4. Discussion

Laccases are ubiquitous enzymes in fungi and higher plants and have been shown to play important roles in developmental cycle of various fungi (Mayer, 1987; Thurston, 1994). There are many reported cases that show a single fungal species able to express more than one laccase enzyme. Different culture conditions, for instance, may lead to the production of different isozymes by the same fungus (Bollag and Leonowicz, 1984, Wahleithner *et al.*, 1996, Palmieri *et al.*, 1997, Farnet *et al.*, 2000). Examples of organisms able to produce more than one laccase isozyme include, *Podospora anserina*, 3 isozymes (Thurston, 1994), *Agaricus bisporus*, 2 isozymes (Perry *et al.*, 1993), *Pleurotus ostreatus*, 3 isozymes (Palmieri *et al.*, 1997) and *Rhizoctonia solani*, 4 isozymes (Wahleitner *et al.*, 1996). As there are multiple laccases predicted in *M .grisea*, distinctions between different laccases would be impossible to draw using crude protein extracts that contain multiple laccases. Relative expression of MGG_08127.5 was highest in the normal growth and MGG_02876.5 showed ~15-fold induction under nitrogen starvation. Therefore heterologous expression, purification and further characterisation were done to study these two laccases of *M. grisea*.

*Mg*Lac1 and *Mg*Lac2 both showed molecular masses ~ 66 kDa. It has been reported that the typical molecular masses for laccases range between 60 and 80 kDa, there are many reported cases, which do not show this typical size (Thurston, 1994). Such laccases are produced by *Phanerochaete flavidoalbans*, 100 kDa (Perez *et al*., 1996), *Agaricus bisporus*, 100 kDa (Perry *et al*., 1993a) and *Aspergillus nidulans*, 110 kDa (Thurston, 1994).

4.1 Enzyme Activity

Ten different aromatic compounds were chosen to study the substrate specificity of *Mg*Lac1 and *Mg*Lac2. The substrates were chosen according to the nature and position of substituents on the phenolic ring. ABTS, a non-phenolic compound and common substrate as well as mediator, was included in the study. ABTS has a higher redox potential than laccase itself and the mechanism by which laccase-catalysed oxidation of ABTS occurs is not yet fully understood (Bourbonnais *et al.*, 1997).

Laccases are known to have *p*-diphenol as well as *o*-diphenol activity, but they mostly have a higher affinity and activity toward the *p*-diphenols (Xu, 1996; Filazzola *et al.*, 1999; Sanchez-Amat and Solano, 1997). This is not the case with the *Mg*Lac1 in the present study.

Compound typified by three hydroxyl groups (pyrogallol) was oxidised efficiently. *Mg*Lac2 had higher affinitiy (according to Km values) toward this compound than towards the compounds with two adjacent hydroxyl groups such as catechol.

*Mg*Lac1 had a higher binding affinity (lower Km) for catechol than pyrogallol. *Mg*Lac1 and *Mg*Lac2 could not oxidise Guaiacol (ortho-substituted phenol), DMP (dimethoxy substituted phenol) and ferulic acid (one methoxy substitution).

Both laccases exhibited very low Km values for ABTS indicating very high binding affinity toward this substrate. From the Vm/Km ratios reported it was apparent that syringaldazine was oxidised efficiently by both laccases but there was no activity towards L-tyrosine, hence proving that they are not Tyrosinases. *Mg*Lac1 and *Mg*Lac2 therefore classify as true laccases according to substrate specificity.

4.2 pH optimum and thermostability studies

The pH optimum obtained for *Mg*Lac1 and *Mg*Lac2 is acidic and representative of typical laccases. The dependence of laccase on pH (Xu *et al*., 1996) usually renders a bell-shaped profile as can be seen for catechol and pyrogallol. This bi-phasic profile is the result of two opposing effects. The first is due to the redox potential difference between a reducing substrate and the Type 1 copper centre of laccase, where the substrates dock. Here the electron transfer rate is favoured for phenolic substrates at a high pH. The second is generated by the binding of a hydroxide anion to the Type 2/Type 3 copper centre of laccase, which inhibits the binding of O_2, the terminal electron acceptor, and therefore inhibits the activity at a higher pH because of the increased amount of OH- ions.

ABTS is the only substrate in our studies that does not adhere to this principle. With ABTS there is rather a monotonic decline than a bell-shaped profile. ABTS is regarded as a non-phenolic substrate in contrast to the two phenolic substrates discussed in the previous paragraph. The oxidation of ABTS to the cation radical by laccase does not involve protons and its redox potential is therefore independent of pH (Xu *et al*., 1996). The contribution of the hydroxide anion therefore leads to a monotonic decline (Xu, 1997).

Experiments to study the effect of temperature on the enzyme revealed that *Mg*Lac1 and *Mg*Lac2 were most thermostable at 30^0C. The typical optimum temperature range reported for laccases is between 50^0C to 60^0C (Luisa *et al.*, 1996). Examples of other laccases with higher temperature optima than what is commonly expected were reported

from the fungus, *Marasmius quercophilus* strain 17 (Farnet *et al.*, 2000) and *Coriolus hirsutus* (Shin and Kim, 1998

4.3 Inhibition studies

*Mg*Lac1 was more susceptible to inhibition by sodium azide and *Mg*Lac2 to cysteine as observed by low IC50 values. Four potential inhibitors were evaluated to test the inhibition properties of laccases. These inhibitors included: sodium azide (NaN_3) that complexes to the coppers in the active site, cysteine that is a sulfhydryl organic compound with a reducing effect on the copper-containing active site of laccase, EDTA that exhibits metal chelating properties and halide (Cl^-). Halides are known to inhibit laccase at the Type 2/3 trinuclear copper site. It is at this site that molecular oxygen is reduced to two molecules of water. The inhibition therefore entails that the oxygen is prohibited from being reduced, causing a break in the terminal electron acceptance, and leading to a decrease in redox potential difference between the two copper sites.

4.4 Other biochemical properties of *Mg*Lac2

Our studies provide evidence that the extracellular laccase *Mg*Lac2 produced by *M. grisea* may be involved in lignin degradation and / or melanin synthesis and / or iron metabolism. It may well be that multicopper oxidase dependent iron uptake systems are present in fungal systems.

A role for laccase in iron metabolism has also been observed in *C. neoformans* (Jung et al., 2006). They speculated that laccase oxidation of ferrous iron may be important during infection in addition to the enzyme's role in melanin production. The fact that laccase is expressed only during very early stages of infection would be consistent with a role in

iron acquisition and in protection of fungal cells from hydroxyl radical attack from host cells during the initial adaptation to the host environment.

4.5 RNA silencing

RNA silencing is an efficient tool for knocking down the expression of a gene of interest. An advantage of the RNA-silencing approach is its possible applicability to essential genes, which cannot be knocked out.

To date, gene knock-down experiments using RNA silencing were mostly carried out using silencing vectors producing hairpin or intron-containing hairpin RNA (hpRNA or ihpRNA) in filamentous fungi (Liu *et al*., 2002; Kadotani *et al*., 2003; Fitzgerald *et al*., 2004; Mouyna *et al*., 2004; Rappleye *et al*., 2004; McDonald *et al*., 2005; Tanguay *et al*., 2006). As construction of a hpRNA- or ihpRNA-expressing vector requires two steps of oriented cloning, its applicability is generally limited to a small or moderate scale.

Antisense repression system of RNA silencing in *M. grisea* was used in thepresent study. The silencing vector pSILENT (Nakayashiki *et al*., 2005) carries *Aspergillus nidulans trpC* (P*trpC*) and *trpC* terminator (T*trpC*) and multicloning sites between the promoters. Transcription of a target gene from promoter produces a pool of antisense RNAs in the cell, which would combine together into long double-stranded RNA (dsRNA) to be processed inside the cell.

4.8 Simultaneous silencing of homologous genes in the genome

As RNA silencing serves as a sequence-dependent gene inactivation system, it is known that transcripts homologous to the target are often suppressed simultaneously. To examine the level and extent of simultaneous silencing of homologous genes in the knock-down transformants used in this study, quantitative reverse transcription polymerase chain reaction (qRT-PCR) was performed.

Because silencing was observed with other multicopper oxidases as well, we checked their nucleotide sequence similarity. Sequence similarity at 7-25 nucleotide length of the laccase genes were observed in the sequence alignment. It has been reported that transcripts with minimum of 7 nt complementary sequence were sufficient to be silenced by siRNAs and short-hairpin RNAs (shRNAs) in a manner reminiscent of target silencing by miRNAs (Jackson *et al*., 2006).

It is noteworthy that the mRNA expression of some non-silenced homologous gene was highly upregulated in the knock-down transformants compared with in wild type. This may due to possible functional compensation against the reduced gene expression of the target and its homologous gene(s), which is also often reported with a functionally related homologue with the target gene in a knock-out mutant (**Nguyen et al., 2008)**.

4.9 Advantages and disadvantages of antisense

The greatest merit of the antisense system is that it allows single-step antisense oriented cloning for vector construction. This advantage includes only fewer cloning steps. Another advantage of simultaneous silencing of more than one gene is its potential use for multiple gene targets. Using hpRNA expressing vectors, simultaneous silencing of multiple endogenous genes has been shown in some fungi (Liu *et al*., 2002; Mouyna *et*

al., 2004). This strategy will be effective when a redundantly functioning gene family is subjected to functional analysis.

One of the advantages of exploring gene function by RNA silencing is its applicability to essential genes. The function of essential genes in fungal pathogenicity remains largely unknown as conventional approaches such as gene disruption or mutant screening are not available because of lethality. For example, imperfect silencing with reduced levels of gene expression showed that calmodulin (CAM) and calcineurin (catalytic subunit), which are known to be essential genes in *S. cerevisiae* or *A. nidulans*, were involved in infection-related developments (sporulation and appressorium formation) in addition to cell growth on rich media (Quoc *et al*., 2008).

The major disadvantages of RNA silencing include the difficulty of data interpretation due to incomplete silencing and/or potential off-target effects, which describe unexpected changes in the expression pattern of genes that have partial sequence similarity to the target. The results obtained with *M. grisea* knock-down transformants in calcium signalling pathway (Quoc *et al*., 2008) were mostly consistent with those previously observed in knock-out transformants of the corresponding gene and/or pharmacological studies in yeast or other fungi. In addition, even though the levels of silencing sometimes differed significantly among knock-down transformants of a certain gene, similar phenotypes, albeit at varying degrees, were usually observed among them. Therefore, the biological processes in which the gene is involved could be clearly determined.

4.10 Characterisation of knock-down transformants

*MgLac*1 knockdown transformants had normal morphology, germination rate and appressorium formation compared with wild-type and was not affected in pathogenicity.

MgLac2 knockdown transformants showed weak cell wall when treated with cell wall degrading enzymes and did not infect the host. In the assay using onion epidermis, it was shown that *MgLac2* knockdown transformants developed appressorium at the tip of the germ tube but could not penetrate into the plant tissue. Fungal mutants lacking cell wall integrity has been shown to be compromised for their infection ability (Jeon *et al*., 2008).

4.11 Membrane laccase

Redution in the number of colonies with gene speific siRNAs of *MGG_07771* was observed. Bioinformatics analysis predicted that *MGG_07771* is a laccase with a transmembrane domain. It was hypothesized that silencing of this laccase leads to weakening of the membrane of *M. grisea*, whih in turn leads to hypersensitivity to the cell-wall-degrading enzyme, miconazole and SDS. Retarded lipid mobilization is also h reported in *M. grisea* mutants with weak cell wall (Jeon *et al*., 2008).

Here we utilised RNA interference as a tool for specific silencing of gene expression in *M. grisea,* as it has been previously reported that direct use of dsRNAs or siRNAs could result in sequence specific suppression of that particular gene (Elbashir et al. 2001).

The results presented here show that siRNAs are powerful agents for mediating gene silencing. The decrease in the number of colonies was achieved without the need to construct vectors and take cultures through laborious transformation and characterization procedures. The down-regulation effect is not heritable, and has inherent variability, but provides the basis of a useful screen of gene functionality where the gene sequence is

known and where there is a readily available test for a phenotypic change. siRNA technology is therefore a faster and simpler approach to down-regulate genes than more conventional methods and can be added to the array of methods for functional analysis of genes in fungi.

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