

Molecular analysis of the *Heterobasidion annosum* Mitogen Activated Protein Kinase (MAPK) HaPMK

Department of Forest Sciences

Nevena Hristozova
hristozova.nevena@gmail.com
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Tiivistelmä — Referat — Abstract The white rot fungus <i>Heterobasidion annosum</i> s.l. is a basidiomycete which is considered to be the most economical important pathogen of conifer trees (<i>Pinus</i> , <i>Picea</i> and <i>Abies</i>) in the northern hemisphere. Presently, the knowledge on the biology and molecular aspects of the <i>Heterobasidion</i> pathosystem is still poor and this is the major set-back in preventing the spread of the pathogen. A deeper investigation at the molecular level of the pathogenicity factors involved during the infection process is very important to better control the disease. Intra-cellular signal-transduction pathways, and in particular the Mitogen Activated Protein Kinases (MAPKs), have been shown to play key roles in the infection cycle in many fungal pathogens, being pivotal in survival, appressorial formation, sporulation and response to various biotic and abiotic stresses. The aim of this study is to characterize a specific <i>H. annosum</i> MAPK, with high sequence homology to FUS3 gene (involved in mating) in <i>S. cerevisiae</i> and with PMK1 gene (involved in appressoria formation) in <i>Magnaporthe grisea</i> . In order to study the function of this MAPK in <i>H. annosum</i> , we performed a complementation experiment in the <i>S. cerevisiae fus3Δ</i> mutant. Expression level profiles, proteomics and immunology studies were used to distinguish between phosphorylated/active and non-phosphorylated/inactive form of the MAPK. Some valuable insights on this kinase cascade in <i>Heterobasidion</i> were discovered, but further studies are required to fully understand its role in the lifecycle of this fungus.			
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1 INTRODUCTION

1.1 *Heterobasidion annosum*

The basidiomycete *Heterobasidion annosum sensu lato s.l.* is a conifer pathogen which is the causative agent of butt and root rot disease. It is a serious problem in the northern hemisphere in managed forests due to its spreading mainly through open wounds (fresh cut stumps) and root to root contacts. Most of the conifers are susceptible to this pathogen and it is thought to be the most economically important decay disease in the temperate forests (Asiegbu, 2005). The total amount of decay caused by *H. annosum* is estimated to be about 90% in Norway spruce in Southern Finland (Tamminen, 1985). The spread of the pathogen in Finland is mainly in the Southern area and biggest losses are observed in the coastal area of the Baltic Sea (Tamminen, 1985)(Moykkynen, 1998). In some severe cases the losses of timber can reach up to 37% (Tuimala, 1979) . Indirect losses in terms of lower timber quality and reduced yield, slow growth compared to healthy trees has been reported (Rönnerberg, 2007). The decayed trees are easier targets of bark beetles (Filip & Morrison, 1998), other pathogens as well as to damage by abiotic stress (Eglè Petrylaitė, 2004). Annual income loss attributed mainly to the low timber quality is thought to be about €800million in Europe (Asiegbu, 2005), of which at least €35 million in Finland (Bendz-Hellgren, M., Lipponen, K., Solheim, H. & Thomsen, I.M, 1998).

H. annosum is a complex of intersterile groups, which according to some authors are still partially interfertile (Lind, Olson, ke, & Stenlid, 2005). The groups belonging to the *H. annosum* complex are named accordingly to their host preference (P, F, and S type depending on the host, Pine, Fir and Spruce respectively). The fungus can spread by both basidio- and conidiospores but the main means of infection is thought to be the basidiospores landing on fresh cut stumps or open wounds on roots and stems, as shown on Figure 1. Only very rarely the spores of *H. annosum* have been shown to infect standing trees through bark wounds (Hodges, 1969). Despite the efforts to breed different varieties of conifers to be selected for disease resistance, some partial resistance can be achieved but no full resistant species has been found so far. To date, little information is available on the plant-pathogen interaction at the molecular level. Unlike in agriculture and horticulture where extensive research has been carried out related to the molecular mechanism of the pathogen infection, on the tree pathosystem the knowledge at the molecular level is still at infancy. One of the main reasons behind

this is the technical difficulties of experiments involving trees, with the time-span of a typical experiment being considerably long. Also, the lack of resistant host tree varieties is a major hindrance.

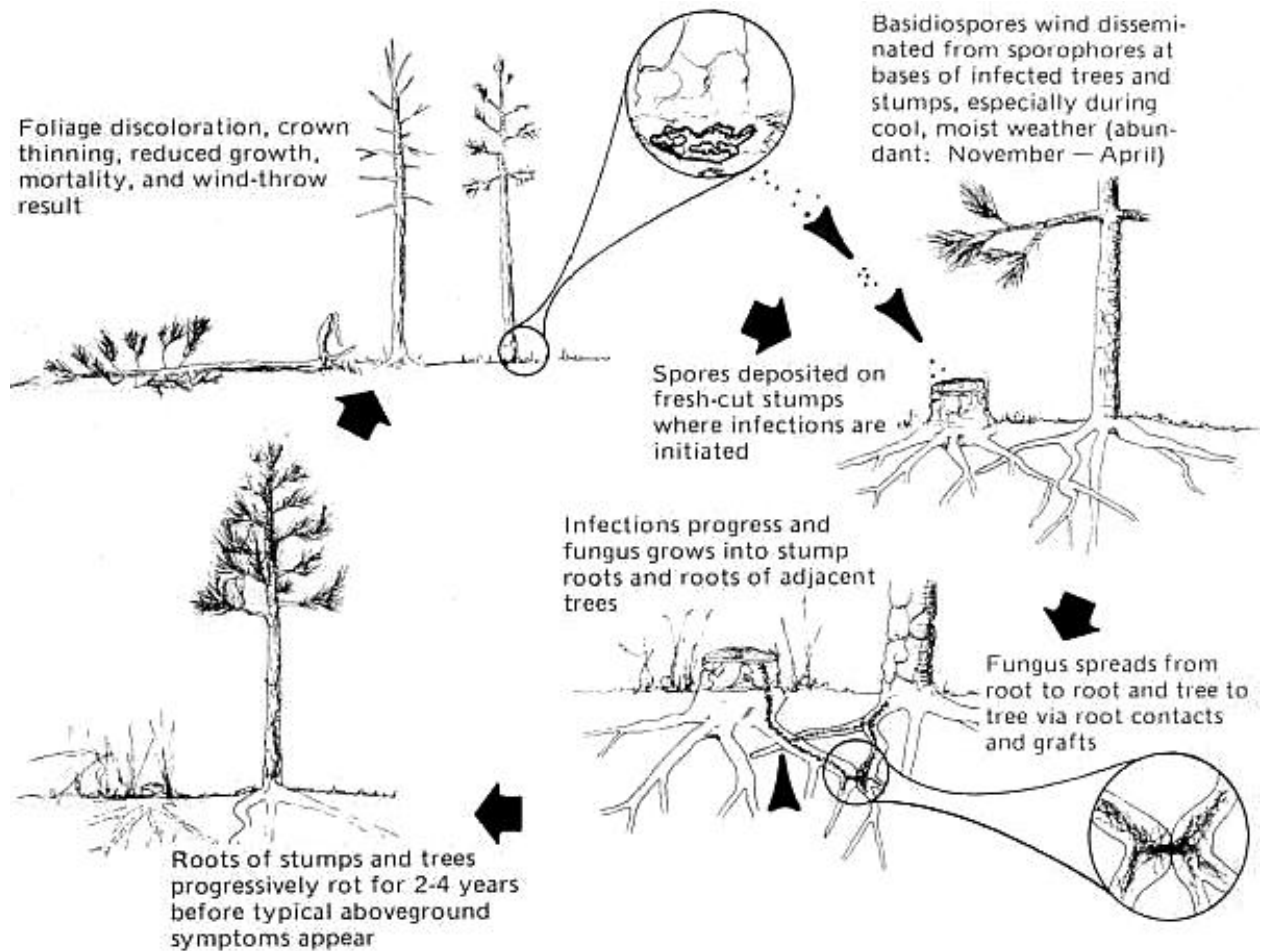


Figure 1. Spreading of *H. annosum* (Florida Department of Agriculture and Consumer Services, 2004).

On the pathogen side more information is available, since manipulation of *H. annosum* is relatively easy in laboratory conditions. Not surprisingly, an important feature of a successful pathogen is the spore viability. The infection cannot be established without a direct contact between the pathogen and the host, which makes the initial adhesion and recognition of the host by the pathogen a crucial step. Once the pathogen has recognized the host, spore germination is followed in many fungal species by the formation of the appressoria, an infection structure which is used by the fungus to breach the plant surface. *H. annosum* has been shown to form appressoria during invasive growth in seedling roots since during the normal infection process it spreads mainly through open wounds where appressoria structure is dispensable (Asiegbu, 2005). The extracellular cell-wall-degrading enzymes are a class of proteins secreted by the fungus which facilitate the penetration into the tree tissues protected by polysaccharides and lignin (Eastwood, 2011). Apart from degrading the plant cell wall and protecting itself from

the toxins released by the tree, the fungus itself secretes low molecular weight toxins whose *in vivo* function is yet to be characterized. Along with the toxins and enzymatic machinery employed in the infection process, also other interesting types of proteins have been shown to be expressed at the initial infection stage e.g. molecules involved in cellular signaling (MAPKs), in metabolism of amino acids, in oxidative phosphorylation, in cellular growth, development and of course in carbohydrate metabolism (Abu, Li, & Asiegbu, 2004).

1.2 Signal transduction pathways – overview

Living cells are not isolated units of life since they constantly interact with other organisms and the surrounding environment by the exchange of chemical compounds (for example small metabolites, signal molecules, information carriers etc) (Lodish et al., 2000). The signal molecules, which are released or sensed by the cell, can have relatively simple chemical structure (for example sugars in induction of catabolic enzymes, ions in muscle contraction, and cellular secretion pathways), or can be represented by more complicated molecules (for example signal peptides, lipids and nucleotides) (Cooper, 2000). Signals can be both intercellular and extracellular. Very often the outcome of a signal perception is the alternation of the gene expression in the cell (i.e. increase/decrease or *de novo* induction/repression of a gene or gene family). The number of intracellular pathways that play an important role in signal transduction in different organisms is quite high. However, they can be categorized into a relatively small number of classes.

1.2.1 Kinase cascades

One of the most conserved signal transduction pathways that can be found in any organism is the so called “molecular circuit” (Figure 2) (Kholodenko, 2010).

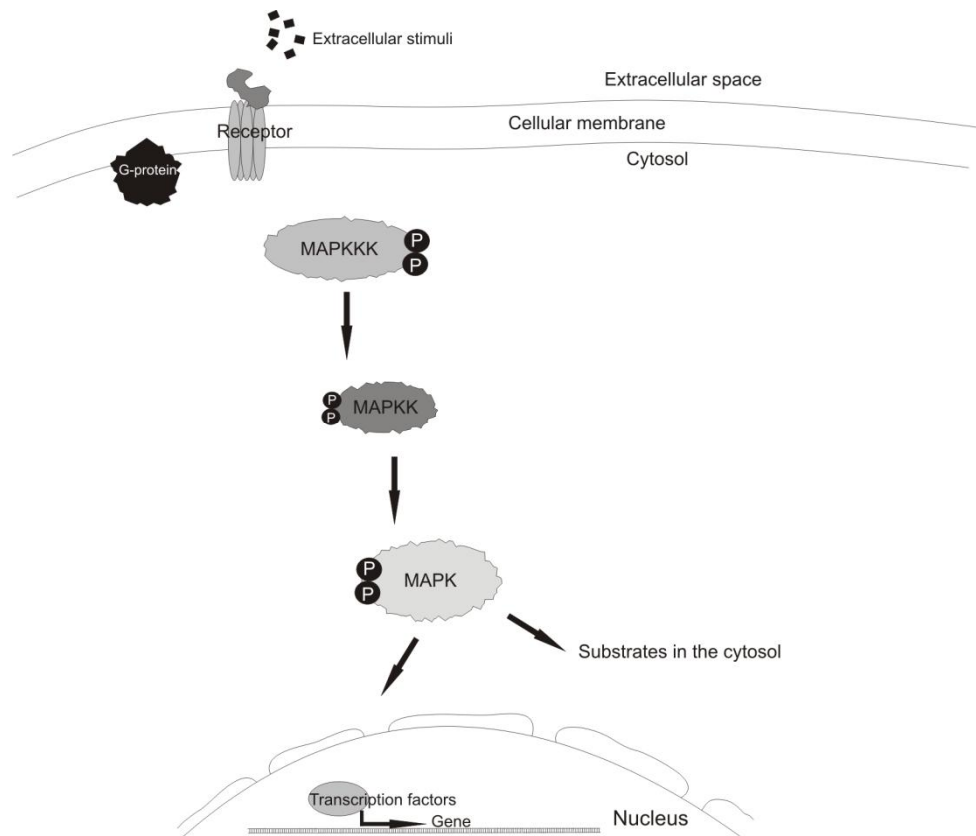


Figure 2: Mitogen Activated Protein Kinase signal-transduction pathways (Kholodenko, 2010)

The extracellular signal is perceived from the environment by a receptor that converts it into a chemical signal, which is then transmitted to an intracellular molecule (Lodish et al., 2000). In some cases, the extracellular signal molecule is physically transported into the cell by a membrane channel and can directly regulate the gene expression. However, in most cases, the signal molecule remains in the cellular surrounding and the information is transduced via other molecules from the extracellular space to the cell interior (Lodish et al., 2000). The signal transduction is performed by membrane-bound protein receptors that are characterized by at least 3 domains: an extracellular domain, which senses the signal (often by specific interaction with the signal molecule), a membrane domain, which docks the whole structure to the plasma membrane and interacts with other membrane molecules with additional functions, and finally the intracellular domain, which represent the catalytic domain (Lodish et al., 2000). The classes of secondary messenger molecules which perceive the signal from the receptor-ligand complex are relatively limited; nevertheless the cell is able to mount a highly diversified response to different extracellular stimuli. Crosstalk between several pathways is also possible via the shared components and the evolutionary plasticity of their domains. One of the most conserved systems for signal transduction is the

activation of protein by direct phosphorylation on their activation site (Lodish et al., 2000).

1.2.2. Ser/Thr protein kinases

Protein kinases are a class of enzymes that catalyze the phosphorylation (addition of a phosphate group) of target proteins leading to modification of their activity (activation, deactivation, change in localization and association with other molecules, change in the protein target stability etc). In particular, Serine-Threonine kinases (ser/thr kinases) are proteins with a conserved catalytic domain that phosphorylates only Ser/Thr residues in a given positions in the target protein. A specific class of ser/thr kinases is called Mitogen-Activated Protein Kinases (MAPK) (Amita & M. Gabriela, April 2004) .

MAPKs are involved in the cellular response and adaptation to environmental signals by activating a variety of responses like apoptosis, cellular division, stress response etc (Amita & M. Gabriela, 2004). A MAP kinase pathway is usually composed of a transmembrane receptor, up to six consecutively activated MAP kinases and a target protein called effector (for example transcription factors (TF), enzymes, proteins of the cellular matrix, gene regulators, histone proteins, etc). MAP kinase pathways are highly conserved indifferent species, from unicellular organism to mammals (Ashworth et al., 1992; Crews, 1993; Nishida, 1994). The signaling cascade begins with the receptor molecule at the plasma membrane that phosphorylates a secondary messenger or directly the MAP kinase kinase kinase (MAPKKK), which in turn phosphorylates a MAP kinase kinase (MAPKK) which finally activates a MAP kinase (MAPK). The MAPK then activates an effector molecule leading to a specific type of cellular response (Fanger, Gerwins, Widmann, Jarpe, & Johnson, 1997)(Siow et al., 1997). In mammalian cells there are at least four different groups of MAP kinase enzymes involved in response pathways to extracellular stimuli (Gupta, 1996). There is a high rate of conservation among them and three of these four groups are Ser-Thr-specific kinases (Amita & M. Gabriela, April 2004). Crosstalk between different pathways is also possible since in some cases several MAPKKK enzymes can activate the same MAPKK due to redundancy in their function. It should be emphasized that the response to a certain stimulus can be a combination of several different pathways, which can also work independently. Often different magnitude of the same signal can trigger different responses. For example, depending on the state of the cell or the cellular type, certain signals might lead to the activation of either apoptotic MAPK pathways or proliferative

pathways (e.g. prolonged activation of the JNK MAPK in some neuronal cells leads to programmed cell death, while transient activation of the same pathway does not result in apoptosis) (N. L. Johnson, 1996; Marty W. Mayo et al., 1997).

1.2.3. Biological function of MAPK pathways

MAPK-mediated cellular responses also play an important role in plant cell defense, development and growth. Since plants are non-motile organisms, they have to adapt quickly to the environment by developing stress responses and pathogen defense mechanisms. Many plant MAPK pathways are triggered by Pathogen-Associated Molecular Patterns (PAMPs), a class of molecules associated with specific group of pathogens which are recognized by pattern recognition receptors and activate the innate immunity (Ausubel, 2005). The response to PAMPs may not be specific but it is often sufficient to overcome an infection, usually by triggering the activation of several MAP kinase pathways involved in several cellular responses. One of the hotspots in plant MAP kinase-mediated immunity are the stomatal cells, which in normal conditions regulate the gas exchange, but also restrict pathogen invasion. It has been shown that closure of the stomatal cells, mediated by phytohormone ABA under drought conditions, is also used in case of pathogen invasion (Asselbergh, 2008) . In both drought and pathogen invasion the activation of the plant response is mediated by the MPK3 MAP kinase (Pitzschke, Schikora, & Hirt, 2009). However, it has not been proven yet whether drought and pathogen invasion trigger the same pathways or the induction of the MPK3 in both of the responses is an outcome of a cross-talk. Bearing in mind the theory of the co-evolution of species, some pathogens evolved mechanisms to overcome plant defense response by hijacking the plant MAPK pathways (Lewis, 1981; Parlevliet, 1986). Some pathogens are able to block the PAMP-receptors by degrading signal-transduction molecules and even by secreting own MAP kinases, which can replace the plant MAPKs thus modulating the plant response to establish the infection process. In general, the plant MAPKs can be grouped into six functional groups. Even though plant species are phylogenetically distant from animals, high sequence similarity has been found among MAPK proteins from species that belong to both kingdoms (Pitzschke et al., 2009).

1.3. The yeast model

The model organism for MAPK-signal-transduction-pathway studies *Saccharomyces cerevisiae* has five different MAPK pathways, one for mating, one for invasive growth, one for cell wall modeling and two for stress responses (Widmann & Gibson, 1999). There is a lot less cross talk between these pathways than previously thought, especially between the FUS3 (the pheromone response pathway) and the Kss1 (responsible for the invasive growth pattern) cascades (Bardwell, Cook, Inouye, & Thorner, 1994). The Kss1 MAPK is able to partially carry out the function of the FUS3 MAPK only under certain conditions, although it was previously thought that those proteins have redundant function. Even though they are shared between different pathways, they are initially activated by different signals and the target proteins they phosphorylate are also different (Gartner, Nasmyth, & Ammerer, 1992). The specificity of the two pathways is guaranteed by a scaffold protein that binds the components of the FUS3 MAPK cascade by specific steric domain interactions. In response to the activation of the FUS3 pathway, specific genes are activated and others are repressed leading to cell growth arrest; Far1, for example, which plays a role in cell cycle arrest and cellular orientation and polarity in the mating process is activated during this process() (VALTZ, 1995)(Shimada, 2000).

A special yeast behavior, called invasive growth, is also controlled by MAPK cascade (including Kss1 MAPK). Once activated in diploid cells the pathway leads to a switch from budding to pseudohyphal growth (i.e. cells do not fully separate but remain attached and elongate, forming hyphae-style structure) allowing the yeast to display a growth type similar to filamentous fungi (Gimeno & Fink, 1992). In the cell wall remodeling signal-transduction pathway, MPK1 represents the MAPK protein. In this pathway there are two MAPKKs involved, which seem to have a redundant function. Some rate of similarity has been found between the protein sequence of the upstream kinase of MAPK and the mammalian protein kinase C. Despite intensive investigation, the pathway has not been fully characterized yet. The two MAPK pathways in yeast which are responsible for stress conditions adaptation are mainly involved in osmotic stress. The “two-component” osmosensor pathway, which is composed of three proteins and it's functionally homologous to the two component system in prokaryotes, is activated by a sensor protein on the cellular membrane. Another stress-pathway, the Sho1 dependent, is also activated by membrane bound receptor. Both of the pathways sense hyperosmolarity and the outcome in both cases is the induction of survival genes,

for example for glycerol synthesis when the cell is exposed to high salt concentration (Maeda & Takekawa, 1995).

A specific MAPK pathway, including the Smk1, is activated during yeast sporulation in diploid cells in starvation conditions. In this case the MAPKKK is thought to be a Sps1 homologue which triggers expression of genes involved in the formation of the spore wall (Mitchell, 1994). Given the fact that yeast are indeed fungi, similarity in many of the processes can be found between budding yeasts and higher fungi, pathogenic or not. Like any other organisms, fungi can sense the environment, whether it is soil, water, air or host tissue and the ways of perceiving and responding to the environmental signals are conserved. For example, many of the cAMP activated cascades and the MAP kinase cascades existing in yeast, plants and animals are also present in *Basidiomycetes*. Recently, in pathogenic fungi several MAPK pathways were proven to be of key importance for the virulence and pathogenesis of the species (Carlile, Watkinson, & Gooday, 2001).

1.4. The fungal models

1.4.1. *Ustilago maydis*

One of the most important plant pathogens is the corn smut *Ustilago maydis* (Figure 3) which is able to cause severe damage to all above ground parts of the plant. The first MAPKK gene characterized is *FUZ3* (the yeast homologue *STE7*) which is thought to be activated in the pathogenesis during conjugation tube production process, filamentous growth and tumor induction (Mayorga & Gold, 1999). Later, the MAPK gene - *ubc3* (the yeast hologue *FUS3*) was also studied. It was shown how the *ubc3* mutants fail to secrete sufficient amounts of pheromones and to form conjugation tubes; the mutant is also unable to perform hyphal fusion without any direct physical contact (Brachmann, Kahmann, Schirawski, & Muller, 2003; Gold, 1999). Probably *ubc3* and *fuz7* MAPKs have partial redundancy since they both are involved in the formation of conjugation tubes. It was also reported that the MAPK pathways and the cAMP signaling pathways in *Ustilago maydis* are tightly interconnected (Kronstad et al., 1998).

1.4.2. *Magnaporthe grisea*

The rice blast *Magnaporthe grisea* is another important plant pathogen that causes severe decrease in yield of rice, wheat, barley and millet. Several MAPK pathways have been characterized in the rice blast also. The *M. grisea* MAP kinase pathways are very similar to those described in yeast even though they are activated by slightly different signals and trigger diverse responses in the two organisms. In the rice blast PMK1 (the yeast homolog FUS3), MPS1 (the yeast homolog SLT2) and OSM1 (yeast homolog HOG1) are thought to be involved in pathogenicity (Dixon et al., 1999; Dixon, Xu, Smirnoff, & Talbot, 1999; J. Xu & Staiger, 1998). The *PMK1* gene is one of the most extensively studied and its role in the fungal germination was well documented (Thines, Roland, & Talbot, 2000). The *PMK1* gene has been shown to have constant low level of expression, but during germination higher levels of expression are observed. *Mps1* mutants show reduced pathogenicity in terms of decreased ability to form penetrating appressoria but they are viable and can still infect rice leaves through wounds. OSM1 is involved in osmoregulatory pathways but unlike in yeast, its activation triggers the accumulation of arabidol instead of glycerol. From this point of view, the *M. grisea* MAPK OSM1 is not only involved in pathogenesis, but it is also dedicated to survival mechanisms under abiotic stress (osmstress) (Kramer, Thines, & Foster, 2009; J. Xu, 2000).

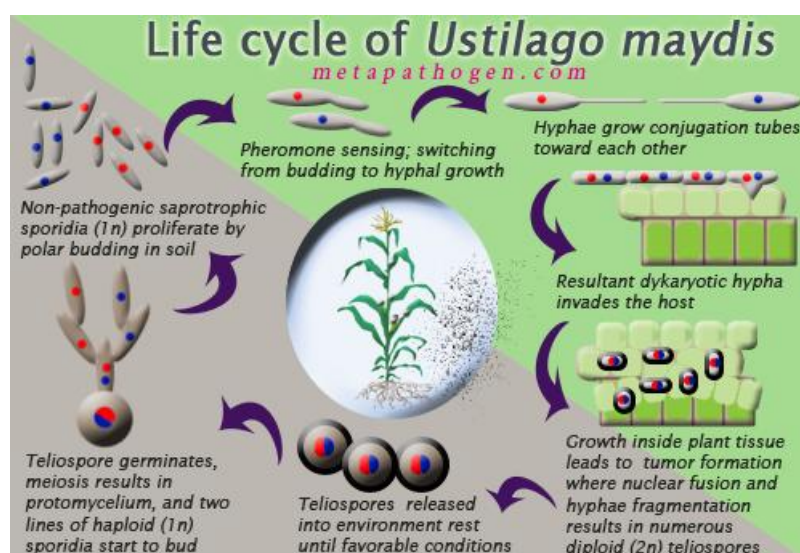


Figure 3: Life cycle of corn smut.

1.5. Studying the MAPK pathways

MAPK pathways are signal transduction pathways that regulate many cellular responses to various signals/conditions in all eukaryotic organisms (Gehart, 2010). Despite the difference and the variation observed both in the number and in the outcome of the MAPK pathways in the organisms studied, great magnitude of conservation can be found between the pathways structure and the sequences of the MAPKs that characterize them. An extracellular signal can trigger a specific cellular response by either *de novo* synthesis or activation of target molecules (Hunter, 1995). Recent studies suggest that MAPK pathways are activated mainly by up-regulation and activation of the components that form the pathways itself (Keyse, 2000). In the case of activation, MAPK proteins are turned on by a specific phosphorylation which is mediated by upstream MAPKKs in the same pathway. The outcome is either phosphorylation of effector molecules or direct transfer of the MAPK protein into the nucleus. Several experiments have demonstrated the GFP- MAPK fusion proteins moving from the cytoplasm to the nucleus upon stress signal perception (Madrid et al., 2006; Stambe, Atkins, Hill, & Nikolic-Paterson, 2003). Unlike the MAPKs, that are thought to be the ultimate targets interacting with the effector molecules downstream, the MAPKK and the MAPKKKs are exclusively restricted to the cytosol of the stressed cells. It is thought that MAPKs move temporarily to the nucleus only to perform their specific function, after that they are transported back to the cytoplasm (Fanger, 1999). MAPK are then deactivated by specific phosphatases which are exclusively present in the cytosol, meaning that MAPKs are usually both activated and deactivated outside of the nucleus (Camps, Nichols, & Arkininstall, 2000). However, both the active and inactive forms of MAPKs have been shown to be able to cross the nuclear double-layered membrane (Lidke et al., 2010). Regarding the up-regulation of the MAPK proteins, it shows a great variation between different organisms and conditions; for example, in the fungal pathogen *Lecanillium fungicola*, a five-fold increase in the expression of the Pmk1-like MAPK has been reported during the fungal infection process (Collopy, 2010).

1.5.1. Functional Analysis

Gene functional assays can be carried by performing both genomic- and proteomic-based studies. From a genetic point of view, one of the most popular methods to determine the activity of a certain gene product is to study the different phenotype in the

presence and in the absence of the gene (most often by knocking-out and afterwards to restore the gene function in the genotype) (Alberts, Johnson, Lewis, & et al., 2002). Knocking-out a gene can be done by deleting the sequence (resulting in total lack of production of the protein of interest) or by introducing a relatively small mutation which is enough to reduce or fully destroy the function of the protein (for example in some enzymes a single amino acid substitution, deletion, addition in the active site can alter or delete the functions of the enzyme). To prove that the observed phenotype is determined by the alteration of the properties of the target protein, the gene coding for that protein should be reintroduced (knock-in) by a reverse mutation (if possible) or by reintroducing a full copy of that gene in the cell. This copy should be expressed and should produce an active, fully functional protein. The gene sequence can be introduced through homologous recombination in the genome or by ectopic expression by transforming the cell with a vector carrying the gene sequence. The restored phenotype is then a sufficient proof that the gene of interest is somehow involved in that specific cellular process. For some organisms like *Heterobasidion annosum*, it is not possible to use the knock-out/knock-in method to study MAPK function due to lack of efficient transformation method. In this case, a complementation assay is an alternative method for the gene function characterization. In species, where gene-manipulation techniques are not optimized it is still possible to clone the gene of interest and to introduce it in another species which is mutant on its homologous gene. This exact type of experiment has been proven to work well for many systems. In *Tuber brochii* such experiment was performed in study of Fus3-like MAPK. A mutated *fus3Δ S. cerevisiae* strain was transformed in order to observe if the expressed protein can restore the mating ability of the yeast (Menotta et al., 2006).

An important aspect of the gene functional studies is the characterization of the activation profile of the protein of interest. When the protein (metabolic enzymes, transcription factors etc.) is activated by *de novo* synthesis, often a peak in the transcript level can be observed shortly after perceiving the signal which triggers their activity. In this case Retro-Transcription quantitative Polymerase Chain Reaction (RT-qPCR) is the most common technique used to quantify the transcript level. Other proteins are only activated post translationally upon signal induction, for example by phosphorylation or partial cleavage (Walsh, Garneau-Tsodikova, & Gatto Jr., 2005). In this case, the most widely used methods to evaluate the magnitude of activation are for example mass-spectroscopy (MS), immunolabeling, and flowcytometry (Hur et al., 2008)(Stevens,

2005). Also, MAPK pathways are often used to trigger a cellular response in stress conditions when a very fast response is required. From this point of view, a direct protein modification guarantees a much faster activation compared to the *de novo* synthesis. Furthermore, MAPKs are indeed targets of the upstream MAPKKs that phosphorylate them only after they are themselves phosphorylated. In *S. cerevisiae* the dual-phosphorylation profile of the FUS3 MAPK has been studied quantitatively by MS to determine both the order (which residue is phosphorylated first) and the extent of the phosphorylation events (R. E. Chen & Thorner, 2007; Raggiaschi). The study shows an increase in the magnitude of the phosphorylation and in the time span in which the molecule stays phosphorylated. To determine and distinguish between different phosphorylated molecules (or even same molecules, phosphorylated in different residues) different antibodies can be produced. To assess the MAPK Serine/Threonine phosphorylation, pSer and pThr-specific antibodies are used to immunoprecipitate the phosphorylated (activated) MAPK from the cellular lysate (Cardinale et al., 2000). Many commercially available antibodies that can be used for this purpose exist. Also, a number of other techniques could be used to quantify the dynamic changes in protein phosphorylation like immunoblotting, fluorescent and radioactive labeling, and PCR.

1.5.2. Bioinformatics: Gene Sequence similarity versus protein function

The considerable sequence similarity that MAPK proteins display can be shown by both functional and statistical methods. Nucleotide and amino acid sequences of homologous proteins can be extracted from databases and aligned together to assess their level of similarity. A popular algorithm for the purpose is the NCBI BLAST interface (Altschul et al., 1990). Certain speculations about the function of the genes/proteins can be derived based on this alignment. For example, high sequence similarity between different sequences belonging to different species often leads to similar tertiary structure, conserved domains, and interaction with rather similar target molecules. However, the real protein function in the cell might be different and the activation mode could be quite diverse between species (Gan et al., 2002). For example, the MAPK proteins FUS3 (*S. cerevisiae*), PMK1 (*M. grizae*), and ERK2 (*H. sapiens*) share significant sequence similarity and are involved in conserved signal-transduction pathways. On the other hand, the signals, the target proteins together with the overall outcome from their activation are different (the pathway is related to the mating/filamentous growth in yeast, to pathogenicity in the rice blast, and to the cellular

growth and differentiation in human) (Lefloch, Pouyssegur, & Lenormand, 2008). Determining whether the similarity between sequences is significant or is simply caused by a random variation, is of major importance while conducting any type of functional studies. One of the most important statistical parameters to evaluate the false-positive rate is the E-value (M. Johnson, 2008). Although the E-value is dependent on the size of the database used, it is representative enough for the initial evaluation of the rate of significance between protein or nucleic acid sequences. When aligning multiple sequences, it is important to have both closely and distantly-related species in the group, since this can provide a result which brings more information on the actual phylogenic connection between the species. Based on sequence alignment, a phylogenetic tree representing the relationship between species can be build (Felsenstein, 1981; Saitou & Nei, 1987). It is important to have in mind that building phylogenetic tree, we assume that all significant sequence similarities arise from the principle of a common ancestry (SOBER & STEEL, 2002). This, however, is not always the case. Similarity between genes might have arisen due to horizontal gene transfer or convergent evolution (Arendt & Reznick, 2008). Building a phylogenetic tree based on separate genetic/molecular sequences is, in its essence, the same bioinformatics procedure as building them based on whole genomes, since they are both based on the molecular evolution – the very core of species evolution itself (Van Valen, 1974). So given that we use the common ancestor to build a phylogenetic tree, the sequences used in this tree are considered homologous and not only similar. Since the signal-transduction pathways involving MAPK proteins are highly conserved among species, the species-specific MAPKs can be considered homologous to each other and can be aligned to assess the level of homology. The amino-acid sequences of the homologous MAPK proteins can be also aligned to give additional information on the magnitude of similarity. The genetic code is degenerative (one amino acid is coded by more than one codon) and in the different kingdoms some of the codons for the same amino acid are preferentially used among others (codon usage) (Rofman, 0704). Under these conditions, a higher similarity could be expected on amino acid levels of homologous sequences, more than on nucleotide levels. Very often if a specific amino acid is replaced with another, the chemical properties are maintained. A codon from the mRNA can be translated in the polypeptide sequence as different amino acid, but it might be still similar by its properties - an aromatic, aliphatic or charged residue – for example the substitution of Val with Leu (Ng & Henikoff, 2006).

2 Objectives

Due to the lack of efficient DNA-transformation system for the basidiomycete *H. annosum*, in this work we decided to use other common molecular techniques to study the HaPMK function.

The following specific aims were addressed in the studies included in this thesis:

- To clone of the basidiomycete *H. annosum* Mitogen Activated Protein Kinase HaPMK;
- To study the HaPMK activation (phosphorylation) in different fungal growth conditions on both transcriptional and protein level, by performing qPCR and Wester blot assays;
- To study the cellular localization of HaPMK in the yeast system upon protein activation.

3 Materials and Methods

3.1 Fungal isolates and growth conditions

3.1.1. *Heterobasidion annosum* strains and growth conditions

The homokaryon *Heterobasidion annosum* P type isolates were kindly provided by K. Korhonen from the Finnish Forest Research Institute (METLA). The stock numbers of the isolates provided are given in Table 1.

Table 1: Stock numbers of *H. annosum* P strains provided by METLA.

#	species	Country	Area	Host	collector
92 153	<i>H. annosum</i> s.s. P	Germany	Bayern	<i>Picea abies</i>	H. Marxmüller
95 259	<i>H. annosum</i> s.s. P	Bulgaria	Sofia	<i>Picea</i> sp.	N. La Porta
97 080	<i>H. annosum</i> s.s. P	Portugal	Madeira	<i>Pinus</i> sp.	A. Hietala
99 075	<i>H. annosum</i> s.s. P	Italy	Trentino	<i>Picea abies</i>	K. Korhonen
03 007	<i>H. annosum</i> s.s. P	Finland	Asikkala	<i>Picea abies</i>	K. Korhonen
04 120	<i>H. annosum</i> s.s. P	Finland	Myrskylä	<i>Picea abies</i>	K. Korhonen

The isolates were grown first on fresh HAGEM media plates (0.5% Glucose, 0.05% NH_4NO_3 , 0.05% KH_2PO_4 , 0.05% $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.5% Malt extract and 2% agar) to check for fungal and bacterial contamination. The isolates were observed under light microscope for homokaryon distinctive features which are the tendency to form distinctively higher amount of aerial mycelia and lack of clamp formations (ref: Dancing genomes: fungal nuclear positioning). For this study the selected homokaryon strains *H. annosum* P-strain 04120 was used. In the experiments, the isolate was grown in 150 ml liquid HAGEM media (0.5% Malt extract and 0.5% Glucose) at room temperature under laboratory condition.

3.1.2 *Saccharomyces cerevisiae* strains and growth conditions

The *Saccharomyces cerevisiae* strain used in this study was kindly provided by Anuj Kumar (Department of Molecular, Cellular, and Developmental Biology, and Life Sciences Institute, University of Michigan). The strain is defined as hyperfilamentous with a genetic background derived from Σ 1278b (Gimeno & Fink, 1992) and genotype *MATa ura3-52 leu2 Δ 0 fus3 Δ* . The strain was plated first on fresh YPD media plates supplemented with 0.2 mg/ml geneticin. All transformed yeast using the pYES2 vector (cat. V825-20, Version K, Invitrogen) with or without insert, were grown on selective SD-U media selective plates (Uracil deficient minimal media, according to Invitrogen manufacturer instruction). To repress the gene cloned into the pYES2 vector, the yeast transformants were grown on selective SD-U liquid media supplemented with 2% glucose. To induce the gene in the pYES2 vector, the yeast transformants were instead grown on selective SD-U liquid media supplemented with 2% galactose and 1% raffinose. The yeast growth conditions were overall set according to pYES2 manufacturer's instruction (Invitrogen).

3.2 Cloning of the FUS3-like HaPMK

3.2.1 cDNA synthesis

In order to study the cellular function of the *HaPMK* (Han-Kss1-2 from the Kss1/Fus3-clade) gene a set of cloning experiments was performed (Hamel, 2012). Total RNA was previously isolated from *H. annosum* mycelia and cDNA was synthesized as follows:

DNase treatment of RNA samples:

1. On ice, the following were mixed in 0.5ml PCR-tubes:

- a. RNA sample _____ 1 μ g
 - b. DNase I, RNase free 10X Reaction buffer _____ 1 μ l
 - c. DNase I, RNase free (1U/ μ l) (Fermentas) _____ 1 μ l
 - d. Nuclease free water _____ to 10 μ l
2. Incubation at 37°C for 30 minutes
 3. 1 μ l of 25mM EDTA (Fermentas) was added to terminate the reaction
 4. The mixture was incubated at 65°C for 10 minutes to inactivate the DNase

Reverse transcription reaction for qPCR:

1. the following were mixed in the same tube where the DNase reaction was made:
 - a. Template RNA (DNA-free) corresponding to 1 μ g total RNA _____ 11 μ l
 - b. Random Hexamers primers (100 μ M 0,2 μ g/ μ l - Fermentas) _____ 1 μ l
 - c. H₂O Nuclease-Free _____ 0,5 μ l
2. The reaction was heated to 65°C for 5 min and cool down quickly on ice.

The mixture was centrifuged and the following were added:

 - a. 5X RevertAid Reverse Transcriptase buffer (Fermentas) _____ 4 μ l
 - b. dNTPs (10mM of each) _____ 2 μ l
 - c. RiboLock RNase Inhibitor (Fermentas) _____ 0.5 μ l
 - d. RevertAid Reverse Transcriptase (200U/ μ l - Fermentas) _____ 1 μ l
3. The content of the tubes was mixed and centrifuged briefly
4. The reaction mixture was then incubated at 25°C for 10 min followed by 60 min at 42°C
5. The reaction was stopped by heating it at 70°C for 5 min.

The synthesized cDNA in 20 μ l total reaction volume was stored at -20°C until used.

3.2.2 Amplification of the *Heterobasidion annosum* MAPK sequence

The *Heterobasidion annosum* putative MAPK sequence (*HaPMK*) was retrieved from the JGI *Heterobasidion* Genome Browser (<http://genome.jgi-psf.org/Hetan2/Hetan2.home.html>) database with TBLASTN search, using the sequence of the *S. cerevisiae* *FUS3* (YBL016W) from the Saccharomyces Genome Database (<http://www.yeastgenome.org/>) as query. The *HaPMK* sequence was then amplified by

PCR using specific primers designed on the gene model found in the *Heterobasidion* Genome Browser. All the primers needed (Table 2) were designed manually over the 5'-end and 3'-end gene regions in order to amplify the full-length *HaPMK* cDNA and to introduce the desired restriction sites for *HindIII* and *NotI* at both sides of the transcript for directional cloning. The reverse primer for the *eGFP* sequence was created to overlap with the 5'-end of the *HaPMK* sequence to be able to fuse the *eGFP* sequence to the 5'-end of *HaPMK* gene.

Table 2. Primer sequences

Primers' name	Sequences
Forward <i>HaPMK</i>	CATAAGCTTATGGCTGACGCTGCGAAAAAG
Reverse <i>HaPMK</i>	CATAGCGGCCGCTTATGTCATGTTTACAGGGTT
Forward <i>eGFP</i>	CTCAGTAAGCTTATGGTGAGCAAGGGCGAGGA
Reverse <i>eGFP</i>	TCGCAGCAGCCATCTTGTACAGCTCGT

3.2.3 Cloning of *HaPMK*

The primers for *HaPMK* amplification were designed to incorporate restriction sites for *HindIII* and *NotI* restriction endonuclease enzymes (Table 2). To determine the optimal primer annealing temperature, a gradient PCR was run. The PCR reaction contained 1 µl template cDNA (see Paragraph II, a), 2.5 µl from each primer (Forward *HaPMK* and Reverse *HaPMK*, 10 µM each, Table 2), 1 µl dNTP mix (10 mM each) and 0.5 µl Phusion DNA polymerase enzyme (Finnzyme), in a final volume of 50 µL. The primers annealing temperature for the gradient PCR was set in the range between 50.4 °C and 60.5°C with the following program: initial denaturation at 98°C/30 sec, 35 cycles of denaturation at 98°C/10 sec - annealing at (gradient 50.4°C to 60.5°C)/30 sec - extension at 72°C/75 sec, and final extension at 72°C for 10 min. The optimal annealing temperature was found to be 57°C. The PCR for final *HaPMK* amplification was then carried out in a 50 µl reaction using 3 µl of *H. annosum* total cDNA (see Paragraph II, a), 2.5 µl from each primer (Forward *HaPMK* and Reverse *HaPMK*, 10 µM each, Table 2), 1 µl dNTP mix (10 mM each) and 0.5 µl Phusion DNA polymerase enzyme (Finnzyme) with the following program: initial denaturation at 98°C/30 sec, 35 cycles of denaturation at 98°C/10 sec - annealing at 57°C/30 sec - extension at 72°C/75 sec, and final extension at 72°C for 10 min. The amplified *HaPMK* cDNA, estimated to be approximately 1200 bp long (based on the JGI model), was purified from the PCR mix with standard 1% agarose gel electrophoresis run at 100 V for 30 min (Cosmo Bio Co.,

Ltd. i-Mupid mini-gel electrophoresis unit). The band on the gel corresponding to the expected *HaPMK* size was purified from the gel with E.Z.N.A.® Gel Extraction Kit (Q-Spin Column, Omega) and eluted with 30 µL nuclease free water.

3.2.4 HaPMK cloning into pGEMT-easy vector

For further cloning into pGEM-T easy vector (Promega), in order to secure single 3' adenine overhangs, the blunt-end *HaPMK* cDNA was treated with 1U DyNAzyme™ II DNA Polymerase (cat. F-501S, Finzymes) in a 50 µl reaction mixture with 0.2 mM dATP (cat. F-561A, Finzymes), and 17 µl of PCR-purified *HaPMK* cDNA. The reaction was then run on gel and the *HaPMK* fragment was purified again as described in the previous section.

The *HaPMK* with adenosine-overhanging was used in a 3:1 molar ratio to the pGEM-T easy vector to produce the pGEM-T-*HaPMK* vector. The ligation was performed according to manufacturer instruction using 60 ng of *HaPMK*. The ligation reaction mixture was incubated at 22°C for 30 min and then transferred to 70°C for 5 min to terminate the reaction.

3.2.5 Fusion PCR

The *HaPMK* gene was fused to the *eGFP* for subsequent subcellular localization. *HaPMK* was amplified from pGEMT-*HaPMK*1 vector using specific primers to include the NotI restriction site at the 3' end of the gene (Table 2). The *eGFP* was amplified using specific primers to include an overlapping region with the *HaPMK* at the 3'-end and the HindIII restriction site at the 5'-end of the reporter gene (Table 2). The two genes were fused together by a “fusion PCR” using Phusion® High-Fidelity DNA Polymerase (cat. F-530S Finnzymes) and the following PCR program: pre-incubation at 98°C for 30 sec, 30 cycles of denaturation 98°C/10 sec - annealing at 58°C/20 sec - extension at 72°C/1 min, and final extension at 72°C for 10 min.

The *eGFP*-*HaPMK* fusion fragment was purified from gel and double digested at both ends using HindIII and NotI (Fermentas) restriction enzymes in a single reaction according to the manufacturer's instruction. The fragment was then ligated into the pYES2 vector (Invitrogen) restricted with the same two enzymes generating the pYES2-GFP-*HaPMK* construct.

3.2.6 *E. coli* transformation

The transformation of *E. coli* DH5 α competent cells with pGEMT-HaPMK and pYES2-eGFP-HaPMK was performed according to the heat-shock method (Froger & Hall, 2007). For the pGEMT-HaPMK plasmid the transformants were selected based on the blue-white test (Ullmann, Jacob, & Monod, 1967) according to pGEMT-easy instruction. For both plasmids the positive colonies were then confirmed with “colony PCR” in 50 μ l final volume. Briefly: cells from the colony to be screened were resuspended in the PCR mix containing 1 μ l of *HaPMK* primers (Forward *HaPMK* and Reverse *HaPMK*, 10 μ M each, Table 1), 1 μ l dNTPs (10 mM each), 5 μ l of 10X buffer and 0.5 μ l DyNAzyme™ II DNA Polymerase (Finnzyme). The reaction was run as follows: initial denaturation at 98°C/30 sec, 35 cycles of denaturation at 98°C/10 sec - annealing at 57°C/30 sec - extension at 72°C/75 sec, and final extension at 72°C for 10 min. Three positive *E. coli* clones carrying the pGEMT-HaPMK and pYES2-eGFP-HaPMK were stored at -80°C in 25% glycerol stocks.

The presence and the correct orientation of *HaPMK* into the vector was confirmed by restriction reaction. For the purpose, the plasmid was isolated from the *E. coli* transformants using GenElute™ Plasmid Miniprep Kit (cat. PLN70, Sigma Aldrich). For the restriction reaction, the enzymes FastDigest® *NotI*, *HindIII*, and also *PstI* (cat. FD0614, Fermentas) which cleaves inside the *HaPMK* sequence were used.

3.2.7 *HaPMK* and *eGFP-HaPMK* cloning into *Saccharomyces cerevisiae* pYES2 expression vector

One microgram of the pGEM-T-HaPMK construct and the *eGFP-HaPMK* fragment (see Paragraph II, e) were treated with FastDigest® *NotI*, *HindIII* (Fermentas) in 20 μ l total reaction volume and incubated for 30 min at 37°C. The fragments produced after the restriction reaction were separated on an agarose gel electrophoresis (1% agarose gel, 100 V, 30 min). The intact 1.2kb *HaPMK* and the 1.7 kb *eGFP-HaPMK* fragment with 5'-end *NotI* and 3'-end *HindIII* sticky ends were purified from the agarose gel with E.Z.N.A.® Gel Extraction Kit (Omega), by eluting with 50 μ L nuclease free water.

The purified *HaPMK* gene and *eGFP-HaPMK* fusion gene were cloned in the pYES2 shuttle vector (cat. V825-20, Invitrogen). The plasmid was also cut with FastDigest® *HindIII* and *NotI* to secure the correct orientation of the cloned fragment. The ligation was performed in 20 μ L reaction with 40 ng pYES2 vector in 3:1 ratio insert/vector, according to T4 DNA-ligase manufacturer's instruction (Fermentas) to produce the pYES2-HaPMK and the pYES2-HaPMK vectors. DH5 α strain of *E. coli* competent

cells were transformed according the standard heat-shock protocol and screened as described for pGEMT-easy vector (see Paragraph II, f). Three positive *E. coli* clones carrying either the pYES2-HaPMK or the pYES2-HaPMK vectors were stored -80°C in 25% glycerol stocks.

3.2.8 *S. cerevisiae* transformation

The hyperfilamentous yeast strain (see Section 3.1.2) was transformed by electroporation as previously described by (Raffaello, Keriö, & Asiegbu, 2012) with the empty pYES2 vector, the pYES2 containing the coding sequence for the *HaPMK* (pYES2-HaPMK), and the pYES2 containing the sequence for the *HaPMK* along with the *GFP* tag in the 5' end (pYES2-eGFP-HaPMK). The cellular localization of the cloned eGFP-HaPMK protein in yeast was assessed by live cell microscopy, following induction with 1% butanol as previously described by Bharucha et al. (2008). The yeast cells were observed using a confocal microscope (Leica TCS SP5II HCS A) with 488nm laser and 63x water objective (the yeast cells were further digitally magnified by a factor 5.4X using the microscope software package).

3.3. *Heterobasidion annosum* experiment for HaPMK induction study

For *H. annosum* HaPMK protein induction study, different treatments were applied to the *H. annosum* 04120 isolate to compare whether they have any influence on the induction and activation of the HaPMK protein. The treatments were as follows:

- water extract of Pine sapwood (Extract S)
- water extract of Pine hardwood (Extract H)
- liquid media where *H. annosum* was grown for 4 weeks (H.a. Media)

The water extracts were prepared from wood chips from the respective part of the stump. The chips were soaked in sterile water for one night with shaking in closed flasks. The extract was filtered through a filter paper disk (cat. 211003, Munktell), freeze-dried, and redissolved in 50 ml of sterile ddH₂O. The liquid media from *H. annosum* liquid culture was recovered by simply filtering out the fungal mycelia using Miracloth membrane (cat. 475855-1R, Merck).

Flasks containing liquid culture of *H. annosum* 04120 were incubated for 4 weeks to obtain a sufficient amount of mycelia and induced with the respective treatment by

pouring 5 ml of Extracts S and H and 150 ml of H.a.Media in the culture flask and incubating at room temperature for 1 h without shaking. The mycelium was harvested by filtering out the culture using Miracloth (cat. 475855-1R, Merck), quickly squeezed, and immediately frozen in liquid nitrogen. Total protein extraction was performed as described by J. M. Chandler et. al (2008) with the following modifications: the frozen mycelia was broken by grinding with mortar and pestle in 750 μ l lysis buffer (0,5 mM Sodium deoxycholate, 20 mM TRIS pH 7.6, 10 mM NaCl, 1 X ProteoBlock™ Protease Inhibitor Cocktail (cat. R1321, Fermentas), and 1 X Halt Phosphatase Inhibitor Cocktail (cat. 78420, Thermo Scientific)).

The mixture was then shaken for 4 min, in cycles of 30 sec, interrupted with 30 sec on ice for cooling down, and then centrifuged at 6000 rpm for 1 min at 4°C. The supernatant was recovered and stored at -80°C until used. Protein quantification was performed according to the Bradford method, using Protein Assay Dye Reagent Concentrate (cat. 500-0006, BioRad), and the standard curve was prepared using BSA standard in concentrations respectively 10, 6, 4, 2 and 1 μ g/ml.

3.3.1 Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS PAGE)

The proteins were separated on a SDS PAGE carried out according to Laemmli protocol (Laemmli), 4% stacking gel and 12% resolving gel. Twenty μ g of total protein were mixed with 4 μ l of 5 X loading buffer in 20 μ l total volume, boiled for 5 minutes, and loaded on the gel. The separation was performed at 20 mA in presence of SDS electrophoresis buffer using SDS-PAGE electrophoresis module (BioRad) until the bromophenol blue reached the bottom of the gel. To assess the size of proteins, a Broad Range Prestained Protein Marker (cat. P7708, New England BioLabs) was used. The gel was stained overnight with PageBlue™ Protein Staining Solution (cat. R0571, Fermentas) to check the equality of protein loading, and the total protein patterns. Proteins were transferred overnight on Amersham Hybond ECL nitrocellulose membrane (cat. RPN303D, GE Healthcare) at 30 V, +4°C, by using 20% methanol transfer buffer with 150 mM glycine and 25 mM Tris-base.

3.3.2 Western Immunoblotting

The *H. annosum* western immunoblotting was performed according to the Cell Signaling Tehcnology instruction manual and meeting the requirements of the membrane used. The membrane was blocked with 5% low fat milk in TBS/T buffer to

avoid unspecific antibody binding. Two different antibodies were used in two distinct assays: Phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) Antibody (cat. 9101S, Cell Signaling Technology), and p44/42 MAPK (Erk1/2) (137F5) Rabbit mAb (cat. 4695S, Cell Signaling Technology). Signal detection was performed by alkaline phosphatase conjugated goat-anti-rabbit antibody (cat. 170-6460, BioRad) according to manufacturer instruction. After signal detection, the membrane was washed with ddH₂O for 10 minutes.

3.4 *S. cerevisiae fus3Δ* experiment for HaPMK induction study

3.4.1 *Protein purification and SDS PAGE separation*

To study the activation and the cellular localization of the HaPMK protein in the *S. cerevisiae* hyperfilamentous *fus3Δ* system, the yeast cells carrying the pYES2-GFP-HaPMK plasmid were treated with 1% butanol for 3-4h (N. Bharucha et al., 2008b) in liquid SC-U media. The butanol treatment has been shown in previous studies to lead to the activation of the MAPK protein in yeast. The purification of total protein from the treated *S. cerevisiae fus3Δ* yeast cells was performed according to the pYES2 user manual. Briefly: yeast cells were grown in liquid SC-U with 2% galactose and 1% raffinose media until OD₆₀₀ = 0,4 was reached, cells were then centrifuged and washed with sterile water. Cells were broken using 100 µl of acid-washed glass beads in 500 µl lysis buffer (0.1 M sodium phosphate, pH 7.4, 1 X ProteoBlock™ Protease Inhibitor Cocktail (cat. R1321, Fermentas), and 1 X Halt Phosphatase Inhibitor Cocktail (cat. 78420, Thermo Scientific)) by repeated vortexing and ice-cooling. The mixture was centrifuged at maximum speed for 10 min. The supernatant, containing total protein extract, was transferred to fresh microcentrifuge tube. Purified protein samples were stored at -80°C until used. Protein quantification was performed according to the Bradford method, using Protein Assay Dye Reagent Concentrate (cat. 500-0006, BioRad), and the standard curve was prepared using BSA standard in concentrations respectively 10, 6, 4, 2 and 1 µg/ml. The proteins were separated on a 12% acrylamide SDS PAGE following exactly the same procedure as previously described (see Section 3.3.1).

3.4.2 *Western Immunoblotting*

The *S. cerevisiae* western immunoblotting was performed according to manufacturer's instruction (Cell Signaling Tehcnology) and by meeting the requirements of the

membrane used. The membrane was blocked with 5% low fat milk in TBS/T buffer to avoid non-specific antibody binding. Two antibodies were used in two distinct assays: Phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) Antibody (cat. 9101S, Cell Signaling Technology), and p44/42 MAPK (Erk1/2) (137F5) Rabbit mAb (cat. 4695S, Cell Signaling Technology). Signal detection was performed by alkaline phosphatase conjugated goat-anti-rabbit antibody (cat. 170-6460, BioRad) according to manufacturer's instruction. After signal detection, the membrane was washed with ddH₂O for 10 minutes.

3.5 RNA purification from *Heterobasidion annosum* and quantitative PCR (qPCR)

3.5.1 RNA extraction and quantification

Heterobasidion annosum homokaryon 04120 cultures were grown on liquid HAGEM media as described in Section 3.1.1, for 4 weeks at 24°C, without shaking, to reach a sufficient amount of mycelia for RNA extraction. They were treated with 5 ml of Extract S and H and 150 ml of H.a.Media, for 1 hour without shaking, as described before. After the treatment, the cultures were filtered briefly, wrapped in aluminium foil and frozen in liquid nitrogen.

The total RNA extraction was performed according to the CTAB protocol (Jones, 1953) with some modifications. To isolate total RNA from the samples, they were homogenised to fine powder in liquid nitrogen and transferred to 2 ml microcentrifuge tube. Nine hundred µl of extraction buffer (2% Cetyl trimethylammonium bromide, 100 mM Tris-HCl, 2 M NaCl, 25 mM EDTA, 2% mercaptoethanol) pre-warmed at 65°C was added to the samples. The tubes were vortexed for 10 min at room temperature. Each sample was extracted twice with one volume of chloroform-isoamyl alcohol (24:1) and centrifuged at 12000 rpm for 10 min at room temperature. The upper aqueous phase was transferred to new 2 ml tube and the samples were precipitated overnight with ¼ volume of LiCl at +4°C. Following a centrifugation at maximum speed for 30 min at +4°C, the pellet was air dried in sterile conditions. The pellet was resuspended in 500 µl of SSTE buffer (1 M NaCl, 0.5% SDS, 10 mM Tris-HCl, 1 mM EDTA).

Further extraction was performed with one volume of chloroform-isoamyl alcohol (24:1) and centrifuged at 12000 rpm for 10 min at room temperature. The upper phase was transferred to new tube and two volumes of absolute cold ethanol was added. The samples were precipitated for 2 hours at -20°C and centrifuged at maximum speed for

30 min at +4°C. The pellet was washed with 70% ethanol and centrifuged again at half speed for 5 min. The pellet of total RNA was then air dried and resuspended in 25 µl DEPC H₂O and quantified with NanoDrop 2000c (Thermo Scientific). The quality of RNA was determined also by performing a RNA agarose gel electrophoresis. For the purpose 1,2% agarose gel in FA buffer (200 mM MOPS, 50 mM Sodium acetate and 10 mM EDTA) and 2.5 mg/ml EtBr was prepared. The RNA samples were preheated with the 5X RNA loading dye to 65°C for 10 min before loading. The separation was performed at 50 V for 90 min in multiSUB gel electrophoresis unit (Clever Scientific ltd). RNA integrity in the denaturing gel was checked with Gel Doc XR+ system (BioRad). One µg of total RNA was retrotranscribed and cDNA was synthesised following the protocol described in previous section (Section 3.2.1).

3.5.2 qPCR

The Ubiquitin ligase and GAPDH gene models in *H. annosum* were found by BLASTp search in the JGI *Heterobasidion* genome browser using the *S. cerevisiae* genes from the Saccharomyces Genome Database (SGD, <http://www.yeastgenome.org/>) as queries, as described in earlier works (Raffaello, T. 2012). Primers for the qPCR were designed using the Universal ProbeLibrary AssayDesign Center (Roche, <http://www.roche.com>) to amplify an internal region of the *HaPMK* transcript. The primers for the Ubiquitin ligase and GAPDH genes were kindly provided by Tommaso Raffaello and Hongxin Chen respectively. The primers and sequences used for qPCR are listed in Table 3.

The cDNA synthesized to be used in the qPCR reaction was diluted 40 times. For each reaction 5.5 µl of the diluted cDNA sample were used in a 15 µl final reaction volume using the LightCycler 480 SYBR Green I Master Mix (cat. 04707516001, Roche). The following cycles were used in the LightCyclerH 480 Instrument II (384 wells plates, Roche): preincubation at 95°C/5 min, 40 cycles of denaturation at 94°C/10 sec - annealing at 59°C/10 sec - extension at 72°C/10 sec, and final extension at 72°C/3 min. A melting curve for each primer was run at the end of the qPCR reaction. The primers efficiency was calculated from the slope of the linear regression line based on a 10 fold serial dilution (5 dilution points) of a control. The Cp values were calculated using the LIGHTCYCLER 480 software, and the *HaPMK* fold change was calculated with the Pfaffl method (Pfaffl, M W. 2001) using GAPDH and ubiquitin ligase as reference genes (qPCR primers listed in Table 3).

Table 3. Primer sequences

qPCR primers	sequence
Ubiq_ligase_For	GGCGACAACATGTTCCAAT
Ubiq_ligase_Rev	AAGACGCCTCCTGCGTAAG
GapDH_shF	ATCGTTGAGGGCTTGATGAG
GapDH_shR	GTGGACGAAGGGATGATGTT
HaPMK For	CATAAGCTTATGGCTGACGCTGCGAAAAAG
HaPMK Rev	CATAGCGGCCGCTTATGTCATGTTTACAGGGTT

4 Results

4.1 Cloning of the FUS3-like HaPMK

The *HaPMK* from *H. annosum* was cloned into the pGEM-T-easy vector system and transformed in *E. coli* DH5 α by heat-shock. The success of the cloning was confirmed by colony PCR, where instead of template DNA, single colonies were picked and resuspended in the PCR mix, containing the specific *HaPMK* primers. Several of the tested colonies were positive and contained the cloned gene as the PCR product was visualized on 1% agarose gel (Fig. 4).

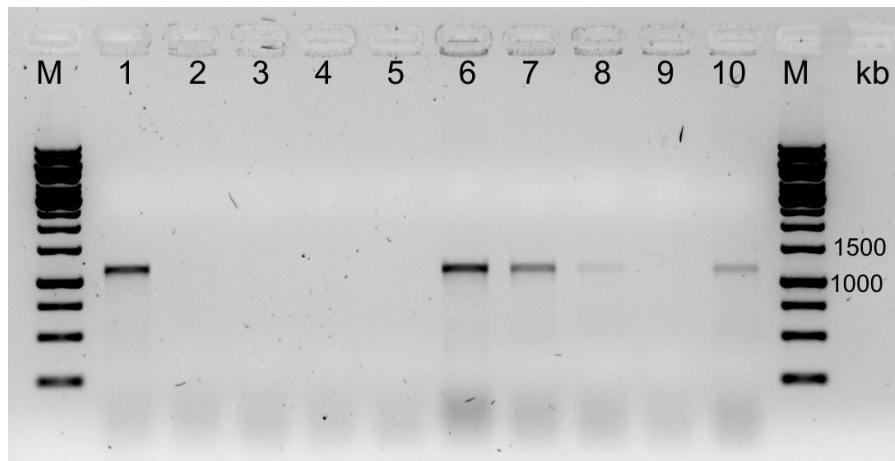


Figure 4. Colony PCR of the transformed *E. coli* colonies and 1% agarose gel electrophoresis. M = 1 kb DNA marker; 1, 6, 7, 8 and 10 = colonies of *E. coli* carrying the *HaPMK* gene (1.2 kb) into pGEMT-easy vector.

The *HaPMK* gene was then cloned into the pYES2 vector and transformed in *S. cerevisiae fus3 Δ* cells by electroporation. Before the yeast transformation, the pYES2-*HaPMK* was purified from *E. coli* DH5 α and was analyzed by performing a double restriction reaction with *NotI* and *HindIII* restriction endonucleases. The reaction

mixture was then visualized on 1% agarose gel, where two distinct bands could be observed, one corresponding to the *HaPMK* gene 1.2 kb and another to the pYES2 plasmid of approximate size 6 kp (Fig. 5).

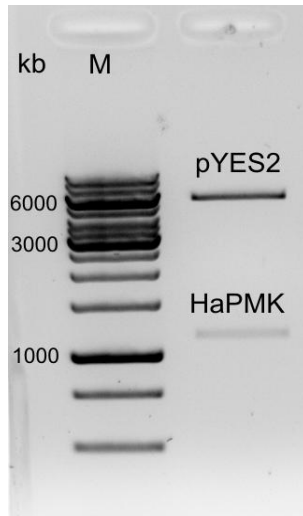


Figure 5. The two distinct bands of the 5,9 kb pYES2 vector and 1200 bp *HaPMK* after the restriction reaction with *HindIII* and *NotI*. M = 1 kb DNA marker; pYES2 = vector for *S. cerevisiae* expression study; HaPMK = *HaPMK* gene insert.

The fusion of the *eGFP* coding sequence and the *HaPMK* gene was performed by “fusion PCR”. The PCR product was then run on 1% agarose gel. The gel displayed a single band with about 1.9 kb in size, which was the expected size of the *eGFP-HaPMK* fusion gene (Fig. 6).

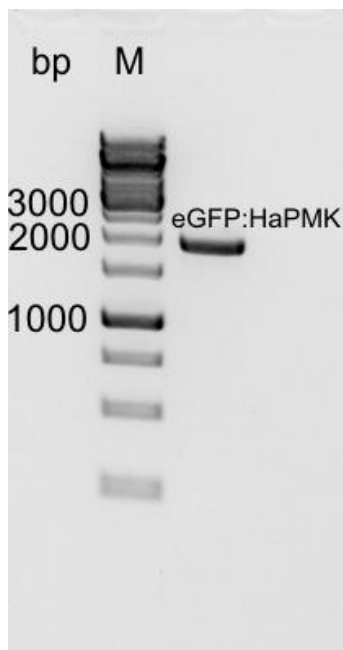


Figure 6. The single 1.9 kb band, corresponding to the fusion eGFP-*HaPMK* gene after “fusion PCR”. M = 1 kb DNA marker; eGFP:HaPMK = fusion *eGFP-HaPMK* gene.

The sequencing of the pGEM-T-HaPMK construct was performed by Institute of Biotechnology at University of Helsinki, Helsinki, Finland. The nucleotide sequence was then translated into protein sequence *in silico* using BioEdit Sequence Alignment Editor (Hebert & Gregory, 2011). Both the nucleotide and the amino acid sequences are presented on Fig. 7. The amino acid sequence was then used to analyze and identify the domains of the HaPMK protein, by using the InterProScan algorithm (<http://www.ebi.ac.uk/Tools/pfa/iprscan/>) (Quevillon et al.,) (Fig. 8).

4.2 Protein extraction and immunoblotting

The proteins extracted from cultures of *H. annosum*, treated with water extracts from pine heartwood, sapwood, and the culture media of another homokaryon replicate were separated on 12% SDS-PAGE. The separation pattern of the *H. annosum* total protein was visible after overnight staining with PageBlue™ Protein Staining Solution (Fig. 9). Total protein sample was also extracted from *S. cerevisiae fus3Δ* transformants, grown on the three variants of culture media: SC-U with glucose, SC-U with galactose, and SC-U with galactose with 1% butanol induction. The protein extract from each sample was separated on 12% SDS-PAGE gels and stained overnight with PageBlue™ Protein Staining Solution (Fig. 10). Western immunoblotting assays were performed both with the protein extracts from *H. annosum* and *S. cerevisiae fus3Δ* transformants using anti-p44/42 MAPK antibody, but after the development of the blotting membranes no band indicating the recognition of the HaPMK protein by this antibody was observed. Thus, western immunoblot with anti-Phospho-p44/42 antibody was performed, after overnight transfer of the proteins from SDS-PAGE gel to Amersham Hybond ECL nitrocellulose membrane. Both the blotting of the *H. annosum* total protein (Fig. 11A) and the *S. cerevisiae fus3Δ* transformants total protein extracts (Fig. 11B) gave multiple bands of different sizes.

1	ATG GCT GAC GCT GCG AAA AAG TCG AAA CAT GGT CAC GCT GCC TCT	45
1	Met Ala Asp Ala Ala Lys Lys Ser Lys His Gly His Ala Ala Ser	15
46	CAG GCT CAG CCT GCA CCA CGA AAG GTT CGG TTC AAC GTA GGC ACA	90
16	Gln Ala Gln Pro Ala Pro Arg Lys Val Arg Phe Asn Val Gly Thr	30
91	CAA TAT CAG GTT CTG GAC GTC GTT GGA GAG GGC GCC TAT GGT ATC	135
31	Gln Tyr Gln Val Leu Asp Val Val Gly Glu Gly Ala Tyr Gly Ile	45
136	GTG TGT TCA GCT GTG CAT CGA CCA AGT GGT CGC AAA GTC GCT ATC	180
46	Val Cys Ser Ala Val His Arg Pro Ser Gly Arg Lys Val Ala Ile	60
181	AAG AAG ATC ATG CCT TTC GAC CAC TCC ATG TTC TGC CTT CGG ACG	225
61	Lys Lys Ile Met Pro Phe Asp His Ser Met Phe Cys Leu Arg Thr	75
226	CTC CGT GAG CTC AAG CTG CTG AAA TTC CTC AGT GAG GCT GGT GTC	270
76	Leu Arg Glu Leu Lys Leu Leu Lys Phe Leu Ser Glu Ala Gly Val	90
271	AGC GAA AAT ATT ATT TCC ATC TTG GAT ATC ATT AAG CCT CCC TCT	315
91	Ser Glu Asn Ile Ile Ser Ile Leu Asp Ile Ile Lys Pro Pro Ser	105
316	ATA GAC GCA TTC AAA GAA GTT TAT CTG ATC CAA GAA CTG ATG GAA	360
106	Ile Asp Ala Phe Lys Glu Val Tyr Leu Ile Gln Glu Leu Met Glu	120
361	ACG GAT ATG CAT CGT GTC ATA CGA ACG CAG GAT CTA TCC GAT GAC	405
121	Thr Asp Met His Arg Val Ile Arg Thr Gln Asp Leu Ser Asp Asp	135
406	CAT GCA CAA TAC TTT GTG TAT CAA ACC CTT CGT GCC TTG AAA GCA	450
136	His Ala Gln Tyr Phe Val Tyr Gln Thr Leu Arg Ala Leu Lys Ala	150
451	TTA CAT TCC GCG GAT GTC ATC CAT CGT GAT CTC AAG CCA TCC AAC	495
151	Leu His Ser Ala Asp Val Ile His Arg Asp Leu Lys Pro Ser Asn	165
496	TTG CTC TTG AAC GCC AAC TGC GAT CTC AAG GTC TGT GAC TTT GGT	540
166	Leu Leu Leu Asn Ala Asn Cys Asp Leu Lys Val Cys Asp Phe Gly	180
541	TTA GCC CGC TCG GTC AAG ACT GCA GAG CCA TCG GGC ACC GAG ACG	585
181	Leu Ala Arg Ser Val Lys Thr Ala Glu Pro Ser Gly Thr Glu Thr	195
586	GGA TTC ATG ACT GAG TAT GTC GCC ACA CGG TGG TAC CGT GCG CCA	630
196	Gly Phe Met Thr Glu Tyr Val Ala Thr Arg Trp Tyr Arg Ala Pro	210
631	GAA ATC ATG CTT ACC TTC AAA CAA TAC ACC AAG GCC ATC GAC GTT	675
211	Glu Ile Met Leu Thr Phe Lys Gln Tyr Thr Lys Ala Ile Asp Val	225
676	TGG TCT GTA GGC TGC ATC CTC GCA GAA ATG CTA TCA GGC AAA CCG	720
226	Trp Ser Val Gly Cys Ile Leu Ala Glu Met Leu Ser Gly Lys Pro	240
721	CTC TTC CCC GGA CGA GAC TAT CAT CAC CAG CTC ACC CTC ATC CTC	765
241	Leu Phe Pro Gly Arg Asp Tyr His His Gln Leu Thr Leu Ile Leu	255
766	GAC GTA CTC GGC ACG CCT ACC CTT GAC GAG TTC TAC GCG ATC ACG	810
256	Asp Val Leu Gly Thr Pro Thr Leu Asp Glu Phe Tyr Ala Ile Thr	270
811	ACG CGC CGC TCG CGG GAC TAC ATC CGT GCG CTG CCG TTC CGG AAG	855
271	Thr Arg Arg Ser Arg Asp Tyr Ile Arg Ala Leu Pro Phe Arg Lys	285
856	AAG AAG CCC TTT ACG CAG CTC TTC CCT AAC GCA AAT CCG AAC GCG	900
286	Lys Lys Pro Phe Thr Gln Leu Phe Pro Asn Ala Asn Pro Asn Ala	300
901	ATC GAT TTC CTC ACT AAG ACC CTG ACA TTC GAT CCA AAG AAG CGG	945
301	Ile Asp Phe Leu Thr Lys Thr Leu Thr Phe Asp Pro Lys Lys Arg	315
946	ATC ACA GTA GAA CAG GCG CTT GCG CAT CCC TAC CTC GAG GCA TAC	990
316	Ile Thr Val Glu Gln Ala Leu Ala His Pro Tyr Leu Glu Ala Tyr	330
991	CAT GAT CCT GAC GAC GAG CCC GTC GCC CCA CCT CTC GAT CCA GAA	1035
331	His Asp Pro Asp Asp Glu Pro Val Ala Pro Pro Leu Asp Pro Glu	345
1036	TTC TTC GAG TTT GAT CTC CAC AAG GAC GAC ATC AGT CGC GAA CAG	1080
346	Phe Phe Glu Phe Asp Leu His Lys Asp Asp Ile Ser Arg Glu Gln	360
1081	TTG AAG GAG CTT CTT TAT GAA GAG ATC ATG AGC TTC AAC CCT GTA	1125
361	Leu Lys Glu Leu Leu Tyr Glu Glu Ile Met Ser Phe Asn Pro Val	375
1126	AAC ATG ACA TAA 1137	
376	Asn Met Thr End	

Figure 7. Nucleotide and amino acid sequences of the cloned *HaPMK* gene from *H. annosum* which was sequenced at the Institute of Biotechnology, Helsinki

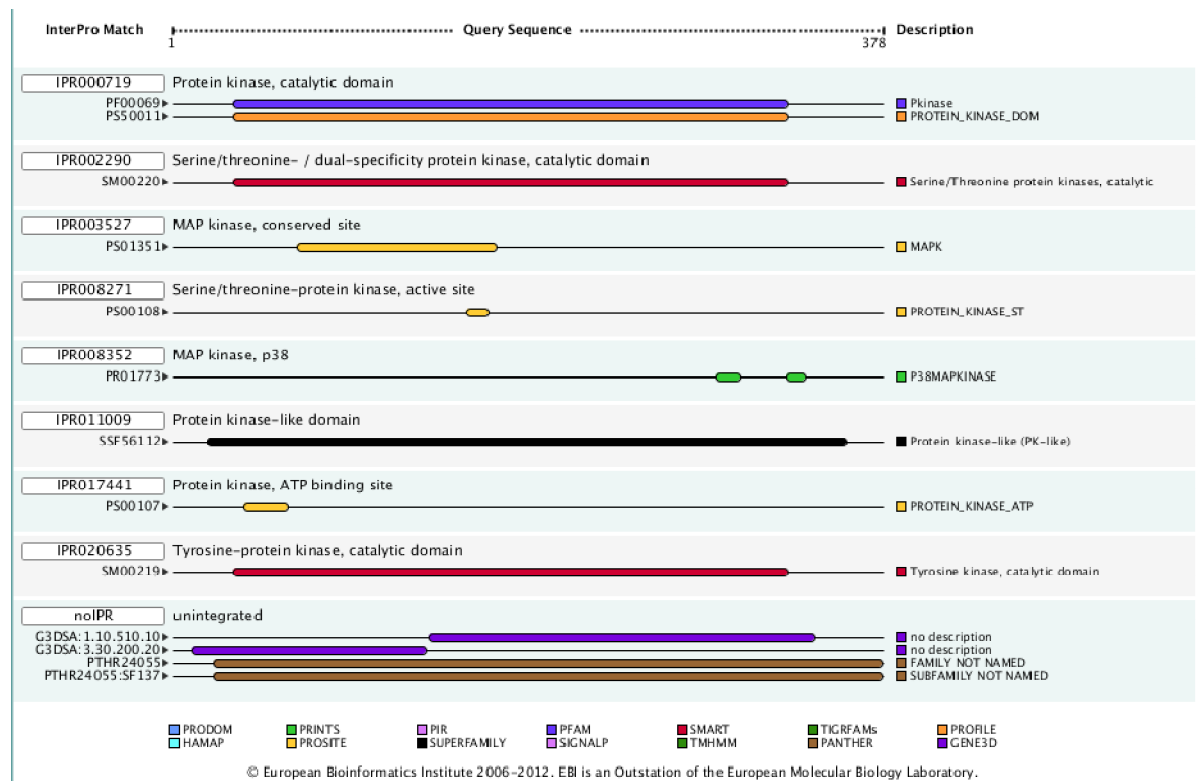


Figure 8. The amino acid sequence of HaPMK protein from *H. annosum* analysed using InterPro scan program (<http://www.ebi.ac.uk/Tools/pfa/iprscan/>). The domains found are all related to the conserved Mitogen Activated Protein Kinase features.

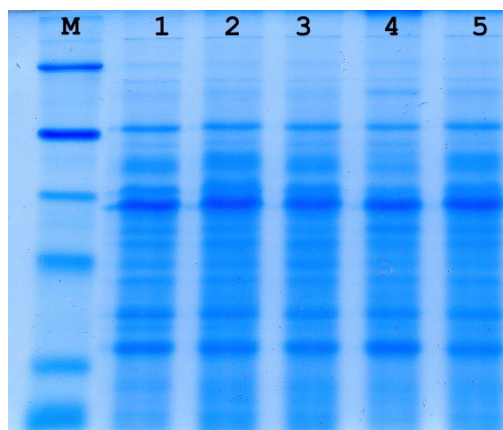


Figure 9. Total protein from *H. annosum* separated on 12% SDS-PAGE. M = molecular protein marker, Broad Range Prestained Protein Marker (kDa 175, 80, 58, 46, 30, 25, 17, 7). Sample loading: 1, 2 = Control; 3 = heartwood treated culture; 4 = sapwood treated culture; 5 = culture treated with *H. annosum* culture media.

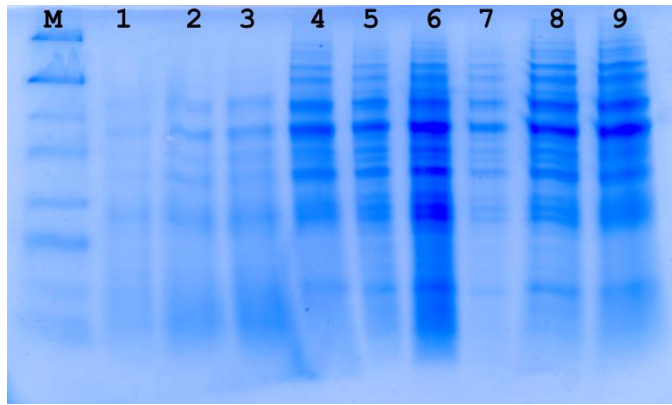


Figure 10. Total protein from *S. cerevisiae fus3Δ* separated on 12% SDS-PAGE. M = molecular protein marker, Broad Range Prestained Protein Marker (kDa 175, 80, 58, 46, 30, 25, 17, 7). **Sample loading:** 1 = *S. cerevisiae fus3Δ* pYES2, grown on glucose; 2 = *S. cerevisiae fus3Δ* pYES2, grown on galactose; 3 = *S. cerevisiae fus3Δ* pYES2, grown on galactose + 1% butanol induction; 4 = *S. cerevisiae fus3Δ* pYES2-HaPMK, grown on glucose; 5 = *S. cerevisiae fus3Δ* pYES2-HaPMK, grown on galactose; 6 = *S. cerevisiae fus3Δ* pYES2-HaPMK, grown on galactose + 1% butanol induction; 7 = *S. cerevisiae fus3Δ* pYES2-eGFP-HaPMK, grown on glucose; 8 = *S. cerevisiae fus3Δ* pYES2-eGFP-HaPMK, grown on galactose; 9 = *S. cerevisiae fus3Δ* pYES2-eGFP-HaPMK, grown on galactose + 1% butanol induction.

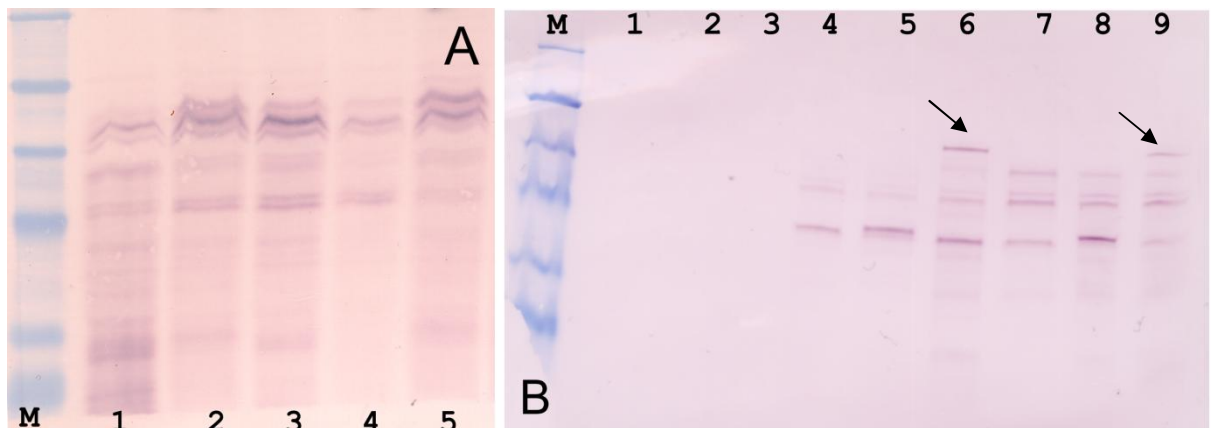


Figure 11. Western Immunoblotting of total protein extracts with anti-Phospho-p44/42 antibody.
A = *H. annosum* homokaryon cultures. M = molecular protein marker, Broad Range Prestained Protein Marker (kDa 175, 80, 58, 46, 30, 25, 17, 7). Sample loading: 1, 2 = Control; 3 = heartwood treated culture; 4 = sapwood treated culture; 5 = culture treated with *H. annosum* culture media.
B = *S. cerevisiae fus3Δ* transformants. M = molecular protein marker, Broad Range Prestained Protein Marker (kDa 175, 80, 58, 46, 30, 25, 17, 7). Sample loading: 1 = *S. cerevisiae fus3Δ* pYES2, grown on glucose; 2 = *S. cerevisiae fus3Δ* pYES2, grown on galactose; 3 = *S. cerevisiae fus3Δ* pYES2, grown on galactose + 1% butanol induction; 4 = *S. cerevisiae fus3Δ* pYES2-HaPMK, grown on glucose; 5 = *S. cerevisiae fus3Δ* pYES2-HaPMK, grown on galactose; 6 = *S. cerevisiae fus3Δ* pYES2-HaPMK, grown on galactose + 1% butanol induction; 7 = *S. cerevisiae fus3Δ* pYES2-eGFP-HaPMK, grown on glucose; 8 = *S. cerevisiae fus3Δ* pYES2-eGFP-HaPMK, grown on galactose; 9 = *S. cerevisiae fus3Δ* pYES2-eGFP-HaPMK, grown on galactose + 1% butanol induction.

4.3 Cellular localization of HaPMK in *fus3 Δ S. cerevisiae*

The fusion protein eGFP-HaPMK was transformed into *S. cerevisiae fus3Δ* mutants using the pYES2 vector system for expression. The transformants were grown on SD-U selective media supplemented with glucose for cellular growth, with galactose for protein over expression, or with galactose + 1% butanol for up to 16 hours in order to

activate the fusion protein and observe its subcellular localisation. The characteristic filamentous growth of the yeast mutant could be observed with a light microscope and the expressed eGFP-HaPMK fusion protein with GFP-filter after being excited with UV light (Fig. 12A and B). The cellular localization of the fusion protein in control and induced with butanol in *S. cerevisiae fus3Δ* pYES2-eGFP-HaPMK transformed cells was observed by a Leica TCS SP5II HCS A confocal microscope after excitement of GFP with 488nm laser and under 63x water objective (Fig. 13A and B).

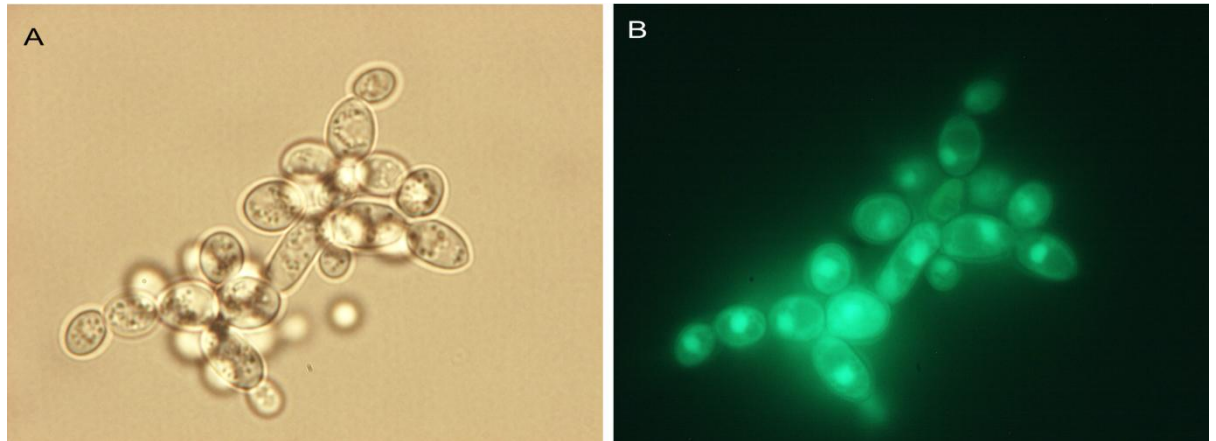


Figure 12. Light microscopy of *S. cerevisiae fus3Δ* pYES2-eGFP-HaPMK transformants: **A = light phase.** **B = GFP**, after excitement with UV light and GFP-filter set.

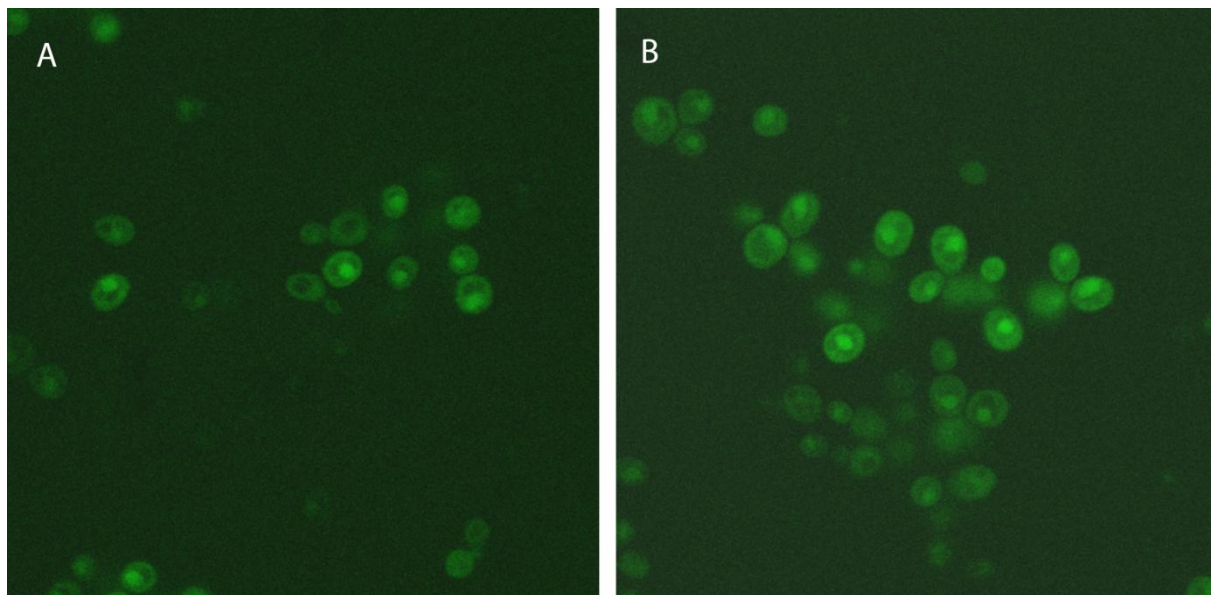


Figure 13. Confocal microscopy of *S. cerevisiae fus3Δ* pYES2-eGFP-HaPMK transformed cells. **A = cells grown on galactose media;** **B = cells grown on galactose media and induced with 1% butanol.**

4.4 RNA purification from *Heterobasidion annosum* and quantitative PCR

To evaluate the expression of HaPMK, total RNA was extracted from 04120 cultures, which were grown in the conditions described in Section 3.1.2.. The quality of the extracted RNA was verified by performing denaturing 1,2% agarose gel

electrophoresis (Fig. 14). The two bands correspond to the 18S and 28S rRNA, which are most abundant in the total RNA pool in eukaryotic cells.

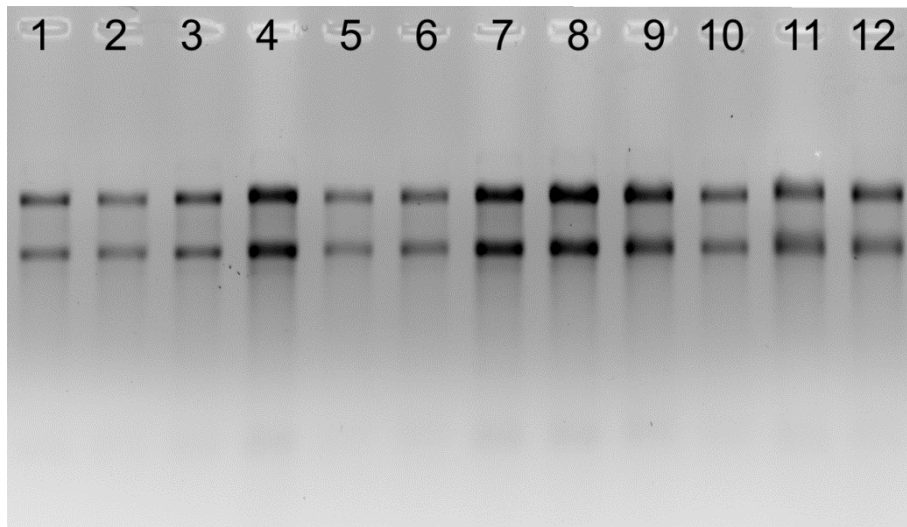


Figure 14. 1,2% denaturing agarose gel to test the total RNA quality after extraction. Sample loading: **1, 2 and 3** = Control; **4, 5 and 6** = RNA from S extract treated *H. a.* 04120 cultures; **7, 8 and 9** = RNA from H extract treated *H. a.* 04120 cultures; **10, 11 and 12** = RNA from H.a.Media extract treated *H. a.* 04120 cultures.

The expression of HaPMK was compared to the two housekeeping genes DAPDH and Ubiquitin ligase by performing a qPCR assay. After analyzing the data from the experiment, no statistically significant change in the accumulation of the HaPMK transcript was observed between the different treatments and the control (Fig. 15).

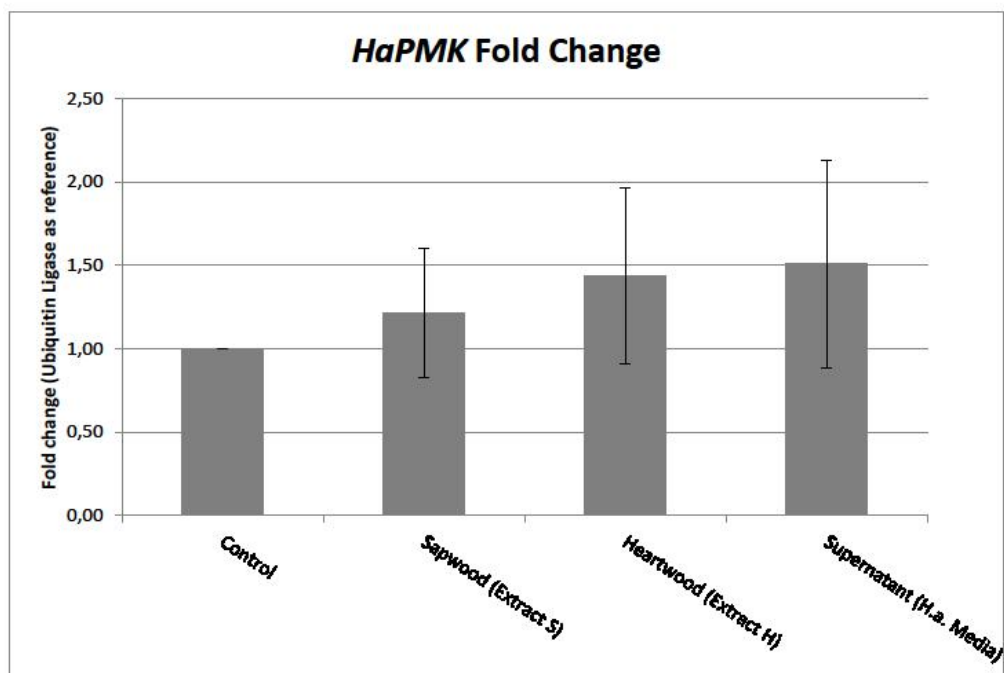


Figure 15. Comparison of the expression of HaPMK and Ubiquitin ligase genes. All samples were treated for 1h with the respective water extracts. The error bars show the standard deviation of the three biological replicates.

5 Discussion

In the present study, a putative pathogenicity gene in the plant-pathogenic fungus *Heterobasidion annosum* was characterized. This basidiomycete is the causative agent of the root and butt rot disease in conifer trees and is considered the most economically important decay fungus in the forests of the temperate climate zone (Asiegbu, 2005). The genome of *Heterobasidion* has been sequenced and annotated through the JGI annotation pipeline (Olson et al., 2012). A total of 11 464 gene models were predicted, but only a very small number have been functionally analysed. Many of the genes involved in the pathogenicity, host recognition, infection, penetration and host invasion belong to this group of non-functionally analysed genes. The genes belonging to the signalling pathways are of particular interest, since the cell signalling is the alarm clock that activates expression of pathogenicity-related genes, when specific signal is present in the cellular environment. The MAPK pathways have been shown to be involved in pathogenicity and to be highly conserved not only in many pathogenic fungi, but throughout the eukaryotes (Widmann & Gibson, 1999). In plant pathogenic fungi, the different MAPK pathways may have different outcomes, depending on the specific life cycle of the fungus, but essentially at least one conserved MAPK pathway is present, which is involved in the pathogenesis. In the rice blast fungus *Magnaporthe oryzae*, the pathogenicity MAPK pathway incorporates the Pmk1 kinase – a homologue of the MAPKs Fus3 and Kss1 in *S. cerevisiae*, and was found to be essential for the formation of appressorium and invasive growth (J. -. Xu & Hamer, 1996). Homologues of the PMK1 gene have been shown to be important for disease development in other phytopathogenic fungi too (Zhao, Mehrabi, & Xu, 2007). For example, *PTK1* is involved in appressorium formation and pathogenicity in *Pyrenophora teres*, which causes blotch on barley (Ruiz-Roldán, Maier, & Schäfer, 2001); the *fmk1* in *Fusarium oxysporum*, a vascular wilt fungus, is vital for root penetration and thus – for pathogenicity (Di Pietro, Garcia-Maceira, Meglecz, & Roncero, 2001); the FUS3 in the citrus pathogen *Alternaria alternata* is essential for conidiation and fungal penetration (Lin, Yang, Wang, & Chung, 2010); the *ubc3* in *Ustilago maydis* is required for filamentous growth and determines the full virulence of this pathogen (Gold, 1999). The *H. annosum* HaPMK sequence is homologous to the mating MAPK Fus3 in *S. cerevisiae*, and respectively - to the already mentioned pathogenicity MAPKs. As a plant pathogen, *H. annosum* also needs to overcome the host protection barrier in order to penetrate into the host tissues and reach the pool of nutrients in the

cells. This pathogen has been shown to form appressorium very rarely as its main points of plant colonization are natural openings or wounded roots and fresh cut stumps (Asiegbu, 2005). Naturally, the question is whether the HaPMK is also part of a pathogenicity signalling pathway in this fungus or perhaps it has a different function. According to previous studies, the HaPMK was found to be expressed during spore germination of *Heterobasidion* and is considered a pathogenicity determinant (Abu et al., 2004). Despite this, the specific signals that activate this pathway are not known, nor the downstream effectors, which are influenced by the activation of the HaPMK. Homologous genes have been shown to take part in formation of penetration structures in other non-appressorium-forming plant pathogens, such as *Mycosphaerella graminicola* (COUSIN et al., 2006), *Botrytis cinerea* (Zheng, 2000) and *Claviceps purpurea* (Mey, Oeser, Lebrun, & Tudzynski, 2002). Since spores germinate whenever they have encountered favourable conditions, the stimulus activating the MAPK pathways that regulate this process are often sensed when the the pathogen has recognised its host. Such signals could possibly be a chemical compound, present on the host surface itself. In order to fully understand the early stages of the pathogenicity process, it is not enough only to determine the signal that activates the specific pathways. It is also necessary to recognise the effector molecules of this pathway, thus revealing the outcome from its activation. Several MAPKs have been shown to influence gene expression by activation of transcription factors in the nucleus or cellular enzymes and cytoskeleton components (Qi, 2005). In order to activate transcription factors, the phosphorylated MAPK molecule has to translocate into the nucleus and for many MAPKs this is common pathway (R. H. Chen, Sarnecki, & Blenis, 1992; P. Lenormand et al., 1993; P. Lenormand, Jean-Marc Brondello, Brunet, & Jacques Pouysségur, 1998). For example, the Pmk1 in *M. grisea* has been shown to translocate into the nucleus, exclusively during the developmental stages of the pathogen (Bruno, Tenjo, Li, Hamer, & Xu, 2004).

Based on these previous studies, the initial experiments focused on the characterization of the *H. annosum* HaPMK as well as possible host stimuli that might have influence on the expression and activation profile. Complementation tests were also done in yeast to observe the cellular localization. As a potential triggering conditions, water extracts from heartwood and sapwood from pine (to mimic the natural conditions of the host for *H. annosum*) were used. Additionally, liquid cultures from another compatible *H. annosum* homokaryon isolate was used to test whether the HaPMK is involved in mating processes, as reported in some other organisms. This

pathway is responsible for mating, a process required before the initiation of the infection cycle (Banuett, 1989). Initial western blot analysis to assess the recognition of the non-phosphorylated form of HaPMK (anti-p44/42), or the phosphorylated form of the protein (anti-phospho-p44/p42) did not produce any positive result. A possible explanation is that the amount of HaPMK was below the threshold for the antibody detection. It is also possible that the proteins were not activated in any of these conditions, or it is equally likely to be a question of phosphorylation and dephosphorylation rather than protein synthesis (Fujiwara & Denlinger, 2007). Thus, we repeated the experiment in an attempt to visualize the phosphorylated HaPMK under the same treatment conditions, using the anti-phospho-p44/p42 antibody. In this case, the antibody recognised a number of targets in all the samples, including the controls, and gave multiple bands on the nitrocellulose membrane. This pattern of recognition might be due to protein degradation during the total protein extraction, which seemed unlikely as the different samples gave practically similar pattern. Additionally, the extraction buffer contained protease inhibitor intended to minimise protein degradation. It is however likely that this monoclonal antibody does not recognise only the HaPMK phosphorylation site. For example, the antibody is supposed to recognise the amino acids at the phosphorylation site based on the human and yeast homologues, but if it was synthesized using synthetic peptide, it is possible that it does not recognise the amino acids of the HaPMK due to sequence variation, and/or recognises several different MAPK proteins and thus the multiple bands.

In order to check the specificity of the anti-p44/p42 antibody against HaPMK, we decided to over express the protein by cloning it in an expression vector and transforming *S. cerevisiae* strain. The strain used was *fus3Δ* – a deletion mutant on the HaPMK homologue Fus3 in yeast. We chose the pYES2 vector, due to its properties – the possibility to clone genes under inducible GAL1 promoter and thus to be able to actively turn on the expression of the heterologous protein, by supplementing the feeding media with galactose (Mumberg, Muller, & Funk, 1994). The results of the western blot assay did not give any bands of recognition, as was in the case of the assay with the protein isolated from *H. annosum* (results not shown). This led us to two possible hypotheses, both of them probably contributing to the lack of success in this assay: first, that the anti-p44/p42 antibody does not recognise the HaPMK sequence; and secondly, that the *S. cerevisiae* strain we used was not suitable for over expression of heterologous proteins. The later one is also supported by years of empirical data in the field of biotechnology that definitely not every yeast strain is suitable for

heterologous protein expression (Romanos, Scorer, & Clare, 1992). On the other hand, when we tested whether the anti-phospho-p44/p42 antibody recognised the over expressed and extracted HaPMK from yeast, we observed multiple bands on the nitrocellulose membrane – a result much similar to the one when the protein was isolated from *H. annosum* cultures and assessed with the same antibody. In this case too, the possibility of protein degradation during extraction is highly unlikely due to the presence of protease inhibitors in the extraction buffer. This led us to conclude that a more probable reason for the multiple bands is the low specificity on the anti-phospho-p44/p42 antibody for the HaPMK protein and possible recognition of other phosphorylated proteins, including other MAPKs.

Despite these drawbacks, a promising aspect was observed in this assay – in the case of extracted total protein samples from the *fus3Δ S. cerevisiae* pYES:eGFP:HaPMK transformants, two of the bands recognised by the anti-phospho-p44/p42 antibody on the nitrocellulose membrane are of the approximate molecular size of the fusion eGFP:HaPMK protein (Figure 11, marked with arrows). According to previous studies, the 1% butanol activates this MAPK pathway in yeast (N. Bharucha et al., 2008c), thus leading to the phosphorylation of the relative MAPK in it. In this study, the band was visible in both butanol induced and non-induced samples, which suggests that HaPMK protein was activated in both of these conditions. It might also be that the HaPMK in this case was activated by another signal from the environment, of which we are not aware, or it has very low expression and is constantly activated.

In order to check whether the HaPMK is properly expressed in the yeast mutants through the pYES2 expression system and its localization in the cell, we assessed the sub-cellular localization of the fusion eGFP:HaPMK protein in the *fus3Δ* mutants. Several earlier studies have shown activated MAPK translocating from the cytosol into the nucleus (Maeder et al., 2007; Qi, 2005). F. van Drogen showed that the yeast homologue (Fus3p) is localized specifically in the nucleus and the shmoo-tip (the mating protrusion in yeast) when the α -mating factor is present in the cellular environment (van Drogen, Stucke, Jorritsma, & Peter, 2001). Another study showed fusion of the Fus3p to cytoplasm bound molecules, which prevent it from translocating into the nucleus upon activation which severely impaired the response outputs (R. E. Chen, Patterson, Goupil, & Thorner, 2010). In other studies, in the filamentous *fus3Δ* yeast strain, the vYFP:Fus3 fusion protein has been located in the nucleus when cells were activated with 1% butanol, and this result has been supported by additional staining of the DNA with DAPI (N. Bharucha et al., 2008a). In the present study,

similar localization of the fusion eGFP:HaPMK was observed in both butanol induced and non-induced cultures. It was found to be mainly localized in a distinct part of the cell, which we believe is the nucleus, since a relatively big dark area observed in the cell is most likely the vacuole. We tried to perform live cell staining with DAPI (DNA binding dye (Williamson, 1979)), but this turned out not to be successful. This was probably due to the lower efficiency of the dye uptake by living cells compared to fixed cells. A similar outcome was observed when we used the FM4-64 fluorescent dye in an attempt to stain the cellular membranes (Cochilla, Angleson, & Betz, 1999; Williamson, 1979) and specifically the nuclear membrane (Zal, Zal, Lotz, Goergen, & Gascoigne, 2006) in order to support our statement that eGFP:HaPMK is indeed localized in the nucleus. The reason for the very low efficiency of both these stains is probably the thick and rigid cell wall of *S. cerevisiae* cells, and the fact that both the dyes are not natural chemical compounds and the binding and uptake of such molecules is usually restricted by the living cell. However, the main result from this experiment is that the HaPMK is apparently localized in the nucleus in both butanol induced cells and in controls, which probably suggests that it is constantly activated. This hypothesis is supported, as discussed previously, by the recognition of the HaPMK protein by the anti-phospho-p44/p42 antibody again in both the induced culture and the control. Apparently, the butanol induction does not specifically activate this MAPK in the yeast system, and some other stimulus may be responsible for the HaPMK activation. The reason for this constant activation and nuclear localization of HaPMK protein in the *fus3Δ* yeast transformants could be that, despite belonging both to the fungal kingdom, *Heterobasidion annosum* and *S. cerevisiae* are quite different biological systems.

Since the western blot and cellular localization did not give unambiguous answers to the questions in what conditions and how HaPMK protein from *H. annosum* reacts, we decided to perform an assay on a transcriptional level to determine whether the HaPMK gene is highly expressed in *Heterobasidion* under the experimental conditions. As already discussed before, in earlier studies HaPMK gene has shown higher expression during the spore germination of *H. annosum* (Abu et al., 2004). Thus, in our study we performed a RT-qPCR, testing whether the treatment of homokaryon *H. annosum* cultures with water extracts from heart- and sapwood, and with the culture media of a compatible culture would result in detectible accumulation of the HaPMK transcript. We compared the amount of HaPMK mRNA with two housekeeping genes – GAPDH (Pancholi, 2003) and Ubiquitin ligase (Hershko, 1983). The internal primers, generated in this work showed very high efficiency, as well as the primers for the

housekeeping genes. The RT-qPCR data analysis though, did not suggest differential expression of the HaPMK compared to the two reference genes in any of the tested conditions and the control. In fact, HaPMK seemed to be expressed less than the GAPDH and more than the Ubiquitin ligase, but after statistical analysis of the data, these differences turned out to be insignificant. Thus, the transcriptional regulation of the HaPMK gene was not influenced by the conditions tested. This would come to support previously expressed hypothesis that the activation of HaPMK is more likely to be on protein phosphorylation/de-phosphorylation level, than on transcriptional level. On the other hand, another probable explanation for lack of differential expression of HaPMK could be that the conditions selected and tested were not the right ones, required to trigger the expression of this kinase and activate the pathway to which it belongs.

6 Conclusion

Heterobasidion annosum root rot is a widely recognised ecological and economical problem in temperate forests in Europe and North America. The need for studying the intrinsic properties which define this organism as a pathogen, causing severe tree decay and economic losses, had only recently been recognised by the scientific community. Thus, this study is in many aspects still in its infancy and faces many obstacles. Some of these obstacles were also experienced in our work trying to characterize a putative pathogenicity related Mitogen Activated Protein Kinase and determine its function and the conditions under which it is active. One of the main drawbacks in gene function studies like this, in the case of *H. annosum* is the lack of efficient DNA transformation system for this fungus. Once such system is established, more direct approach to revealing the precise HaPMK function can be used, for example, making single gene knock-out mutants. Until then, there is still room for improvement of already existing methods, like the ones used in this work. The western blot assay could be further improved, for example testing other antibodies which recognise homologue to the HaPMK proteins, and ideally by producing specific monoclonal antibody against purified HaPMK protein. For over expression of the heterologous HaPMK in yeast, other yeast strains with different genetic background can be tested – some that are more suitable for heterologous expression. Also, for studying the cellular localization of the cloned protein in the yeast system, it is worth to try improving the live staining protocols for several fluorescent dyes, which would provide more definite answers to

the question on where HaPMK is localized and when. The transcriptional assay would provide extremely valuable information on the expression level of HaPMK and MAPKs in general, once the right testing conditions are determined. All in all, figuring out the right conditions in which HaPMK gene expression is triggered, more precisely the exact signal, and the HaPMK protein activated, will be the turning point in further research on the function of this gene and its role in the pathogenicity of *Heterobasidion annosum*. By continuing the research in this field, there is a high probability that *Heterobasidion* could more widely be used as a model pathogen for conifer-pathosystem which can provide valuable information on not only the biology of similar species, but also on possible disease management and prevention.

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