Synopsis of the thesis entitled

ANALYSIS OF PATHOGENICITY IN *MAGNAPORTHE GRISEA*

Submitted by

KAMINEE RANKA

For the award of the Degree of

DOCTOR OF PHILOSOPHY

Department of Microbiology and Biotechnology Centre

Faculty of Science, M. S. University of Baroda,

Baroda-390 002

2008

Introduction:

Outbreaks of rice blast disease are a serious and recurrent problem in all rice-growing regions of the world, and the disease is extremely difficult to control. Rice blast, caused by the fungus *Magnaporthe oryzae,* is therefore a significant economic and humanitarian problem. It is estimated that each year enough rice is destroyed by rice blast disease to feed 60 million people.

Infections occur when fungal spores land and attach themselves to leaves using a special adhesive released from the tip of each spore. The germinating spore develops an appressorium, a specialized infection cell, which generates enormous turgor pressure (up to 8MPa) that ruptures the leaf cuticle, allowing invasion of the underlying leaf tissue. Subsequent colonization of the leaf produces disease lesions from which the fungus sporulates and spreads to new plants. When rice blast infects young rice seedlings, whole plants often die, whereas spread of the disease to the stems, nodes or panicle of older plants results in nearly total loss of the rice grain. Different host-limited forms of *M. oryzae* also infect a broad range of grass species including wheat, barley and millet. Recent reports have shown that the fungus has the capacity to infect plant roots.

M. oryzae outbreaks are controlled through the application of potentially hazardous fungicides and the use of resistant cultivars. The fungus has been able to develop resistance to many of these cultivars, because it is highly variable. An understanding of early events of the infection is of paramount importance if durable control measures are to be developed.

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:

i. Enzymes for degradation of the plant cell wall and cuticle.

ii. Proteins with carbohydrate substrate-binding domains, with a role in attachment and colonization of plant tissue.

iii. Pathogen effector proteins which fungus secretes directly into host plant cells to perturb host cell signalling or suppress the plant innate immune system.

Plant cell wall and cuticle degrading proteins are cutinases, cellulase, xylanases, laccases etc. Several of these genes are significantly up-regulated during infection-related development.

Biological role of fungal laccases are uncertain, they could be involved in:

(i) Melanin polymerization

(ii) Lignin degradation

(iii) Oxidation – reduction of plant toxins

Laccase is a copper- containing enzyme that catalyzes the oxidation of a phenolic substrate by coupling it to the reduction of oxygen to water.

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), suggesting that it might play a role during the infection of rice plant.

The present work focuses on the identification and characterisation of two laccase genes from *M. oryzae.*

Objectives:

1. Identification of putative laccase gene(s) in *Magnaporthe oryzae.*
2. Silencing of laccase gene(s) in *Magnaporthe oryzae.*
3. Characterisation of antisense transformants.
4. Heterologous expression, purification and characterisation of laccase(s).

Work Done

Using bioinformatics tools, 12 sequences with copper binding domain were obtained, which were further refined by using Signal P and Wolf PSORT, tools for predicting subcelluar localization of proteins. 9 sequences were found to be secretory. Multiple alignment and Clustal W program were used to create a phylogram.

Expression profiling of 12 laccases was carried out using real time quantitative PCR (qPCR). The analysis showed that Mgg_08127.6 (*MoLac1*) and Mgg_02876.6 (*MoLac2*) was highly expressed in the normal condition and nitrogen starved condition respectively. Therefore, these two laccases were selected for further characterisation.

PCR amplification of coding region of *MoLac1* and *MoLac2* were done with the gene specific primers. The desired amplicon of 2.2 Kb and 2 Kb respectively were obtained and cloned in antisense orientation under trypsin promoter to obtain the plasmid pTrp-MoLac1 and pTrp-MoLac2. Further, they were mobilized to pCAMBIA1305.2 to obtain pTrp-MoLac1-T and pTrp-MoLac2-T.

The *Agrobacterium* strain LBA4404 (pSB1) harbouring pTrp-*MoLac1*-T and pTrp-*MoLac2*-T, were used to transform the fungus as described (Mullins et al. 2001). Transformants were selected on hygromycin to a final concentration of 200 µg/ml. Untransformed *M. oryzae* was kept as a control which did not grow on hygromycin containing medium. The transformants were maintained as monoconidial isolates to get pure cultures.

Southern blot analysis of the antisense transformants of *MoLac1* and *MoLac2* was carried out. Transformants with single copy integration were used for further characterisation.

Quantitaive analysis of the relative transcript levels of *MoLac1* and *MoLac2* were performed using qPCR. The qPCR analysis showed 1.5±0.04 to 8.17±0.15 and 2.99±0.1 to 8.1±0.2 fold reduction in the level of *MoLac1 and MoLac2* transcripts respectively, in antisense transformants compared to the untransformed wild type B157.

Detached leaf infection assay were performed using spores of antisense transformants of *MoLac1* and *MoLac2.* The a*MoLac1* were able to cause infection like the untransformed fungus, whereas a*MoLac2* were unable to cause infection unlike the untransformed fungus.

Coding regions of *MoLac1* and *MoLac2* were cloned into a yeast expression vector 'pEG (KT)' (Mitchell et al. 1993) so that they were translationally 'in frame' with Glutathione-S-Transferase (GST) gene. Yeast strain, S288C, was transformed with the recombinant plasmid by 'one-step transformation' method .The GST laccase fusion protein was purified from total protein extracts by affinity chromoatography following induction of expression by the addition of galactose (4% w/v) a GST purification module as per the manufacturer's instructions (Amersham, Buckinghamshire, England). The purified fraction of MoLac1 and MoLac2 were checked on SDS-PAGE. A single band of desired molecular weight was observed in

both laccases. The purified fusion protein fraction of MoLac1 and MoLac2 were used to raise polyclonal antibodies in rabbit.

Based on the presense of N terminal signal sequence, MoLac1 and MoLac2 were predicted to be extracellular. Immunoblot analysis indicated that MoLac1 and MoLac2 were extracellular.

The GST fusion protein was used to study substrate specificity. ABTS, 2, 6-DMP, Syringaldazine, Guaiacol, Catechol, Hydroquinone, Pyrogallol, Phloroglucinol, Ferulic acid and Tyrosine were used for this study. MoLac1 showed activity towards substituted phenols in the order: meta > ortho > para. MoLac2 showed activity towards substituted phenols in the order: para > ortho > meta.

The influence of pH on laccase activity was studied spectrophotometrically. Three different substrates were used to determine the optimum pH of laccases, because it is known that laccases have different optimum pH values for different substrates. The three substrates used were: ABTS, Catechol and Pyrogallol. The pH optima were determined over a range of pH 3 to 9. The 0.1 M Britton- Robinson buffer was used for the entire pH range. This buffer was made by mixing 0.1 M boric acid, 0.1 M acetic acid and 0.1 M phosphoric acid and adjusting the pH with 0.5 M NaOH. The different assays for the different substrates were conducted as described under assay methods. All assays were done in triplicate. MoLac1 optimum pH is found to be in between 4-5 and that of MoLac2 is between 4-4.5.

The effect of temperature on laccase activity was determined spectrophotometrically following the laccase-catalysed oxidation of 1 mM DMP for 2 minutes at temperatures ranging 25 ^{o}C to 80 °C at 5 °C intervals. Britton Robinson buffer (0.1 M) was used for all the reactions, at the optimum pH for catecol of each laccase. The substrate was incubated for at least 20 minutes at the different temperatures before the enzyme was added to start the reaction. Optimum temperature of MoLac1 and MoLac2 was found to be 80 ^{0}C and 70 ^{0}C, respectively.

Inhibition studies were performed by preincubation of the enzyme with the inhibitor substance for a certain time (usually 2-10 min) to ensure that the inhibition is complete (Johannes and Majcherczyk, 2000). The subsequent kinetic measurement was started by the addition of proper substrate and then their decline or the formation of the product was monitored. Four potential inhibitors (sodium azide, cysteine, EDTA and Cl-,) were evaluated to test the inhibition properties. MoLac1 and MoLac2 was observed in the Cysteine was found to be the most efficient inhibitor of both laccases and chloride was the least effective.

Lignin degradation potential was estimated by diluting 20 ml of a 0.2% (wt/vol) Remazole brilliant blue dye solution (in H_2O) to 4 ml with pH 4.5 citrate-phosphate buffer. Laccase (400 U) was added, and decolorization was assayed after 0, 10, 20, 30, 45, 60, 75 and 90 min at 23 ^{0}C. Decolorization was monitored by diluting 0.5 ml of the dye solutions to 2 ml with a 10 mM sodium azide solution in 10 mM pH 4.5 citrate-phosphate buffer. The ratio of absorbance (*A*591/*A*500) was measured for the azide-dye solution. Decolorization assays were performed in triplicate, and

absorbances were compared with that of a control without added laccase. Both the laccases showed lignin degradation potential.

Polymerisation of 1,8-DHN was measured by the addition of 15 U of laccase to a 2-ml solution of 1 mM DHN in 5 mM pH 4.5 citrate-phosphate buffer in 50% ethanol (Edens et al. 1999). The polymerization solution was incubated at 23°C and monitored spectrophotometrically by scanning the solution from 320 to 520 nm at 0 to 120 min. An identical control solution without the enzyme that auto-oxidized was also scanned. MoLac2 showed the ability to polymerise DHN.

References:

Dean, R. A., Talbot, N. J., Ebbole, D.J., Farman, M.L., Mitchell,T.K., Orbach,M.J., Thon,M., Kulkarni, R., Xu,J.R., Pan,H., Read,N.D., Lee, Y.H., Carbone,I., Brown,D., Oh,Y.Y., Donofrio,N., Jeong,J.S., Soanes,D.M., Djonovic,S., Kolomiets, E., Rehmeyer,C., Li,W., Harding,M., Kim,S., Lebrun,M.H., Bohnert, H., Coughlan,S., Butler,B., Calvo,S., Ma,L.J., Nicol,R., Purcell,S., Nusbaum,C., Galagan,J.E. &. Birren,B.W. 2005. The genome sequence of the rice blast fungus Magnaporthe grisea. *Nature* 434: 980 – 986.

Iyer, G and Chattoo, B. B. 2003. *FEMS Microbiology Letters* 227: 121 -126.

Mullins E.D., Chen X., Romaine P., Raina R., Geiser D.M. and Kang S. 2001. *Agrobacterium* mediated transformation of *Fusarium oxysporum*: An efficient tool for Insertional Mutagenesis and gene transfer. The Amer. Phytopath. Soc. 91(2): 173-180.

Mitchell DA, Marshall TK, Deschenes RJ. 1993. Vectors for the inducible expression of glutathione S-transferase fusion proteins in yeast. Yeast 9: 715–723

Johannes C and Majcherczyk A. 2000. Laccase activity tests and laccase inhibitors. J. biotechnol. 78: 193-199.

Edens WA, Goins TQ, Dooley D and Henson JM. 1999. Purification and characterization of a secreted laccase of *Gaeumannomyces graminis* var. *tritici*, Appl. Environ. Microbiol. 65: 3071-3074

----------	----------	----------
Research Guide	**Head** **Dept of Microbiology**	**Dean** **Faculty of Science**