Review of literature

Magnaporthe grisea classification:

Kingdom	Fungi
Phylum	Ascomycota
Class	Sordariomycetes
Order	Sordariomycetes incertae sedis
Family	Magnaporthaceae
Genus	Magnaporthe
Species	*M. grisea*

Rice blast disease

Rice blast is the most important disease that affects global rice production. Its importance to food security is underlined by the fact that rice contributes 23% of the calories consumed by the global human population and is the most important food product in Asia, where 55% of the world's population lives and 92% of rice is grown and consumed. Population growth has been rapid in the rice-growing regions of the world, creating an increase in demand for rice of 3% per year. Indeed, a recent analysis by the International Food Policy Research Institute indicates that rice production will need to increase 38% by 2030 to feed the expanding human population and will need to be cultivated on less ground as more arable land is lost to housing and industry (Wilson and Talbot., 2009).

There are over 80 reported rice diseases and some of them have been shown to be a major limitation to rice yield in different rice ecosystems (Mew., 1991). Rice blast is perhaps the most widely distributed plant disease, as it occurs in 85 countries world wide (Ou., 1972) and causes 70-80% of crop loss during an epidemic season (Ou., 1985; Bonman *et al*., 1991). Blast was first reported in Asia more than three centuries ago and is now present in over 85 countries. It was probably first recorded as *rice fever disease* in China in 1637 and was later described as *imochi-byo* in Japan in 1704, and as *brusone* in Italy in 1828 (IRRI., 2002). During 2003, in India, rice blast was responsible for losses of more than 266,000 tons of rice, which was about 0.8% of the total yield. In Japan, the disease affects approximately 865,000 hectares of rice fields each year. In the Philippines, rice fields may suffer more than 50% yield losses each year caused by rice blast (IRRI., 2003).

In recent years, rice blast epidemics have occurred in China — where 5.7 million hectares of rice were destroyed between 2001 and 2005 — Korea, Japan, Vietnam and the United States. The need for a better understanding of this disease becomes clear if we consider the poor durability of many blast-resistant cultivars of rice, which have a typical field life of only 2–3 growing seasons before disease resistance is overcome, and increasing energy costs, which affect fungicide and fertilizer prices. Rice blast control strategies that can be deployed as part of an environmentally sustainable plan for increasing the efficiency of cereal cultivation are therefore urgently required (Wilson and Talbot., 2009).

M. grisea infects more than 50 types of grasses, including economically important crops like barley, wheat, rice, and millet, but individual field isolates are limited to infect one or few host species (Valent., 1990). *M. grisea* produces spots or lesions on leaves (leaf blast), nodes (node blast), neck of panicle (neck rot) or other parts of the panicles (panicle blast). The lesions are elliptical with more or less pointed ends. The center of the lesions is usually gray or whitish with a brown margin. The shape and the color of the lesion depend on the environmental conditions, age of lesion and the degree of susceptibility. The lesions on the neck can result in complete loss of crop (Talbot., 1995). Some blast infected rice plants are shown in **Figure 1**.

M. grisea is highly adaptable to environmental conditions and can be found in irrigated lowland, rain-fed upland, or deepwater rice fields (Rao., 1994). In spite of great deal of research into the pathogen and the disease, blast still remains to be a serious constraint in rice production in all irrigated and upland environments. Control strategies like the use of resistant cultivars and application of fungicides have not allowed complete eradication of the disease. The fungus has been able to develop resistance to both chemical treatments and genetically resistant rice cultivars developed by plant breeders. As such, a detailed understanding of the infection mechanism may help in the development of new strategies to control this disease.

The rice blast fungus can be found referenced in the literature under several names. *Pyricularia oryzae* was used to refer to the asexual stages of rice blast fungus as it was found in the field. The rice pathogen was morphologically indistinguishable from pathogens of other hosts, and the entire group was defined under the name *Pyricularia*

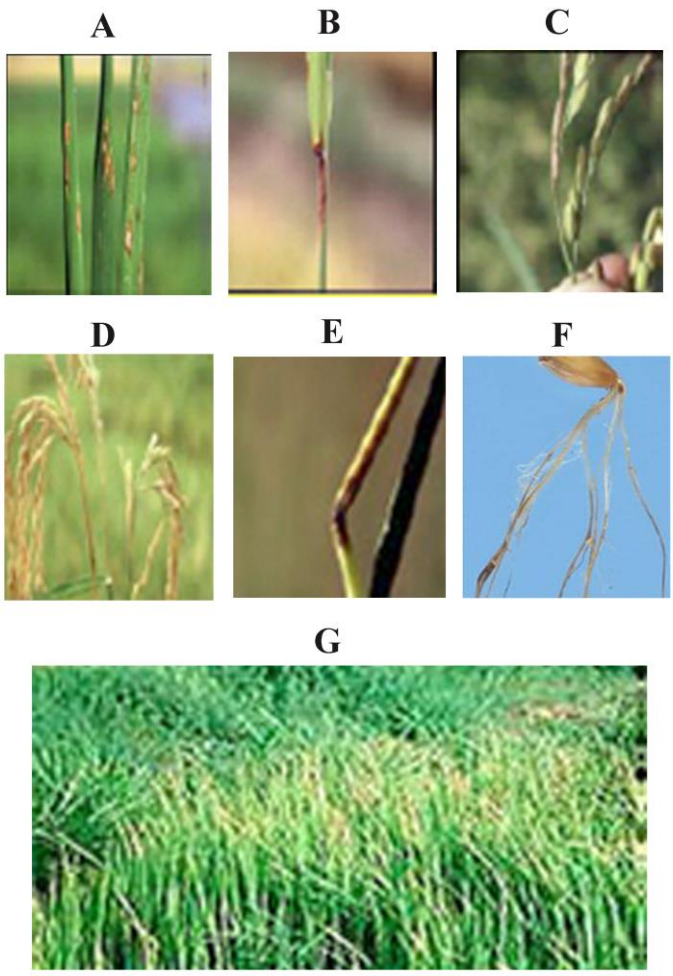

Figure 1: Blast infected rice plants. A: Leaf blast, B: Collar blast, C: Panicle blast, D: Neck blast, E: Node infection, F: Root infection, G: Blast infected rice plants

The center of origin of rice and rice blast is proposed to be the Uttar Pradesh hills of the Indian Himalayas (Kumar *et al*., 1999). Fertile strains of both mating types are found (or *Piricularia*) *grisea*. The sexual stage was named *Magnaporthe grisea* until it was shown by phylogenetic analysis and interstrain fertility tests that *Magnaporthe* isolates should be separated into species that infect *Digitaria* spp. (crab grass) (*M. grisea*), hereas *M. oryzae* collectively refers to the other characterized isolates, including the rice pathogen (Ebbole., 2007).

from this region, whereas few studies have found fertile hermaphroditic strains of rice-infecting isolates outside of this region. Furthermore, evidence for on-going sexual recombination was found in this population based on genotyping, despite the fact that the sexual stage has never been confirmed in the field. The genetic relationship between rice pathogens and pathogens of other hosts suggests a single ancient invasion of rice (Couch *et al*., 2005). Tracing the center of origin for *M. oryzae* prior to its acquisition of rice as a host remains an interesting question. The rice pathogen appears to have shifted to other grass hosts that grow in association with rice cultivation; however, there is no indication that subsequent shifts have occurred from these grasses back to rice (Ebbole., 2007).

Rice blast fungus as a model system to study plant pathogen interaction

M. grisea is a haploid filamentous ascomycete and has become an excellent model organism for studying fungal phytopathogenicity and host-pathogen interactions (Valent., 1990; Tucker and Talbot., 2001). *M. grisea* can be cultured on defined media and various genomic and cDNA libraries are available and genetic maps of the fungus have been developed (Nitta *et al*., 1997; Nishimura *et al*., 1998; Zhu *et al*., 1997; Farman and

Leung., 1998; Zhu *et al*., 1999). Transformation protocols have been developed and various auxotrophic and drug resistance markers are available (Valent and Chumley., 1991; Parsons *et al*., 1987; Kachroo *et al*., 1997). A large international strain collection and various probes for population genetic studies are also available (Babujee and Gnanamanickam., 2000; Hamer *et al*., 1989). The early stages of the plant infection process including germination, appressorium formation, and penetration can be studied *in vitro* using artificial surfaces (Bourett and Howard., 1990). Infections can be carried out in a variety of hosts (Lau *et al*., 1996) and the infection process can be followed biochemically or cytologically, allowing the analysis of infection deficient mutant phenotypes (Heath *et al*., 1992; Bourett and Howard., 1990).

Infection cycle and appressorium development

M. grisea can reproduce both sexually and asexually. *M. grisea* also has an infectious life cycle, which is asexual. Conidia are produced on aerial conidiophores and dispersal of the spore inoculum is primarily by air and splash dispersal. The variability of the fungus and the propensity for new races to emerge in the field make its control and management difficult.The infection life cycle begins with the landing of asexual spores called conidia on the host surface followed by infection typical of foliar pathogen. In the presence of high humidity, contact between the spore and the leaf cuticle induces release of mucilage from the spore apex, enabling the conidium to attach to the leaf surface (Hamer *et al*., 1988). Once attached, the spore germinates by rapid growth of a germ tube. The tip of the

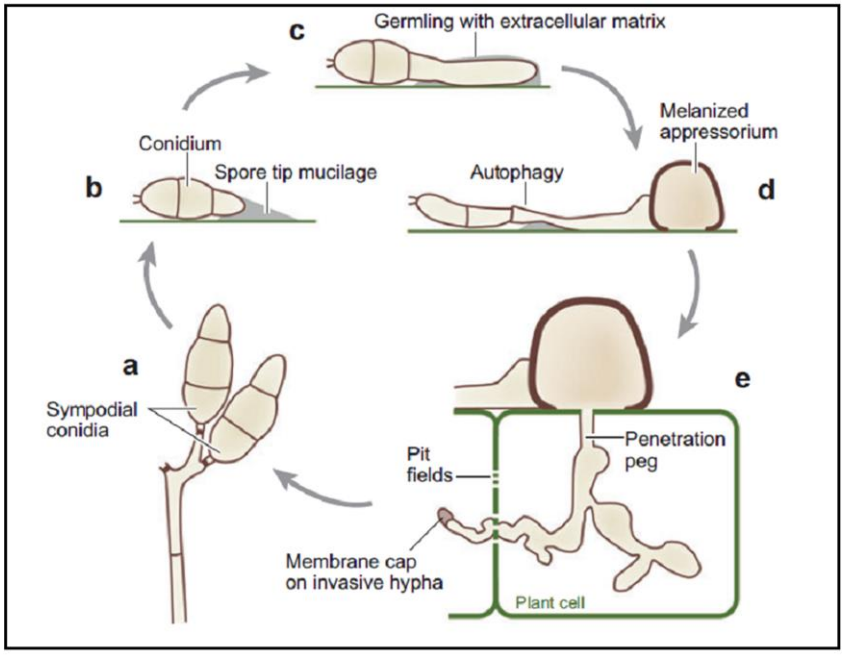

Figure 2: Disease cycle of *Magnaporthe grisea*. (*a*) Conidia are arranged in a sympodial fashion on the aerial conidiophore. (*b*) Conidia adhere to the host surface using mucilage stored in a compartment at the spore tip. (*c*) Conidia germinate and produce a hyphal filament that is sheathed in an extracellular matrix that adheres to the host surface. (*d)* The melanized appressorium is formed and contains the autophagocytized contents of the conidium and germ tube. (*e*) A penetration peg employs turgor pressure to penetrate the plant cuticle and cell wall. The penetration peg gives rise to infection hyphae that have a bulbous appearance and ramify within the cell. Movement into the next cell occurs specifically at clusters of plasmadesmata, called pit fields. The hyphae constricts to a narrow diameter as it passes through the plant cell wall. Invasive hyphae have a membrane cap composed of membrane lamellae that may function in protein secretion into the cytoplasm of the living host cell. (Ebbole., 2007)

elongating germ tube enlarges and forms a dome-shaped, melanin-pigmented infection structure called the appressorium within 4–6 hours (**Figure 2**).

The word appressoria is derived from the latin "apprimere" meaning "to press against". Its role was originally thought to be exclusively in attachment to plant surface, but is now understood to be important in penetration. Appressorium development can be induced by various signals, such as the presence of cutin monomers (Gilbert *et al*., 1996), wax compounds (DeZwaan *et al*., 1999; Uchiyama *et al*., 1979) starvation stress (Jelitto *et al*., 1994; Talbot *et al*., 1997), hydrophobicity of the leaf surface and addition of external cAMP (Lee and Dean., 1993, 1994). Appressoria attach firmly to the surface and then generate enormous turgor pressure (8 MPa) used to rupture the cutical layer (Howard *et al*., 1991). Appressorial turgor generation is accompanied by *CPKA* (catalytic subunit of protein kinase A) and *PMK1* (mitogen activated protein kinase 1) dependent mobilisation of carbohydrate and lipid reserves, which accumulate in developing appressoria before being degraded rapidly in mature cells prior to plant infection (Thines *et al*., 2000; Weber *et al*., 2001). Carbohydrates and lipids are converted into glycerol in the appressorium. The melanin layer within the appressorium maintains the glycerol content, retarding its efflux during turgor development (de Jong *et al*., 1997; Howard and Ferrari., 1989). Therefore, melanin deficient mutants do not generate appressorial turgor and are non pathogenic (Chumley and Valent., 1990). The breaching of the plant cell wall by the fungus also appears to involve polarisation of the cytoskeleton to the point of infection and localised cell wall modification (Bourett and Howard., 1990; Bechinger *et al*., 1999). Once inside the plant, invading hyphae swell and fill the host epidermal cells within 24

hours. Penetration of neighboring epidermal or parenchymal cells occurs within 48 hours. Approximately 4 days after inoculation, the first visible symptoms of infection may be observed.

Sexual reproduction occurs when two strains of opposite mating types meet and form a perithecium in which ascospores develop. Once released, ascospores can develop appressoria and infect host cells. Mating is governed by alternate alleles of the mating type locus, Mat1. Although the generation of fully fertile laboratory strains has facilitated genetic analysis of this fungus (Valent and Chumley., 1991), sexually fertile rice pathogens are extremely rare.

Root infection by *M. grisea*

Phytopathogenic fungi can be divided broadly into two types: those infecting leaves and stems of plants and those proliferating in root tissue (Agrios., 1997). *M. grisea* can not only infect the foliar region of the plant but also infects cereal roots indicating that it retains the capacity to infect distinct plant tissue types and develop a range of infection structures (Dufresne and Osbourn., 2001). The mode of root infection is the same as most root infecting fungi; it grows long hyphae that form an infection pad to gain entry to the root's interior. The melanised appressoria associated with classical foliar infection are not observed on the surface of rice roots when either conidia or mycelium are used as inoculum. In contrast, hyphal swellings resembling the hyphopodia of root-infecting fungi are evident at infection sites (Sesma and Osbourn., 2004). Once embedded in the root the fungus can produce resting structures. The blast fungus can also invade the

plant's vascular system, growing inside the xylem and phloem and blocking the transport of nutrients and water from the roots, and produce lesions on aerial plant parts.

Nitrogen starvation of *M. grisea*

Several studies have suggested that lack of nutrients is one of the signals that control expression of pathogenicity factors in various fungal pathogens of plants (Snoeijers *et al*., 2000) and humans (Lengeler *et al*., 2000). Starvation stress has also been implicated as a key influence on fungal gene expression during growth of *M. grisea* within the host plants (Talbot *et al*., 1997). It has been proposed that a subset of signaling pathways that regulate fungal pathogenicity have been co-opted from those involved in nutrient sensing and subsequent fungal response (Alspaugh *et al*., 1997; Pellier *et al*., 2003).

In a study by Talbot and coworkers in 1997, it was shown that nitrogen starvation-stress induced expression of a large number of genes expressed during growth of the fungus in plant tissue, particularly during disease symptom outbreak. Strains carrying mutations at the *NUT1* locus controlling nitrogen source utilization, or the *NPR1* and *NPR2* loci which regulate nitrogen metabolism and pathogenicity, produced only residual senescent activity on host. It was concluded that nitrogen starvation stress may act as one of the inductive cues for disease symptom expression during rice blast infections.

Melanin production and deficiency

The deposition of a cell wall layer composed of melanin represents the final and functional stage of appresorium formation. This melanin layer is homogenous, positioned outside the plasma membrane and imparts dark pigmentaion to the appresorium. The dark

brown to black pigment, produced by *M. grisea* is polymerised from the polyketide precursor 1,8-dihydroxynapthalene and has been suggested to be catalysed by a phenol oxidase, such as laccase (Bell and Wheeler., 1986). Mutational analysis has defined three unlinked biosynthetic genes ALB1, RSY1 and BUF1 respectively for albino, rosy and buff appearances in culture (Chumley and Valent., 1990). Melanin deficient mutants of *M. grisea* fail to infect intact host plants, but the same mutants successfully infect plants that have been wounded by abrading the leaf surface. This suggests that the enzyme related to the conversion of 1,8-DHN to melanin could serve as potential target for inhibitors which the directly or indirectly relates to pathogenesis.

Extracellular enzymes in penetration

The genome of a rice pathogenic strain of *M. oryzae*, 70-15, was sequenced through a whole-genome shotgun approach. It has seven chromosomes. The total length of all sequence contigs is 39.5 (Mb). Within the *M. oryzae* genome, 12,841 genes are predicted with protein products, ~80% of which are hypothetical proteins and ~20% are predicted proteins. It has a large and complex secretome, 739 proteins are predicted to be secretory (Dean *et al*., 2005).

The secretory proteins can be classified as enzymes for degradation of the plant cell wall and cuticle, proteins with carbohydrate substrate-binding domains, with a role in attachment and colonization of plant tissue, pathogen effector proteins which fungus secretes directly into host plant cells to perturb host cell signaling or suppress the plant innate immune system.

The cell walls of plant act as barrier to and as source of nutrition for pathogens, and as a reservoir of signal molecules that regulate plant defense, growth and development (Hahn *et al.*, 1989; Darvill *et al.*, 1992; Zablackis *et al.*, 1996). In order to penetrate the cell wall and use the cell wall's building blocks as nutrients, most fungal and bacterial pathogens secrete cell wall polysaccharide cleaving endoglycanases when infecting plant tissues (Cooper *et al.*, 1988; Dean and Timberlake., 1989; Southerton *et al*., 1993; Walton., 1994; Kolattukudy *et al*., 1995; Peltonen., 1995; Howard and Valent., 1996; Xu and Mendgen., 1997). Direct penetration of the plant cuticle by the fungal pathogens could hypothetically be achieved by mechanical or enzymatic means. Many fungi produce extracellular degradative enzymes such as cutinases, cellulase, xylanases, proteases, amylases and oxidative enzymes such as laccases and peroxidases. Cutinase is clearly essential for penetration of the cuticle in some pathogenic interactions. In *Fusarium solani* f.sp.pisi on pea (Maiti and Kolattukudy., 1979) and *Colletotrichum gloeosporioides* on papaya (Dickman *et al.*, 1982), antibodies against cutinase prevent infection through an intact plant cuticle. Cutinase deficient mutants of *Fusarium solani* f.sp.pisi (Koller *et al.*, 1982; Dantzig *et al.*, 1986) and *Colletotrichum gloeosporioides* (Dickman and Patil., 1986) show reduced pathogenicity. Penetration of the plant surface by these two fungal plant pathogens occur by way of undifferentiated hyphae in contrast to the elaborate specialised infection structures, called appresoria, employed by *M. grisea*. The rice blast fungus secretes a number of extracellular enzymes such as cutinase (Sweigard *et al*., 1992), xylanases (Wu *et al*., 1995), whose role have been evaluated in pathogenesis. Cutinase deficient mutants and xylanases mutants, under the

conditions idealised for infection, infect the host plant as efficiently as the parent, indicating that these genes are dispensable for pathogenesis.

Laccases have been shown to be an important virulence factor in many diseases caused by fungus, e.g. *Cryphonectria parasitica* (severe chestnut blight), *Gaeumannomyces graminis* (severe root disease), *Cryptococcus neoformans* (laccase knock out mutants are non pathogenic). High laccase activity in culture filtrate of *M. oryzae*, 24 hours after spore germination, was detected (Iyer and Chattoo., 2003). This suggested that it may play a role in pathogenesis, because during the infection of rice plant, genes that are expressed early, such as MPG1, MPS1 may fulfill various functions in pathogenesis in concert with other genes such as laccase.

Fungal laccases

Introduction

Laccase (EC 1.10.3.2, p-diphenol oxidase) is one of a few enzymes that have been studied since the nineteenth century. Yoshida first described laccase in 1883 when he extracted it from the exudates of the Japanese lacquer tree, *Rhus vernicifera* (Thurston., 1994; Levine., 1965). In 1896 laccase was demonstrated to be a fungal enzyme for the first time by both Bertrand and Laborde (Thurston., 1994; Levine., 1965). Laccase is a member of the large blue copper proteins or blue copper oxidases, which comprise a small group of enzymes. Other enzymes in this group are the plant ascorbate oxidases and the mammalian plasma protein ceruloplasmin (Thurston., 1994; Xu., 1996; Ducros *et al*., 1998).

Laccases are either mono or multimeric copper-containing oxidases that catalyse the one-electron oxidation of a vast amount of phenolic substrates. Molecular oxygen serves as the terminal electron acceptor and is thus reduced to two molecules of water (Ducros *et al.*, 1998). The ability of laccases to oxidise phenolic compounds as well as their ability to reduce molecular oxygen to water has lead to intensive studies of these enzymes (Jolivalt *et al.*, 1999; Xu., 1996; Thurston., 1994). The biotechnological importance of these enzymes can also be attributed to their substantial retention of activity in organic solvents with applications in organic synthesis.

Laccases have widespread applications, ranging from effluent decolouration and detoxification to pulp bleaching, removal of phenolics from wines and dye transfer blocking functions in detergents and washing powders, many of which have been patented (Yaver *et al*., 2001). The biotechnological application of laccase has been expanded by the introduction of laccase- mediator systems, which are able to oxidise non-phenolic compounds that are otherwise not attacked and are thus able to degrade lignin in kraft pulps (Bourbonnais and Paice., 1990).

Occurrence and location

Laccase is the most widely distributed of all the large blue copper-containing proteins, as it is found in a wide range of higher plants and fungi (Leontievsky *et al.*, 1997) as well as in bacteria (Diamantidis *et al*., 2000). Laccases in plants have been identified in trees, cabbages, turnips, beets, apples, asparagus, potatoes, pears, and various other vegetables (Levine, 1965). Laccases have been isolated from Ascomyceteous, Deuteromyceteous

and Basidiomyceteous fungi (Assavanig *et al*., 1992). In the fungi, Ascomycetes and Deuteromycetes have not been a focus for lignin degradation studies as much as the white-rot Basidiomycetes. Laccase from *Monocillium indicum* was the first laccase to be characterised from an ascomycete showing peroxidative activity (Thakker *et al.*, 1992). The white-rot basidiomycetes are the most efficient degraders of lignin and also the most widely studied. The enzymes implicated in lignin degradation are: lignin peroxidase, which catalyses the oxidation of both phenolic and non-phenolic units, manganese-dependant peroxidase and laccase, which oxidises phenolic compounds to give phenoxy radicals and quinines; glucose oxidase and glyoxal oxidase for H_2O_2 production and cellobiose-quinone oxidoreductase for quinone reduction (Kirk and Farrell., 1987; Thakker *et al.*, 1992). The different degrees of degradation of lignin with respect to other wood components depend on the environmental conditions and the fungal species involved. It has been demonstrated that there is no unique mechanism to achieve the process of lignin degradation and that the enzymatic machinery of the various microorganisms differ. *Pleurotus ostreatus,* for instance, belongs to a subclass of lignin–degrading microorganisms that produce laccase, manganese peroxidase and veratryl alcohol oxidase but no lignin peroxidase (Palmieri *et al.*, 1997). *Pycnoporus cinnabarinus* has been shown to produce laccase as the only ligninolytic enzyme (Eggert *et al.*, 1996) and *P. sanguineus* produces laccase as the sole phenol oxidase (Pointing and Vrijmoed., 2000).

In plants, laccase plays a role in lignification and in fungi, laccases have been implicated to be involved in many cellular processes, including, delignification, sporulation, pigment

production, fruiting body formation and plant pathogenesis (Thurston., 1994; Yaver *et al*., 2001). Only a few of these functions have been experimentally demonstrated (Eggert *et al*., 1998).

Ligninolytic enzymes have mostly been reported to be extracellular but there is evidence in literature of the occurrence of intracellular laccases in white-rot fungi (Schlosser *et al*., 1997). Intracellular as well as extracellular laccases were identified for *Neurospora crassa* (Froehner and Eriksson., 1974). Froehner and Eriksson suggested that the intracellular laccase functioned as a precursor for extracellular laccase as there were no differences between the two laccases other than their occurrence.

It is difficult to say how many ascomycete species produce laccases as no systematic search has been undertaken. In addition to plant pathogenic species, laccase production was also reported for some soil ascomycete species from the genera *Aspergillus, Curvularia and Penicillium* (Banerjee and Vohra., 1991; Rodriguez *et al*., 1996; Scherer and Fischer., 1998), as well as some freshwater ascomycetes (Abdel- Raheem and Shearer., 2002; Junghanns *et al*., 2005). However, the enzyme from *Aspergillus nidulans* was unable to oxidize syringaldazine (Scherer and Fischer., 1998) and the enzymes from *Penicillium spp.* were not tested with this substrate, leaving it unclear if they are true laccases.

Yeasts are a physiologically specific group of both ascomycetes and basidiomycetes. Until now, laccase was only purified from the human pathogen *Cryptococcus (Filobasidiella) neoformans*. This basidiomycete yeast produces a true laccase capable of oxidation of phenols and aminophenols and unable to oxidize tyrosine (Williamson.,

1994). The enzyme is tightly bound to the cell wall and contributes to the resistance to fungicides (Zhu *et al*., 2001; Ikeda *et al*., 2003). A homologous gene has also been demonstrated in *Cryptococcus podzolicus* but not in other heterobasidiomycetous yeasts tested (Petter *et al*., 2001) and there are some records of low laccase-like activity in some yeast species isolated from decayed wood (Jimenez *et al*., 1991). The production of laccase was not demonstrated in ascomycetous yeasts, but the plasma membrane-bound multicopper oxidase Fet3p from *Saccharomyces cerevisiae* shows both sequence and structural homology with fungal laccase. Although more closely related to ceruloplasmin, Fet3p has spectroscopic properties nearly identical to fungal laccase, the configuration of their type-1 Cu sites is very similar and both enzymes are able to oxidize Cu1 (Machonkin *et al*., 2001; Stoj and Kosman., 2003).

Laccase-catalysed reactions

Substrate oxidation by laccase is a one-electron reaction generating a free radical. As one electron oxidation of a substrate is coupled to a four-electron reduction of oxygen the reaction mechanism cannot be straightforward (Thurston., 1994). The initial product is typically unstable and may undergo a second enzyme-catalysed oxidation or otherwise a non-enzymatic reaction such as hydration, disproportionation or polymerisation. The bonds of the natural substrate, lignin, that are cleaved by laccase include, Cα - oxidation, Cα-Cβ cleavage and aryl-alkyl cleavage (**Figure 3**).

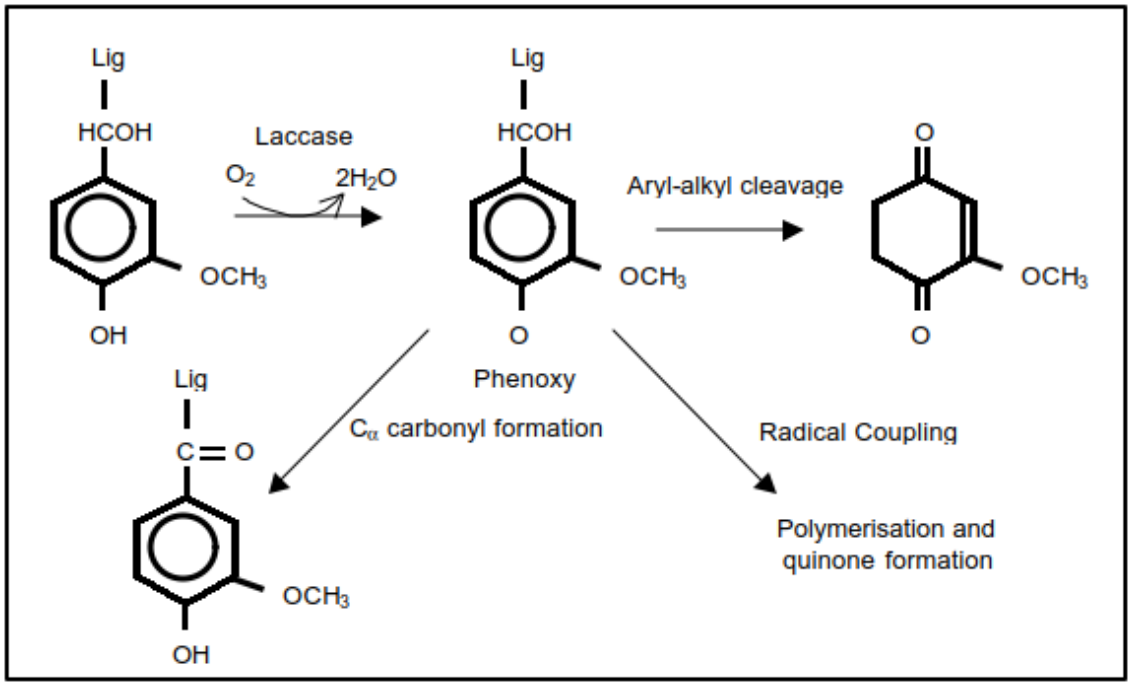

Figure 3: Laccase-catalysed oxidation of phenolic groups of lignin (Archibald *et al*., 1997).

Classification according to substrate specificity

Laccase (EC 1.10.3.2) as mentioned in the introduction, is a blue copper protein, but also falls within the broader description of polyphenol oxidases. Polyphenol oxidases are copper proteins with the common feature that they are able to oxidise aromatic compounds with molecular oxygen as the terminal electron acceptor (Mayer., 1987). Polyphenol oxidases are associated with three types of activities:

(i) Catechol oxidase or *o*-dipenol: oxygen oxidoreductase (EC 1.10.3.1)

(ii) Laccase or *p*-diphenol: oxygen oxidoreductase (EC 1.10.3.2)

(iii) Cresolase or monophenol monooxygenase (EC 1.18.14.1)

(Mayer., 1987)

These different enzymes can therefore be differentiated on the basis of substrate specificity (Walker and McCallion., 1980). There is, however, difficulty in defining laccase according to its substrate specificity, because laccase has an overlapping range of substrates with tyrosinase. Catechol oxidases or tyrosinases have *o*-diphenol as well as cresolase activity (oxidation of L-tyrosine). Laccases have ortho and paradiphenol activity, usually with more affinity towards the second group. Only tyrosinases possess cresolase activity and only laccases have the ability to oxidise syringaldazine (Thurston., 1994; Eggert *et al.*, 1996). There has only been one report of an enzyme exhibiting both tyrosinase and laccase activity (Sanchez-Amat and Solano, 1997).

The second difficulty in defining laccase according to substrate specificity is that laccases are not specific for their substrate range, as it varies from one organism to another. Thurston (1994) stated in a review that hydroquinone and catechol are good laccase substrates, but that guaiacol and 2,6-dimethoxyphenol (DMP) are often better, but not

always. Para-phenylenediamine is a common substrate and syringaldazine is a unique substrate for laccase only. Thus, laccase oxidises polyphenols, methoxy–substituted phenols, diamines and a vast range of other compounds (Thurston., 1994).

Neurospora crassa laccase (Germann *et al*., 1988) only effectively oxidises para and ortho-diphenols with the exception of phloroglucinol. Laccase from *Pyricularia oryzae* preferred phloroglucinol as a substrate above other substituted monophenols (Alsubaey *et al.,* 1996). Laccases from *Cerrena unicolor* and *Trametes versicolor* oxidise meta-substituted phenols but to varying degrees. Laccase from *Cerrena unicolor* oxidises para-substituted phenols to the greatest extent (Filazzola *et al.*, 1999) while *Trametes versicolor* laccase oxidises ortho-substituted phenols to the greatest extent (Jolivalt *et al.*, 1999). An immobilised commercial laccase was shown to be able to degrade meta, ortho and para-substituted methoxyphenols, chlorophenols and cresols, but the substituted phenols from these three types of phenols are oxidised in different orders and to different extents (Lante *et al.*, 2000).

Many different reactions have been reported to be catalysed by laccases from different fungi. A comparative study concerning properties of fungal laccases indicated that all the laccases in the study had the ability to oxidise methoxyphenolic acids but to different degrees. The oxidation efficiencies of the laccases were also dependant on pH (Bollag and Leonowicz., 1984). Laccases were also shown to be able to decarboxylate vanillic acid to methoxyquinone (Ander and Eriksson., 1978). Two lignin derived hydroquinones, namely 2-methoxy-1,4-benzohydroquino ne and 2,6- dimethoxy-1,4-benzohydroquinone were oxidised by laccase from *Pleurotus eryngii* (Guillen *et al.,* 2000). The auto

oxidation of the semiquinones produced by the laccase-catalysed reaction leads to the activation of oxygen. 2,6-dimethoxy-1,4-benzohydroquinone was oxidised more efficiently than 2-methoxy-1,4-benzohydroquinone by laccase (Guillen *et al.,* 2000). This correlates to the higher affinity of laccase for DMP than for guaiacol. Leonowicz *et al.* (1985) used fractionated lignosulphonates (peritan Na) to prove that laccases have the ability to polymerise and depolymerise certain substrates. The products of laccase-catalysed reactions often lead to polymerisation through oxidative coupling. Oxidative coupling reactions of such products result from C-O and C-C coupling of phenolic substrates and from N-N and C-N coupling of aromatic amines (Medvedeva *et al.*, 1995; Hublik and Schinner., 2000). Laccase from *Rhizoctonia practicola* was used to demonstrate the ability of laccase to catalyse the coupling of two differently halogenated phenols, 2,4-dichlorophenol and 4-bromo-2- chlorophenol. The laccase catalysed reaction led to the formation of three dimmers with asymmetric formation (Bollag *et al.*, 1979).

Industrial processes such as paper bleaching produce organochlorine compounds. These compounds include chlorinated phenols, catechols and guaiacols. Laccase from *Coriolus versicolor* has been shown to dechlorinate tetrachloroguaiacol andrelease chloride ions (Iimura *et al.*, 1996).

Laccases from *Trametes villosa* and *Trametes hirsuta* have the ability to modify fatty and resin acids to a certain degree. The amount of linoleic, oleic and pinolenic acids were reduced for fatty acids and the amount of conjugated resin contained in resin acids was decreased (Karlsson *et al.*, 2001). Laccase also has the ability to cleave an etheric bond of the substrate, glycol-b-guaiacyl ether (a model lignin compound).

Phenolic compounds that are oxidised very slowly by laccase have recently been used to increase the storage stability of laccase activity for *Trametes versicolor* (Mai *et al.*, 2000). The increased stability of laccase could have technological importance, as there are so many potential applications for laccase.

Structure of laccase enzymes

Studies of purified enzymes

Laccases are monomeric or multimeric copper-containing enzymes. An example of a multimeric enzyme is the laccase produced by *Podospora anserina*, which has a tetrameric structure with identical subunits. The typical laccase has a relative molecular mass (Mr) of 60 000 to 80 000 and is 15-20 % glycosylated (Thurston., 1994; Luisa *et al.*, 1996), there are many exceptions, however. Exceptions include laccases produced by *Monocillium indicum* (Mr = 100,000), *Agaricus bisporus* (Mr = 100,000), and *Aspergillus nidulans* (Mr = 110,000) (Thakker *et al.*, 1992; Perry *et al.*, 1993a; Thurston., 1994).

There are many reported cases that show that a single fungal species may express more than one laccase enzyme. The white-rot fungus *P. ostreatus* produces at least eight different laccase isoenzymes (Baldrian, 2005). Different culture conditions may also lead to the production of different isozymes by the same fungus (Bollag and Leonowicz., 1984; Wahleitner *et al.*, 1996; Palmieri *et al.*, 1997; Farnet *et al.*, 2000).

Active site

Other than laccase that typically contains four copper atoms per monomeric molecule, ceruloplasmin and ascorbate oxidase contain more than four copper atoms per molecule.

Therefore, laccase provides the simplest system to study active site structure and reactivity of multicopper oxidases (Germann and Lerch., 1986).

Three types of copper can be distinguished using UV/visible and electroparamagnetic resonance (EPR) spectroscopy. Type 1 copper is responsible for the blue colour of the protein at an absorbance of approximately 600 nm and is EPR detectable, Type 2 copper does not confer colour but is EPR detectable and Type 3 copper consists of a pair of copper atoms in a binuclear conformation that give a weak absorbance in the near UV region but no detectable EPR signal (Thurston., 1994). The Type 1 Cu site is present as a Cu (II) species for the resting enzyme (Ducros *et al*., 1998). The Type 1 Cu is usually coordinated to two nitrogens from two histidines and sulphur from cysteine (**Figure 4**). It is the bond of Type 1 Cu to sulphur that is responsible for the characteristic blue colour of typical laccase enzymes. The geometry is described as distorted trigonal bipyramidal coordination with a vacant axial position where the substrate docks (Ducros *et al*., 1998). The coordination is unusual as it is intermediate between the preferred coordination states for Cu (I) and Cu (II) species. A leucine residue is present but is too far away to be directly coordinated. The Cu is therefore only coordinated to three atoms (Ducros *et al.*, 1998).

The sequences of several cloned laccase genes were compared with that of ascorbate oxidase for which a crystallographic structure was already known at the time of the

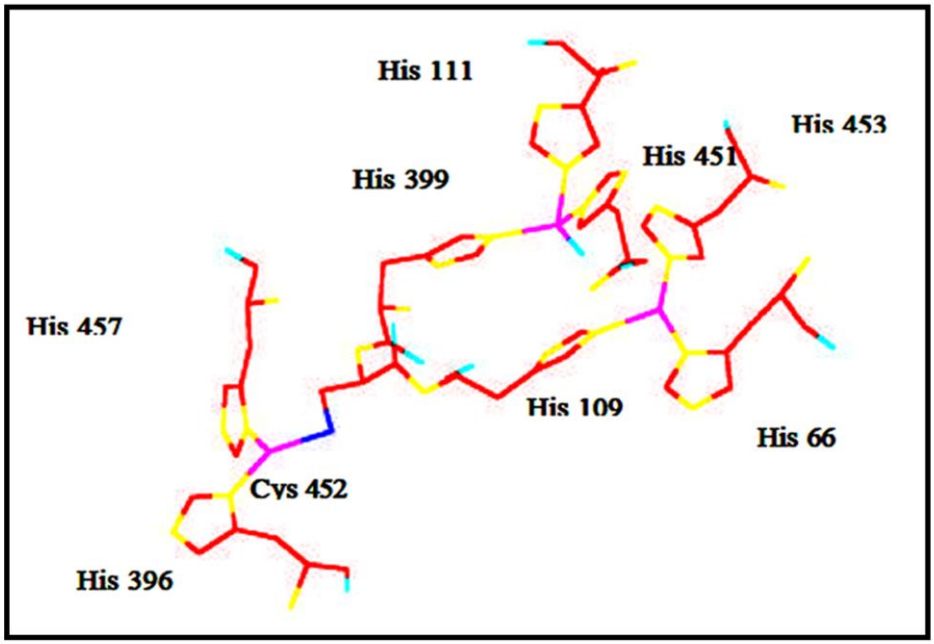

Figure 4: A ball-and-stick model depicting the coordination of copper atoms and ligands in the active site of Cu-2 depleted *Coprinus cinereus* laccase (Ducros *et al*., 1998). Copper atoms are in green, sulphur in yellow and oxygen in red.

particular study (Leontievsky *et al.*, 1997). The structure showed that type 2 and type 3 coppers are close together in a trinuclear centre (Leontievsky *et al.*, 1997). In 1998 a crystallographic structure of laccase (**Figure 5**) was reported for the copper-2 depleted laccase from *Coprinus cinereus* (Ducros *et al*., 1998). The comparative studies (Leontievsky *et al.*, 1997) and the findings by Ducros and co-workers coincided. The copper atoms of the T2/T3 sites are coordinated to eight histidines, which are conserved in four His-X-His motifs. The two T3 atoms are coordinated to six of the histidines (**Figure 4**) while the T2 atom is coordinated to the remaining two. A hydroxide ligand bridges the pair of T3 atoms (**Figure 4** in red), and because of its strong anti-ferromagnetic coupling it is responsible for the phenomenon of the T3 pair being EPR silent (Ducros *et al*., 1998).

The cloned sequences of various laccases also show that the 10 histidine and 1 cysteine residues that are copper ligands in ascorbate oxidase are conserved in all laccase sequences known to date except one from *Aspergillus nidulans* that has a methionine ligand of type 1 copper (Leontievsky *et al.*, 1997). These conserved cysteine and histidine residues serve as a pathway for the transport of electrons from the T1 Cu site where electrons are extracted from phenolic substrates to the trinuclear site that serves as the binding site of dioxygen where the electrons are required for dioxygen reduction (Ducros *et al*., 1998).

The crystal structure of laccase (Ducros *et al*., 1998) shows that laccase is a monomeric molecule that consists of three cupredoxin-like domains (**Figure 5**) that result in a

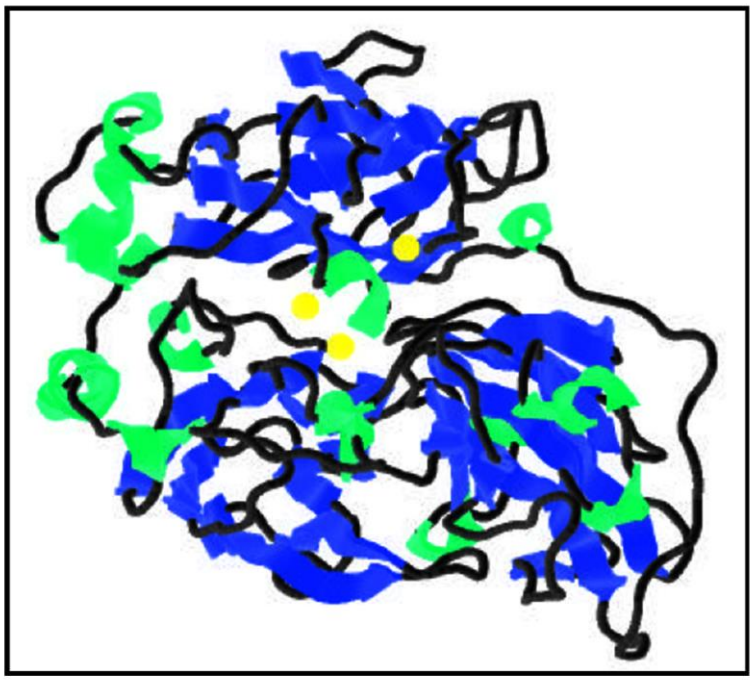

Figure 5: Crystallographic structure of the Cu-2 depleted laccase for *Coprinus cinereus* (Ducros *et al.*, 1998).

globular structure. The molecular architecture, as for all blue copper oxidases, contains β-barrel domains (**Figure 6.A**). The third domain has a β-sandwich conformation (**Figure 6.B**) as well as four short helical regions. The exact nature of the reaction mechanism of laccases that involves the reduction of dioxygen to water and the concomitant four one-electron oxidations of reducing substrates remains controversial. 'Two-site-ping-pong-bi-bi'-kinetics has been proposed. This involves a multi-product, multi-substrate reaction, where the release of initial products is necessary before new substrates are bound by the enzyme (Ducros *et al.*, 1998).

Xu (1996) did a comparative study with different fungal laccases and a range of substrates including, phenols, anilines and benzenethiols. He proved that the first transfer of one electron between substrate and enzyme was governed by the outer sphere mechanism. The steric effect of small ortho-substituents such as methyl or methoxy groups was found to be of little importance when compared to the electronic effect (Xu., 1996). Xu (1996) estimated the type 1 copper site of laccase to be approximately 10 Å in depth.

Active site exceptions

Although most laccases adhere to these phenomena there are certain highly purified laccases that do not show these typical characteristics. Not all laccases are reported to possess four copper atoms (Thurston., 1994) per monomeric molecule. One of the laccases from *Pleurotus ostreatus* is said to confer no blue colour and was described by the author to be a white laccase (Palmieri *et al.*, 1997). It was determined by atomic

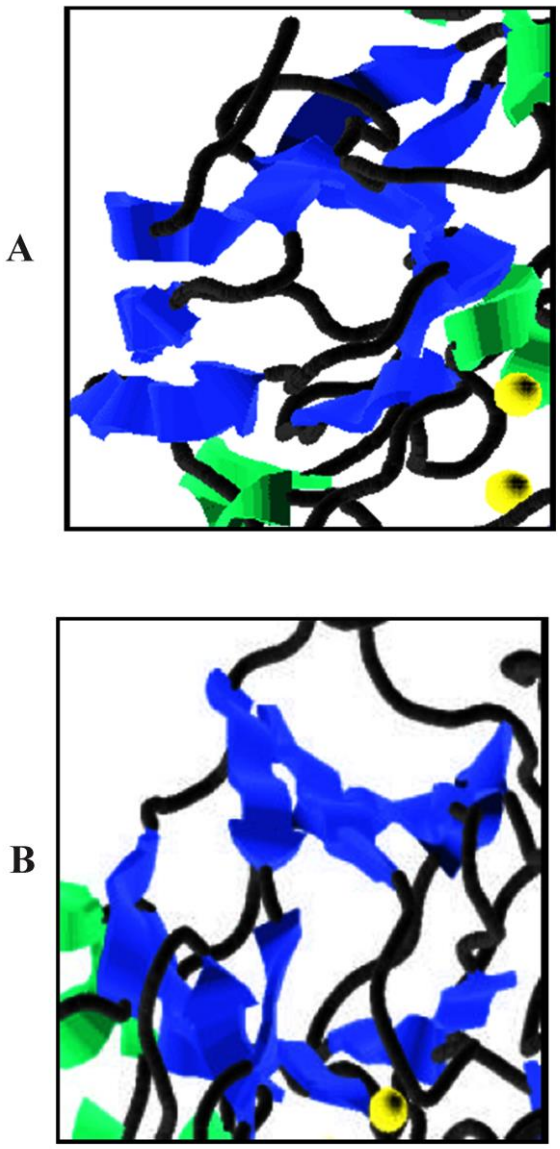

Figure 6: β-barrel (A) and β-sandwich (B) conformations from the cupredoxin like domains described by Ducros and co-workers for *C. cinereus* laccase (Cu-2 depleted). (Ducros *et al.*, 1998).

absorption that the laccase consisted of 1 copper atom, 1 zinc atom and 2 iron atoms instead of the typical four coppers.

Certain laccases have been found to be yellow or yellow-brown rather than the blue colour that is expected for laccases (Leontievsky *et al.*, 1997). Yellow laccases and blue laccases from the same organism had similar copper contents. It was proposed that yellow laccases, under normal aerobic conditions, did not maintain their copper centres in the oxidised state of resting enzymes. The binding of low molecular mass phenolic material from lignin degradation could contribute to such a change of enzymatic property. The explanation has not yet been verified (Leontievsky *et al.*, 1997).

cDNA and gene sequences

The first gene and/or cDNA sequences were recorded for laccase from the Ascomycete fungus, *Neurospora crassa* (Germann and Lerch., 1986). Further sequences were published from 1990 onwards. These included laccases from *Aspergillus nidulans* (Aramayo and Timberlake., 1990), *Coriolus hirsutus* (Kojima *et al*., 1990), *Phlebia radiata* (Saloheimo *et al*., 1991), *Agaricus bisporus* (Perry *et al*., 1993b), *Pycnoporus cinnabarinus* (Eggert *et al*., 1998), *Coriolus versicolor* (Mikuni and Morohoshi., 1997), *Trametes versicolor* (Jönsson *et al*., 1997), *Podospora anserina* (Fernandez-Larrea and Stahl., 1996), *Coprinus congregatus* (Leem *et al*., 1999), *Ganoderma lucidum, Phlebia brevispora, Lentinula edodes, Lentinus tigrinus* (D'Souza *et al*., 1996).

The sequences mostly encoded polypeptides of approximately 520 to 550 amino acids (including the N-terminal secretion peptide). The one cysteine and ten histidine residues involved in the binding of copper atoms were conserved for laccases and this is also

similar to what is found for sequences from ascorbate oxidase. The difference between laccases and ascorbate oxidase in the copper-binding region is that ascorbate oxidase exhibits the presence of a methionine ligand, which is not present in the laccase sequences. The absence and presence of the methionine ligand has led to interesting studies of mutagenesis conducted by Xu and coworkers (Xu *et al*., 1998; Xu *et al*., 1999).

Mutagenesis of the active site

Various models have been generated to correlate the Cu site structure and the molecular properties of laccase. In particular, it has been postulated that the coordination geometry and ligands of the type-I Cu might determine the redox potential of this site. Many laccases were shown to have a leucine or methionine residue at the position corresponding to that of the T1 Cu site (Thurston., 1994; Ducros *et al*., 1998; Aramayo and Timberlake., 1990; Leontievsky *et al.*, 1997). Xu and coworkers (1998) observed that *Trametes versicolor* laccase that has a high redox potential (0.8 V) presented a phenylalanine residue instead of methionine or leucine and predicted that it might be responsible for the high redox potential.

In 1996 Xu and his co-workers showed that three high redox laccases had a leucine-glutamate- alanine tripeptide, rather than the valine-serine- glycine tripeptide found in low redox laccases. The position of the tripeptide corresponds to the T1 pocket and serves as part of the substrate-binding pocket (Xu *et al.*, 1996). The effects of the triple mutation on the redox potential, suggest that the substrate binding pocket and the electron transfer pathway from the substrate to the T1 Cu were affected. They thus proved that it might be possible to regulate laccase catalysis by targeted engineering (Xu *et al.*, 1998).

A pentapeptide was also targeted in the vicinity of the T1 copper site of a low and a high redox laccase. A leucine residue was replaced by a phenylalanine residue at the position corresponding to the T1 Cu axial ligand. No significant effects could be elucidated (Xu *et al.*, 1998).

Applications of laccase in biotechnology

A vast amount of industrial applications for laccases have been proposed and they include paper processing, prevention of wine decolouration, detoxification of environmental pollutants, oxidation of dye and dye precursors, enzymatic conversion of chemical intermediates and production of chemicals from lignin. Before laccases can be commercially implemented for potential applications, however, an inexpensive enzyme source needs to be made available (Yaver *et al*., 2001). Two of the most intensively studied areas in the potential industrial application of laccase are the delignification or biobleaching pulp and the bioremediation of contaminating environmental pollutants (Schlosser *et al*., 1997).

Laccases of fungi attract considerable attention due to their possible involvement in the transformation of a wide variety of phenolic compounds including the polymeric lignin and humic substances. So far, more than a 100 enzymes have been purified from fungal cultures and characterized in terms of their biochemical and catalytic properties. Most ligninolytic fungal species produce constitutively at least one laccase isoenzyme and laccases are also dominant among ligninolytic enzymes in the soil environment. The fact that they only require molecular oxygen for catalysis makes them suitable for

biotechnological applications for the transformation or immobilization of xenobiotic compounds.

Antisense repression as a method to alter gene function

Antisense repression is a means to alter gene function that works at the level of mRNA rather than at the level of the genomic locus (Inouye., 1988; Kuss and Cotter., 1999; Persidis., 1999). Antisense repression starts by having single-stranded RNA that is complementary to the target mRNA. The antisense RNA binds to the target mRNA to form an RNA±RNA duplex, and the target mRNA is inactivated, possibly as a result of degradation or inability to access the translational machinery (Liu and Carmichael, 1994; Kumar and Carmichael., 1998).

Antisense repression has several features that can be complementary to the standard methods of gene inactivation using targeted disruption. In conventional gene disruption strategies, especially in an organism whose genome has many introns, the genomic locus must be cloned. Antisense repression does not require the isolation of the genomic locus, and gene inactivation can be accomplished by using relatively short pieces of coding sequence rather than entire genes. Another advantage is that antisense repression does not require homologous integration, and it may be especially useful for other fungal organisms in which rates of homologous recombination are very low. Another potential use for antisense repression is the simultaneous inactivation of multiple loci by using a single antisense molecule that targets a sequence common to all of the target mRNAs. For example, a single antisense transcript affected the expression of all three discoidin I genes in *Dictyostelium,* a feat that otherwise would have required three separate rounds of

targeted disruption (Crowley *et al*., 1985). In addition, a single antisense oligonucleotide targeted against the multicopy gene encoding gp90 of *Trypanosoma cruzi* was able to inhibit function *in vivo* (Malaga & Yoshida., 2001). Genes essential for vegetative growth can be identified with antisense repression (Liu *et al*., 1992; Tentler *et al*., 1997), and this was also recently demonstrated in *Candida albicans* (De-Backer *et al*., 2001). Proving that genes are essential to an organism is extremely complicated since they cannot be easily identified using conventional gene knockout strategies. However, genes essential to the organism may be ideal targets for the development of new antifungal drugs since inhibition of function would confer fungicidal rather than fungistatic activities. Antisense repression is not only useful as a laboratory tool, but has also been used *in vivo* as an antibiotic and to alter host gene function. There is currently an FDA approved medication consisting of antisense oligonucleotides for the treatment of cytomegalovirus (CMV) retinitis (Perry and Balfour., 1999), and there are clinical trials in humans that are using antisense oligonucleotides to treat leukaemia and other malignancies (Kuss and Cotter., 1999). Furthermore, investigators have injected antisense oligonucleotides into experimental animals to alter gene expression of both the host (McKinley *et al*., 2000) and the pathogen (Chakraborty *et al*., 1999).

RNA interference (RNAi) as a method to alter gene function

RNA interference (RNAi) is used to suppress gene function at the post-transcriptional level in several cell systems (Cottrell and Doering 2003; Marques and Williams 2005) including fungi (Kadotani et al. 2003). The mechanism of gene suppression by RNAi is increasingly well understood (Cottrell and Doering 2003; Chi et al. 2003; Catalanotto et

al. 2004) and involves the silencing RNAs (siRNA) assembling into endoribonuclease-containing complexes known as RNA-induced silencing complexes (RISCs). The siRNA strands subsequently guide the RISCs to complementary RNA molecules where they cleave and destroy any single-stranded RNA in the cell with a matching sequence (Hutvagner and Zamore 2002; Hammond et al. 2001; Bagasra and Prilliman 2004; Kariko et al. 2004).

Technically there are two main methods for initiating gene silencing by RNA. The conventional method involves the introduction of DNA plasmid constructs that, after transcription in vivo, will produce long hairpin RNAs (Kadotani et al. 2003) whilst the siRNA approach involves the direct introduction of synthetic double stranded RNA molecules of around 22 nucleotides long (Liang 2005).

Although several studies have shown that RNAi functions in plants (Waterhouse et al. 1998), mammals (Marques and Williams 2005), nematodes (Tavernarakis et al. 2000) and fungi (Nakayashiki 2005) there has been little, but increasing, use of this molecular tool in down-regulating genes of filamentous fungi, e.g. in Neurospora crassa (Cogoni and Macino 1999) and Magnaporthe oryzae (Kadotani et al. 2003). Genes from plants (Qi et al. 2004) and mammalian cell lines (Elbashir et al. 2001) have hitherto been shown to be down-regulated by using small siRNA duplexes.

RNAi pathway can be experimentally harnessed to silence virtually any gene of interest with tightly controlled specificity by introducing dsRNAs or small interfering (siRNA) containing targeted gene sequences into cells or organisms and the detailed analysis of

resulting loss of functions and altered phenotypes represents the most direct and most readily interpretable method for experimentally elucidating the cellular function of genes.

In this study we describe the adaptation of antisense repression and RNA interference to the study of plant-pathogenic fungus *Magnaporthe grisea*. The availability of this technique should facilitate future molecular studies using *M. grisea*, and we also propose that antisense repression and RNA interference can be applied to other plant-pathogenic fungi. Antisense repression has already proven itself to be valuable in the diploid *Candida albicans*, where a single transformation event has resulted in altered gene function (DeBacker *et al*., 2001). Antisense repression and RNA interference may also be valuable in molecular studies of other fungi where genetic manipulations are much more technically difficult.

Phylogenetic analysis of laccase and related multicopper oxidases

A phylogenetic analysis of amino acid sequences of more than 350 multicopper oxidases (MCOs) from fungi, insects, plants, and bacteria, done by Hoegger and co-workers (2006), provided basis for a refined classification of this enzyme family into laccases sensu stricto (basidiomycetous and ascomycetous), insect laccases, fungal pigment MCOs, fungal ferroxidases, ascorbate oxidases, plant laccase-like MCOs, and bilirubin oxidases. Analyses of the completely sequenced fungal genomes showed that the composition of MCOs in the different species can be very variable. Some species seem to encode only ferroxidases, whereas others have proteins which are distributed over up to four different functional clusters in the phylogenetic tree.

The composition of the multicopper oxidases of different fungal taxonomic groups seems to be quite variable. Half of the basidiomycete and filamentous ascomycete belong to the

laccase sensu stricto clusters. The other sequences of both basidiomycetes and filamentous ascomycetes are distributed over the fungal pigment multicopper oxidases, ferroxidases, and ascorbate oxidases clusters or belong to no cluster. In contrast, MCOs from the ascomycetous yeasts belong almost all to the ferroxidases.

11 multicopper oxidases from *M. grisea* were included in this study. Among these multicopper oxidases 4 belonged to Ascomycetes laccases cluster, 2 belonged to fungal pigment (melanin) multicopper oxidases and fungal ferroxidases each, 1 belong to ascorbate oxidase cluster and 2 did not fall in any cluster.

	Basidiomycetes				Filamentous ascomycetes						Ascomycetous yeasts											Zygomycete
Cluster[a]	Cci	Cne	Pch	Uma	Ani	Ncr	Fgr	**Mgr**	Cgo	Cim	Ego	Sce	Cal	Cgl	Cgu	Clu	Ctr	Kla	Dha	Yli	Spo	Ror
Total MCOs	17	6	5	6	7[b]	10	13	**11**	7	2	3	3	5	3	3	3	3	3	3	3	1	5
Basidiomycete laccases	17																					
Ascomycete laccases					2	8	5	**4**	4	1												
Fungal pigment MCOs (melanin DHN)				2	3		3	**2**							1	1			1			
Fungal ferroxidases		4	1	1		2	2	**2**	2		2	2	5	2	2	2	3	2	2	3	1	1
Fungal and plant ascorbate oxidases				1	2		1	**1**	1													4
Not in any cluster		2	4	2			2	**2**		1	1	1		1				1				
Genes of high affinity iron uptake pathways																						
ftr1 homologue(s)	−	+	+	+	−	+	+	**+**	+	−	+	+	+	+	+	+	+	+	+	+	+	+
ftr1 homologue(s) clustered with MCOs[c]	−	2	1	1	−	1	2	**1**	1	−	−	−	−	−	−	−	−	−	−	1	1	1
sid1/*sidA* homologues	+	−	−	+	+	+	+	**+**	+	+	−	−	−	−	−	−	−	−	−	−	+	−[d]

Table: Number of sequences from complete fungal Multicopper oxidase multigene families in the different clusters and presence of homologues of representative genes of the high affinity iron uptake pathways. (Hoegger et.al., 2006)

Valderrama and co-workers (2003) reconstructed the fungal laccase loci evolution from the comparative analysis of 48 different sequences. The topology of the phylogenetic trees indicated two pieces of information, that a single monophyletic branch exists for fungal laccases and that laccase isozyme genes may have evolved independently, possibly through duplication-divergence events.