Functional characterization of gene(s) involved in pathogenicity of the rice blast fungus *Magnaporthe oryzae*

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1. INTRODUCTION

1.1 *Magnaporthe oryzae* as a model system to study host-pathogen interactions

Magnaporthe oryzae, a hemi-bio-trophic phytopathogenic fungus, is the major causal agent of the blast disease in rice and other important cereal crops worldwide. Almost one-half of the world population is dependent on rice as their staple food crop. So, it is a matter of great concern to study and control this disease. Importantly, the rice-blast pathosystem is considered as a model system to study host-pathogen interactions [1] due to various salient features like – economic importance, genetic tractability, well established laboratory practices to study the infection related development and ease in genetic transformation.

The infection cycle of *M. oryzae* [2], upon landing onto plant leaf surface, starts with conidial germination in the presence of moisture, followed by perception of specific cues on the host tissue to undergo development of a specialised infection structure called appressorium. The enormous turgor pressure generated inside the appressorium helps the fungus in penetrating the host cuticle. It is generally believed that, while the intracellular turgor pressure contributes to the mechanical force, the localised loosening of the host/plant cell wall underneath the appressorium is carried out by certain plant cell wall digesting enzymes secreted by the fungal pathogen. Once inside the first host cell, the fungal pathogen uses different strategies to evade plant immunity to colonise the tissue, leading to the disease progression.

1.2 *Magnaporthe oryzae* Feruloyl Esterases (FAEs)

Plant cell walls are a pivotal battleground between microbial pathogens and their plant hosts. Microbial pathogens secrete an array of cell-wall-degrading enzymes (CWDEs) to depolymerize the non-cellulosic polysaccharides of primary cell walls. *M. oryzae* forms the appressorium which generates mechanical turgor pressure and via penetration peg it breaches the rice leaf cuticle and hence fungus is able to establish itself on the plant host.

Some group of CWDEs like endo-xylanases [3] and cellulases [4] have been fairly studied in *M. oryzae* and their role in fungal virulence, both in vertical penetration and horizontal expansion has been demonstrated. These CWDEs act on various complex polymers of the plant cell wall such as xylan and cellulose and degrades them into their monomeric constituents that can be readily absorbed and utilized by the fungus. However, one such group of enzymes which is predicted to

play an important role in fungal virulence but not studied in great detail in *M. oryzae* is Feruloyl Esterases (also called Ferulic Acid Esterases, FAEs). It has been shown that feruloyl esterase genes are required for full pathogenicity of the apple tree canker pathogen *Valsa mali* [5].

Also, based on the genome sequence [6], nine members of FAE gene family have been predicted in *M. oryzae* as compared to its non-pathogenic counterparts like *Neurospora crassa* have only one and *Aspergilus nidulans* have three FAE genes.

Feruloyl Esterases (FAEs; EC 3.1.1.73), a subclass of carboxylic acid esterases (EC 3.1.1.1), are a group of enzymes involved in the degradation of xylan and pectin, from which they release ferulic acid and other aromatic acids. The enzymatic reaction catalysed by FAE is as follows –

Feruloyl-polysaccharide + H_2O → Ferulate + polysaccharide

The plant cell-wall is mainly composed of three polysaccharides: cellulose, hemicellulose (consists of xylan and xylan derivatives) and pectin. A mesh-like interconnected network of cellulose microfibrils and hemicellulose is embedded within the pectin matrix. These three components (viz. cellulose, hemicellulose and pectin) are interconnected to each other via an ester bond formed between diferulate and the polysaccharide. This mesh-like network is required for providing strength and maintaining integrity of the plant cell-wall. Also, this ester-bond between diferulate and polysaccharide provides elasticity and plasticity to the plant cell-wall. FAEs hydrolyse these ester bonds (formed between diferulate and polysaccharide) and thus dissociate the complex into ferulate and the constituent polysaccharide. Breakage of the ester bonds leads to weakening of the plant cell-wall, which actually acts as a barrier protecting from its microbial pathogens.

Molecular analysis of FAE from *Aspergillus spp.* has revealed that many of these enzymes is modular, comprising a catalytic domain and a non-catalytic cellulose-binding domain (CBD) [7]. The major obstacle in identification of the cell wall depolymerases is that most of their expression is under tight regulation, i.e. catabolite product repression and substrate induction. Some researchers have predicted that MoFAE, working with other inhibiting protein, may also be indirectly involved in the fungus-plant interaction by producing structure-specific oligosaccharide fragments that are recognized by the plant host as elicitor signal molecules. RT-PCR analysis showed abundant FAE mRNA at later stages of lesion formation and expansion, which may implicate the function of MoFAE in the development of infection hyphae and the proliferation of fungus inside the plant cells [8].

Thus, considering the life cycle of the fungus and the predicted role(s) of FAEs as CWDEs during penetration and post-penetration colonisation by the fungus, it was of much interest to study the role(s) of FAEs in the rice-blast fungal pathogen.

2. OBJECTIVES

1. Determining the secretory nature of putative FAEs
2. Analysis of gene expression levels of putative FAEs under different conditions
3. Investigating the role of FAEs in pathogenesis

3. RESULTS

3.1 Determining the secretory nature of feruloyl esterases

We presumed that *M. oryzae* FAEs would function outside the cell; and hence it was important to study the secretory nature of these fungal enzymes.

3.1.1 *In silico* analysis of secretory signal peptide in FAEs by SignalP tool

To check the presence or absence of secretory signal peptide, the amino acid sequences of FAEs were analysed using SignalP bioinformatics program, which predicts the presence and location of conventional secretory signal peptide cleavage sites in a given amino acid sequence. Our analysis showed a likely presence of the conventional secretory signal peptide in seven out of nine FAEs in *M. oryzae*.

3.1.2 Confirmation of secretory nature of selected FAEs by yeast signal trap system

In order to validate the results of SignalP analysis, two putative *FAE* genes were randomly selected and subjected to Yeast Signal Trap (YST) [9] system using pYST vector. The two FAE genes were then individually cloned in frame with the *SUC2* invertase gene without its native signal peptide, in the vector pYST. The recombinant plasmid was used to transform the budding yeast strain (*Saccharomyces cerevisiae*) DBYα2445, which does not carry its native *SUC2* invertase gene. Thus, neither the untransformed yeast strain nor the yeast transformed with the backbone vector would be able to grow on selection plate containing sucrose as the sole carbon source. However, the yeast transformed with the FAE gene, presumably with an inherent signal peptide sequence, cloned upstream to Suc2 invertase gene in pYST would be able to secrete Suc2 invertase and hence utilize sucrose as the sole carbon source in the selection medium. Indeed, the yeast transformants harboring the FAE genes-containing plasmids grew on sucrose agar, confirming the presence of signal peptide and thereby the secretory nature of proteins encoded by genes MGG_05529 and MGG_07294.

3.1.3 Determination of the effect of plant host-extract on the fungal FAE enzyme activity

The feruloyl esterase enzyme activity was checked in the intracellular and secretory fractions of fungal culture treated with rice leaf extract for 72 hrs. The preparation of host-extract, its treatment to the fungus and the spectrophotometry-based FAE enzyme assay were essentially carried out as described previously [10; 11]. Briefly, the assay involves FAE-based conversion of a specific substrate, 4-nitrophenyl ferulate (4-NPF) to 4-nitrophenol, which is a coloured product detected at 410 nm. Specific activity of the samples, in terms of μM product (4-nitrophenol) per mg protein, was determined against standard curve generated using commercially available spectrophotometry grade 4-nitrophenol (4-NP).

Importantly, unlike the intracellular fraction, secretory fraction of the host extract treated culture showed significantly higher (~6 fold) FAE activity when compared to the untreated culture, indicating the induced feruloyl esterases activity by the components present in the rice leaf extract.

3.2 Analysis of expression levels of FAEs under different growth conditions

Expression of cell-wall degrading enzymes including feruloyl esterases (FAEs) is believed to be tightly regulated under certain conditions. To understand the likely mechanism, we decided to identify the growth conditions or stages at which these FAEs are up- or down-regulated.

3.2.1 Levels of FAE transcripts under different vegetative culture conditions

Gene expression profiling of FAEs under different vegetative culture conditions was carried out by quantitative real-time PCR (qRT-PCR). Following conditions were used to study the effect on the expression levels of all the nine FAEs:

N_2 starvation	mimics nutrient stress condition during infection
Cutin monomers	promote appressorium formation
Pectin	component of primary plant cell-wall
Xylan	component of primary plant cell-wall
Ferulic acid (FA)	component released from polymers like pectin & xylan
N-acetylglucosamine (NAG)	amino sugar, basic unit of chitin (fungal cell wall)
Dextrose	control

The wild type *M. oryzae* (B157) conidia were grown in liquid complete medium for 3 days, followed by transfer to medium with either of the aforementioned compound/condition and incubation at 28 ^{o}C under shaking for 48 hrs. Total RNA was isolated from the fungal biomass, followed by cDNA

preparation using M-MuLV reverse transcriptase and oligo(dT) primers. The qRT-PCR was performed and the relative fold change in the transcript levels ($2^{-\Delta\Delta Ct}$ values) of all nine FAEs was estimated under respective growth conditions when compared to the control condition. β-tubulin was used as an endogenous control for this experiment.

FAE transcript levels in all the test conditions, except xylan- and NAG-treated cultures, were largely unaltered when compared to control. While, almost all the FAE genes were found to be repressed with xylan as the sole carbon source, treatment with a fungal cell wall component NAG led to induced FAE gene expression in *M. oryzae*. We inferred that the down-regulation of FAE expression in the presence of xylan could be due to catabolite repression.

3.2.2 Gene expression levels of FAEs during different pathogenic growth phases

To study the gene expression levels of FAEs during different stages of infection, fungal conidia (20 µL each of 10^5 conidia/mL) were inoculated on detached barley leaf pieces placed on kinetin (2 mg/L) agar plates. The plates were incubated at 28° – 30 °C and the inoculated leaf portions were harvested at specific time points (12, 24, 48, 72 and 96 hours post inoculation) signifying different stages of fungal infection cycle. Total RNA was extracted from the points of inoculations of each leaf and was used for cDNA preparation. The primers and PCR conditions used were same as those followed in the previous experiment. Transcript levels of FAE genes at different stages of infection cycle were estimated by qRT-PCR and compared to those from the vegetative fungal culture grown in complete medium and β-tubulin as an endogenous control. Sample prepared from barley leaves inoculated with gelatine solution was used as a negative control for this experiment. Almost all the FAE genes showed upregulation at different phases of pathogenic life cycle. Importantly, one FAE gene (MGG_08737) was significantly upregulated during pre-invasive appressorial development and host penetration stages (12 and 24 hpi) and remained elevated during the subsequent host colonization (48 hpi). Therefore, we chose the FAE gene MGG_08737 for our further studies.

3.3 Investigating the role of FAE in pathogenesis

Given the upregulation of the FAE gene (MGG_08737) before and during penetration, it was worth studying its role in pathogenesis of *M. oryzae*. In this direction, we generated a gene-deletion mutant of the FAE gene and characterised it further.

3.3.1 Deletion of FAE gene (MGG_08737) in *M. Oryzae*

A gene deletion construct was prepared by double-joint PCR [12] approach using hygromycin phosphotransferase (HPT) gene as a selectable marker. Double-joint PCR is a recombinant PCR technique wherein 5' and 3' flanking sequences of the gene (MGG_08737) were fused with the HPT gene cassette by two rounds of PCR. Third round of PCR was performed using the nested primers to get a specific amplification product. Amplified product was confirmed by RE digestion. This recombinant PCR construct was further purified by Na-acetate/ethanol precipitation and used for fungal transformation using *M. oryzae* protoplasts.

Fungal transformants were selected on YEG agar plates containing 200 µg/mL hygromycin. Transformants were first screened by PCR using gene-specific primers. Out of a total 72 transformants screened, one showed the desired PCR product. Selected transformant was further confirmed by PCR using two different sets of primers: i) 5'flank-Forward & HPT-Reverse and ii) HPT-Forward and 3'flank-Reverse. Selected transformant showed desired PCR-amplified product using both the sets of primers.

Selected deletion transformant was further analysed for the absence of transcript corresponding to MGG_08737 by RT-PCR. In this experiment, total RNA was extracted and subjected to 1st strand cDNA synthesis using oligo(dT) primers and subsequent PCR with gene-specific primers. As expected, no transcript was detected in the selected transformant when compared to the WT and an ectopic transformant.

Next, the selected transformant was confirmed for site-specific integration and copy number of the selectable marker gene, by Southern blot hybridization. Genomic DNA was extracted from the WT and *fae*Δ strain and further subjected to restriction digestion with HindIII or PstI. The HPT gene, used as a probe, was labeled and DNA detection was performed using Alkphos and CDP-star kit (GE healthcare ltd.) following manufacturer's instructions. Southern blot hybridization analysis confirmed deletion of FAE orf in the mutant with a single insertion event of the selectable marker gene.

3.3.2 Phenotypic characterization of the *fae*Δ mutant

After confirmation of the FAE gene deletion, phenotype of the *fae*Δ mutant was studied in comparison with the wild type strain. We found that the vegetative growth and asexual development (conidiation) was unaltered in the *fae*Δ mutant when compared to the WT. Similarly, the early

pathogenic event i.e. appressorial development was comparable to that in the WT. However, interestingly, the *fae*Δ strain was found to be largely impaired in penetration of the host cuticle, where only ~10% of the mutant appressoria showed invasion of rice cells. Importantly, these invading appressoria further failed to elaborate the invasive hyphae as assessed by detached rice sheath and leaf assays. Thus, the *fae*Δ strain was significantly non-pathogenic on rice plants when compared to the wild type *M. oryzae*. Given that the FAE activity on the plant cell wall would release ferulic acid and oligosaccharide molecules, we wondered whether exogenous supply of these compounds would rescue the *fae*Δ mutant during host invasion. Indeed, exogenously supplied ferulic acid and glucose individually rescued the *fae*Δ phenotype, and supported the invasive growth of the mutant strain inside the host tissue. This indicated that the released ferulic acid and/or sugar molecules, upon FAE function, might play a key role providing antioxidant property and energy, respectively, during host invasion.

4. ONGOING WORK

4.1 *In silico* analysis of FAE genes in different host-specific isolates of *M. oryzae*

To check whether the nine feruloyl esterase genes are diverged in different host-specific *M. oryzae* isolates, we are performing BLAST analysis to find the homologs in different *M. oryzae* strains isolated from various host plants. We would then perform a 'Multiple Sequence Alignment' using ClustalW for all the nine genes, followed by construction of a phylogenetic tree using the MEGA tool. The evolutionary history of the nine genes would then be inferred using maximum-likelihood method. Genetic variations in terms of SNPs would also be studied using the reference genome of 70-15 strain of *M. oryzae*.

5. CONCLUSION

Findings from this study highlights an important function for the fungal feruloyl esterase, particularly coded by MGG_08737, in targeting the plant cell wall at the time of penetration and to accumulate ferulic acid and sugar molecules as antioxidant and energy source to enable host colonisation by the blast fungus *Magnaporthe oryzae*.

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