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In Silico Genomics of Fungal Oxidoreductase Genes

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ACADEMIC DISSERTATION

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To my family; for their limitless love and support

and

to the late Dr. Dezsö Péteri; for the chess games and encouraging words

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ABBREVIATIONS

aa	Amino acid(s)
Ca^{2+}	Calcium ion
CAZy	Carbohydrate-Active EnZymes database
CAZymes	Carbohydrate-active enzymes
CFGP	Comparative fungal genomics platform
Cu^{2+}	Copper ion
DAMPs	Danger (damage)-associated molecular patterns
dpi	Days post-inoculation
Duox	Dual oxidase
EF-Tu	Elongation factor TU
ER	Endoplasmic reticulum
ETI	Effector triggered immunity
ETS	Effector triggered susceptibility
FAD	Flavin adenine dinucleotide
Fe^{2+}	Ferrous iron, oxidative state 2+
Fe^{3+}	Iron ion, oxidative state 3+
fPoxDB	Fungal peroxidase database
Fre	Ferric reductase(s)
FRO	Ferric-chelate reductase(s)
FSD	Fungal secretome database
GC	Guanine and Cytosine nucleotide(s)
GFP	Green fluorescence protein
H_2O	Hydrogen dioxide, water
H_2O_2	Hydrogen peroxide
HR	Hypersensitive response
LRR-RLK	Leucine-rich repeats – receptor like kinase
LRR-RLP	Leucine-rich repeats – receptor like protein
MAMPs	Microbe-associated molecular patterns
Mn^{2+}	Manganese ion, oxidative state 2+
MSA	Multiple sequence alignment(s)
NADPH	Nicotinamide adenine dinucleotide phosphate
NB-LRR	Nucleotide-binding domain leucin-rich repeats protein
NGS	Next-generation sequencing
Nox	NADPH oxidase
NoxA, B, C	Fungal Nox isoform A, B, C

NoxD	Stablizing subunit of the fungal Nox complex
NoxR	Regulatory subunit of the fungal Nox complex
PAMPs	Pathogen-associated molecular patterns
PRR	Pattern recognition receptor
PTI	PAMP triggered immunity
Rbohs	Respiratory burst oxidase homologues (Nox homologues)
ROS	Reactive oxygen species
TM	Transmembrane
TMH	Transmembrane helix
TMHs	Transmembrane helices
wpi	Weeks post-inoculation

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. Choi, J., **Détry, N.**, Kim, K.T., Asiegbu, F., Valkonen, J.P. & Lee, Y.H. 2014, "fPoxDB: fungal peroxidase database for comparative genomics", *BMC Microbiology*, vol. 14, p117.

II. **Détry, N.**, Choi, J., Kuo, H.C., Asiegbu, F. & Lee, Y.H. 2014, "*In silico* sequence analysis reveals new characteristics of fungal NADPH oxidase genes", *Mycobiology*, vol. 42, p241-248.

III. Kuo, H.C., **Détry, N.**, Choi, J. & Lee, Y.H. 2015, "Potential roles of laccases on virulence of *Heterobasidion annosum* s.s.**"**, *Microbial Pathogenesis*, vol. 81, p16-21.

Author contribution:

I. The author and CJ developed the pipeline for acquiring peroxidase genes. The author and CJ manually and semi-automated conducted data analysis, interpretation and curation of the retrieved peroxidases, especially the ancestral NADPH oxidases. KKT contributed to the data analysis. The author and CJ wrote the article. LHY conceived the study and contributed to the experimental design. LHY, AF, VJP and KTT contribute to the drafting of the article.

II. The author planned the experiment. The author and CJ conducted data analysis and interpreted the results of ancestral NADPH oxidases. The author wrote the manuscript. LHY conceived the study and contributed to the experimental design. LHY, AF and KHC, contribute to the drafting of the article.

III. The author participated in the planning of the experiments. The author and KHC conducted the data analysis. CJ provided support on *in silico* identification of laccase-encoding genes and phylogenetic analysis. KHC wrote the article. LHY conceived the study and contributed to the experimental design. The author and LHY contributed to the drafting of the article.

ABSTRACT

Oxidoreductases are found in all living organisms and play essential roles in housekeeping, perception of environmental stress, plant-pathogen interactions, defense reactions, and pathogenicity. In particular, laccase, peroxidases and NADPH oxidase, have been implicated in virulence of phytopathogenic fungi in pathogenicity. Despite its relevance towards plant microbe interaction, the identification and comparative analysis of fungal peroxidase-encoding genes at the genomic level have been limited by the lack of a bioinformatics platform as well as paucity of information on transcript profiling of potential candidate oxidoreductase genes.

In this project, one of the primary tasks addressed was the construction and development of a new fungal peroxidase database (fPoxDB). The availability of fPoxDB platform facilitated comparative and evolutionary studies of fungal peroxidases at the genomic level. The database contains 6,113 peroxidase genes of 25 gene families from 331 genomes. The archived genes were subjected to pre-computational analyses using eight different bioinformatic tools: SignalP 3.0, SecretomeP 1.0f, TargetP 1.1b, predictNLS, ChloroP 1.1, TMHMM 2.0c, PSortII, and InterPro Scan. Similarity search tools including HMMER, ClustalW, Blast, and BlastMatrix are provided on the platform. A web interface enables researchers to browse the database via either “species” or “class”. Graphics support the work and enhance user-friendliness. Retrieved data can be exported to other family web-systems including the comparative fungal genomics platform (CFGP).

In parallel to the above study, the second project was focused on *in silico* comparative analysis fungal NADPH oxidase (Nox) genes sequences. Nox proteins are transmembrane enzymes found in most eukaryotic organisms and influence many biological processes by generating reactive oxygen species (ROS). In fungi, Nox enzymes play roles in pathogenicity, such as the weakening of plant cell walls by ROS. The enzymes exhibit high sequence similarities to the ferric-reductases (Fre) and ferric-chelate reductases (FRO) proteins, which are involved in reduction of Fe^{3+} to Fe^{2+} for iron uptake. A total of 34 eukaryotic genomes, covering 28 fungal, one Oomycota, three animal and two plant species, were subjected to bioinformatic analysis. The results indicate that the properties of fungal Nox genes differ from those of the human and plants, providing novel insights that will enable more accurate identification and characterization of the fungal genes.

In the third project, as not much is known is about precise role of oxidoreductase encoding genes in pathogenesis, we explored expression profiling of a typical oxidoreductase gene, laccase, in *Heterobasidion* – conifer pathosystem. Laccases are multi-copper oxidoreductases catalyzing the oxidation of phenolic substrates, and they play diverse roles in plants and fungi. In fungi, laccase have been shown to be involved in pathogenicity, as well as in lignin degradation. To understand the potential roles of laccases of the forest pathogen *Heterobasidion annosum*, a total of 18 laccase genes was identified in this fungus and phylogenetically analyzed. The expression levels of the genes, and laccase activities, during growth of *H. annosum* on its host in the presence

or absence of additional carbon source, such as glucose and sucrose, were investigated. Based on increased transcript expression levels eight laccases were considered to be potentially involved in *H. annosum* virulence.

In summary, we provide the research community with a database dedicated to fungal peroxidases, and our *in silico* analysis affords new insights in the structure of fungal NADPH oxidases. Lastly, we present experimental evidence that some *H. annosum* laccases might be involved in virulence during infection of non-suberized Scots pine seedlings.

1. INTRODUCTION

1.1 Fungi and their lifestyle

Fungi are eukaryotic organisms that can be found in nearly all habitats on Earth, and have an essential impact on the environment as well as on other organisms (Blackwell, 2011). Several fungi play an important ecological role in Earth's carbon cycle. For example, the basidiomycetous *Phanerochaete chrysosporium* and *Postia placenta* are capable of decomposing plant material like lignin or cellulose and making bound carbon available again (Lundell et al., 2010; Wymelenberg et al., 2010). On the other hand, symbiotic, arbuscular and mycorrhizal fungi are known to play important roles in plant nutrition and health. Arbuscular and mycorrhizal fungi like *Gigaspora margarita* (Akiyama et al., 2005) and *Laccaria bicolor* (Martin et al., 2008) colonize solely plant roots and support their host with water, phosphor, nitrogen and other inorganic nutrients, in return for glucose. Moreover, they also enhance overall fitness of plants, provide abiotic stress resistance, and connect co-existing plants with each other and reallocate nutritional resources between them (Singh et al., 2011; van der Heijden et al., 2014). The endophytic fungi, *Epichloë festucae* and *Neotyphodium* species, are known to support ryegrass growth and providing abiotic stresses protection against salinity, drought, and soil acidity (Singh et al., 2011). Fungi have been also a beneficial source as secondary metabolite producers for the development of penicillin (Blackwell, 2011), the cholesterol reducers statins (Barrios-González & Miranda, 2010), and immune-suppressant cyclosporine used for transplantations (Colombo & Ammirati, 2011). However, despite their importance and benefits, several fungi can also be dangerous and devastating pathogens. Fungal pathogens like *Cryptococcus neoformans* (Loftus et al., 2005) and *Candida albicans* (Molero et al., 1998) cause severe infections in immunocompromised human patients. In plants, fungal pathogens are known to cause tremendous economically damage to agriculture and forestry industry. Examples include *Magnaporthe oryzae*, the causative agent of rice blast disease (Kim et al., 2014), *Puccinia triticina*, causing severe damage to wheat globally (Bolton et al., 2008), *H. annosum*, the causal agent of root- and butt-rot (Woodward et al., 1998), *Fusarium graminearum*, which is causing fusarium head blight and infecting a broad range of cereal species (Kazan et al., 2012), and *Fusarium oxysporum* (Di Pietro et al., 2003; Michielse & Rep, 2009) and *Botrytis cinerea*, causing the "grey mold" disease (González-Fernández et al., 2014), which can infect more than 100 different plant species (Dean et al., 2012). Biotrophic phytopathogenic fungi, such as *P. triticina* and *Melampsora larici-populina*, derive nourishment from living host tissue (Vleeshouwers & Oliver, 2014), while necrotrophs like *Sclerotinia sclerotiorum* (Kim et al., 2011), feast on dying host cells. Moreover, fungi are not limited to only one lifestyle and have shown to be able to switch from one to another during the course of infection. Examples are hemibiotrophic fungi, such as *M. oryzae*, *Phytophtera infestans* and *Colletotrichum higginsianum* (Guttman et al., 2014; Mendgen & Hahn, 2002; Münch et al., 2008),

which exhibit a switch from biotrophic to necrotrophic lifestyle (Vleeshouwers & Oliver, 2014). A more extreme lifestyle change is known for *H. annosum*, which is capable of switching from saprotrophic to necrotrophic growth and *vice versa* (Garbelotto & Gonthier, 2013). A further interesting aspect of fungi is their ability to produce a wide and diverse range of metabolites and enzymes including oxidoreducatases particularly laccase, peroxidases and NADPH oxidase, many of which have been implicated in virulence or pathogenicity. Despite their abundance and relevance, the identification and comparative analysis of oxidoreductase genes at the genomic level have been hampered by the limited bioinformatics tools. Furthermore, whether the pattern, copy number and distribution of fungal gene families encoding oxidoreductases are related to their life style remains to be clarified.

1.2 Oxidoreductases

Oxidoreductases (EC 1.-.-.-) constitute the most abundant class of enzymes in nature and occur in all living organisms including fungi. Enzymes belonging to this group catalyze oxido-reductions by transferring electrons or protons from a donor or redox equivalent towards an electron acceptor or redox equivalent; redox equivalents are capable of acting either as donor or acceptor. The oxidoreductase class has 25 subclasses and many sub-subclasses according to data stored in ExplorEnz – The Enzyme Database (http://www.enzyme-database.org/class.php; McDonald *et al.*, 2007; McDonald *et al.*, 2009; NC-IUBMB, 2010). Assignment to subclass depends on donor or acceptor which may be chemical groups, e.g. CH-OH, cofactors, such as NAD^+, FAD^+, or NADP, chemical agents, metal ions, specific proteins, e.g. iron-sulfur proteins, oxygen, hydrogen, or other chemical compounds (McDonald & Tipton, 2013; NC-IUBMB, 2010). In total, the Enzyme Database, http://enzyme.expasy.org (Bairoch, 1999), has over 1,600 relevant entries, reflecting the abundance of the class and highlighting the diverse functions of such enzymes.

Involvement and examples of oxidoreductases in living organisms can be found in a multitude of different reaction steps covering, but are not limited to, anaerobic or aerobic respiration, biosynthesis, degradation of complex polymers, and defense reactions. In anaerobic respiration pathways, such as in the methanogenesis of hydrogenotrophic Archaea the formylmethanofurano dehydrogenase (EC 1.2.99.5), methylenetetrahydromethanopterin reductase (EC 1.5.98.2), and CoB-CoM heterodisulfid reductase (EC 1.8.98.1) play important roles in the reduction of carbon dioxide to methane for energy generation (Bobik & Wolfe, 1988; Buan & Metcalf, 2010; Shima *et al.*, 2000; Shima *et al.*, 2002). Whereas in the denitrification, or dissimilatory nitrate respiration, of nitrate to dinitrogen in *Pseudomonas aeruginosa*, all four enzymes involved belong to the oxidoreductases: nitrate reductase (EC 1.7.99.4), nitrite reductase (EC 1.7.2.1), nitric oxide reductase (EC 1.7.2.5) and nitrous oxide reductase (EC 1.7.2.4) (Arai, 2011; Palmer *et al.*, 2007). In *Desulfovibrio vulgaris*, the last two terminal steps of the dissimilatory

sulfate reduction are catalyzed by adenosine-5'-phosphosulfate reductase (EC 1.8.4.8) and dissimilatory sulfite reductase (EC 1.8.99.3) (Grein *et al.*, 2013; Lee *et al.*, 1973). In the aerobic respiration, the glyceraldehyde 3-phosphate dehydrogenase (EC 1.2.1.12) during glycolysis, as well as the pyruvate dehydrogenase (EC 1.2.4.1), isocitrate dehydrogenase (EC 1.1.1.41 and EC 1.1.1.42), α-ketoglutarate dehydrogenase (EC 1.2.4.2), succinic dehydrogenase (EC 1.3.5.1), and malate dehydrogenase (EC 1.1.1.37) in the citric acid / Krebs cycle, are involved in the energy generation by usage of glucose (Reece *et al.*, 2011). Additionally, the ferredoxin-NADPH oxidase (EC 1.18.1.2) in plants and cyanobacteria is involved in the photosystem in order to generate a proton gradient for energy generation (Berg *et al.*, 2002). Regarding biosynthesis, oxidoreductases are involved in the Calvin cycle of plants, NADPH:glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.13) (Berg *et al.*, 2002; Wedel & Soll, 1998), the fatty acid synthesis, 3-ketoacyl-ACP reductase (EC 1.1.1.100) and Enoyl-ACP reductase (EC 1.3.1.9) (Berg *et al.*, 2002; Wakil *et al.*, 1983), and in the cholesterol synthesis of animals (Reece *et al.*, 2011). In the latter, more than seven oxidoreductase, such as hydroxymethylglutaryl-CoA reductase (EC 1.1.1.34), lanosterol 14α-methyl demethylase (EC 1.14.13.70), and sterol methyl oxidase (EC 1.14.13.72), are essential for the synthesis of cholesterol from acetyl-CoA (Berg *et al.*, 2002; Risley, 2002). Classic examples regarding oxidoreductases that are involved in degradation of complex polymers, are peroxidases (EC 1.11.-.-), such as lignin peroxidase (EC 1.11.1.4), manganese peroxidase (EC 1.11.1.3), and versatile peroxidase (EC 1.11.1.6) from Basidiomycota species, which are capable of breaking down lignin (Camarero *et al.*, 1999; Floudas *et al.*, 2012; Tien & Kirk, 1983).

NADPH oxidases (EC 1.6.3.1) are widely found in eukaryotes where they play essential roles in defense against pathogens by catalyzing reactive oxygen species (ROS) formation (Bedard & Krause, 2007). Moreover, due to the fact that the function and application of ROS varies greatly in nature, depending on both the organism and the respective cellular localization of it, NADPH oxidases are also indirectly involved in other important and physiological roles as a source of ROS generation. For example, ROS may be involved in inflammation (Bedard & Krause, 2007), but also in signaling (Kovtun *et al.*, 2000), cell development (Foreman *et al.*, 2003), host defense and hypersensitive response (HR) (Torres *et al.*, 2002), and programmed cell death (Breusegem & Dat, 2006).

Over the past 50 years, oxidoreductases have received a great deal of attention in the context of biotechnology. Some of these enzymes have been shown to possess potential industrial application. For example, Tzanov *et al.* (2003) showed that an enzymatic pre-treatment of cotton with laccases (EC 1.10.3.2) can enhance whiteness in a conventional bleaching process, and potentially reduce the dosage need of hydrogen peroxide for textile bleaching. While a laccase from *Trametes hirsuta* showed the ability of detoxifying certain dyes, and exhibit a higher thermal stability when immobilized on alumina (Abadulla *et al.*, 2000). Moreover, some oxidoreductases

have also been potentially implicated for food and beverages applications. Andersson *et al.* (2002) tested different industrial lamination processes to bind glucose oxidase (EC 1.1.3.4) on tetra pack packages in order to scavenge oxygen to improve food shell life. Whereas Gokoglan *et al.* (2015) constructed an amperometric biosensor using pyranose oxidase (EC 1.1.3.10) to detect glucose content in beverages. Interestingly, recently Dutta *et al.* (2015) reported the use of an FAD dependent glycerol-3-phosphate dehydrogenase (EC 1.1.5.3) for the possible development of an electrochemical immunosensor. This enzyme-labeled immunosensor would be suitable for medical purposes in detecting heart related diseases (Dutta *et al.*, 2015).

The vast involvement of oxidoreductase in biological functions certainly makes them a very fascinating object for study. Regarding fungi, laccases are a very interesting and important oxidoreductase sub-subclass for study due to the fact that they are involved in a variety of factors that can contribute to fungal pathogenicity and virulence.

1.3 Laccases

Laccases (EC 1.10.3.2, benezenediol: oxygen oxidoreductases), first described from the lacquer tree *Rhus vernicifera* (Yoshida, 1883), are multi-copper oxidases that catabolize a variety of aromatic ring structures, e.g. *p*-diphenols, but not tyrosine, via reduction of molecular oxygen to water (Giardina *et al.*, 2010; Hildén *et al.*, 2009; Thurston 1994). The general structure of laccases is rather diverse, but the structure of the active site seems to be well-conserved in fungal laccases (Mayer & Staples, 2002). Laccases usually have three copper ions (T1, T2, and T3) co-ordinated with histidine residues (Giardina *et al.*, 2010, Solomon *et al.*, 1996). The T1 copper is also termed the “blue copper”, imparting the characteristic blue color. The lack of the T1 copper is a feature of the so-called “yellow” or “white” laccase (Baldrian, 2006). The absence of T1 copper in some laccases has caused some authors to question if such laccases can in fact be termed “true” laccases, although they can oxidize phenols. The term “laccases with unusual spectral properties” has been suggested as more appropriate (Giardina et al., 2010).

Laccases are commonly found in higher plants, fungi, insects, and microorganisms (Giardina *et al.*, 2010; Mayer & Staples, 2002; Ranocha *et al.*, 2002). Plant laccases have been suggested to be involved in lignin polymerization, but experimental proof was missing (Boudet 2000; Ranocha *et al.*, 2002). Recently, experimental studies in *Arabidopsis thaliana* and *Populus trichocarpa* provided evidence that laccases are in fact involved in lignification (Berthet *et al.*, 2011; Lu *et al.*, 2013; Zhao *et al.*, 2013).

Fungal laccases have been more intensively studied, and exhibit various physiological functions, including lignin degradation (Arora & Sharma, 2010; Thurston, 1994), an involvement in virulence (Lin *et al.*, 2012), pathogenesis (Zhu *et al.*, 2001; Zhu and Williamson, 2004), conidial pigmentation (Clutterbuck, 1972), and morphology (Chen *et al.*, 2004; Giardina *et al.*, 2010). In

C. neoformans, a pathogen that infects immunocompromised patients, two paralogue laccases are expressed, *CNLAC1* and *CNLAC2*. The former is bound to the cell wall and produces melanin (Zhu *et al.*, 2001), which protects the pathogen against antibiotics, ROS, and phagocytosis (Williamson *et al.*, 1998; Zhu and Williamson, 2004). The latter has been shown to be cytoplasmic, as well as partially cell wall localized, and also being involved in melanin synthesis (Missall *et al.*, 2005). Loss of *lac3* of *Cryphonectria parasitica*, the causal agent of Chestnut blight, reduced virulence, but no morphological change was observed (Chung *et al.*, 2008).

Laccases have also become the focus of industrial and biotechnological attention. The enzymes exhibit low substrate specificity, a requirement for molecular oxygen as electron acceptor, a pH optimum between 4–6, extracellular stability, and biodegradability (Baldrian 2006; Couto and Herrera 2006; Hildén *et al.*, 2009). Currently, several commercial products containing laccases are available. Examples include DeniLite® and Suberase® from Novozymes, Denmark. DeniLite® is used in denim fading (Novozymes, 2013), while Suberase® is used in pre-treatment of cork stopper production for wine bottles (United States Patent and Trademark Office, US serial number 75816334, https://tsdr.uspto.gov/; Xu, 2005). Moreover, potential applications of laccases include degradation of pollutants and dyes (Abadulla *et al.*, 2000; Zeng *et al.*, 2011), soil and water bioremediation by removing polycyclic aromatic hydrocarbons (Chakroun *et al.*, 2010; Pozdnyakova *et al.*, 2006), medical diagnostics by differentiation between morphine and codeine (Bauer *et al.*, 1999), and development of anti-cancer drugs (Wellington & Kolesnikova, 2012; Wellington *et al.*, 2013). A more detailed survey of possible applications, and environmental aspects, has been explained in Mayer & Staples (2002), Baldrian (2006), Xu (2005), Couto & Herrera (2006) and Fernández-Fernández *et al.* (2013).

1.4 Peroxidases

Peroxidases (EC 1.11.-.-), with around 28 sub-subclasses, use peroxide as acceptor in their enzymatic reactions according to data stored in ExplorEnz – The Enzyme Database (http://www.enzyme-database.org/class.php; McDonald *et al.*, 2007; McDonald *et al.*, 2009; NC-IUBMB, 2010). Some of the best characterized enzymes in this subclass include NADH (EC 1.11.1.1) and NADPH peroxidase (EC 1.11.1.2), cytochrome-c peroxidase (EC 1.11.1.5), catalase (EC 1.11.1.6), peroxidase (EC 1.11.1.7), manganese peroxidase (EC 1.11.1.13), lignin peroxidase (EC 1.11.1.14) and versatile peroxidase (EC 1.11.1.16).

Peroxidases are divided into the mammalian (now assigned: peroxidase-cyclooxygenases) (Zamocky *et al.*, 2008) and plant superfamilies (nonanimal peroxidases) (Hofrichter *et al.*, 2010; Lundell *et al.*, 2010). The latter is further divided into three classes: the intracellular peroxidases (Class I), the extracellular fungal peroxidases (Class II), and the extracellular plant peroxidases (Class III) (Banci, 1997; Conesa *et al.*, 2002). Most peroxidases contain protoporphyrin IX, heme,

with an iron ion in an oxidation state of +3 at rest (Banci, 1997; Conesa *et al.*, 2002; Hofrichter *et al.*, 2010). Class II peroxidases possess characteristic post-translational modifications, including covalently attached glycans, disulfide bonds, and ionically bound calcium (Hofrichter *et al.*, 2010). The glycans increase enzyme solubility and stability; the latter two modifications are essential to ensure structural integrity (reviewed in Conesa *et al.*, 2002).

The catalytic cycle of a heme peroxidases features several oxidation steps (Figure 1), starting when a hydrogen peroxide molecule (H_2O_2) reacts with the heme of the enzyme's active site. This reaction leads to the release of water and oxidation of the iron ion (Fe^{3+}) to oxyferryl (Fe^{4+}=O). Additionally, a porphyrin cationic radical is formed (N•+). This state is termed Compound I. Next, a substrate molecule (S) is oxidized to the radical form ($S^{\bullet}$) with reduction of the cationic radical of Compound I to form Compound II. Compound II subsequently oxidizes another substrate molecule, thus creating another radical substrate, and releases water upon reduction of the ferric ion in the active center to the resting state (Figure 1) (Banci, 1997; Dolphin *et al.*, 1971; Poulus, 2014).

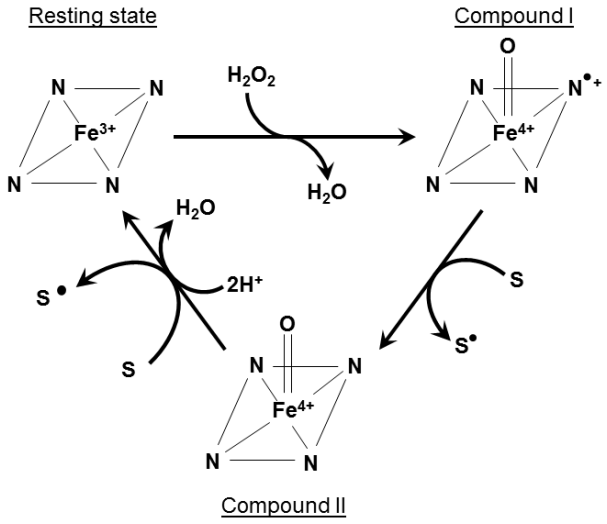


Figure 1: Catalytic cycle of a heme peroxidase

The catalytic cycle of a heme peroxidase starts when a hydrogen peroxide molecule (H_2O_2) is reacting with the heme of the enzyme's active site. Here, hydrogen peroxide (H_2O_2) is cleaved heterolytically, leading to the release of water (H_2O), the oxidation of the ferric ion (Fe^{3+}) to oxyferryl (Fe^{4+}=O), as well as the removal of an electron from the ferric ion. Furthermore, a second electron is removed from the porphyrin and a porphyrin cationic radical ($N^{\bullet}$+) is formed. This state is termed Compound I. In the next reaction step, the porphyrin cationic radical ($N^{\bullet}$+) oxidizes a substrate molecule (S) by removing an electron, generating a substrate radical ($S^{\bullet}$) and reducing the porphyrin cationic radical ($N^{\bullet}$+). This state is termed Compound II. In the last step, the oxyferryl (Fe^{4+}=O) oxidizes another substrate molecule (S), and generating another substrate radical ($S^{\bullet}$). During this reaction, two hydrogen atoms are used to release the oxygen from the reduced ferric ion in the form of water (H_2O). This state is termed resting state (Banci, 1997; Dolphin *et al.*, 1971; Poulus, 2014).

Since the first description of the horseradish peroxidases (EC 1.11.1.7) from *Armoracia rusticana*, over 100 years ago, the chemistry and structure of peroxidases have been extensively investigated (Poulus, 2014). For the last couple of decades, focus has been on potential industrial,

environmental (Peralta-Zamora *et al.*, 1998), and medical therapeutic applications (Greco *et al.*, 2000; Tupper *et al.*, 2004), as well as on drug synthesis, biosensor development (Tang *et al.*, 2003; Tang *et al.*, 2008; Zhao *et al.*, 2011), electron microscopy imaging (Martell *et al.*, 2012), biodetoxification (Inoue *et al.*, 2015; Wang *et al.*, 2011), and biodegradation of aromatic pollutants (Rodríguez *et al.*, 2004).

Potentially useful enzymes include lignin peroxidase, manganese peroxidase, and versatile peroxidase; the latter enzyme can catalyze both reactions, whereas the oxidation of Mn^{2+} is optimal at pH 5 compared to aromatic compounds at pH 3 (Heinfling *et al.*, 1998; Husain, 2010; Ruiz-Dueñas *et al.*, 2001). Lignin peroxidase, manganese peroxidases, and versatile peroxidase from various fungal white rot species in the phyla Basidiomycota have been under intensive investigation and show to be able to breakdown several recalcitrant aromatic ring structures, e.g. those of dyes, xenobiotics, pesticides, and other environmental contaminants (Acevedo *et al.*, 2010; Baratto *et al.*, 2015; Inoue *et al.*, 2015; Wang *et al.*, 2011).

Although, the potential benefits for industrial applications of these peroxidases have been shown, currently, none are used in commercial-scale applications. At present, a limiting factor is the cost-efficiency. The cost-efficiency is poor because enzyme yields remain low, and enzyme stability and function depend on environmental conditions, for example, the end-products of the enzymatic reaction may inactivate the enzyme (Husain, 2010; Moilanen *et al.*, 2015). Despite such challenges, it is clear that research will continue to focus on improving the cost-efficiency. Moreover, the need of saving energy, and reducing and sanitizing wastewater, will be a strong motivator for the potential usage of peroxidases in the future, as industrial companies will need to stay economically viable as well as environmentally viable. An excellent example is the wood and paper industry, which still uses large amounts of hazardous chemicals at high temperatures for pulp delignification (Xu, 2005). Here, potential future application of lignin peroxidase, manganese peroxidase, and/or versatile peroxidase would allow the industry to create more value under milder reaction conditions, both saving energy and reducing the amounts of chemicals needed.

Due to the importance, usefulness and potential benefits that fungal peroxidases hold, it is very important to develop and provide researcher with a bioinformatic platform specialized on fungal peroxidase in order to support the potentially identification of new candidate peroxidases for study and analyses.

1.5 NADPH oxidases

NADPH oxidases (Nox) (EC 1.6.3.1) are transmembrane proteins found in most eukaryotic species, and generate ROS by transporting electrons across the membrane to oxygen. The current accepted structure for Nox isoforms has been deduced from biochemical studies based on human Nox2 ($gp91^{phox}$), and it is assumed to be similar in other species (Bedard & Krause, 2007). The

enzymes contain highly conserved domains, including NADPH- and FAD-binding domains, six α-helical transmembrane (TM) domains, and four iron-binding histidines in TM helical (TMH) domains. TMH domains 3 and 5 each contain two histidine residues which are 13 and 12 residues apart from each other, respectively (Finegold *et al.*, 1996; Sumimoto, 2008). Glycosylation has been shown to occur in the human Nox2 and Nox4 isoform, and Duox 1 and 2 (Figure 2) (Bedard & Krause, 2007).

Seven different Nox isoforms (Nox1-5, Duox1 and Duox2) have been found in human. Nox1-4 require the subunits $p22^{phox}$, $p40^{phox}$, $p47^{phox}$, $p67^{phox}$ (also called "activator subunit"), as well as the GTPase Rac for functionality (Bedard & Krause, 2007; Siegmund *et al.*, 2013; Torres & Dangle, 2005). Nox5, Duox1 and Duox2 possess an EF-Hand and have been shown to be activated solely by Ca^{2+} stimulation (Bánfi *et al.*, 2004; Ameziane-El-Hassani *et al.*, 2005; Sumimoto *et al.*, 2011). Nox homologues in plants, called Respiratory burst oxidase homologues (Rbohs), possess in general an EF-Hand domain that is regulated by binding Ca^{2+} (Bedard *et al.*, 2007; Sagi & Fluhr, 2001).

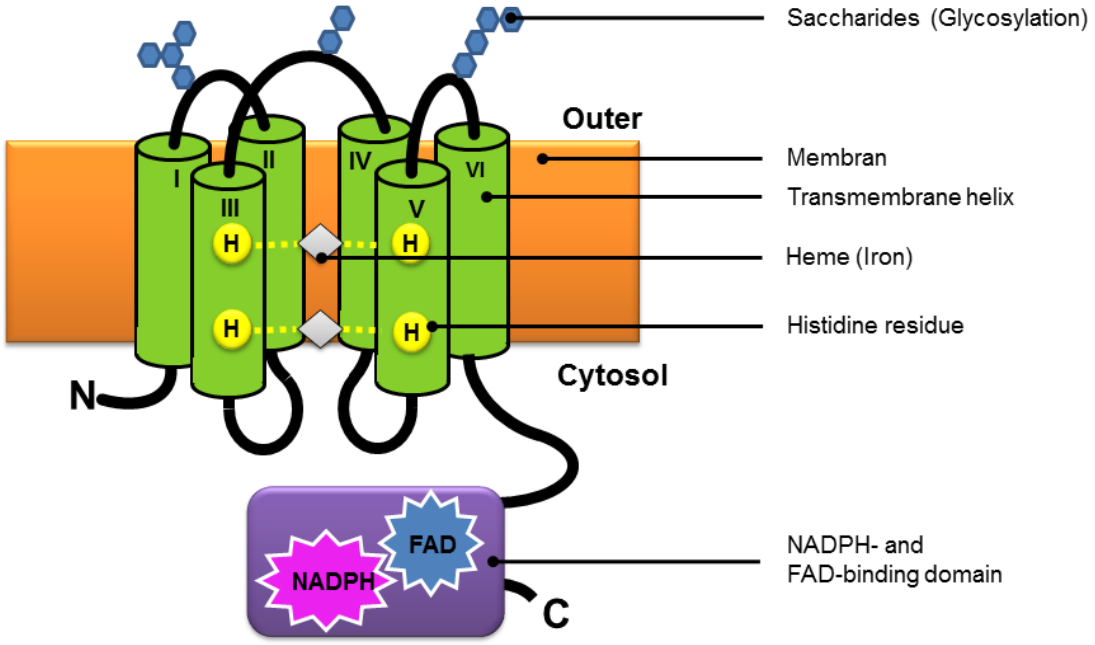


Figure 2: Currently accepted structure of the catalytic subunit of the NADPH oxidase complex for human Nox1-5 and fungal Nox isoforms NoxA, B and C.

The structure of the catalytic subunit encompasses the following conserved domains: a NADPH- and FAD-binding domain which are located at the C-terminal region, six ∝-TMHs, and four iron-binding histidines. Two histidines are located in TMH 3 and TMH 5 respectively. The distance between each histidine pair is supposed to be highly conserved and spanning 13 aa in TMH 3 and 12 aa in TMH 5, and assumed to be essential for correct iron localization. Glycosylation has been shown to occur in the human Nox2 and Nox4. In addition, the human Nox5 and fungal NoxC isoform possess a Ca^{2+}-binding EF-hand domain, and located at the N-terminal region in human. The EF-hand is not depicted in this figure (Bedard & Krause, 2007; Sumimoto, 2008).

In fungi, three Nox isoforms are found: NoxA, B, and C. NoxC is structurally and phylogenetically closely related to animal Nox5/Duox, while NoxA and NoxB are closer to human Nox2 (Lacaze *et al.*, 2015). NoxA and B require the regulatory subunit NoxR, Cdc24, the polarisome protein Bem1, and the RhoGTPase RacA for activation (Lacaze *et al.*, 2015; Takemoto *et al.*, 2011). NoxR is a homolog of the human Nox regulatory unit $p67^{phox}$, and required for Nox functionality as shown for *Neurospora crassa* (Cano-Domínguez *et al.*, 2008). Furthermore, experiments showed that NoxR physically interacts with Bem1 and the GEF protein Cdc24. Thus, it was proposed that Bem1 acts as "organizer" like the mammalian "organizer subunit" $p47^{phox}$, and substitutes $p40^{phox}$ and $p47^{phox}$ in fungi (Bedard & Krause, 2007; Lacaze *et al.*, 2015; Takemoto *et al.*, 2011). Recently, the homologue of the mammalian stabilizing subunit $p22^{phox}$ was found in fungi and named NoxD (Lacaze *et al.*, 2015; Siegmund *et al.*, 2015). Interestingly, NoxD physically interacts only with NoxA, but not with NoxB, as shown in a study with *B. cinerea* (Siegmund *et al.*, 2015). In addition, the tetraspanin Pls1 was proposed to associate with NoxB and having a $p22^{phox}$-like role. Although clear evidence for physical interaction is still missing, mutation studies showed similar phenotypes for Pls1 and NoxB in *B. cinerea* and *Podosporina anserina* (Lacaze *et al.*, 2015; Lambou *et al.*, 2008; Siegmund *et al.*, 2013). Contrary to NoxA and B, NoxC possess an additional EF-hand domain that is assumed to be regulatory in nature. Due to the fact that NoxC possess an EF-Hand domain, it most likely does not require any organizer or activator subunit compared to NoxA and NoxB.

Nox are involved in a multitude of essential biological processes due to its ROS generating ability. In fungi, Nox are known to be involved in cell development and differentiation (Dirschnabel *et al.*, 2014; Lara-Ortíz *et al.*, 2003), sexual reproduction (Cano-Domínguez *et al.*, 2008; Malagnac *et al.*, 2004), hyphae fusion (Dirschnabel *et al.*, 2014) and pathogenicity (Egan *et al.*, 2007; Kim *et al.*, 2011). For example, *Aspergillus nidulans* NoxA mutants were unable to develop cleistothecia as well as ascospores (Lara-Ortíz *et al.*, 2003). Similar results were obtained by Dirschnabel *et al.* (2014). Here, Nox1 and NoxR mutants of *Sordaria macrospora* were not only incapable of developing fruiting bodies, but also had a defect in hyphae fusion, while ascospores of Nox2 mutants lost the ability to germinate (Dirschnabel *et al.*, 2014). Also, Cano-Domínguez *et al.* (2008) showed that the deletion of NOX-1 in *N. crassa* lead to a decrease in asexual development and female sterility, next to a reduction of hyphal growth, while NOX-2 deletion resulted in spores that were unable to germinate. In *P. anserina*, Nox are important for the differentiation of fruiting bodies as well as sexual reproduction (Malagnac *et al.*, 2004). Moreover, Nox1 and Nox2 of *M. oryzae* play roles in appressorium formation and growth of aerial hyphae, and thus are essential for pathogenicity (Egan *et al.*, 2007). Ryder *et al.* (2013) further showed that Nox in *M. oryzae* are also involved in the F-actin cytoskeletal remodeling of the appressoria pore, and polarized growth of the hyphae during infection. In *S. sclerotiorum*, SsNox1 is involved in oxalate production which correlates with virulence, as oxalate has been shown to be able to

suppress the plants oxidative outburst (Cessna *et al.*, 2000; Kim *et al.*, 2011). Interestingly, a *noxA* mutant of *E. festucae* lost the ability to maintain a mutualistic symbiotic interaction with its host, suggesting that Nox are also involved in symbiosis (Scott *et al.*, 2007; Tanaka *et al.*, 2006).

In human and plants, Nox have been associated with inflammation and apoptosis (Vlahos *et al.*, 2011), HR and programmed cell death (Torres *et al.*, 2002), and cell development (Foreman *et al.*, 2003).

A very interesting study by Dickinson *et al.* (2011) showed that Nox2-mediated H_2O_2 generation within the brain is essential for signaling maintenance and proliferation of neural stem cells in mice; expanding the involvement of Nox in biological processes. Furthermore, although the ROS generation is often associated with stress related functions for an organism, here it demonstrated the essential need for neurogenesis.

Although much knowledge about the involvement and effects of Nox on host and pathogens have been accumulated over the last decades, the identification of Nox genes and isoforms via bioinformatic approaches has been rather difficult. This is owed to the fact that ferric reductases (Fre) and ferric-chelate reductases (FRO) share high sequence similarities and homologous domains with Nox genes. In *Saccharomyces cerevisiae*, Fre are important for iron reduction and uptake (Dancis *et al.*, 1990), while FRO in plants reduce ferric chelates in the rhizosphere, making ferrous iron (Fe^{2+}) available for plants (Robinson *et al.*, 1999; Jeong & Connolly, 2009).

1.6 Impact of genomics and bioinformatic platforms

In 1996, the first eukaryotic genome *S. cerevisiae* was fully sequenced (Goffeau *et al.*, 1996). Since then, several fungal genome sequencing initiatives have been launched including the "1,000 fungal genomes" project (http://genome.jgi-psf.org/programs/fungi/1000fungalgenomes.jsf). Such efforts have continuously increased the numbers of fungal genomic sequences, facilitated by the constant development of ever-faster sequencing methods, including next-generation sequencing (NGS) technology (Schmidt & Panstruga, 2011). Structural genomics, the study of the genome structures, such as heterochromatization, alternative splicing, single-nucleotide polymorphism, has been essential not only to assemble genomes from accumulated NGS data but also for automated annotation of genes, introns, exons, transposons, and assignments of gene loci. Functional genomics, the study of function and expression of a gene or multiple genes on a genome-wide level, has played an important role in linking structural genomics data to gene expression and/or phenotypic data (Kapushesky *et al.*, 2011), allowing the conduct of comparative studies exploring not only evolutionary questions (Hagen, 2000; The Tomato Genome Consortium, 2012) but also genome plasticity and dynamics (Gardiner *et al.*, 2012; Ma *et al.*, 2010; Manning *et al.*, 2013).

As more genomic data become available, bioinformatic platforms containing these genomes, and also bioinformatic tools permitting *in silico* sequence studies, are required. Manual analyses and comparisons of sequences with each other can be less convenient as well as leading to a deceleration of research progress. Databases such as the comparative fungal genomics platform (CFGP) (Park *et al.*, 2008; Choi *et al.*, 2013), the "comparative platform for small secreted proteins" (OrysPSSP) (Pan *et al.*, 2013), the "comparative platform for green plant genomics" (Phytozome) (Goodstein *et al.*, 2012), the "Carbohydrate-Active EnZymes database" (CAZy) (Cantarel *et al.*, 2009; Lombard *et al.*, 2014) and the fungal secretome database (FSD) (Choi *et al.*, 2010) support the research progress by facilitating research analyses and comparisons.

Genomics is associated with certain challenges, including the constant need for development of new algorithms and specific programs/tools to tackle various problems. Also, the project scope may be specialized or computational power and/or memory inadequate. Additionally, as not all researchers are familiar with computer science, databases require graphic user interfaces rather than command lines (Hu *et al.*, 2011). Good visualization of raw data can provide a better understanding of results for researchers, and potentially lead to new insights and discoveries (Saraiya *et al.*, 2005). Another basic challenge in the creation of databases featuring specific gene families is the pipeline set-up, since the pipeline must first be manually created. The general approach is to retrieve all available annotated and, if possible, curated sequences of the relevant protein family. Subsequently, multiple sequence alignment is performed, followed by manual trimming and curating of the alignment, which is then used to create a "hmmer" profile (Eddy, 1998). Such profiles are convenient and are amenable to later modification. The created profile can be applied to all genomes in a database. Another approach for a pipeline set-up is by searching for domains using InterPro Scan in fungal genomes. Subsequently, retrieved sequences are manually curated to eliminate false-positives or errors and the remaining genes are stored in the database.

1.7 Role of comparative genomics in understanding fungal lifestyles

Comparative genomics aims "to identify all differences among genomes, and then elucidate which sequence differences are responsible for phenotypic shifts in organisms" (Hu *et al.*, 2011). Comparative pathogenomics focuses on the mechanism of pathogenicity by identifying pathogenicity/virulence factors among pathogens.

With the introduction of NGS, providing faster and cheaper high-throughput sequencing of whole-genomes, comparative genomics and pathogenomics have been under constant development and growth. Although, the vast accumulation of sequence data has created new challenges, such as the need for development of novel algorithms to handle this data, it has also offered the

opportunity to explore and compare genomes of non-pathogens and pathogens on a wider level (Hu *et al.*, 2011).

Over the past years, comparative pathogenomics studies revealed that the genomes of pathogens are very dynamic and that life-style associated genes can only be found in the respective fungal pathogen genomes. In fungi, genes involved in pathogenicity are often clustered in supernumerary chromosomes (Harimoto *et al.*, 2007), telomeric regions (Chen *et al.*, 2007; Fedorova *et al.*, 2008), and transposable elements (Ma *et al.*, 2010), allowing gene transfer between organisms of the same species as well as different species. Such transfer even seems to be able to occur between species of different kingdoms (Fitzpatrick *et al.*, 2008; Gardiner *et al.*, 2012; Schmidt & Panstruga, 2011; Schmitt & Lumbsch, 2009). This possible rapid exchange of genes or even chromosomes between species within a biotope is certainly of a great concern for the agriculture and medical field, as it might lead to the development/rise of new resistant fungal pathogens or multi-antibiotic resistant bacterial strains. Although this might be a worrying scenario, comparative pathogenomics offers the opportunity to develop appropriate counter-measures (Hu *et al.*, 2011). For example, comparative genomics and pathogenomics have been used in identifying potential new vaccine targets (Seib *et al.*, 2012).

Traditionally, studies on pathogenicity have focused on a defined number of genes. Today, genomics and bioinformatics, combined with high-throughput phenotypic screening, allow identification and analysis of pathogenicity genes on a much broader scale (Jeon *et al.*, 2007; Talbot, 2007). Novel genes and gene functions have been described, for example in *Magnaporthe oryzae*, after development of a functional genomics and informatics platform (Jeon *et al.*, 2007). The availability of such platforms, equipped with both genome sequences and bioinformatic tools, has steadily grown over the years and is very likely to continue.

Another aspect that underlines the vast application possibilities, as well as the constant development of pathogenomics, is the capability to investigate gene expression of species that have two different lifestyles (Olson *et al.*, 2012). This also includes species that have a lifestyle switch during the course of infection. O'Connell *et al.* (2012) have showed that gene expression patterns changed during host-pathogen interactions and lifestyle switches. In *C. higginsianum*, genes for secondary metabolites were highly upregulated in the early stage of infection, followed by upregulation of carbohydrate-active enzymes, such as cutinase and pectinase, during appressorium formation. The upregulation of secondary metabolite genes continued through-out the biotrophic stage, whereas gene expression of lytic enzymes and plasma membrane transporters were expressed during the transition to necrotrophy. Furthermore, a comparison of *in vitro* and *in planta* appressorial gene expression revealed substantial differences. These differences lead to the conclusion that appressoria are also involved in sensory function, in order to prepare the pathogen for the imminent host invasion (O'Connell *et al.*, 2012).

Given the results of O'Connell *et al.* (2012), future analysis of other pathogenic as well as fungal species with saprotrophic, symbiotic, or necrotrophic lifestyles will certainly hold the key to new and exciting discoveries.

1.8 "Hide and seek" – molecular elements involved in plant-pathogen interactions

Every living organism must be constantly prepared to defend itself against pathogens in order to survive. Survival relies on an effective immune system, capable of identifying pathogen and warding them off. Here, plants and animals have developed different immune systems to detect and protect against such attacks. Animals have a somatic adaptive immune system featuring rapid movement of defender cells to points of attack (Abbas *et al.*, 2012), and plants have evolved an innate immune system with a wide recognition repertoire. The plant immune system can detect pathogens at two different levels (Dodds & Rathjen, 2010), as comprehensively illustrated in the "zigzag model" (Figure 3) of Jones & Dangl (2006).

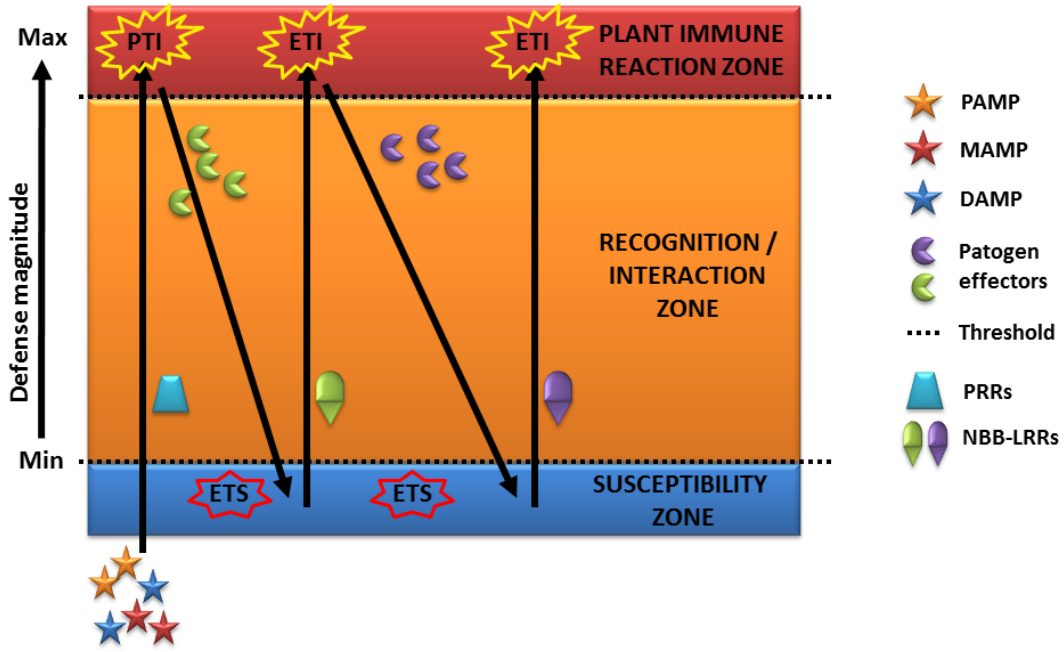


Figure 3: Zigzag model depicting the possible recognition and interaction steps during a pathogen infection in its respective host plant.

In the first step, PAMPs, MAMPs and/or DAMPs are recognized by plant PRRs and triggered PTI, causing a plant immune reaction. In the second step, pathogen effectors that have specifically evolved in order to avoid PTI lead to ETS, and potential causing disease. In the third step, effector recognition by NB-LRRs leads to triggering ETI and inducing a plant immune reaction. In the fourth step, pathogens have evolved pathogen effectors which inhibit NB-LRRs to avoid recognition which results in an ETS. In the fifth step, plants have developed NB-LRRs which are capable of detecting the new developed pathogen effectors. Thus, ETI is triggers and the plant potentially survives and wards off the pathogen infection. Abbreviations: PAMP, pathogen-associated molecular pattern; MAMP, microbe-associated molecular pattern; DAMP, danger-associated molecular pattern; PRRs, pattern recognition receptors; NB-LRRs, nucleotide-binding domain leucine-rich-repeat proteins. Modified from Jones & Dangle (2006).

The first level of detection involves pattern recognition receptors (PRRs) located on the cell surface that recognize pathogen-associated molecular patterns (PAMPs), microbe-associated molecular patterns (MAMPs), and danger (damage)-associated molecular patterns (DAMPs) (Boller & He, 2009; Zipfel 2008). The PRRs are transmembrane receptors, and can be classified into LRR-RLKs (leucine-rich repeats–receptor like kinases) and LRR-RLPs (leucine-rich repeats–receptor-like proteins) (Segonzac & Zipfel, 2011). PAMPs are defined as: "conserved, usually structural, molecules common to pathogenic organisms" (Dodds & Rathjen, 2010). The well-studied examples of PAMPs include the flagellin (Felix *et al.*, 1999; Gómez-Gómez & Boller, 2000; Zipfel *et al.*, 2004) and chitin of fungi (Kaku *et al.*, 2006; Miya *et al.*, 2007), elongation factor TU (EF-Tu) of bacteria (Zipfel *et al.*, 2006), and various cell wall glucosides (Nürnberger & Brunner, 2002; Zipfel 2008). Since molecular patterns like flagellin or EF-Tu are not limited to only pathogenic microbes, the term MAMPs is used to extend the definition of PAMPs, thus including also non-pathogenic microbes (Zipfel, 2008). DAMPs include all molecules associated with danger or damage to the plant, and include cell wall and cuticular fragments (Dodds & Rathjen, 2010). As PAMPs, MAMPs, and DAMPs can induce immune responses, these materials are often grouped under the umbrella term "elicitors".

When an elicitor is recognized by the corresponding PRR on the outer surface of a plant cell, PAMP-triggered immunity (PTI) develops to halt colonization (Jones & Dangl, 2006). To avoid recognition by the plant, pathogens have developed effectors, termed virulence contributors (Jones & Dangl, 2006). These effectors support infection by either evasion of recognition or disrupt downstream signaling to render PTI ineffective (Zipfel, 2008), such as the AvrPto effector in *Pseudomonas syringae* (Xiang *et al.*, 2008). Another example is HopAI1 of *P. syringae*, capable of inhibiting two mitogen-activated protein kinases in *A. thaliana*, leading to the suppression of flagellin flg22 induced PTI (Zhang *et al.*, 2007). In such instances, effector-triggered susceptibility (ETS) is induced and the pathogen may cause disease in the compromised host (Jones & Dangl, 2006; Dodds & Rathjen, 2010).

The second level of detection is effector recognition by intracellular nucleotide-binding domain leucine-rich-repeat proteins (NB-LRRs) (Eitas & Dangle, 2010). If an effector is recognized, effector-triggered immunity (ETI) is induced, stimulating HR, characterized by programmed cell death and development of disease resistance (Jones & Dangl, 2006; Boller & Felix, 2009). If a pathogen evolves new effectors that can inhibit one or more of the NB-LRRs involved in recognition, ETS is induced once more (Jones & Dangl, 2006; Thomma *et al.*, 2011). To counter such new effectors, plants must develop new NB-LRRs in order to survive.

Thus, plants and pathogens are engaged in a continuous arms race (Boller & Felix, 2009; Dodds & Rathjen, 2010) featuring both detection and avoidance. The host response to infection (either a PTI or an ETI) is always accompanied by an oxidative burst. ROS generation imposes

oxidative stress on an invading pathogen. Biotrophic and hemibiotrophic fungi have evolved sophisticated scavenging systems to negate the effects of host-generated ROS, while necrotrophic fungi do not necessarily require such a system (Heller & Tudzynski, 2011). Peroxidases and catalases are oxidoreductases that can detoxify ROS and are known to play roles in fungal pathogenicity (Heller & Tudzynski, 2011).

Although the "zigzag" model seeks to outline the features of the evolutionary and molecular arms race between pathogens and plants, the protein partners involved, and their molecular interactions, remain poorly defined. However, over the past decade, many fascinating details have emerged. For example, for *A. thaliana* (Sinapidou *et al.*, 2004), *Oryzae sativa* (Ashikawa *et al.*, 2008), and *Tricitum* spp. cultivars (Loutre *et al.*, 2009), it has been shown that not one NB-LRR, but a NB-LRR pair is required in order to confer full resistance towards a corresponding pathogen effector (Eitas & Dangle, 2010). While in tomato it was shown that the NB-LRR protein Prf forms a complex with the protein kinase Pto in order to recognize the effectors AvrPto and AvrPtoB from *P. syringae* pv. *tomato*. Moreover, the Prf-Pto complex was capable of forming a multimeric complex, which also included the integration of other Pto-like kinases, and thus expanding the recognition of other *P. syringae* effectors (Gutierrez *et al.*, 2010).

1.9 Conifer pathogen *Heterobasidion annosum*

Heterobasidion annosum sensu lato (s.l.) is the causal agent of root and butt root diseases of conifers in the Northern Hemisphere, and is considered to be the most destructive fungus in economic terms (Asiegbu *et al.*, 2005; Woodward *et al.*, 1998). The annual loss caused by degradation of timber-quality wood and infection of healthy trees, rendering such trees unusable in the wood and paper industry, is estimated to be around 35 million Euro (€) in Finland alone and over 790 million € in the European Union overall (Asiegbu *et al.*, 2005; Garbelotto & Gonthier, 2013; Woodward *et al.*, 1998). In 2012, the whole genome of *H. irregulare* was sequenced and published, not only because it represents an economically important fungus, but also in order to identify molecular elements that are involved in balancing between the saprotrophic and necrotrophic lifestyle (Olson *et al.*, 2012).

H. annosum s.l. is a species complex which includes three intersterile groups in Eurasia and two restricted to North America. In Eurasian, the species *H. annosum* sensu stricto (s.s.), *H. abietinum,* and *H. parviporum* occur, while *H. irregulare* and *H. occidentale* appear in North America (Garbelotto & Gonthier, 2013). Interestingly, each species has initially been classified based on their host preferences, the Eurasien species are also classified into P-, S-, and F-type (Niemelä & Korhonen, 1998). In Eurasia, *H. parviporum* (S-type) is tightly linked to the Norway spruce (*Picea abies*) and *H. abietinum* (F-type) to the silver fir (*Abies alba*), while *H. annosum* s.s. (P-type) possesses the broadest host spectrum, favoring pines (*Pinus* spp.) over other conifers and

certain broad-leaved tree species. In North America, *H. occidentale* exhibits a wide host preference, including the tree genera *Tusga, Picea,* and *Abies*, whereas *H. irregulare* usually infects junipers (*Juniperus* spp.), pines, and the cedar (*Calocedrus decurrens*) (Garbelotto & Gonthier, 2013).

Most *Heterobasidion* species have saprotrophic lifestyles, although some *H. annosum* s.l. species are necrotrophic (Garbelotto & Gonthier, 2013). Necrotrophic species primarily infect freshly cut stumps or dead trees via basidiospore dispersal (Rishbeth, 1951a;b; Garbelotto & Gonthier, 2013). Wounds on stems or roots have also been suggested as possible entry points of infection. However, Rishbeth (1951b) was the first to observe that growth of *H. annosum* on stumps was replaced by other fungi later identified as *Phlebiopsis gigantea* (Rishbeth, 1951b). This initial finding formed the basis for the development of *P. gigantea* as a biocontrol agent (Rotstop) against *H. annosum*. Although infection of trees may occur via open wounds, *H. annosum* is primarily colonizing stumps via airborne sexual spores called basidiospores (Gonthier & Thor, 2013; Redfern & Stenlid, 1998) and the incidence of stump colonization is depending on the stump diameter (Morrison & Johnson, 1999).

Secondary infection occurs after successful colonization of freshly cut stumps or dead trees. *H. annosum* grows from the stump along the roots to infect healthy trees via root-to-root contact (Asiegbu *et al.*, 2005; Garbelotto & Gonthier, 2013; Rishbeth, 1951b). Thus, the infection strategy of *H. annosum* features primarily saprotrophic actions on dead trees or fresh stumps. A switch to the necrotrophic lifestyle occurs after further colonization, when healthy trees are encountered via root-to-root contact and during invasive growth.

2. AIMS OF THIS STUDY

Oxidoreductases are strongly implicated as components of plant-fungal interactions. In plants, oxidoreductases play essential roles in immune reactions, including ROS creation by NADPH oxidases and strengthening of physical barriers. On the contrary, fungi use the enzymes to weaken and degrade the plant cell wall, and to detoxify plant-generated ROS. The lack of a bioinformatics platform containing fungal peroxidase genes and analytical tools has limited research at the genomic level. Thus, the aims of this study were:

1) To develop a pipeline allowing retrieval from, and identification of, fungal peroxidase genes in the bioinformatics portal CFGP (Choi *et al.*, 2013), and to add this information to a newly constructed fungal peroxidase database (**I**), to facilitate comparative and evolutionary studies on fungal peroxidases at the genomic level.

2) To investigate structural and sequence differences on genes encoding fungal NADPH oxidases and their isoforms by comparative and evolutionary analysis at the kingdom level (**II**).

3) To explore the roles played by laccases during *H. annosum* infection, by profiling the expression levels and laccases activities during infection of pine seedlings by fungi grown on different carbon sources (**III**).

3. HYPOTHESES

The first hypothesis is that the structure of fungal Nox isoforms is rather diverse and may differ from the commonly accepted Nox structure which is based of the human Nox ($gp91^{phox}$). The second hypothesis is that *H. annosum* laccase expression levels and activities are not under catabolite repression and potentially involved in infection of *Pinus sylvestris* seedlings.

4. MATERIALS AND METHODS

The bioinformatic tools, species and methods used in this study are summarized in Table 1 and Table 2.

For example, the tool HMMER was used for searching and identification of peroxidases and NADPH oxidase (Nox) protein sequences (**I**, **II**). Using HMMER requires first to create a sequence alignment of protein sequences, which are then entered into *hmmbuild* in order to create an hmmer profile. This profile can then be applied on a sequence containing database and the retrieved sequences will be scored based on E-value.

TMHMM2 was used to predict transmembrane helices in Nox (**II**). The usage of HMMER provided an advantage as it incorporates several rules, like that cytoplasmic side are often positively charged, when searching protein sequences for potential transmembrane helices.

Table 1: Bioinformatic tools used in this study.

Methods	*Publications*
BLAST	I
BLASTMatrix	I
ClustalW	I, II
ChloroP 1.1	I
HMMER	I, II
InterPro scan	II
MEGA5	II
MEGA6	III
NetCGly 1.0	II
NetNGly 1.0	II
NetOGly 3.0	II
PSortII	I
SecretomeP 1.0 f	I
SignalP 3.0	I
TargetP 1.1b	I
T-Coffee	I
TMHMM2	II

Table 2: Species and methods used in this study.

Species	*Strain/Genotype*	*Publications*
Heterobasidion annosum **P-type**	Isolate 03012 (Kari Korhonen, METLA, Finland)	III
Pinus sylvestris	Svenska Skogsplantor (Saleby FP-45, Sweden)	III
Methods		***Publications***
cDNA synthesis		III
Enzyme activity assay (laccase)		III
Infection assays		III
qRT-PCR		III
Isolation of DNA and RNA		III

5. RESULTS AND DISCUSSION

5.1 fPoxDB (I)

5.1.1 "Fetch the peroxidases!" - Pipeline construction and evaluation

Despite the significance of peroxidases, and the vast literature thereon, genome-level analysis has been compromised by the lack of a dedicated peroxidase platform for comparative genomics. fPoxDB was created to remedy this defect, and features bioinformatic analysis tools behind a user-friendly interface, facilitating comparative studies and efficient data-handling.

In order to set up a pipeline for fPoxDB, a set of fungal peroxidase and selected oxidoreductase protein sequences was manually retrieved from Peroxibase (Fawal *et al.*, 2013). The selected oxidoreductase protein sequences included the "ancestral NADPH oxidase" (Nox) genes, the respiratory burst oxidase homolog (Rboh) from plants, and dual oxidase (Duox) from mammals. Nox genes share high-level sequence similarities with ferric reductases (Fre) and ferric-chelate reductases (FRO). Thus, Fre or FRO are often wrongly designated as Nox enzymes, confounding analyses. Nox enzymes are divided into three gene families: Nox A, B, and C (Lacaze *et al.*, 2015; Sumimoto, 2008). Rboh and Duox were included as these are Nox homologues as well as they belong to the Nox family (Bedard *et al.*, 2007; Bedard & Krause, 2007; Sagi & Fluhr, 2001), and in order to make those available for future studies, e.g. phylogenetic analysis (**II**). Also, the regulatory subunit of NoxA and NoxB, NoxR, was added (Cano-Domínguez *et al.*, 2008; Lacaze *et al.*, 2015; Scott, 2015; Sumimoto, 2008). Although NoxR per se is not a peroxidase, rather regulating Nox, we included this gene/protein because of the importance thereof.

After manual retrieval of all relevant protein sequences, T-Coffee was used to perform multiple sequence alignment of each gene family (Di Tommaso *et al.*, 2011). Manual curation of Nox genes occurred by separating them into NoxA, B and C. Separation were based on the results of a phylogenetic analysis (**Figure 1 in II**). Trimming of fungal peroxidase sequences and oxidoreductase sequences occurred in cases where overhanging sequences and/or interspersed gaps within the alignment were found. The refined alignments were entered into *hmmbuild* (version 2.3.2) to create sequence profiles (Eddy, 1998). Optimization of the identification accuracy was sought by focusing only on highly conserved sequences shared by most proteins in each family.

The hmmer profiles identified 6,113 putative peroxidase genes from 331 genomes stored in CFGP (CFGP 2.0; http://cfgp.snu.ac.kr) (Choi *et al.*, 2013) (**Figure 1 in I**). The accuracy of the pipeline was evaluated to determine whether positive and negative hits were distinguished. The first hmmer analysis retrieved 310 putative Nox sequences, of which phylogenetic analysis showed that only 102 sequences were genuine Nox sequences and the rest were Fre or FRO sequences (see 5.2.1; **II**). This showed that the initial Nox hmmer profile was inadequate, since Fre or FRO genes could be misinterpreted as Nox genes. The Nox hmmer profile for each isoform was re-

created, using the 102 sequences. This recreation improved the accuracy of Nox identification (see 5.2.1; **II**).

Pipeline accuracy was tested using positive and negative protein sets, the sequences of which were obtained from the UniProtKB/SwissProt database (Apweiler *et al.*, 2013). The positive set included 77 protein sequences from eight peroxidase gene families, and the negative set 236 non-peroxidase protein sequences, such as laccases and other oxidoreductases, eliminate false-positives. No significant hits were obtained with the negative set and every member of the positive set was predicted. These results validated the pipeline's accuracy and discrimination power. This underlines also, that Nox were the only sequences which were difficult to be identified due to their high sequence similarity with Fre and FRO.

The general outline and approach for the construction of a pipeline ready for identification and possible annotation of protein sequences depends on a variety of factors. These factors can include the scope of the project, the availability of prediction software and algorithms, and functional annotated available sequences, but are not limited to those.

One possible approach can be the manual retrieval of sequences, the creation of multiple sequence alignments (MSA), possible manual curation of such alignments, creation of hmmer profiles from MSA and search for similar sequences within a database. This was applied in the case for the fungal peroxidase database (fPoxDB), similarly to the protein families database (Pfam) (Punta *et al.*, 2012). Another approach can involve the usage and combination of already available prediction programs to construct a hierarchical pipeline, which was used in the FSD (Choi *et al.*, 2010). Although the pipeline construction of fPoxDB and FSD differ from each other, each pipeline has its own advantage for the respective goal. In fPoxDB, the creation of hmmer profiles offers an advantage that these are very amenable to change and improvement based on the results. This was shown for the set-up of a second hmmer profile for Nox, as the first hmmer profiles potentially predicted Fre/FRO as Nox. Also, hmmer profiles hold the advantage that only gene specific sequences will be retrieved, supporting the identification accuracy. For instance, the sequence structure of peroxidases is completely different compared to those of Nox. Thus, hmmer profiles based on Nox sequences will highly unlikely identify peroxidase genes as Nox genes, and vice versa. In FSD, the availability of different prediction programs for secretory proteins provided the opportunity to search for and compare identified sequences, supporting the identification accuracy. Also, the usage of subcellular localization predication programs, such as predictNLS (Cokol *et al.*, 2000), PSortII (Nakai & Horton, 1999) and TargetP 1.1b (Emanuelsson *et al.*, 2000), as well as the transmembrane prediction program TMHMM2 (Sonnhammer *et al.*, 1998), supported the elimination of false-positive results.

Regarding the "seed alignment", it is essential for any bioinformatic approach and analysis to obtain high quality sequences, and if possible, a vast amount of sequences (Punta *et al.*, 2012).

To determine the quality of a sequence, most strategies rely on statistical approaches, such as the sequencing depth and sequencing coverage, the quality scores of the base calling software, and the average GC content (Anvar *et al.*, 2014). Base calling software, like Phred, originally was used to assign nucleotide bases towards chromatogram peaks and shapes during Sanger sequencing (Ewing *et al.*, 1998; Ewing & Green, 1998). Moreover, Phred also assigns quality scores to each nucleotide base, reflecting the error probability logarithmically that this base is incorrectly called. Nowadays Phred-like quality scores are used to assess the accuracy of sequencing platforms, and therefore reflect the sequence quality. Sequencing depth and sequencing coverage also provide an information upon the overall quality of a sequence as these indicate the amount of times a region has been sequenced and how much of the sequenced region was covered. For example, a sequence can still contain errors, but when multiple reads of the same sequence are available the overall error possibility is reduce dramatically, thus improving the sequence quality. Also, if the sequence region is not covered 100%, there might be the possibility that sequence information is missing (Sims *et al.*, 2014). The GC content is influencing the sequence quality, as it was shown that the coverage of sequencing platforms is bias based on the GC content across the genome. Coverage bias can lead to over- or under-representation of genomic regions, and in the latter case can result in either missing/incomplete sequence region, or to miss out on important genomic features, such as the potential of identifying single-nucleotide polymorphism (Ross *et al.*, 2013).

The availability of vast amount of functionally annotated sequence data for fungal peroxidases presented an excellent opportunity for the usage of hmmer profiles for pipeline construction. Hmmer usage provided accuracy and efficiency, while requiring limited computational resources. For fungal Nox, less functionally annotated sequence data is available and possible future identification of new sequence structure might reveal differences from those currently known. Here, also hmmer profiles were a good choice as they are amenable to change and future functionally annotated sequences will be easily included into these profiles in order to improve the pipeline's accuracy and identification capability for Nox.

5.1.2 Use of the database and associated tools

The increasing numbers of full-genome sequences renders it challenging to absorb all information and analyze data efficiently. Thus, databases that can both handle vast amounts of data and feature easy-to-use interfaces are essential (**Figure 3 in I**). fPoxDB is such, featuring a menu and browsing system, and including the bioinformatic tools BLAST (Altschul *et al.*, 1990; Johnson *et al.*, 2008), HMMER (Eddy, 1998), BLASTMatrix (Choi *et al.*, 2013), and ClustalW (Larkin *et al.*, 2007), as well as SignalP 3.0 (Bendtsen *et al.*, 2004a), TMHMM (Sonnhammer *et al.*, 1998), PSortII (Nakai & Horton, 1999), predictNLS (Cokol *et al.*, 2000), ChloroP 1.1 (Emanuelsson *et al.*, 1999), SecretomeP 1.0f (Bendtsen *et al.*, 2004b), TargetP 1.1b (Emanuelsson *et al.*, 2000), and

InterPro scan (Hunter *et al.*, 2012). Additionally, a virtual personal storage hub termed “Favorite Browser” allows convenient data storage and selection, and is connected to other web-based systems, including FSD (Choi *et al.*, 2010). The connection to CFGP allows extension of comparative studies, where 27 additional bioinformatic tools are available, such as PHYML (Guindon *et al.*, 2010; Guindon & Gascuel, 2003), SigCleave (Rice *et al.*, 2000), RPSP (Plewczynski *et al.*, 2008), and NetCGly (Julenius, 2007).

Moreover, the database structure of fPoxDB, being a three-tier system, and connected to CFGP (Choi *et al.*, 2013) as part of the gene family databases provides overall good advantage for system administrators. For example, when all data is stored in CFGP, maintenance or update requirement on the available genomes needs only to occur in CFGP. All other gene family databases which are connected to CFGP, like FSD or fPoxDB, will be automatically updated. Furthermore, the “Favorite” folders provide a significant advantage for user, as all data and job results/analyses can be stored there. Additionally, the connection of CFGP and other gene family databases offers any data stored in the “favorite” folders to be also available in all other gene family databases. This provides the user with the opportunity to use different database depending on their needs. Also the visualization of data supports the user in gaining better insights as well as to develop new ideas/theories.

5.1.3 Analyses of PeroxiBase and CAZy DB compared to fPoxDB

Each publicly available research database is specialized for a defined need and purpose. Bearing in mind, that every database not only has different goals and different strategies, but also different methods, and different thresholds, to name a few, this certainly makes comparative analyses extremely difficult (Lombard *et al.*, 2014).

The PeroxiBase is designed to study and comprehend evolutionary relationships of peroxidases of all living organisms. The provided tools, such as CIWOG (Wilkerson *et al.*, 2009) and GECA (Fawal *et al.*, 2012), for phylogenetic analyses and visualization are rather specialized as they focus on the identification of common introns within peroxidase sequences (Fawal *et al.*, 2013). Another general goal of PeroxiBase is to acquire and centralize all available and newly identified peroxidases. Here, a goal overlap with fPoxDB occurs, as one of fPoxDB aims is to collect and identify all peroxidases within sequenced fungal genomes. Moreover, fPoxDB also features phylogenetic analysis possibilities for fungal peroxidases. Although, such an overlap of goals and tools could be seen as competition is merely a reflection of different approaches of identifying and analyzing peroxidases, and rather support researchers with opportunity to choose based on their goals and needs.

The CAZy database on the other hand is focusing on linking sequence information, substrate specificity, and structural information of carbohydrate-active enzymes found in Bacteria,

Archaea, Eukaryota and Viruses. Classification of CAZymes into families is based on sequence similarities, and into subfamilies based on substrate specificity. Additionally, available structural information can be found in the respective enzyme entries (Lombard *et al.*, 2014).

5.2 *In Silico* sequence analysis reveals new characteristics of fungal NADPH oxidase genes (II)

5.2.1 "Shaping the phylogenetic tree" – Nox, Fre and FRO

"Ancestral NADPH oxidase" sequences were retrieved from PeroxiBase and used to create hmmer profiles, as described in 5.1.1 (**I**), which were in turn used to search 28 fungal, one oomycete, two plant, and three animal genomes. A total of 310 putative Nox sequences was retrieved and subjected to phylogenetic analysis. A phylogenetic tree was constructed to explore potential relationships among known and putative genes (**Figure 1 in II**). Surprisingly, only 102 sequences were Nox genes; the others were Fre or FRO genes. Thus, the hmmer profiles of "ancestral NADPH oxidase" sequences in PeroxiBase identified Fre or FRO sequences as Nox sequences. Hence, new hmmer profiles were created for each Nox isoform, based on the 102 sequences identified as true Nox sequences. The identification of 102 true Nox sequences was based on the fact that these sequences were cladded closely together with functionally characterized Nox. The tree also showed that the frequency of appearance of Nox, Fre, and FRO genes varied among kingdoms. Plants have more Rbohs than Fre or FRO genes; the reverse is true in fungi.

Zhang *et al.* (2013) conducted an evolutionary study of the ferric reductase domain superfamily and showed that Fre, FRO and Nox probably originate from a bacterial ferric reductase domain, that had undergone consistent extension of its domain with other components, such as regulatory modules or parts of the active complex. Additionally, the study also showed that the high number of gene copies within a species is a result of extensive gene gain and loss during evolution (Zhang *et al.*, 2013). This would explain to some extent the discrepancy in the amount of Nox, Fre and FRO between kingdoms, as Fre originated earlier than Nox. Another explanation could be that fungi require only a few Nox in order to control their multicellularity and interactions with their environment, while plants require more. For example, plant Rbohs have been shown to be involved in more than just plant defense (Torres *et al.*, 2002), such as cell-to-cell communication (Miller *et al.*, 2009), symbiotic nodule formation (Marino *et al.*, 2011), and mechanosensing (Monshausen *et al.*, 2009; Suzuki *et al.*, 2011).

NoxA was found in most fungal species, followed in terms of frequency by NoxB and C. As expected, NoxC was present only in the Ascomycota, but NoxA and B were present in most fungal taxa. Noticeable, in nearly all fungal species, only one gene for each isoform occurred. This is in accordance with the currently available literature, describing the occurrence of only one Nox gene

for each isoform in most fungi, while NoxC has only been described in Ascomycota (Aguirre & Lambeth, 2010; Brun *et al*., 2009; Cano-Domínguez *et al.*, 2008; Dirschnabel *et al.*, 2014; Egan *et al.*, 2007; Lara-Ortíz *et al.*, 2003; Malagnac *et al.*, 2004).

No Nox genes were identified in *S. cerevisiae*, *Schizosaccharomyces pombe*, *C. albicans*, *C. neoformans*, *Ustilago maydis,* or *Rhizopus oryza.* All Nox, Fre, and FRO genes were absent from *Phycomyces blakesleeanus* and *Encephalitozoon cuniculi*. Reasons for the absence of these genes could either be due to gene-lost during the evolutionary process, poor quality of the genomes or that genome assembly was not good enough. Also, there is the possibility that these genes are actually found in the respective genomes, but their sequence difference was too high compared to the hmmer profiles and therefore not identified. The genome size as a possible factor for the occurrence or absence of Nox, Fre and FRO genes could only be applied for *E. cuniculi*, since it possessed the smallest genome compared to all other fungi. Here, the small genome of *E. cuniculi* reflects a strong host-dependency together with an evolutionary process that has led to multiple gene losses (Katinka *et al.*, 2001; Peyretaillade *et al.*, 2011). Furthermore, a search for connection in relation to the lifestyle of fungi did not provide any conclusive explanation. Here, Nox, Fre and FRO occurred in fungi which were having similar lifestyles to those fungal species that were lacking Nox, Fre and FRO.

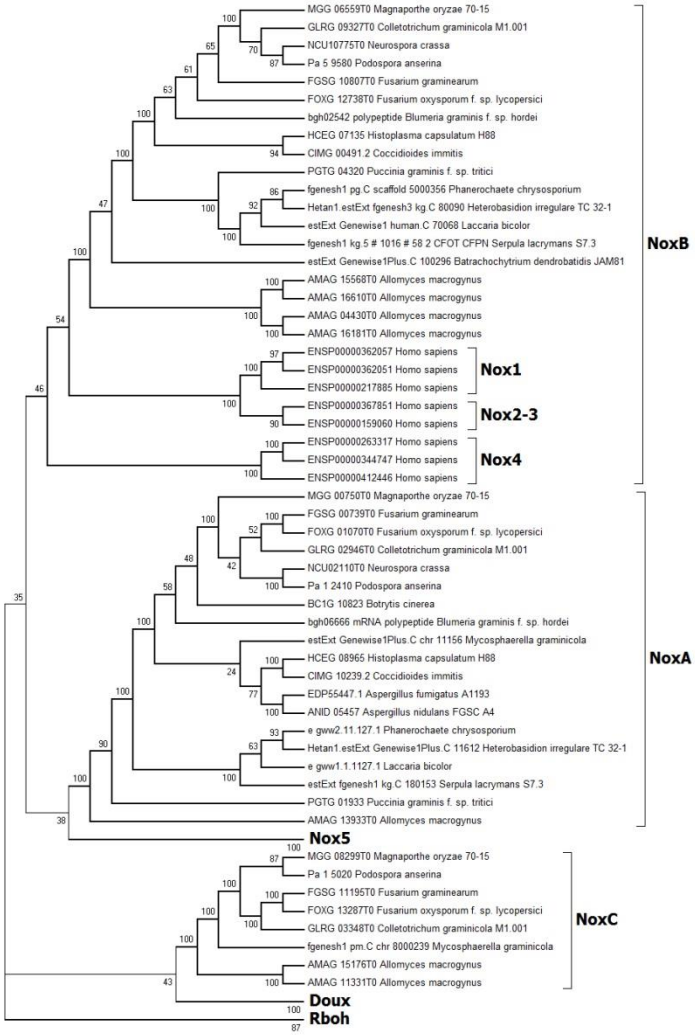
Interestingly, Rinnerthaler *et al.* (2012) showed experimentally that the YGL160W encoding gene of *S. cerevisiae* was capable of producing superoxide and was not involved in reduction and uptake of iron. When YGL160W was added to a phylogenetic tree containing 310 sequences, YGL160W was closely linked to "orf19.5634" (FRP1), an annotated ferric reductase gene from *C. albicans* (Baek *et al.*, 2008), within the Fre clade (unpublished data). As YGL160W was not identified as a Nox, rather linking closely to an annotated Fre, it could be therefore assumed that this "yeast Nox" is an ancestral gene reflecting the early evolutionary divergence of Nox, Fre, and FRO. This assumption would also be supported by the study of Zhang *et al.* (2013), where it was presumed that Nox development occurred in very early eukaryotes, and originating from a ferric reductase. Furthermore, the occurrence of a yeast Nox sharing high sequence similarity to a Fre gene opens up the possibility that a similar gene could be found in the species *S. pombe*, *C. albicans*, *C. neoformans*, *U. maydis, R. oryzae*, *P. blakesleeanus*, or *E. cuniculi*. Future studies could focus on identifying potential genes sharing a high sequence similarity with the yeast Nox and then targeting them for functional characterization.

It must be emphasized that phylogenetic analysis allowed Nox isoforms to be separately grouped (**Figure 1 in II**). NoxA and B clades were closely associated, and species from the phylum Ascomycota and Basidiomycota were represented. In contrast, the NoxC clade was more remote and separately cladded from NoxA and B, and contained Ascomycota species and one Blastocladiomycota species. Similar results regarding the cladding of the three fungal Nox isoforms

were obtained when a second phylogenetic tree was constructed for 102 Nox sequences (Figure 4). Also, in fPoxDB only 17 fungal genomes are predicted to have NoxC, while NoxA is predicted in 139 and NoxB in 128 fungal genomes respectively (http://peroxidase.riceblast.snu.ac.kr). Thus, based on our findings and the results from Brun *et al.* (2009), it seems that NoxC is under weaker evolutionary pressure than NoxA or B, and has most likely been lost in the respective fungal species.

Figure 4. Phylogenetic tree for 102 Nox sequences.

A neighbor-joining tree constructed from 102 Nox sequences. The numbers at each node indicate the bootstrap support with 10.000 repetitions. Clades are named respective to the occurrence of the characterized sequence(s). Locus name and species reference name are shown within the clades of NoxA, B, and C. Human Nox1-4 are included in NoxB clade. Clades for Nox5, Duox and Rboh have been collapsed. As seen NoxA and B clade are closely associated and Ascomycota as well as Basidiomycota are represented. NoxC is cladded separately and farther distant from NoxA and B, while more closely to the Duox and Rboh clades. Nox5 is cladded closely to NoxA.



In terms of the essential NoxR proteins, which regulate NoxA and B (Segmüller *et al.*, 2008), no systemic annotation was available. Therefore, hmmer sequence profiles were created to retrieve NoxR genes, which were subjected to phylogenetic analysis (**Figure 3 in II**). The results showed that NoxR was present in all species that had NoxA and B, hence consistent with a regulatory role of NoxR. NoxR was also found in two species, *R. oryzae* and *P. blakesleeanus*, that did not possess any Nox isoforms. This could indicate that there might be either Nox with yet unknown sequences structure, as mentioned before, or that in these species Nox were lost during evolution and NoxR genes are still remaining. Additionally, *Allomyces macrogynus* had three NoxR isoforms, whereas all other species had only one.

5.2.2 Structural analysis of Nox – TMHs and di-histidine motifs

After documenting the distribution and numbers of Nox and NoxR isoforms in fungal species, the focus was on two highly conserved features of relevant domains: TMH domains and distances between conserved histidines. In human, Nox isoforms have been extensively studied, and predicted to have six TMH domains, and Duox seven, while the histidine distances in Nox are 13 aa (TMH 3) and 12 aa (TMH 5) (Bedard & Krause, 2007; Sumimoto, 2008). On the contrary to human Nox, for fungal Nox less information is available regarding their TMH domains and histidine distances. To our knowledge, only in the study from Lara-Ortíz *et al.* (2003) a TMH domain prediction was performed and NoxA in *A. nidulans* was predicted to have six TMH domains.

The average numbers of TMH domains in fungal Nox isoforms varied among phyla. NoxA of Ascomycota species had in average five TMHs, NoxB seven or eight, and NoxC five. In the Basidiomycota, NoxA isoforms had five to seven TMHs, and NoxB seven. Notably, when the human Nox isoforms were subjected to TMH domain analysis, a difference of one or two of such domains were observed compared to the information available from the literature. This may reflect the usage of different predictive tools. For example, a hydropathy plot was used to predict four to six TMH domains in human Nox2 ($gp91^{phox}$) (Bedard & Krause, 2007). Hydropathy plot predicts transmembrane helices by quantifying the hydrophobicity of amino acids in a protein sequence. TMHMM2 in comparison is incorporating several rules when predicting transmembrane helices, and can provide better results. For instance, it takes into account that transmembrane proteins have alternating cytoplasmic and non-cytoplasmic loops which limit the possible transmembrane helices, while it also includes that amino acids located on the cytoplasmic side are often positively charge (Krogh *et al.*, 2001).

One challenge concerning Nox is the localization. Even in the case for human Nox which is one of the best studied Nox, a clear localization has been difficult due to the fact that Nox do not have a clear localization signal (Laurindo *et al.*, 2014; Siegmund *et al.*, 2013). Thus, another possible explanation for the difference in the amount of TMHs could be related to the localization of Nox within fungi organelles. Initially, it was proposed by Bretscher & Munro (1993) that the length and shape of a transmembrane domain influences its localization. This assumption was confirmed by the study of Sharpe *et al.* (2010), showing that the transmembrane domain length of internal membrane proteins differs from membrane proteins located in the plasma membrane. In fact, the length of transmembrane domains increases from the ER towards the plasma membrane. The difference in transmembrane domain length is based on the lipid composition influencing the bilayer thickness of the different organelles. For example, sterols and sphingolipids occur more frequent in the plasma membranes of eukaryotes, than in ER or Golgi, and thus supporting the thickness of this bilayer (Sharpe *et al.*, 2010; Simons & Sampaio, 2011).

In fungi, it was shown the average length for transmembrane domains in the ER is 20.6 aa, in Golgi 19.2 aa, in endosomes and the trans-Golgi network 23.6, whereas in the plasma membrane 27.0 aa (Sharpe *et al.*, 2010). When comparing the transmembrane domain length of the fungal Nox isoforms in our study, it revealed an average length of 23 aa for nearly all transmembrane domains (unpublished data). Based on this result, the location of the Nox isoforms most likely would be either in the endosomes or in the trans-Golgi network. However, it was experimentally shown that the "yeast Nox" Yno1p was located at the perinuclear endoplasmic reticulum (ER) by using a green fluorescence protein (GFP) fusion protein (Rinnerthaler *et al.*, 2012). Similar localization results were shown for the Nox of *B. cinerea* and *P. anserina* (Lacaze *et al.*, 2015; Siegmund *et al.*, 2013). GFP-fusion proteins of BcNoxA and BcNoxB in *B. cinerea* showed an ER, as well as nuclear envelop, and occasionally plasma membrane localization. Moreover, BcNoxA was observed to be mainly localized within the nuclear envelop, while BcNoxB was mostly in the ER (Siegmund *et al.*, 2013). These localization results create a contradiction regarding the observed transmembrane domain length of 23 aa. Here, the average length should have been 20.6 aa in order to support a localization within the ER or nuclear envelop, or 27.0 aa for a plasma membrane localization. Therefore, it might be that the interaction of the transmembrane domains residues with the lipids in the ER bilayer favors the respective localization. The amino acid composition of transmembrane domain affecting the localization of transmembrane proteins was shown by Sharpe *et al*. (2010).

Regarding the amount of TMH, it could be proposed that the amount would be important for two functions. First, it would provide a stringent localization of the Nox isoforms, dependent on the amino acid composition. Second, it could possibly be involved during the Nox complex formation with the regulatory subunit NoxR, Cdc24, Bem1, RhoGTPase Rac and NoxD or Pls1. The theory of hydrophobic matching describes the interaction of lipid bilayers with the hydrophobic residues of membrane proteins, which can result in distortion of the bilayer as well as in a conformation change of the membrane protein (Mouritsen, 2011). Here, a possible conformation change caused by the amount of TMH interacting with the bilayer lipids of the catalytic subunit NoxA or NoxB could be required for the interaction with the other Nox complex components, leading to the formation of the Nox complex.

In NoxA of the Ascomycota and Basidiomycota, the distances were 3 aa in TMH domain 3 and 11 aa in TMH domain 5. In addition, another di-histidine motif was found in TMH domain 1 in all Ascomycota species, except in *M. oryzae* and *A. nidulans*, with a distance between 2-8 aa. This latter motif is entirely novel, and may be functionally important.

NoxB of Ascomycota and Basidiomycota had the same motif as NoxA, with a distance of 3 aa in TMH domain 3 and 11 aa in TMH domain 5. Moreover, in Ascomycota NoxB an additional histidine residue was found in TMH domain 3 with a distance of 13 aa. TMH domain 3 of NoxC of the

Ascomycota and Rboh of *P. infestans* contained three histidine residues separated by 13 and 3 aa, and the TMH domain 5 motif had a separation distance of 12 aa (**Figure 2 in II**).

In the literature, histidines were shown to play a crucial role in coordinating the heme within the human Nox2 as well as in the yeast Fre. Point-mutation studies on histidines within the transmembrane helices of human Nox2 or yeast Fre led to a loss of function as well as inability to ligand heme (Biberstine-Kinkade *et al.*, 2001; Finegold *et al.*, 1996). Also, several authors argued that the histidine distance of 13 aa and 12 aa, respectively, would also be important in order to localize the heme above each other and allow the electron transfer across the membrane (Biberstine-Kinkade *et al.*, 2001; Finegold *et al.*, 1996; Sumimoto, 2008). However, our findings showed significant differences in the distances between the di-histidine motifs in fungi compared to the known literature. Currently, it remains to be determined to what extend these variations might contribute to or affect the Nox structure and function. Point mutation studies on the respective histidines in fungal Nox isoforms would be needed to further elucidate the function.

5.2.3 Nox Glycosylation

Glycosylation is essential in many biological processes ranging from cell-cell recognition, protein stabilization, supporting solubility, over to protection from proteases (Crocker & Feizi, 1996; Deshpande *et al.*, 2008; Goto, 2007). Since, no information on fungal Nox glycosylation is available, the glycosylation pattern was analyzed. N-glycosylation has been documented only in human Nox and Duox (Bedard & Krause, 2007).

Glycosylation analysis revealed that the NoxB isoforms of the Ascomycota and Basidiomycota were N-glycosylated and that the Basidiomycota NoxB isoforms had higher numbers of predicted glycosylation sites. Interestingly, no fungal Nox isoforms were predicted to be C- or O-glycosylated.

In fungi, O-glycosylation is commonly found in secretory proteins and supports proteins solubility and stability. It is also playing a role in fungal morphology and differentiation (Goto, 2007). As Nox are transmembrane proteins, this probably explains the lack of O-glycosylation found thereof in our study. It has been shown that C-glycosylation is a feature of the human RNase 2 (Hofsteenge *et al.*, 1994), in the type I cytokine receptor family and thrombospondin type-1 repeat superfamily in human (Ihara *et al.*, 2015). Although, C-glycosylation has been found in most eukaryotes, but not in bacteria or yeast, its functions in proteins is still unclear (Corfield & Berry, 2015; Ihara *et al.*, 2015). To the best of our knowledge, no C-glycosylation in human or fungal Nox has been documented in the literature. Since the function of C-glycosylation is still unclear, and none has been described in Nox to-date, the absence of it in Nox might indicate that it is not an essential feature for protein functionality.

N-glycosylation is important concerning processing and maturation of proteins in the ER and the Golgi compartment. N-glycans on proteins can support the folding, protein conformation, as well as the functionality of the protein (Mohorko *et al.*, 2011; Rosnoblet *et al.*, 2013). The fact that only the fungal NoxB isoforms showed N-glycosylation is intriguing. Although, a clear explanation for this cannot be given, it might be possible that N-glycosylation could support the protein stabilization during the NoxB complex formation. Here, interaction with the regulatory subunits as well as the stabilizer subunit Pls1 could require additional protein stabilization via N-glycosylation in order to keep the ROS generation functionality of the Nox complex.

To elucidate the correct function of N-glycosylation for NoxB, future experimental studies would be required focusing on mutating the respective sequence sites (e.g. aspargine-X-serine/threonine). To-date, such a mutation analysis studies has been performed only on human Nox2 by Wallach & Segal (1997). The study revealed that 3 out of 5 predicted N-glycosylation sites in human Nox2 were glycosylated. Another aspect which needs to be further elucidated is the higher amount of predicted N-glycosylation sites for Basidiomycota species compared to Ascomycota species. Given the experimental results from Wallach & Segal (1997), it is possible that only a small fraction of the predicted sites will be actually glycosylated.

The absence of glycosylation in NoxA and C is another fascinating aspect of fungal Nox, as human Nox are glycosylated. Since glycosylation is also important for trafficking within the cell, it raises the question why NoxA and C are not glycosylated.

Thus, N-glycosylation may be a unique feature of fungal NoxB, and can be used to differentiate NoxA from NoxB within the Basidiomycota, as these two proteins have similar conserved histidine distances, which makes it difficult to distinguish them if their sequences are not subject to a phylogenetic or comparative analysis.

5.3 Potential roles of laccases on virulence of *Heterobasidion annosum* s.s. (III)

5.3.1 Laccase identification in *H. irregulare* and phylogenetic analysis

In total, 21 laccase-encoding gene models from *H. irregulare* were retrieved from JGI (http://genome.jgi-psf.org/Hetan2/Hetan2.home.html) and CFGP (http://cfgp.riceblast.snu.ac.kr/main.php) (Choi *et al.*, 2013), and termed *HaLCC1* to *21*. Three of these genes, *HaLCC7*, *HaCLL17* and *HaLCC19*, were either incomplete in their sequence or incorrect predicted laccase sequences. The remaining 18 genes featured three multi-copper oxidase domains and a signal sequence, with the exception of *HaLLC9* and *HaLCC16* having an additional L-ascorbate oxidase domain (**Figure 1a in III**).

Phylogenetic analysis of 236 laccase-encoding sequences from 26 species belonging to the phylum Basidiomycota showed that the *H. irregulare* genes could be assigned to four groups. Most laccases from all species were of group I (**Figure 1a, b in III,**).

5.3.2 Laccase expression during host infection and under different carbon sources

As a preliminary step to understand the potential roles of laccases in the virulence of *H. annosum* s.s., qRT-PCR was used to measure laccase expression levels during fungal infection of Scot pine (*Pinus sylvestris*) seedlings.

Of the 18 laccase genes tested, 8 (*HaLCC6, HaLCC9, HaLCC12, HaLCC15, HaLCC16, HaLCC18, HaLCC20,* and *HaLCC21*) were strongly upregulated 1 week post-inoculation (wpi), and more than half remained upregulated at 2 wpi. Four genes were not expressed (*HaLCC1, HaLCC2, HaLCC4,* and *HaLCC14*) and two genes were downregulated (*HaLCC8* and *HaLCC10*) (**Figure 2a in III**). Thus, the upregulated laccases are potentially involved in the early steps of host infection, while the others might be involved in saprotrophic growth of the fungus. However, as all primers were designed based on the reference of *H. irregulare* sequences, but were used to amplify *H. annosum* s.s. cDNA, it is possible that the apparent non-expression of some genes reflects only the fact that the primers were not specific enough to properly anneal to the target cDNA.

Six laccase genes were selected for further examination based on their expression patterns after infection of host plants, and phylogenetic considerations. The expression levels of these genes upon growth on different carbon sources were analyzed; all genes showed to be upregulated by glucose, followed by cellulose (**Figure 2b in III**). The results suggest that these laccases are not subject to carbon catabolite repression. Carbon catabolite repression has been consider as an energy saving response (Piscitelli *et al.*, 2011; Ronne, 1995) and shown for the laccases of the basidiomycete I-62 (Mansur *et al.*, 1998), *Trametes sp.* AH28-2 (Xiao *et al.*, 2006), and in *Trametes pubescens* (Galhaup *et al.*, 2002; Piscitelli *et al.*, 2011). Cell wall-degrading enzymes, such as xylanase, pectinase and β-1,3-glucanase, of other plant pathogenic fungi were also shown to be under carbon catabolite repression (Tonukari *et al.*, 2000).

5.3.3 Laccase activity and virulence of *H. annosum* s.s. under different carbon sources

Two further experiments were performed to explore the effects of different carbon sources on laccase activity and virulence.

First, laccase activities in culture media were measured daily, and dramatic increases in activity were evident 5-7 days post-inoculation (dpi), peaking at 12 dpi, and decreasing slightly

thereafter. Notably, laccase activities were higher when cellulose, glucose, or sucrose served as carbon source, compared to cellobiose (**Figure 2c in III**). The positive effect of different carbon sources on laccase activity was also shown in other fungal species. In *Pycnoporus sanguineus*, highest laccase activity was observed when fructose or sucrose was available in the culture media (Eugenio *et al.*, 2009), whereas in *Trametes trogii* with sucrose or glucose (Zeng *et al.*, 2011), in *Trametes versicolor* with cellobiose or mannitol (Mikiashvili *et al.*, 2005), in *Morchella crassipes* with mannose (Kanwal & Reddy, 2011). In the basidiomycete I-62, high laccase activity was seen with glucose or fructose (Mansur *et al.*, 1997), in the white rot fungus *WR-1* with starch or glucose (Revankar & Lele, 2006), in *Pleurotus ostreatus* 493 with mannitol or D-gluconic acid sodium salt, in *P. ostreatus* 494 with glucose or mannitol, and in *P. pulmonarius* as well as *P. eryngii* var. *eryngii* with xylan or D-gluconic acid sodium salt (Stajić *et al.*, 2006). Taking these studies together, it seems that the highest laccase activity is dependent on each fungus's carbon preference (Eugenio *et al.*, 2009). Thus in the case of *H. annosum* it is glucose.

Second, the virulence of *H. annosum* s.s, infecting Scots pine seedlings after growth on different carbon sources, was investigated. Growth on glucose was associated with the highest mortality, followed by growth on cellobiose (**Figure 2d in III**). Thus, carbon source promote the growth of *H. annosum* s.s. as well as lead to an induced laccase activity.

The finding that certain laccase genes are not under carbon catabolite repression is interesting and also logical: If a pathogen infects the host, colonization needs to occur fast before the host immunity system creates sufficient strong responses. Physical barriers such as plant cell walls are designed to ward, limit or slow down the colonization of pathogens. To break down these plant cell structures cell wall degrading enzymes such as laccases are essential to further grow within the host. Here, the benefits of additional carbon sources like sugars are obvious for growth as well as production of cell wall degrading enzymes. Additional carbon sources could be made available during the degradation of cell wall structures and components, or by using the host's sugar transporters. An example of the pathogen *P. syringae* pv. *tomato* acquiring and manipulating the sugar transporters has been previous shown (Chen *et al.*, 2010). It remains to be determined if *H. annosum* is capable of manipulation sugar transporters. A future study could focus on the sugar transporters in Scots pine seedlings and the gene expression levels of those during infection.

Furthermore, eight laccases were strongly upregulated, while the remaining other laccases showed no expression or were down-regulated. It is very likely to assume that the either down-regulated or not expressed laccases in our study might be induced under different conditions, such as different nitrogen sources, different metals, or occurrence of aromatic compounds. For instance, laccase gene expression levels have been document to be induced by the appearance of metals, particularly by Cu^{2+} (Fonseca *et al.*, 2010; Saparrat *et al.*, 2010). Development and tissue

dependent gene expression for laccases *lacc1*, *lacc6*, *lacc7*, *lacc8* and *lacc12* was shown in *P. ostreatus* (Pezzella *et al.*, 2013), while the effects of different nitrogen sources on laccase induction in *Pleurotus sajor-caju* (Soden & Dobson, 2001), in *P. ostreatus* 493 and *P. eryngii* var. *eryngii* (Stajić *et al.*, 2006), in *Pycnoporus sanguineus* (Eugenio *et al.*, 2009), in *T. trogii*, *Trametes villosa* and *Coriolus versicolor* var. *antarcticus* (Levin *et al.*, 2010). Also, fungal toxins, such as Amphotericin B can lead to induction of specific laccase genes, as shown for *lcc1* and *lcc2* in *Coriolopsis rigida* LPSC No. 332 (Saparrat *et al.*, 2010). Additionally, aromatic compounds, such as caffeic acid in *Coprinus comatus* (Lu & Ding, 2010) and pyrogallol in *Cerrena unicolor* (Elisashvili *et al.*, 2010), have been shown to induced laccase activity. Moreover, lignin derivatives have been shown to induce laccase production and are often added to growth media (Piscitelli *et al.*, 2011). Bearing in mind that scot seedling roots are not suberized or woody could therefore provide another explanation for laccases that are down-regulation or not expressed.

Additionally, a study by Yakolev *et al.* (2013) showed that 12 out of 19 laccases genes found in the genome of *H. irregulare* where upregulated during growth in the reaction zone. The reaction zone is a barrier between infected heartwood and healthy sapwood formed by the host. Here, accumulation of phenolic compounds occurs in order to slow down the colonization of the pathogen towards the sapwood. The results implicate that these laccases genes are potentially involved in the detoxification process, and *vice versa* induced by the occurrence of phenolic and aromatic compounds (Yakovlev *et al.*, 2013). Similar results were found for the one laccase gene occurring in *H. parviporum*. The respective laccase gene was up-regulated during the early stage of colonization and later down-regulated after most host cells were dead, implicating a possible role towards detoxification of host related defense substrates (Karlsson *et al.*, 2007).

Taking this into consideration, future studies would need to address the laccase gene expressions levels and laccase activity of *H. annosum* under other conditions, such as different nitrogen sources, different aromatic compounds as well as different metals, in order to see their effects.

6. SUMMARY AND FUTURE PERSPECTIVE

Bioinformatics and comparative genomics have evolved rapidly over the past decades. Automation of next-generation sequencing (NGS) techniques has yielded vast amounts of data. The question is: How can the data be efficiently managed and analyzed in a user-friendly manner? This is particularly important for the handling of fungal genome data and in order to identify potential new gene candidates that might be involved in pathogenicity or lifestyle related.

My first project primarily concentrated on the development of fungal Peroxidase Database (fPoxDB). This is the first database to combine a fungal genomics platform with analytical tools, an easy-to-use interface, and a connection to the comparative fungal genomics portal (CFGP). It facilitates comparative genomics studies and thus providing the research community with the opportunity to study and compare peroxidases among fungal species. New fungal genomes will be added as they become available and novel bioinformatic tools will be incorporated once available.

In parallel to the above, fungal NADPH oxidase genes were further investigated. Fungal NADPH oxidase genes have shown to be essential for pathogenicity, development and maintaining symbiosis. Sequence comparisons of fungal NADPH oxidase genes afforded new insights on gene distribution among fungal species, and also showed that Nox structure varied among fungal taxa. Furthermore, bioinformatic analysis supported the separation of Nox from Fre and FRO, which are often wrongly misinterpreted as Nox genes because of high sequence similarity. Stringent hmmer sequence profiles facilitating future Nox identification were constructed. Future work should feature analysis of more fungal genomes and potential substitution mutation studies on histidines.

My third project focused on laccases which play essential and diverse roles in during the life cycle of fungal pathogens and their hosts. To explore the role of laccases of the devastating conifer pathogen *H. annosum*, the fungus was grown on different carbon sources, and laccase gene expression and activity was studied during the course of infection of pine seedlings. Eight of the 18 laccases were strongly upregulated during the first week post inoculation (wpi), and half of the enzymes maintained high-level expression to 2 wpi. Also, laccase levels rose dramatically by 12 dpi. Furthermore, the laccases of *H. annosum* were not under carbon source repression. These results afford intriguing insights into laccase expression and the effects of carbon sources on enzyme levels. Future studies could focus on investigating the laccases expression under different conditions, such as nitrogen sources or different aromatic compounds, in order to see their effects on pathogenicity.

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- Winston Churchill

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