC 32-1, monokaryotic	I
Heterobasidion annosum s.s.	Isolate 03012, heterokaryotic	II, III, IV
Saccharomyces cerevisiae wild type BY4742	MATa; *his3*Δ1; *leu2*Δ0; *lys2*Δ0; *ura3*Δ0	III
Saccharomyces cerevisiae *hog1*Δ YLR113W	MATa; *his3*Δ1; *leu2*Δ0; *lys2*Δ0; *ura3*Δ0; YLR113W::*kan*MX4	III

4. RESULTS AND DISCUSSION

4.1. *H. irregulare* genome (I)

The size of the *H. irregulare* genome is 33.6 MB, and it is divided into 15 scaffolds, of which 6 are complete chromosomes. In total, 13321 genes were automatically predicted in the JGI genome browser v 2.0, and 1756 genes were manually curated (May 2013). The genome statistics are summarised in Table 3.

Table 3: *Heterobasidion irregulare* genome statistics (Supporting Table 8 (I))

Main genome scaffold (#)	15
Main genome contig (#)	18
Main genome scaffold sequence (Mb)	33.6
Main genome contig sequence (Mb)	33.6
Main genome scaffold N/L50 (Mb)	6/2.6
Main genome contig N/L50 (Mb)	6/2.3
Number of scaffolds > 50 KB	14
Main genome in scaffolds > 50 KB (%)	100

The genome size and gene number are similar to other sequenced white-rot basidiomycetes (Martinez et al. 2004, Stajich et al. 2010, Ohm et al. 2010). The genome is characterised by the presence of transposable elements (TEs); Gypsy-like elements are the most frequent, representing 9.3% of the entire genome. Transposable elements are present in three major regions (two in scaffold 12 and one in scaffold 1) containing genes involved in pathogenesis. Many of these genes are also orphans with no homology to genes in other fungi. The high density of TEs and the presence of orphan genes indicate the strong selective pressure at these three *H. irregulare* pathogenicity loci. Two other groups of genes have been annotated with respect to reproduction (*MAT-A* and *MAT-B* loci) and saprotrophic growth. The mating type locus *MAT-A* encodes homeodomain (HD) transcription factors in which one of the typical HD pairs (HD2) has been replaced, while *MAT-B* encodes 5 transmembrane pheromone receptors and 3 putative pheromone genes. The genome of *H. irregulare* encodes a broad spectrum of CAZys (Table 4).

Table 4: Summary of genes encoding glycosyl hydrolases (GH), polysaccharide lyases (PL) and carbohydrate esterases (CE) in *Heterobasidion irregulare* compared to other fungi (Supporting Table 16 (I))

	GH	*PL*	*CE*
Heterobasidion irregulare	179	7	18
Phanerochaete chrysosporium	181	4	17
Laccaria bicolor	163	7	17
Coprinopsis cinerea	211	13	51
Schizophyllum commune	240	16	30
Magnaporthe grisea	232	5	49

Manual annotation of all MAPK pathway components in *H. irregulare* indicates a high level of conservation. Using the protein sequences from the model organism *S. cerevisiae,* five MAPK pathways in *H. irregulare* were annotated: the pheromone pathway *FUS3/KSS1*, the high osmolarity pathway *HOG1*, the cell integrity pathway *MPK1*, the calcium/calcineurin signalling pathway, and the cAMP pathway (Rispail et al. 2009, Gustin et al. 1998).

4.1.1. The pheromone pathway *FUS3/KSS1* (I)

The pheromone pathway in *S. cerevisiae* is triggered by pheromone binding to the cognate receptors Ste2p and Ste3p. No Ste2p homologue could be found in the *H. irregulare* genome. Basidiomycetes (i.e., *U. maydis* and *C. neoformans*) lack type α receptors (Rispail et al. 2009). Ste3p from *S. cerevisiae* shows similarity to five proteins (protein IDs 147162, 181128, 171777, 181123 and 147163) that are located in a cluster on scaffold 7. These 5 genes are a characteristic of the mating locus *MAT-B* in the bipolar mating type *H. irregulare*.

Upon interaction between the ligand (pheromone) and the receptor, a heterotrimeric G protein dissociates (Alspaugh, Perfect & Heitman 1997, Regenfelder et al. 1997). In *S. cerevisiae*, the inhibitory Gα subunit is called Gpa1p (Rispail et al. 2009, Hamel et al. 2012). A Gpa1p homologous protein characterised by a small G protein α domain is present in the *H. irregulare* genome (protein ID 33983). Two other G proteins are also present (protein ID 57348 and 31682).

In the *S. cerevisiae* pheromone pathway, Cdc24p (a guanidine exchange factor, GEF) activates Cdc42p (a GTPase), which relays the signal to the subsequent elements of the pathway (Johnson 1999, Levin 2005). Cdc24p (protein ID 150672) and Cdc42p

(protein ID 154562) homologues are also present in the *H. irregulare* genome. As a typical guanidine exchange factor, Cdc24p possesses a DH domain and a pleckstrin-like domain, while Cdc42p is characterised by a Ras GTPase domain (Johnson 1999). The *H. irregulare* genome contains a Ste50p (protein ID 441190) homologue that is predicted to function as an adaptor between Cdc42p-Ste20p and the MAPK Ste11p (Jung et al. 2011).

A multiple alignment of the Ste50p proteins from several basidiomycetes (*Coprinopsis cinerea, Laccaria bicolor, P. chrysosporium*, *H. irregulare*, *U. maydis* and *Cryptococcus neoformans* serotypes A, B, and D) revealed that this basidiomycete adaptor protein includes an SH3 domain that is not present in the ascomycetes (Figure 3).

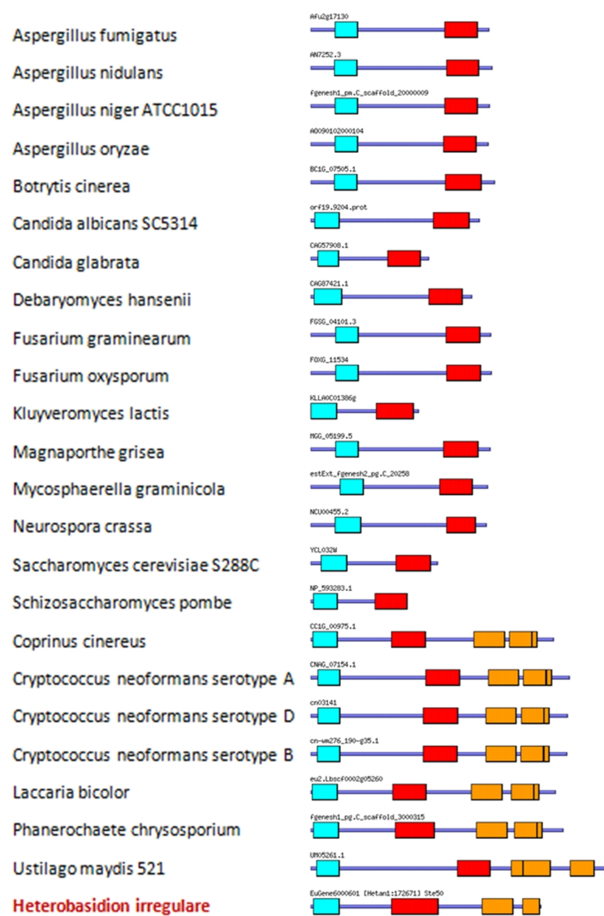

Figure 3: Ste50 proteins from basidiomycota and ascomycota. Light-blue box = SAM domain, red box = kinase domain, orange box = SH3 domain (Olson et al. 2012).

The Ste5p homologue is a scaffold protein that binds Ste11p, Ste7p, and Fus3p (Malleshaiah et al. 2010), and it is absent from *H. irregulare* and all other basidiomycetes analysed. It is also absent from other fungi, including *Candida albicans*, *Schizosaccharomyces pombe*, *Aspergillus fumigatus*, *Fusarium graminearum*, *M. grisea*,

Neurospora crassa, *Rhizopus oryzae*, and *U. maydis* (Rispail et al. 2009). Despite the absence of Ste5p, a predicted Bem1p orthologue (protein ID 155511) with a SH3 domain which interacts with Ste5p in *S. cerevisiae* (Lyons et al. 1996) is present in the *H. irregulare* genome. There is also a Ste20p homologue (protein ID 64508), which is a Cdc42p-activated signal transducing kinase of the PAK (p21-activated kinase) family.

In *S. cerevisiae*, the central core of the pheromone pathway includes three MAPKs: a MAPKKK Ste11p, a MAPKK Ste7p, and a MAPK Fus3p/Kss1p (Gustin et al. 1998, Rispail et al. 2009, Hamel et al. 2012). The SAM domain in Ste11p interacts with the SAM domain in the upstream protein Ste50p (Grimshaw et al. 2004). Homologues to Ste11p (protein ID 43369) and Ste7p (protein ID 147766) are present in *H. irregulare* as single copies, while Fus3p/Kss1p is present in three paralogues (protein ID 67530, 148775, and 59364). In a recent review, the authors suggest a unified nomenclature for the MAPKs in different fungi, giving the names *HaKSS1*, *HaKSS2*, and *HaKSS3* for the three *FUS3/KSS1* paralogues in *H. irregulare* (Hamel et al. 2012).

4.1.2. The high osmolarity glycerol pathway *HOG1* (I)

Fungi perceive changes in the environmental osmolarity using a multistep phosphorelay mechanism activated by the histidine kinase (HK) Sln1p (Posas et al. 1996). A BLASTP search of the *H. irregulare* genome using the *S. cerevisiae* Sln1p protein sequence found one copy of an Sln1p homologous protein (protein ID 389878). However, this protein has no predicted transmembrane domains, while the Sln1p sensor in *S. cerevisiae*, which belongs to Class VI HK, is characterised by 2 domains spanning the plasma membrane (Posas et al. 1996, Rispail et al. 2009, Catlett, Yoder & Turgeon 2003). The *H. irregulare* HK belongs to Class III, which is characterised by several HAMP domains that are thought to regulate protein function (Catlett, Yoder & Turgeon 2003). The intermediate Ypd1p and the response regulator Ssk1p are also part of the two-component signal transduction in *S. cerevisiae* (Li et al. 1998). Both are present as single copies in *H. irregulare* (protein IDs 332169 and 47010 for the Ypd1p and Ssk1p homologues, respectively).

The *S. cerevisiae* HOG pathway can be activated by the association of the proteins Sho1p and Mbs2p at the plasma membrane. One predicted Sho1p homologue is present in *H. irregulare*, although its similarity to the yeast Sho1p is quite low. Interestingly, no Mbs2p is present in *H. irregulare*. The presence of Sho1p in the absence

of any known interacting partner suggests that this branch of the osmostress pathway in *H. irregulare* might be regulated by a novel mechanism. The core components of the HOG osmoregulation pathway are all present in the *H. irregulare* genome. There is one copy of each of the following proteins: MAPKKK Ssk2p (protein ID 167185), MAPKK Pbs2p (protein ID 169311) and MAPK Hog1p (protein ID 153508) (Brewster et al. 1993, Rispail et al. 2009). The HOG pathway MAPK proteins in *H. irregulare* have been confirmed by an independent study (Hamel et al. 2012).

4.1.3. The *MPK1* cell integrity pathway (I)

The cell integrity pathway in *S. cerevisiae* is responsible for the cell wall integrity during environmental stress and its rearrangement during growth and fungal development (Levin 2005). In *S. cerevisiae*, the pathway is activated by several transmembrane proteins, namely, Wsc1p, 2p, and 3p (Verna et al. 1997). The *wsc1*Δ mutation displays stronger heat-shock sensitivity than *wsc2*Δ and *wsc3*Δ, suggesting partial redundancy of the principal *wsc1*Δ protein (Verna et al. 1997). No members of the *WSC* gene family are present in the *H. irregulare* genome. Although these genes are also absent from *U. maydis*, they are present in most ascomycetes (Rispail et al. 2009). In *S. cerevisiae*, an O-glycosylated receptor and membrane protein called Mid2p is involved in the cell integrity pathway in response to pheromone treatment, and it is also involved in cytokinesis (Philip, Levin 2001, Berlin, Paoletti & Chang 2003). However, no *MID2* homologous genes are found in the *H. irregulare* genome. The key protein of the cell wall integrity pathway is the G-protein Rho1p (Levin 2005), and we reported the presence of a Rho1p homologue (protein ID 157027) in the *H. irregulare* genome.

Several other proteins regulate Rho1p activity, including the GEF Rom1/2p (which promotes the exchange between GDP and GTP on Rho1p) and several GAP proteins (which promote the GTPase activity of Rho1p), such as Bem2p and Sac7p (Ozaki et al. 1996, Schmidt, Schmelzle & Hall 2002). Two copies of the Rom1/2p homologue (protein ID 50012 and 478624) and one copy each of Bem2p (protein ID 39880) and Sac7p (protein ID 55223) are present. The cell integrity pathway core is characterised by the MAPKKK Bkc1p (protein ID 407058), the MAPKK Mkk1p (protein ID 181084), and the effector MAPK Slt2p/Mpk1p (protein ID 181095) (Gustin et al. 1998). The *H. irregulare* genome also contains Mkk1p (Mkk2, protein ID 320731) and Slt2p/Mpk1p paralogues (protein ID 324063) (Hamel et al. 2012).

4.1.4. The Ca^{2+} pathway (I)

Of all the pathways described so far, the Ca^{2+} signalling pathway is the least characterised. The Ca^{2+} pathway is involved in a broad range of cellular responses and processes in yeast (Cyert 2001). There are three major membrane proteins involved in Ca^{2+} metabolism in *S. cerevisiae*: Cch1p, Mid1p (Locke et al. 2000), and Fig1p (Muller et al. 2003). The large transmembrane protein Cch1p (protein ID 37626) is present in the *H. irregulare* genome, and it is characterised by 18 transmembrane domains. A Mid1p homologue (protein ID 174557) is also present with a signal peptide but without a transmembrane domain as the protein is anchored by a GPI-anchor (Locke et al. 2000). No Fig1p homologue was found in the *H. irregulare* genome, and this observation supports the hypothesis that Fig1p homologous proteins are only present in ascomycetes (Rispail et al. 2009). Several Ca^{2+}-binding proteins are encoded in the *H. irregulare* genome, although one (protein ID 148960) has the highest similarity with the *S. cerevisiae* calmodulin protein (Ca^{2+} binding protein) Cmd1p. One of the targets of this calmodulin protein is calcineurin, which exists in two isoforms (Cmp2p and Cna1p) in *S. cerevisiae*. In *H. irregulare*, two isoforms of calcineurin are also present (protein ID 66200 and 148882). The regulatory subunit of calcineurin A is also present as a single copy (protein ID 151005) (Cyert 2003).

Ca^{2+} homeostasis in S. *cerevisiae* is regulated by several vacuolar Ca^{2+} channels. Two copies and one copy of the calcium-transporting ATPases Pmr1p and Pmc1p, respectively, are present in the *H. irregulare* genome. Interestingly, a BLASTP search with the *S. cerevisiae* Vcx1p sequence reveals 5 paralogues of the vacuolar Ca^{2+}/ H^{+} exchanger Vcx1p, which is involved in the control of *S. cerevisiae* cytosolic Ca^{2+} concentration. Some Ca^{2+} transporters are expanded in filamentous fungi compared with yeast (Rispail et al. 2009). The presence of many paralogues in this class of transporter may reflect a need for more flexible adaptation to the environment in filamentous fungi compared with yeasts.

4.1.5. The cAMP-PKA pathway (I)

The cAMP-PKA pathway is characterised by the presence of different conserved proteins in eukaryotes, in which adenylate cyclase (AC) is the central component (Shemarova 2009, Pukkila-Worley, Alspaugh 2004). Adenylate cyclase converts ATP to cyclic-AMP (cAMP), which acts as an intracellular second messenger (Shemarova 2009).

The high degree of conservation in eukaryotes is reflected by the presence of all the proteins in this pathway in *H. irregulare*. Its AC (protein ID 169870) displays not only the adenyl cyclase domain but also the leucin rich repeat (LRR), which is important for the function of this protein. Phylogenetic analysis of AC protein sequences from different fungi revealed distinct AC sequences in the Ascomycota, Basidiomycota, and Oomycota (Figure 4). AC is important in many fungal processes, including morphogenesis in *U. maydis* (Gold et al. 1994), the yeast-to-hyphal growth transition in the opportunistic human pathogen *C. albicans* (Zou et al. 2010), and growth, morphogenesis and appressorium formation in *M. oryzae* (Adachi, Hamer 1998). cAMP levels are reduced by the conversion of cAMP to AMP, which is catalysed by a phosphodiesterase (Ma et al. 1999). There are two putative phosphodiesterases in the *H. irregulare* genome. Phosphodiesterase class I (PDE I, protein ID 164096) has a low affinity for cAMP, while PDE II (protein ID 181115) is a high-affinity enzyme.

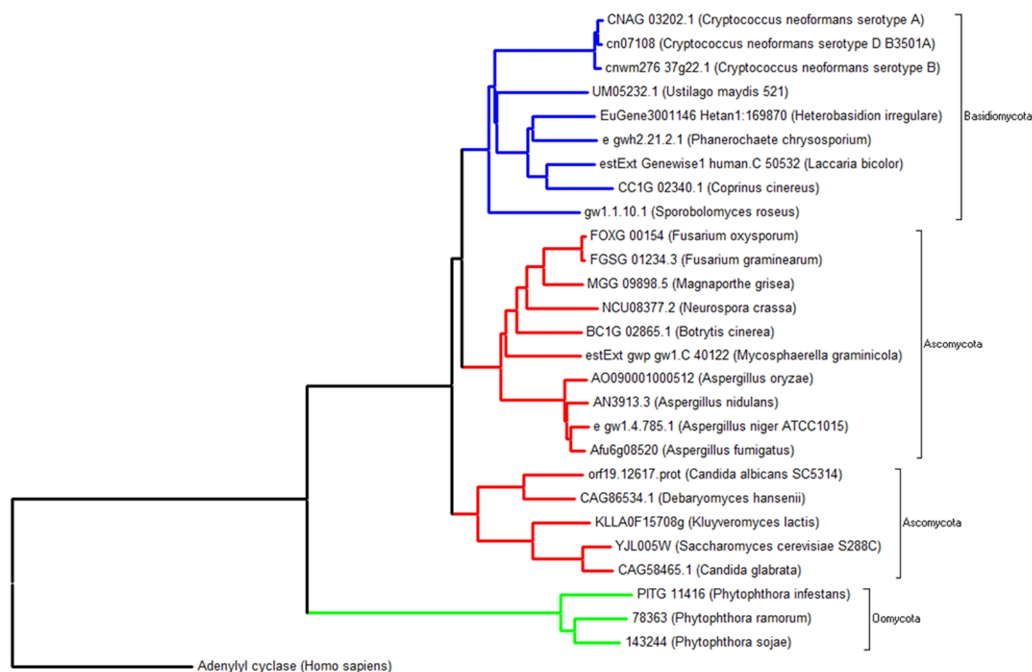

Figure 4: Phylogenetic analysis of adenylate cyclase (AC) from ascomycetes, basidiomycetes, and oomycetes (Olson et al. 2012) (the tree was created using MEGA4 software with the neighbour-joining tree analysis).

Increased levels of cAMP activate a specific kinase called cAMP-dependent protein kinase A (PKA), which is responsible for the downstream regulation and activation of target proteins involved in pseudo-hyphal growth in response to nutrient starvation (Pan, Heitman 1999). In yeast, PKA is formed by one regulatory subunit called

Bcy1p and three catalytic subunits (Tpk1p, 2p, and 3p) with redundant functions (Cannon, Tatchell 1987, Pan, Heitman 1999). The *H. irregulare* genome contains one gene homologous to the yeast PKA regulatory subunit (protein ID 63309). Although in *S. cerevisiae* there are three catalytic subunit genes, *H. irregulare* has only two (protein IDs 68091 and 310777). Because *S. cerevisiae TPK3* is a paralogue of *TPK1* that originated from a whole-genome duplication event, it is possible that in the absence of duplication, only two genes are commonly present in filamentous fungi.

4.2. Gene expression and adaptation of *H. annosum* s.s. exposed to abiotic stresses and during saprotrophic growth on pine wood materials (II)

We used microarray technology as a high throughput technique to investigate the transcriptional response of *H. annosum* s.s. exposed to several abiotic stresses (salt osmotic stress, variation in temperature, oxidative stress, and starvation) and during saprotrophic growth on different pine woody materials (bark, sapwood, and heartwood).

As described in the introduction, *H. annosum* s.s. is a white-rot fungus capable of growing as a necrotroph within living tree tissues, but it also exists as a saprotroph within dead wood material. Furthermore, stump surface colonisation, an important route of entry to a forest tree stand, poses remarkable challenges to fungal survival. Indeed, the open wound of the stump surface is exposed to harsh environmental conditions, which must be overcome by *H. annosum* s.s. to successfully invade the tree stump. We decided to investigate the transcriptional response of *H. annosum* s.s. mycelia exposed to oxidative stress (5 mM H_2O_2 for 60 min), salt stress (0.5 M of either NaCl or $CaCl_2$ for 60 min), high and low temperatures (8°C and 27°C for 3 weeks), and nutrient starvation (growth in glucose-poor media for 5 days). We also examined transcriptional changes that characterise the fungus during saprotrophic growth on pine sapwood, pine heartwood, or pine bark.

4.2.1. Cluster analysis and gene expression (II)

The normalised and filtered microarray data were visualised by hierarchical clustering **(II, Supporting Information Figure 1)**. The three biological replicates for each condition clustered together, indicating the consistent transcriptional pattern and

reproducibility of the experiments. The sample clustering displayed an evident separation between the abiotic stress samples (temperature, salts stress, nutrient starvation, and oxidative stress) and the samples from saprotrophic growth (pine bark, heartwood, and sapwood) **(II, Supporting Information Figure 1)**. The separation between these two groups of samples suggests diverse gene regulation in response to different environmental conditions. Furthermore, conditions characterised by similar transcription profiles were grouped together as pairs: nutrient starvation with H_2O_2, NaCl with $CaCl_2$, and 8°C with 27°C. Similar stress conditions were characterised by similar transcription profiles, suggesting an analogous fungal response **(II, Supporting Information Figure 1)**.

The expression levels of the total number of transcripts detected in the microarray were filtered to obtain a list of up-regulated and down-regulated genes to be used in further analysis. The ten most induced genes in each condition are summarised in **II, Supporting Information Table 11**. Comparison of the up-regulated genes in the paired conditions revealed that 57 genes are up-regulated in both hydrogen peroxide and nutrient starvation, 39 in both NaCl and $CaCl_2$ and 13 in both 27°C and 8°C. In pine wood, as many as 529 genes were commonly induced in bark, sapwood, and heartwood **(II, Figure 1)**. Interestingly, 448 genes were induced specifically in sapwood, almost 4 times more than those specific for heartwood or bark. Because *H. annosum* s.s. grows preferentially in pine sapwood (Stenlid, Redfern 1998), the higher number of specifically induced transcripts in this part of the tree suggests an evolutionary adaptation for sapwood relative to heartwood and bark, where the concentration of fungitoxic and fungistatic compounds (for example, phenolics) is higher (Stenlid, Redfern 1998).

4.2.2. Response of *H. annosum* s.s. during temperature variation (II)

The exposure of *H. annosum* s.s. to 8°C for 3 weeks induced several genes in the MFS-1 and P450s gene families. Despite the few significantly induced genes (44) in *H. annosum* s.s. exposed to 27°C for 3 weeks, MFS-1 and P450 were the gene families most up-regulated also in this condition. qPCR of selected P450 transcripts indicated that some were regulated only in cold conditions, while another was induced in both temperatures **(II, Figure 2 A)**. Cytochrome P450 is a superfamily of monooxygenases that oxidise a broad variety of substrates in eukaryotic cells (Ichinose 2013, Meunier, de Visser & Shaik 2004). These enzymes are enriched in fungi and especially white-rot fungi, which must metabolise many substrates that potentially inhibit fungal growth in the native wood

(Ichinose 2013). In the *H. irregulare* genome, there are 129 predicted cytochrome P450s (Olson et al. 2012) with specific functions that are difficult to assess by sequence analysis alone. Because the media composition was not changed during the temperature shift, our data suggest that some cytochrome P450s are important for adaptation to cold or warm conditions, most likely by targeting intracellular metabolites that arise during these stresses.

In eukaryotic cells, changing the lipid composition of the plasma membrane by increasing the amount of unsaturated fatty-acids is one type of adaptation to cold conditions (Robinson 2001). The qPCR data confirmed the induction of a putative lipid methyltransferase (**II, Figure 2 A**) similar to *S. cerevisiae PEM2*, which converts phosphatidylethanolamine to phosphatidylcholine (Kodaki, Yamashita 1987). This result indicates a general structural adaptation of the fungal plasma membrane to cold conditions. It should also be emphasised that *H. annosum* s.s. lives in boreal and temperate forests where conditions are cold and freezing for most of the year.

4.2.3. Response of *H. annosum* s.s. to salt osmotic stress (II)

We investigated the *H. annosum* s.s. transcriptional adaptation to salt osmotic stress by suddenly exposing the organism to 0.5 M NaCl or $CaCl_2$ for 60 min. The time point was chosen based on the functional study of HaHog1p MAPK, which showed a strong activation at 60 min after osmotic stress induction in NaCl (Chapter 4.3). Although the HaHog1p phosphorylation was strongly induced in NaCl compared with $CaCl_2$ media, the number of genes statistically induced was higher in calcium (415) than in sodium (89). Many more protein kinases were induced in Ca^{2+} compared with Na^+ rich media, suggesting the specific influence of calcium in intracellular signalling. Indeed, the induction of one predicted calcium/calmodulin-dependent protein kinase I, CAMK1, was confirmed by qPCR **(II, Figure 2 B)**. The Na^+/Ca^{2+} exchanger involved in Ca^{2+} homeostasis and similar to *S. cerevisiae VCX1* was also up-regulated in $CaCl_2$. Moreover, a gene involved in osmotic stress response (the predicted glycerol-3-phosphatase similar to the *GPP2* gene in *S. cerevisiae*) was also induced in calcium-rich media **(II, Figure 2 B)**. The activation of genes involved in glycerol accumulation, as mediated by the phosphorylation of Hog1p, is well characterised in yeast (Hohmann 2002). The high conservation of the HOG pathway components in the related species *H. irregulare*, the activation of HaHog1p upon salt osmotic stress exposure (Chapter 4.3), and the induction

of a predicted glycerol-3-phosphatase **(II, Figure 2 B)** suggest that the osmotic adaptation of *H. annosum* s.s. is similar to that of *S. cerevisiae*.

Interestingly, a putative tyrosinase gene was only induced in Ca^{2+} **(II, Figure 2 B)**. In fungi, tyrosinases are key enzymes involved in the biosynthesis of melanin, which provides a barrier against a diverse variety of abiotic stresses and contributes to fungal pathogenicity (Bell, Wheeler 1986, Halaouli et al. 2006, Langfelder et al. 2003). The induction of this transcript and the observation that the infected tree is characterised by a high deposition of Ca^{2+} ions in the infected area (Nagy et al. 2012) prompts the intriguing speculation that *H. annosum* s.s. enhances the melanisation of the cell wall to efficiently colonise the host tissue. Interestingly, one of the five predicted pheromone receptors, a Ste3p homologue, was down-regulated in $CaCl_2$ media compared with the control **(II, Figure 2 B)**. Ste3p is a receptor protein possibly involved in the activation of the pheromone pathway (Chapter 4.1.1) (Rispail et al. 2009). Because no information is available about the real function of these receptors in *H. annosum* s.s., it is difficult to formulate any hypothesis based on our data. Taken together, the high level of calcium deposits in the infected tree tissues and the down-regulation of this receptor in high-concentration Ca^{2+} media suggest that calcium might have a role in regulating the pheromone pathway in *H. annosum* s.s.

4.2.4. Response of *H. annosum* s.s. to oxidative stress and starvation (II)

Selected up-regulated genes during starvation (low glucose media) and oxidative stress (5 mM H_2O_2) were confirmed by qPCR, and genes putatively involved in the glyoxylate cycle (malate synthase, citrate synthase, and isocitrate lyase, **II, Figure 2 C**) and autophagy (2 predicted phosphatidylinositol-4-phosphate 5-kinase (PIP5K) enzymes, **II, Figure 2 C**) were induced specifically in these conditions. The activation of enzymes involved in the glyoxylate cycle has been reported in pathogenic fungi, including *C. albicans* when it lacks nutrients in the surrounding environment (Lorenz, Fink 2001). The importance of autophagy in overcoming nutrient deprivation resides in the ability of the fungus to recycle cellular components (proteins, lipids, carbohydrate, etc.) to survive in nutrient-poor media (Khan et al. 2012). In nutrient starvation, *H. annosum* s.s. was characterised by a strong induction of protein kinases and fungal transcription factors **(II, Figure 2 C)**. The induction of transcripts related to genes involved in signalling, such as

PKs and TFs, indicates the activation of intracellular pathways that counteract the lack of carbon sources to survive in a nutrient-poor environment.

The total number of induced transcripts in selected gene families is reported in Figure 5 (**II, Figure 3**). The representative gene families are protein kinases (PKs), glycosyl hydrolases (GHs), major-facilitator-superfamily 1 (MFS-1s), sugar transporters (STs), transcription factors (TFs), ABC transporters (ABCs), carboxyl esterases (CEs), and cytochrome P450s (P450s). The number of up-regulated genes with > 2-fold changes in H_2O_2 (104) was considerably less than in nutrient starvation conditions (862) and only the cytochrome P450 family seems to be preponderant (Figure 5). Indeed, the induction of a cytochrome P450 and also of a flavin oxidoreductase involved in intracellular redox activity was confirmed by qPCR **(II, Figure 2 C)**. However, the biological significance of these genes in oxidative stress should be further investigated.

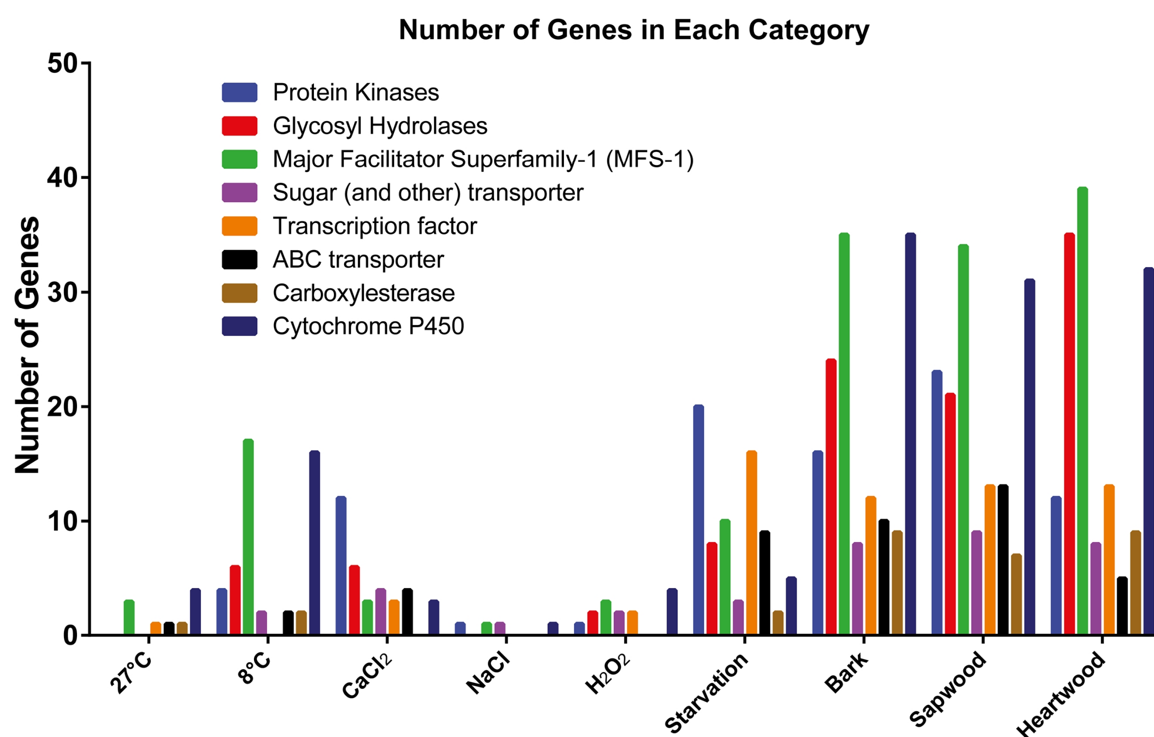

Figure 5: Total number of induced transcripts in selected gene families for each condition included in the microarray study **(II, Figure 3)**.

4.2.5. *H. annosum* s.s. adaptation during saprotrophic growth on pine wood (II)

As previously discussed, *H. annosum* s.s. is a basidiomycete that grows as a necrotroph on pine tree species (*P. sylvestris*). However, its ability to grow as a saprotroph on dead wood material is also well documented (Daniel, Asiegbu & Johansson

1998, Woodward et al. 1998). To understand the molecular mechanisms of adaptation during saprotrophic growth, we profiled the transcriptome of *H. annosum* s.s. after it was grown for 3 months on different pine wood materials, including bark, sapwood, and heartwood. Our attention was focused particularly on understanding the induction of the genes that are involved in wood degradation during saprotrophic growth.

The filtered, up-regulated genes included three major families of induced transcripts that characterise pine bark, sapwood, and heartwood: major facilitator superfamily 1 (MFS-1), cytochrome P450 (P450), and glycosyl hydrolases (GH) (Figure 5). PKs were also preponderant among the gene families, especially in pine sapwood. MFS-1 is a large gene family of transporters characterised by extremely diverse roles in the cell (Law, Maloney & Wang 2008). MFS-1s are either very specific for their substrate, or they accept a diverse range of molecules. MFS-1s are responsible for the membrane transport of ions, sugars, amino acids, and peptides but also toxic molecules such as drugs; thus, these proteins are implicated in drug resistance (Borges-Walmsley, McKeegan & Walmsley 2003). Because there are 140 MFS-1 predicted genes in the *H. irregulare* genome (Olson et al. 2012), it is very difficult at this stage to assign any specific function based only on sequence similarity. However, the induction of many members during saprotrophic growth on wood material indicates their biological importance during fungal growth. Some MFS-1 members use the electrochemical gradient of H_3O^+ to pump toxic compounds out of the cell using an antiporter mechanism (Borges-Walmsley, McKeegan & Walmsley 2003). Because *H. annosum* s.l. has a strong capacity to acidify the media, the creation of an H_3O^+ gradient across the membrane could be used to export toxic metabolites that may arise from the wood degradation process from the cell using MFS-1 transporters.

Cytochrome P450s were also strongly induced during wood degradation. These proteins are most likely necessary to detoxify toxic compounds (for example, phenolics) that are generated by the lignin degradation process and that are also present naturally as a tree defence against pathogen attacks (Ichinose 2013, Meunier, de Visser & Shaik 2004). The high numbers of P450s in other wood-degrading fungi, for example, 106 members in *S. commune* (Ohm et al. 2010) and 113 in *P. chrysosporium* (Martinez et al. 2004), and the high expression of many of them in *H. annosum* s.s. during saprotrophic growth on pine wood material indicate their important role in wood decomposition.

We finally focused our attention on the expression of genes involved in wood degradation. As described in the introduction, carbohydrate active enzymes involved in

wood degradation are glycosyl hydrolases, carbohydrate esterases, and polysaccharide lyases (http://www.cazy.org/) (Cantarel et al. 2009). *H. annosum* s.s. most likely depolymerises highly recalcitrant lignin as the genome of the closely related species *H. irregulare* has several members of the class II fungal peroxidises (8) and multi-copper oxidases (17) (Floudas et al. 2012).

The up-regulated genes that are predicted to be CAZy enzymes and specific for pine bark, sapwood, or heartwood are listed in **II, Supporting Information Table 12**. Pine heartwood material induced more CAZy specific transcripts (18) compared with sapwood (8) and bark (8). We also performed a cluster analysis with the expression level of all the GH genes predicted in the genome. The results indicated that most of the GH transcripts were strongly induced only when *H. annosum* s.s. was grown on wood material **(II, Supporting Information Figure 2)**. Interestingly, the expression pattern of some of the GH genes seemed to be tissue specific. Some genes were induced in both heartwood and bark, while others were mainly induced in heartwood. Moreover, the expression of the 10 predicted members of the GH61 family was strongly induced only in pine heartwood **(II, Supporting Information Figure 2)**. We finally confirmed the induction of selected GH genes by qPCR. Twenty two predicted GH genes belonging to the families GH61, GH28, GH3, GH12, GH1, GH5, GH10, GH15, GH18, GH30, GH35, GH45, GH53, and GH88 were significantly induced in at least one woody material compared with the control **(II, Figure 4)**. The GH61 glycosyl hydrolase family is interesting because its members lack the typical glycosyl hydrolase mechanism of action (Harris et al. 2010). GH61 genes are present in the genome of many ascomycetes and basidiomycetes, white- and brown-rots, litter decomposers and pathogens (Źifčáková, Baldrian 2012), and this particular glycosyl hydrolase family is induced during wood colonisation by *H. irregulare* (Yakovlev et al. 2012). Although the exact function and contribution to the wood degradation process is still unknown, the strong and selective induction of the expression is a good indication of the importance of GH61 genes during saprotrophic growth. GH28s are involved in pectin degradation, and the expansion of this family in necrotrophic fungi has been described (Sprockett, Piontkivska & Blackwood 2011). We observed that some members of the GH28 family were expressed in all woody materials, while others were more strongly induced in bark and heartwood than in sapwood **(II, Figure 4)**. Differences in the physical and chemical properties of the substrate may cause differences in gene expression. We also confirmed the induction of two members of the GH12 family **(II, Figure 4)**. GH12 enzymes degrade hemicellulose,

a structural component of the plant cell wall that is rich in xyloglucans, which are targeted by this GH family of xyloglucan hydrolases (Sandgren, Stahlberg & Mitchinson 2005). Given the similar protein lengths and sequences of these two GH12 proteins, there are no apparent reasons for their tissue specificity. Finally, the gene expression of other glycosyl hydrolases was also validated by qPCR. Based on the statistical significance, some GHs are selectively expressed only in heartwood (GH1, GH5, GH10, and GH45), others in bark and heartwood (GH3, GH30, GH53, and GH88), and only one each in sapwood and bark (GH18), sapwood and heartwood (GH15), and in all woody materials (GH35) (**II, Figure 4**). The qPCR analysis performed on 22 GH genes confirmed the observation made in the hierarchical cluster analysis for many other GH genes. From these results, it is evident that sapwood does not induce the same amount of GH genes compared with bark and heartwood. One explanation is that sapwood is characterised by more soluble and accessible carbon sources as it comprises the living part of the tree. In this growth condition, the induction of GH genes may be inhibited because their expression and synthesis represent a metabolic and fitness cost for the fungus.

Within the enzymatic machinery for lignin degradation, 3 multi-copper oxidases (MCO) and 6 oxidoreductases were found to be specifically induced during saprotrophic growth (**II, Figure 5**). Recent studies of the lignin degradation capacity of *H. irregulare* have identified 8 class II fungal peroxidases and 17 MCO laccases (Floudas et al. 2012). Selected laccase transcripts are more abundant in reaction zone cultures than in heartwood and lignin (Yakovlev et al. 2013). In our microarray study, 3 out of the 19 MCOs tested were specifically and strongly induced during saprotrophic growth on pine wood; these MCOs deserve further investigation. As previously reported, there are no differences in the expression of 6 short-MnPs in heartwood versus reaction zone (Yakovlev et al. 2013). In contrast, we found that some oxidoreductases (IDs 108376, 106089, 181068 and 101580) had considerably higher expression in heartwood than in bark and sapwood, while another gene (ID 127157) had higher expression only in bark (**II, Figure 5**). The different species used (*H. irregulare* vs. *H. annosum* s.s.) and the diverse substrates (Norway spruce reaction zone/heartwood vs. pine bark/sapwood/heartwood) may account for the different results. However, in both works, few selective oxidoreductases are up-regulated when the fungus was grown on heartwood compared with the control, indicating that these genes are important for colonisation and wood degradation in the host tree (Yakovlev et al. 2013).

4.3. Functional study of the MAPK *HaHOG1* and its involvement in stress response (III)

In this study, the growth of *H. annosum* s.s. in the presence of high osmotic and oxidative conditions was investigated. Because no stable and effective transformation system is currently available for *H. annosum* s.s., the putative function of the *HOG1* homologous gene (gene name: Hetan1.estExt_fgenesh2_pm.C_10041) was investigated using the heterologous host *S. cerevisiae hog1*Δ.

4.3.1. *H. annosum* s.s. growth in high osmotic and oxidative conditions (III)

H. annosum s.s. was grown in culture plates supplemented with NaCl, KCl, $MgCl_2$, and $CaCl_2$ at concentrations ranging from 0 M to 0.5 M for 20 days. The results indicate that *H. annosum* s.s. can tolerate salt osmotic stress up to 0.5 M for all the salts, but growth inhibition was stronger in the presence of divalent cations **(III, Figure 1)**. The 1 M concentration was tested for all salts, but the fungus did not grow after 20 days of incubation (data not shown). *H. annosum* s.s. was then exposed to different concentrations of hydrogen peroxide (H_2O_2, 0 mM to 5 mM), which resulted in a concentration-dependent inhibition of fungal growth **(III, Figure 2)**. Our results indicate that *H. annosum* s.s. tolerated salt and oxidative stress at lower levels than many other fungal pathogens. Several strains of *Debaryomyces hansenii*, a yeast-like fungus, tolerate remarkably high levels of osmotic stress up to 2 M NaCl and 2.5 M KCl and oxidative stress up to 10 mM H_2O_2 (Michan et al. 2013). The plant pathogen *Botrytis cinerea* survives in higher osmolytes and oxidants in the media, up to 1.5 M NaCl and 10 mM H_2O_2, compared with *H. annosum* s.s. (Segmüeller et al. 2007). The corn pathogen *U. maydis* tolerates up to 1 M NaCl while still showing cell growth (Gabriela Salmerón-Santiago et al. 2011).

Despite the conservation of all HOG pathway components in these fungi, remarkable differences in tolerance to osmotic and oxidative stresses are reported. The differences observed can be ascribed to different fungal lifestyles (yeast or filamentous form) and hosts (human or plant pathogen), which together require diverse levels of adaptability. Additionally, the HOG pathway activates various target genes that differ between fungi. Finally, the structure of the cell wall, which balances the intracellular turgor pressure generated by a hyperosmotic environment, may have distinct features that influence its resistance in different fungi.

4.3.2. Up-regulation of genes involved in the osmotic stress response (III)

The activation of the HOG pathway during osmotic stress in *H. annosum* s.s. was investigated by quantifying the expression of key pathway genes **(III, Figure 3)**. The *H. annosum* s.s. *GPD1* homologue (protein ID 121564) was induced approximately 2-fold in osmotic conditions compared with the control. *GPD1* is implicated in the yeast osmotic stress response, and its transcript increases in cells exposed to 1.2 M NaCl (Albertyn et al. 1994). Given the high conservation of the HOG pathway in fungi, this result is not surprising, but there is little similarity between the yeast and *H. annosum* s.s. Gpd1p protein sequences. The *STL1* homologue (protein ID 122112) was induced by osmotic stress. The *STL1* gene in *S. cerevisiae* encodes a membrane glycerol proton symporter, and the transcript is also induced (transiently) in hyperosmotic conditions (Ferreira et al. 2005). A relatively strong induction in *H. annosum* s.s. (close to a 3-fold change) was observed for the heat shock protein 78 (*HSP78*) homologous gene. *HSP78* encodes a mitochondrial heat shock protein that is strongly up-regulated under osmotic stress (Posas et al. 2000). We also reported the induction of the *GRE2* homologous genes in *H. annosum* s.s., although this induction was lower compared with other genes. *GRE2* encodes a reductase that is up-regulated by hyperosmotic stress in yeast (Garay-Arroyo, Covarrubias 1999).

In fungi, a high concentration of cations in the surrounding environment dramatically influences intracellular ion homeostasis. For this reason, we also quantified the transcripts of three ATPase pumps possibly involved in controlling the intracellular ion concentration, *ENA1* (P-type ATPase sodium pump) (Haro, Garciadeblas & Rodriguez-Navarro 1991), *PMR1* (Ca^{2+}/Mn^{2+} P-type ATPase) (Antebi, Fink 1992), and *PMC1* (Vacuolar Ca^{2+} ATPase) (Cunningham, Fink 1994). There was no significant variation in the transcript levels of these genes when *H. annosum* s.s. was exposed to NaCl, KCl, and $MgCl_2$. However, *PMC1* was strongly induced when *H. annosum* s.s. was exposed to $CaCl_2$ **(III, Figure 4)**. Thus, this ATPase may be involved in the vacuolar sequestration of Ca^{2+} to maintain a low cytoplasmic Ca^{2+} concentration. The lack of *ENA1* induction was surprising, especially in the mycelia exposed to high Na^+ or K^+. In *U. maydis*, this type of ATPase is important in alkaline media with high levels of Na^+ and K^+ (Benito et al. 2009). *H. annosum* s.s. effectively acidifies the media, and in the presence of a high $[H_3O^+]$, it is possible that other sodium and potassium transporters (such as

electroneutral Na^+/H^+ and K^+/H^+ antiporters) are involved in ion homeostasis (Benito et al. 2009).

4.3.3. Cloning and functional study of the *HaHOG1* gene (III)

As mentioned before, the HOG pathway is activated in yeast not only in response to osmotic stress but also in other conditions, including oxidative stress (Ikner, Shiozaki 2005, Brewster et al. 1993). To investigate the role of the *HOG1* homologue in *H. annosum* s.s., the candidate gene Hetan1.estExt_fgenesh2_pm.C_10041 (*HaHOG1*) was cloned and expressed in the heterologous host *S. cerevisiae hog1*Δ. The phenotype of the yeast deletion mutant *hog1*Δ was characterised by growing the yeast cells in osmotic stress conditions and in a high hydrogen peroxide concentration. Compared with wild type, the yeast *hog1*Δ displayed remarkable growth inhibition in 0.5-1.0 M NaCl, KCl, $CaCl_2$, and $MgCl_2$. Growth inhibition was also observed when *S. cerevisiae* was exposed to oxidative stress in 4 mM H_2O_2.

The results indicated that the *H. annosum* s.s. *HaHOG1* gene complemented the function of the *S. cerevisiae HOG1* gene. The osmotolerance and oxidative tolerance was restored in the yeast *hog1*Δ carrying the *HaHOG1* sequence **(III, Figure 5 and Figure 6)**. The complementation effect could be seen particularly when *HaHOG1* gene expression was induced in galactose-supplemented media. Interestingly, the yeast mutant complemented with *HaHOG1* grew in media supplemented with 1 M NaCl or KCl, while *H. annosum* s.s. was completely inhibited at that salt concentration. Aside from the highly conserved HOG pathway, osmotolerance in fungi is mediated by other chemical, biological, and genetic properties that may not be well conserved in different species of fungi and that may cause variation in the osmotic response of divergent species.

The oxidative stress response has also been investigated in several human pathogenic fungi that are exposed to the oxidative burst of macrophages from the human immune system. Some authors have linked the function of the *C. albicans HOG1* homologue to oxidative stress tolerance, providing evidence that the *hog1* mutant is more sensitive to many oxidants (Alonso-Monge et al. 2003). In *A. fumigatus*, the *sakA* mutant (the *HOG1* homologue) displays increased sensitivity to hydrogen peroxide (Du et al. 2006). In plant pathogenic fungi, *HOG1* is involved in the oxidative stress response in the necrotrophic grey mould *B. cinerea*, whereas Δ*sak1* shows impaired growth in hydrogen peroxide media compared with the wild type (Segmüeller et al. 2007). Our results

regarding *S. cerevisiae hog1*Δ mutant sensitivity to H_2O_2 are consistent with previous reports (Rep et al. 2001). Furthermore, *HaHOG1* cDNA complemented *S. cerevisiae* Δ*hog1* growth inhibition at 4 mM H_2O_2. The complemented mutant yeast grew better in oxidative conditions than the wild type, most likely because of the high amount of recombinant protein HaHog1p synthesised under the control of the GAL1 inducible promoter in the pYES2 vector.

4.3.4. Phosphorylation of the MAPK HaHog1p (III)

Activation of the *S. cerevisiae* MAPK Hog1p is mediated by the upstream MAPKK Pbs2p, which phosphorylates Hog1p on conserved Thr-X-Tyr residues (Widmann et al. 1999). To investigate the activation of HaHog1p in *H. annosum* s.s., the phosphorylation level of this protein was studied under osmotic and oxidative conditions. The high level of conservation, especially in the residues that are phosphorylated by the MAPK, allowed us to use a commercially available monoclonal antibody (mAb) to perform western blot analysis. The Phospho-p38 MAP Kinase (Thr180/Tyr182) rabbit mAb detects endogenous levels of the Hog1p homologue p38 MAPK only when there is dual phosphorylation at Thr180 and Tyr182. We observed strong and rapid activation of HaHog1p within 3 to 10 min upon hyperosmotic stress induction with monovalent salts (0.5 M NaCl and KCl, **III, Figure 7**). However, the western blot did not clearly show the phosphorylation of HaHog1p in the presence of divalent salts (0.5 M $CaCl_2$ and $MgCl_2$, **III, Figure 7**). In contrast, the pattern of HaHog1p phosphorylation during oxidative stress indicated a rapid and strong activation that reached a maximum at 3 min followed by a gradual signal decrease.

Hog1p homologue phosphorylation has been studied in many fungi using NaCl as a stressor. A similar pattern of activation is observed in *C. neoformans* serotype D, where the phosphorylation signal increases after 1 min of NaCl osmotic stress (Bahn et al. 2005). In the osmotolerant yeast *Torulaspora delbrueckii*, TdHog1p is phosphorylated in the presence of 0.5 M NaCl within 1 to 10 min (Hernandez-Lopez, Randez-Gil & Prieto 2006). In the citrus pathogen *A. alternata*, AaHOG1 is also phosphorylated in the presence of 0.6 M NaCl, although no time points are included in this study (Lin, Chung 2010). However, much less information is available about the activation (i.e., phosphorylation level) of the Hog1p homologous proteins in the presence of divalent salts, such as $CaCl_2$ and $MgCl_2$, in filamentous fungi. In *H. annosum* s.s., it is unclear if

HaHog1p was activated after exposure to 0.5 M $CaCl_2$ and $MgCl_2$, and this ambiguity could be caused by several factors. For example, the higher hyperosmotic stress generated by divalent salts compared to monovalent salts causes a strong intracellular perturbation that requires more time for *H. annosum* s.s. to recover and mount a specific response. Alternatively, it was reported that in tree tissues infected by *H. annosum* s.s.*,* the concentrations of Ca^{2+} and Mg^{2+} are increased compared with non-infected areas (Oliva, Romeralo & Stenlid 2011). It has been proposed that the highly saline environment of the colonised tree tissue (especially the high Ca^{2+} content) is a way for the tree to counteract *H. annosum* s.s. infection (Nagy et al. 2012). This high saline environment could therefore inhibit the activation of HaHog1p preventing in this way the fungal response.

4.3.5. Cellular localisation of HaHog1p in *S. cerevisiae* (III)

HaHOG1 was also fused with the reporter gene *GFP*, thus creating a GFP-HaHog1p fusion protein that was expressed in the *S. cerevisiae hog1*Δ mutant. The yeast transformed with the *GFP-HaHOG1* construct was then exposed to 0.2 M NaCl, KCl, $CaCl_2$, and $MgCl_2$ and 5 mM H_2O_2 to study the cellular localisation of the *H. annosum* s.s. MAPK. *H. annosum* s.s. HaHog1p translocated into the nucleus within 30 min of osmotic stress induction (**III, Figure 8)**. However, no clear nuclear accumulation was observed in the presence of hydrogen peroxide. Localisation in the nuclear compartment was confirmed by DAPI nuclear stain (Figure 6).

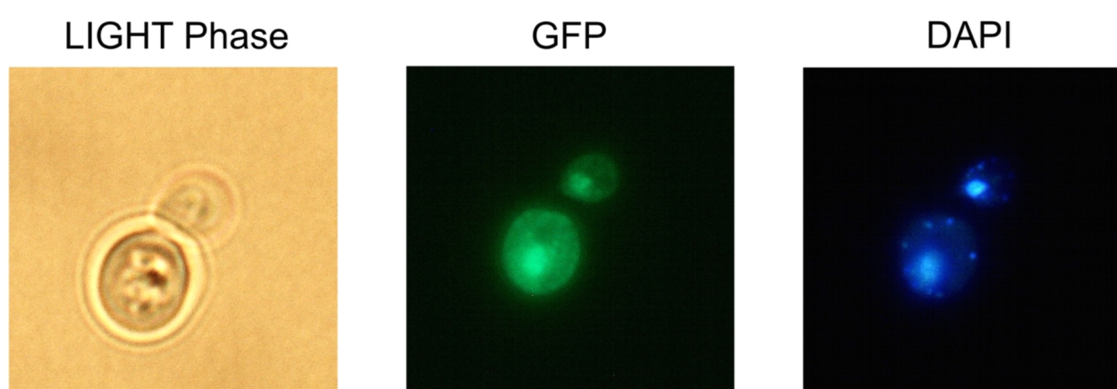

Figure 6: The GFP-HaHog1p fusion protein translocates into the nucleus of the *S. cerevisiae hog1*Δ deletion strain under osmotic stress (0.2 M NaCl).

Several studies have shown the nuclear localisation of the phosphorylated form of Hog1p. In yeast, Hog1p shuttles between the cytoplasm and nucleus, accumulating

preferentially in the nucleus upon acute osmotic stress (0.4 M NaCl) (Reiser, Ruis & Ammerer 1999). In filamentous fungi, homologous Hog1p MAPKs translocate to the nucleus when the mycelia was exposed to osmotic (Bahn et al. 2005) and oxidative stress (Lin, Chung 2010). Although we showed HaHog1p nuclear localisation during osmotic stress, no clear accumulation was seen under oxidative stress in this yeast system. The nuclear localisation of Hog1p was investigated in yeast exposed to the oxidant tert-butyl alcohol (t-BOOH) (Bilsland et al. 2004). Although a certain level of nuclear signal is seen compared with the unstressed yeast cells, the nuclear localisation is not as strong and clear as during hyperosmotic stress (Bilsland et al. 2004). The absence of both a clear localisation during oxidative stress and strong phosphorylation of HaHog1p in *H. annosum* s.s. exposed to divalent salts (despite the nuclear localisation in the same conditions in the yeast system) could suggest a different type of regulation/response in *H. annosum* s.s. compared with *S. cerevisiae*. The different types of fungal growth and cell morphology (single cell vs. filamentous form) may account for the discrepancy between the activated, phosphorylated protein, and the cellular localisation of HaHog1 in *S. cerevisiae*.

4.4. Validation of reference genes for gene expression studies in *H. annosum* s.s. (IV)

The lack of an efficient transformation system for *H. annosum* s.l. to create valid deletion mutants to be phenotypically characterised has pushed the field toward gene expression studies by real-time quantitative PCR (qPCR). The published genome sequence of *H. irregulare* has allowed researchers to design specific primers to target genes whose expression level can now be easily quantified in different experimental conditions to answer defined biological questions. Despite the fact that the transcriptional level of a gene does not always correlate with its specific activation or involvement in that particular condition (the case of the MAPKs and their post-transcriptional activation is a good example), qPCR and gene expression studies are considered an extremely valuable approach to investigating gene regulation in different experimental conditions (Bustin 2000). There have been several studies quantifying transcripts in *H. annosum* s.l. before the genome sequence was available (Iakovlev et al. 2004, Karlsson, Stenlid & Olson 2007, Yakovlev et al. 2008), and others followed afterwards (Yakovlev et al. 2012,

Yakovlev et al. 2013). qPCR is a sensitive technique for nucleic acid quantification. Because it has rapidly evolved, it requires a rational, precise approach to produce valid and scientifically meaningful results (Bustin et al. 2009).

One of the most important aspects of qPCR is the selection of the reference genes to be used for normalising data. The reference gene expression must be stable in the specific experimental conditions that characterise the study (Bustin et al. 2009). The choice of reference genes may deeply affect the results, thus influencing the overall conclusions of the study. Because there were no previously validated reference genes for *H. annosum* s.s., we determined the stability of the transcript level of 11 different genes during saprotrophic growth on pine wood. Because reference genes are usually involved in basic cellular metabolism and other conserved processes, the selection of the reference genes was based on scientific data available for other organisms (Vieira et al. 2011, Hacquard et al. 2011, Yan, Liou 2006, Nicot et al. 2005, Bohle et al. 2007). To assess gene stability, we used two different Excel-based programs, BestKeeper (Pfaffl et al. 2004) and NormFinder (Andersen, Jensen & Ørntoft 2004). Because BestKeeper can analyse a maximum of 10 genes, we decided to run the qPCR validation of the *H. annosum* s.s. candidate genes in two separate runs (Group 1 and Group 2). The same groups were also analysed separately with NormFinder to compare the results obtained with both computing methods. Finally, the stability of all 11 genes was assessed globally with NormFinder, which can accommodate more than ten genes in a single analysis.

The BestKeeper results are summarised in article **IV, Table 2** (Group 1) and **Table 3** (Group 2). The stability ranking of the reference genes based on the standard deviation of their Cq values (SD [±Cq]) was as follows (from the least stable to the most stable): Cyt C < GBP < Mann transf < Actin < RiboS23 < Alfa Tub < Tryp metab for Group 1 and GAPDH < RNA Pol3 TF < Ubiq Lig < RNA Pol2 TF for Group 2. The NormFinder results are shown in article **IV, Figure 1** (Group 1) and **Figure 2** (Group 2). Based on NormFinder calculations, the stability ranking (from the least stable to the most stable) for Group 1 was GBP < Cyt C < RiboS23 < Mann transf < Actin < Alfa Tub < Tryp metab and for Group 2 was GAPDH < Ubiq Lig < RNA Pol2 TF < RNA Pol3 TF. Additionally, the global stability ranking for all the reference genes given by NormFinder is shown in article **IV, Figure 3** with the following ranking: GAPDH < Ubiq Lig < RiboS23 < RNA Pol3 TF < GBP < Tryp metab < Mann transf < RNA Pol2 TF < Cyt C < Alfa Tub < Actin.

NormFinder assigned the lowest value (higher stability) to Tryp met and RNA Pol 3 TF when Groups 1 and 2 are analysed separately. Based on the SD, BestKeeper ranks Tryp met as the most stable in Group 1, while RNA Pol 3 TF does not have the smallest SD in Group 2. However, both genes are indicated by BestKeeper as stable genes based on the correlation analysis (p-value = 0.001 for RNA Pol 3 TF, p-value = 0.011 for Tryp met).

Interestingly, GAPDH is ranked as the most unstable gene by NormFinder. With BestKeeper, the GAPDH SD is the highest of all 11 genes (SD ($\pm C_q$) = 0.96) despite the significance of the correlation analysis (p = 0.001). GAPDH has been used extensively as a reference gene in gene expression studies for qPCR data normalisation, but its stability is not optimal in several organisms and different experimental conditions (Yadav et al. 2012, Dheda et al. 2004, Chen, Fessehaie & Arora 2012, Gopaulchan, Lennon & Umaharan 2013). It should be emphasised that the stability ranking of the 11 genes in *H. annosum* s.s. determined in this study is valid only in the experimental conditions where they have been assessed. We selected a wide series of genes and primer pairs that can be considered every time a new gene expression study has to be optimised for *H. annosum* s.s. However, if the conditions and samples are very different from those described in this work, the reference genes selected from this list require a new validation step to ensure that the most stable genes are used for qPCR normalisation.

5. SUMMARY AND CONCLUSIONS

H. annosum s.l. is a necrotrophic white-rot basidiomycete that causes one of the most destructive conifer diseases in the northern hemisphere. Its genome sequence, available in 2009 and published in 2012, has contributed to our understanding of the molecular mechanisms that regulate the *H. annosum* s.l. fungal lifestyle. Most of the members of the MAPK pathways are present and conserved in *H. irregulare*. However, some components are less conserved, and some are present in higher numbers, especially in the upstream part of the regulatory pathways (i.e., pheromone receptors at the cell membrane level) and at the level of the MAPK effectors (i.e., *HaKSS1* MAPKs). One of the most conserved pathways in *H. irregulare* is the *S. cerevisiae* HOG osmolarity pathway, in which the *HOG1* gene represents the MAPK effector. In this study, the sensitivity of *H. annosum* s.s. to osmotic and oxidative stresses and the activation of the *HaHOG1* gene were investigated. *H. annosum* s.s. is characterised by a higher osmotic and oxidative sensitivity compared with other fungi, although the HaHog1p is activated in osmotic (NaCl and KCl) and oxidative (H_2O_2) conditions. It was also demonstrated that HaHog1p translocates to the nucleus of *S. cerevisiae* upon osmotic stress induction. Finally, selected genes that were induced upon osmotic stress in other fungi were also up-regulated in *H. annosum* s.s.. Taken together, these results support the hypothesis that a conserved mechanism of osmotic stress adaptation is activated in *H. annosum* s.s. by high concentrations of cations, although further functional characterisation should be performed on the more divergent upstream and downstream elements of the HOG pathway.

Given the results from the *HaHOG1* gene studies, the fungal transcriptional response of *H. annosum* s.s. was investigated in a broader range of abiotic stresses and also under saprotrophic growth on different pine woody materials using a microarray approach. Selected genes were validated by qPCR to assess the quality and reproducibility of the large microarray dataset, and this required reference genes for qPCR normalisation. An extensive validation of 11 potential *H. annosum* s.s. reference genes was performed. Candidate reference gene expression stability was assessed during saprotrophic growth and analysed using two software products (BestKeeper and Normfinder). Of the 11 genes validated, two were chosen, Tryp met (Tryptophan metabolism) and RNA Pol3 TF (RNA Polymerase III Transcription Factor), to validate the microarray dataset by qPCR.

The microarray data analysis suggests several mechanisms of *H. annosum* s.s. adaptation to different abiotic stresses. During starvation, when nutrient availability is limited, the fungus activates the glyoxylate cycle and apparently recycles carbon-containing cellular components by enhancing autophagy. The induction of kinase and transcription factor genes is most likely related to the activation of signalling pathways involved in stress adaptation to nutrient starvation. In contrast, cold adaptation involves the up-regulation of cytochrome P450 and major facilitator superfamily (MFS-1) genes, suggesting that *H. annosum* s.s. reacts to toxic metabolites generated by these conditions. During osmotic stress, genes involved in ion homeostasis, such as vacuolar transporters and glycerol metabolism, again suggest the activation of the HOG pathways in which the *H. annosum* s.s. HaHog1p MAPK was shown to be activated.

During saprotrophic growth on different pine wood materials (bark, sapwood, and heartwood), *H. annosum* s.s. induced the expression of many genes belonging to the cytochrome P450 and MFS-1 protein families. The pine wood is degraded by the overexpression of many glycosyl hydrolases genes, of which GH61, GH12, and GH28 were particularly up-regulated during this process, especially for the heartwood degradation. Additionally, the growth on wood material specifically induced the expression of some members of the multi-copper oxidase (MCO) and oxidoreductase gene families.

In conclusion, *H. annosum* s.s. is able to survive abiotic stresses and to engage the wood degradation process via a careful metabolic balance, which allows the fungus to successfully invade and colonise its natural host.

6. FUTURE PERSPECTIVES

As already noted in this work, the availability of the genome sequence of *H. irregulare* will definitely elucidate the molecular mechanisms that control the biology of this fungus. Despite the lack of an efficient transformation system or a reverse genetics approach to generating mutants, the model organism *S. cerevisiae* is a supporting tool that provides valuable information about the function of a specific gene of interest.

The possible function of another MAPK that exists as three paralogues in *H. annosum* s.l. and whose orthologues have been reported to be important for other plant pathogenic fungi is currently being studied. Many questions remain about the interaction between *H. annosum* s.l. and its host. How are these MAPKs regulated during the pathogen-tree interaction? Are there any specific molecules in the host that trigger the activation of these MAPKs? Which receptors on the *H. annosum* s.l. plasma membrane are involved in this type of signalling? Which genes (transcription factors, structural proteins, effectors, etc.) are activated by these pathways? Although the core of these intracellular pathways (for example, the module formed by the three MAP kinases) is characterised by a certain degree of conservation, the upstream (i.e., receptors) and the downstream components (transcription factors, effectors, and other proteins important for the fungal pathogenesis) may have diverged such that a high level of specialisation supports *H. annosum* s.l. in its specific ecological niche.

In the absence of a transformation system, the integration of different molecular biology tools, including next generation sequencing (NGS), qPCR, proteomics, metabolomics and other high throughput approaches, will identify candidate genes involved in pathogenesis. Finally, identification of the genes that are important for fungal pathogenicity and unique to *H. annosum* s.l. is fundamental to developing new specific approaches to controlling root and butt rot disease.

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I tried to do my best. It doesn´t matter if you are a tall tree on the top of the mountain or a small bush down in the valley. Just try to be the best tree or the best bush.

Helsinki, November 2013

Tommaso

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